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A cell-type specific and enhancer-dependent silencer in the regulation of the expression of the human urokinase plasminogen activator gene

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ABSTRACT

A transcriptional silencer has been identified in the 5′ regulatory region of the human urokinase plasminogen activator (uPA) gene. This region is able to block transcription from the human u-PA as well as the rabbit β-globin promoters in a cell type specific and orientation independent way. The silencer is enhancer dependent and is active in two cell lines (HeLa and CV-1) which produce little if any uPA, but not in the high uPA producer PC3. Silencing activity and enhancer dependence can be separated: the silencing activity has been localized to the DNA fragment −660 to −536, while the enhancer dependence is located in the −536 to −308 fragment. The DNA sequence of the silencer region contains an element that closely resembles the TGF-β responsive negative element TIE.

INTRODUCTION

Cell migration and invasion are processes that occur at many stages during embryonic development and also in adult life in response to physiological or pathological stimuli. Examples of such processes include trophoblast implantation, neuronal migration, angiogenesis, myogenesis and muscle regeneration, tumor invasiveness and metastasis. In these processes the extracellular protease plasmin, formed upon activation of the inert zymogen plasminogen, is used by cells for regulated degradation of a variety of extracellular proteins that connect the cells to the extracellular matrix, the basement membrane and other cells (1−9). Plasmin is formed by the action of two enzymes: tissue-type (t-PA) and urokinase-type (uPA) plasminogen activators. The latter is dealt with in the present paper. Plasmin is mostly active in tissues in a cell surface-bound form thanks to the presence of cellular receptors for both uPA and plasminogen (10, 11).

Active plasmin has a very short half life due to the widespread presence of a large excess of alpha-2 antiplasmin inhibitor (12). For this reason the main regulation of the plasminogen activating pathway occurs at the level of plasmin production, i.e. at the level of uPA. uPA synthesis is inducible in all cells provided the right inducer is chosen (reviewed in ref. 5). In the entire organism uPA is present in a limited number of cells in a few organs (13). Classic experiments have shown the inducibility of uPA synthesis in several cultured cells by transformation with oncogenic viruses (14). In the living organism, however, it has been proposed that the expression of the uPA gene is strictly down regulated in most cells and is induced by a variety of agents (growth factors, phorbol esters, hormones, differentiation factors) in both normal and neoplastic tissues (5).

We have previously studied the expression of the human and mouse uPA genes by fusing the uPA promoter to reporter genes and testing promoter activity by transfection in a variety of neoplastic, uPA-producing cells. The following information has been obtained. 1. One and the same start site for transcription is utilized by the endogenous gene as well as by transfected uPA-reporter genes fusions, in both basal and induced transcription (15−17; P. Verde, personal communication). 2. Expression of both the mouse and human promoters requires the action of an enhancer located at about 2 kB from the transcription start site (16, 17). The action of the enhancer is regulated by proteins like c-*jun*, c-*fos* and c-*ets* (17; Verde, P. personal communication). 3. A silencing activity located between −1870 and −1572 in the human uPA promoter appears to be active in some u-PA producing cells and thus possibly serves the function to modulate the activity of the enhancer (16).

Indirect evidence indicates that in cells that do not produce uPA the main regulatory mechanism may be a negative one. First, in all cases studied, the level of uPA mRNA is increased upon cycloheximide treatment (18−20). Although this might indicate also a modification of the half life of the uPA mRNA, available data suggest that this effect is at least in part on the rate of transcription (20). Second, in LLC-PK1 cells, in which uPA production is dependent on calcitonin or cyclic AMP addition (21), recessive calcitonin-independent mutants have been isolated (22).

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In an effort to identify DNA sequences with silencing functions active in cells that do not produce uPA, we have studied the expression in HeLa and CV1 cells of gene fusions between the human uPA promoter and the rabbit β-globin reporter gene. We have found that at $-660/-536$ of the 5′ flanking region of the human uPA promoter, a strong silencer is present which is only evident in the presence of the enhancer and acts in the two cell lines that do not produce uPA but is not active in at least one cell line in which uPA is over-produced.

MATERIALS AND METHODS

Cell lines

Simian CV1 cells were obtained from Dr. Paolo Amati (Rome, Italy). Human HeLa S3 cells (23) and human prostatic carcinoma PC3 (24) were obtained from ATCC. Cells were grown in Dulbecco modified minimal essential medium supplemented with 10% foetal calf serum, glutamine, penicillin and streptomycin.

Plasmid DNA constructions

We have modified the OVEC-1/OVEC-REF system (25) to analyse transcription started at the human uPA gene promoter. This system exploits the rabbit β-globin gene as a reporter and employs a single probe to simultaneously detect and differentiate sample and reference RNA in transiently transfected cells, using S1 mapping. The internal control OVEC-REF utilizes the SV40/β-globin promoter system. In order to analyze uPA-driven transcription, we have inserted uPA promoter sequences upstream of the β-globin gene in the OVEC-1 vector. In addition, we have eliminated the first and part of the second β-globin exon. This modification results in a 29 nucleotides difference in the sequences of sample and reference transcripts, which allows them to be simultaneously detected with the same probe and differentiated by electrophoresis. In the following description the number before βG refers to the 5′ end of the human uPA sequences. EN indicates the presence of the upstream uPA enhancer. When the orientation is opposite to that in the natural condition, the symbol rev is used. The orientation and the junctions of plasmids have been checked by DNA sequencing in the case of p-73-βG and p-85-βG. In the other cases an accurate restriction enzyme analysis was employed. The structure of the DNA templates is shown in Figure 1A.

1. *p-73-βG.* Vector OVEC-1 is a modified pUC18 plasmid containing the Bam HI−KpnI fragment of the rabbit β-globin gene downstream from convenient cloning sites (25). The minimal promoter of uPA, i.e. the EcoRI-SmaI fragment from puPACAT-73 (16) carrying from −73 to +29 of the human uPA gene, was blunt ended by treatment with the Klenow DNA polymerase fragment, adapted with BamHI linkers, and inserted in OVEC-1 at the BamHI site.

2. *p-85-βG.* The Eco 47III−SmaI fragment (−85 to +29) from p-uPACAT-245 (16) was filled in with Klenow DNA polymerase fragment, adapted with BamHI linkers and cloned in the BamHI site of the OVEC-1 plasmid.

3. *p-73-βG-EN and p-85-βG-EN.* To insert the uPA enhancer in p-73-βG and p-85-βG plasmids, the EcoRI/OxaNI fragment (−2212/−1824) from puPACAT-2212 (16) was filled in with the Klenow fragment of DNA polymerase, adapted with HindIII linkers and inserted in p-73-βG downstream of the rabbit β globin gene at the HindIII site.

4. *p-2212-βG.* The EcoRI/SacII fragment from p-uPA-CAT-2212 (16) containing the region −2212 to −14 of the uPA promoter was cloned in the SmaI/SacII digested p-85-βG. This plasmid reconstructs the wild type sequence of the human uPA promoter from −2212 to + 29.

5. *p-660-βG, p2x660-βG, p-rev660-βG, p-660-βG-EN, p2x660-βG-EN, p-rev660-βG-EN.* The −660 to −85 EcoRI/Eco47III fragment from puPACAT-660 (16) was filled in at the EcoRI site by treatment with the Klenow fragment of DNA polymerase and cloned in the SmaI site of the p-85-βG-EN plasmids in both orientations and in a tandem repeat. All constructions have also been made in an enhancerless form by digestion with HindIII (which excises the enhancer fragment) and subsequent ligation.

6. *Deletions and substitutions in p-660-βG and p-660-βG-EN.* All deletions were first made in enhancer-containing constructs and then the enhancer-less constructs obtained by deleting the enhancer region with HindIII. Deletion Δ_1 was constructed by treating p-660-βG-EN with SacI and EcoRV (cleaving at −660 and −537), blunt ending the SacI terminus with T4 polymerase and ligating the resulting DNA. Deletion Δ_2 was obtained digesting p-660-βG-EN with EcoRV and SacII (i.e. deleting from −537 to −14) and then inserting an (EcoRI-blunt-ended) EcoRI/SacII fragment (−301/−14) from puPACAT-301 (16). Deletion Δ_3 was constructed cloning the blunt ended EcoRI /PpuMI fragment (−660/−308) of puPACAT-660 (16) in the SmaI site of the p-85-βG-EN plasmid and choosing the correct orientation after restriction enzyme analysis. Construct Δ_2S contains a pUC18 fragment of similar size substituting the uPA promoter fragment deleted in Δ2. The PvuII/EcoRI fragment from pUC18 (nucleotides 450−628) and the EcoRI/Sac II fragment from puPACAT-301 (−301/−14) (16) were cloned in EcoRV + SacII-digested p-85-βG-EN.

7. *Construction of OV-SIL.* The uPA silencer region (EcoRI-BamHI fragment −660 to −85) of p-660-βG-EN was blunt-ended with T4 DNA polymerase and ligated with the blunt ended OVEC-REF DNA(25) previously linearized with KpnI. In this way, the uPA silencer was inserted at the 3′ end of the β-globin gene in a construct in which the latter is driven by the SV40/β-globin regulatory region.

Transfection

Calcium phosphate precipitation was carried out as previously described (16) using $6-7\times10^5$ cells per 100 mm dish in DMEM supplemented with 10% foetal calf serum. Medium was changed after 12 hrs, and the cells analysed about 48 hrs post transfection. In most experiments, 0.5 μg/plate of internal control DNA (OVEC REF) was co-precipitated with 10 μg/plate of the sample DNA. In the case of OV-SIL, 1 or 5 μg of DNA were coprecipitated with 15 μg of p-73-βG-EN DNA. In all cases four 10cm plates were transfected with each individual construct and all cells pooled to prepare RNA.

Isolation of cytoplasmic RNA from cultured cells

The medium was aspirated and cells washed with phosphate buffered saline, scraped and transferred to an Eppendorf tube, spun 5 min in refrigerated centrifuge at 1,500 rpm. The cell pellet was suspended in 140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.6, 0.5% Nonidet P-40, 0.015% (w/v) Macaloid, lysed by vortexing and then left on ice for 5 min. After centrifugation the supernatant was treated with SDS and proteinase K, incubated 30 min at 37°C or 5 min at 60°C, extracted twice with phenol/chlorophorm/isoamyl-alcohol and ethanol precipitated. The ethanol precipitated RNA was resuspended in sterile water, incubated 30 min at room temperature with RNase-free DNase

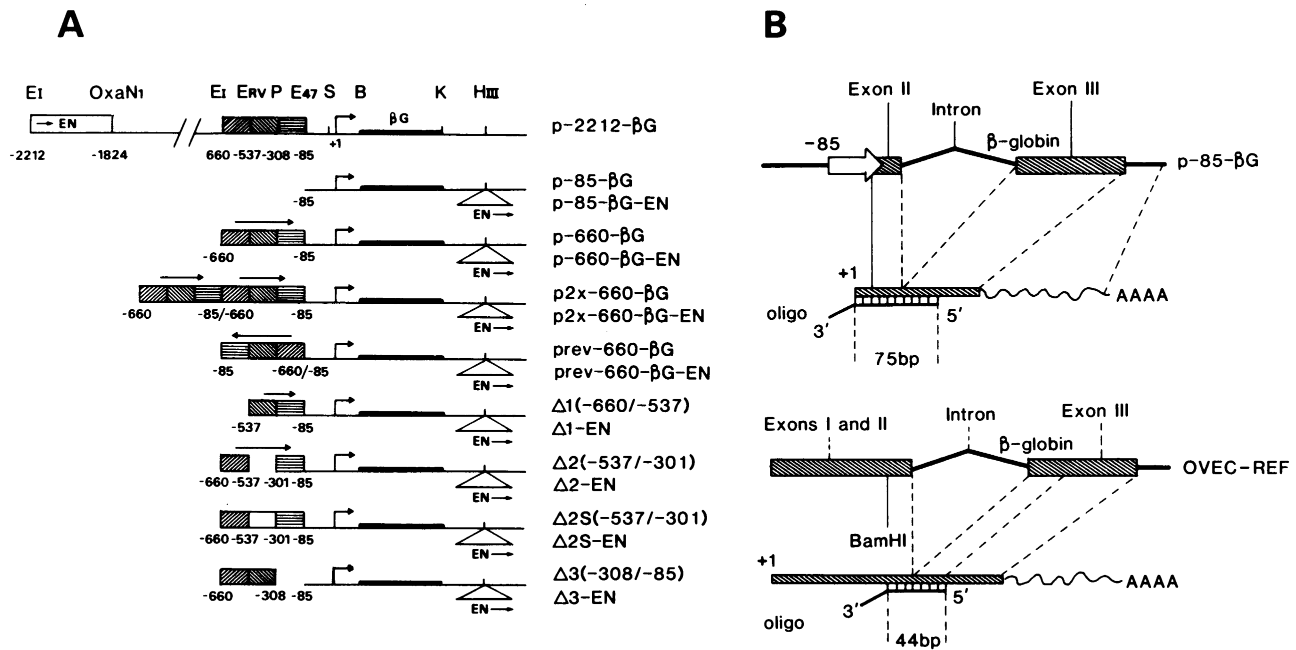

Figure 1. Structure of the DNA templates and schematic representation of the S1 mapping strategy. **A.** Structure of the DNA templates. The same set of constructs was prepared and tested, using both p-73-βG and p-85-βG as uPA minimal promoter. Here only the set with p-85-βG is shown. The basis for the nomenclature is explained in the text (see Methods section). Each construct was prepared in two version one with the enhancer (EN) and one without enhancer. The arrow indicates the orientation of the enhancer; in cloning the enhancer, the EcoRI-OxaNI fragment was used depicted as EN in the −2212-βG template. The −660/−85 reghion is shown made up of three different parts and the arrow indicates its orientation. The empty box in Δ2S and Δ2S-EN represents a 178 bp fragment from pUC18 (see Methods). With the exception of OxaN1, restriction enzymes used to generate the various constructions are indicated by the following symbols: **E**: EcoRI; **ERV**: EcoRV; **P**: PvuII; **E47**: Eco47III; S:Sac2; **B**: BamH1; **K**: Kpn1; **HIII**: HinDIII. The numbers at the bottom indicate the position of the restriction sites within the human uPA 5′ flanking region (16). **B.** S1 mapping strategy. The 5′ labeled 94 bp long oligonucleotide used as a probe (see Methods) can hybridize to both sample (uPA driven) and reference (β-globin driven) transcripts. The different sequence at the 5′ end of the transcripts ensures a different protection by the RNA extracted from transfected cells. The empty arrow in p-85-βG indicates the uPA promoter. The uPA-promoter driven transcript will give raise to a 75 bp protected fragment, while the β-globin promoter-transcript will protect a 44 bp fragment of the probe.

I, extracted once with phenol/chlorophorm/isoamylacohol, ethanol-precipitated and resuspended in 50−100 μL of sterile water.

S1 Mapping

An 89 bp synthetic probe has been used for S1 mapping. Its sequence is: ATATACGCCCAGCGTCGTGTCAGCCTCTG-GGGTCGGGCCTCGGGGCCTAGGACTCTTGAAGTCCG-AGGACCCGTTGCACGACCAACAA-5′ (the bold **T** corresponding to the uPA transcription initiation site). The chemically synthesized probe was labeled at the 5′ end with polynucleotide kinase and γ^{32}P-ATP (6000 Ci/mmole) under standard conditions. The scheme of the products expected in the S1 protection analysis is shown in Figure 1B. 25 to 50 μg RNA (corresponding to about 1/8 of the total RNA extracted), and the probe (40 to 100 Kcpm) were coprecipitated, the air-dried RNA pellet resuspended in 10 μl hybridization buffer (80% deionized formamide, 40 mM Pipes pH 6.4, 400 mM NaCl, 1 mM EDTA), heated for 5−10 min at 85−95°C, and hybridized 4 to 12 h at 37°C. The samples were cooled on ice, supplemented with 300 mM sodium acetate pH 4.5, 3 mM $ZnSO_4$, 10 μg/ml calf thymus DNA and 70−150 U of S1 nuclease (Boehringer, Manheim), digested 1 h at room temperature, then treated with 5 μl proteinase K (10 mg/ml) and 7 μl 10% SDS. The sample is then incubated 30 min at 37°C, extracted once with phenol/$CHCl_3$ /Isoamylalcohol and precipitated with EtOH. The samples are resuspended in Tris-EDTA buffer containing 90% formamide, 0.025%, bromophenol blue and xylene cyanol, heated 3 min at about 90°C, and loaded onto 8 M urea/10% polyacrylamide (19:1) gel, with Msp I digested ^{32}P-labeled PBR322 DNA as a MW marker. Molecular weight markers were labeled with Klenow DNA polymerase and α-^{32}P-CTP or with calf intestinal phosphatase, polynucleotide kinase and ^{32}P-γ-ATP.

RESULTS

A silencer located in the −660/−73 region of the human uPA promoter

As a basic uPA promoter construct, we have employed in this study a plasmid clone in which the proximal region of the promoter, containing essentially the TATA box and the Sp1 binding sites (16), is cloned upstream of the rabbit β-globin gene (Fig. 1A). Two types of constructions were prepared in which either 73 or 85 nucleotides upstream of the uPA transcription start site were used (p-73-βG and p-85-βG): since the results obtained were identical in both cases, we shall mostly show the data obtained with p-85-βG. Transfection experiments with the internal control of the rabbit β-globin gene under the SV40 promoter, were analyzed by S1 protection using a synthetic oligonucleotide that recognizes both the uPA and the SV-40-driven β-globin transcripts. The 5′-end-labeled oligonucleotide (98 bp) gives raise to 75 and 44 bp S1-protected

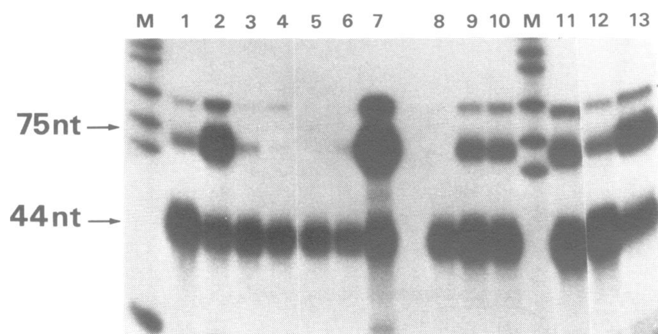

Figure 2. S1 mapping analysis of the silencing activity in the 5′ flanking region of the human uPA gene. Total RNA was extracted from CV1 cells transfected with the various templates and the presence and the amount of the transcripts identified by quantitative S1 mapping. Lanes marked M indicate molecular weight markers. The arrows indicate the migration of the 75 and 44 nucleotides protected fragments, which identify the uPA-βG and the reference OVEC-REF transcripts, respectively. Lanes **1** and **12**: p-85-βG; Lane **2** and **13**: p-85-βG-EN; Lane **3**: p-660-βG-EN; Lane **4**: p-660 rev-βG-EN; Lane **5**: pΔ2-βG-EN; Lane **6**: pΔ3-βG-EN; Lane **7**: pΔ1-βG-EN; Lane **8**: p-Δ2-βG; Lane **9**: p-Δ3-βG; Lane **10**: p-Δ1-βG; Lane **11**: p-660-βG.

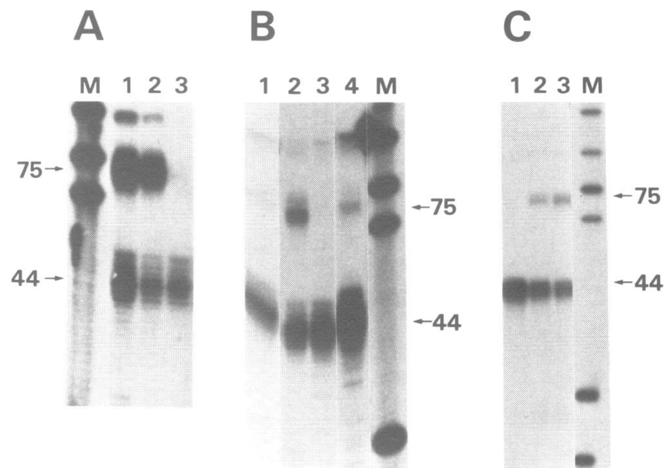

Figure 3. Cell-type specificity of the −660/−85 uPA silencer. The experiment was carried out as described in Figure 2 and in the Methods section. Symbols as for Figure 2. Panel **A**, CV1 cells. Lane **1**: p-73-βG-EN; Lane **2**: p-85-βG-EN; Lane **3**: p-660-βG-EN. Panel **B**, HeLa cells. Lane **1**: p-73-βG; Lane **2**: p-73-βG-EN; Lane **3**: p-660-βG-EN; Lane 4: p-2212-βG. Panel **C**, PC3 cells. Lane **1**: p-73-βG; Lane **2**: p-73-βG-EN; Lane **3**: p-660-βG-EN.

bands with the RNAs driven from the uPA and the SV40 promoter, respectively (Figure 1B).

Upon transfection in simian CV1 cells, a weak expression from the p-85-βG construction was observed, which was drastically increased in the presence of enhancer (compare lanes 1 and 2, or 12 and 13, Figure 2). Interestingly, when the region uPA −660/−85 was inserted upstream in the p-85-βG-EN construct, transcription from the uPA promoter was drastically reduced in a orientation-independent way (compare lane 2 with lanes 3 and 4, Figure 2). In the absence of the enhancer, however, the −660/−85 fragment had no silencing effect on the p-85-βG transcript, having if anything a slightly enhancing effect (compare lanes 11 and 12, Figure 2). The region −660/−85, therefore, appears to contain an enhancer-dependent silencing activity. While the −660/−85 fragment strongly reduced transcription of the p-85-βG-EN template, the presence of a tandem copy of this fragment completely eliminated transcription from the uPA promoter (data not shown).

Cell-line specificity and sub-regional mapping of the proximal uPA silencer

We have tested the cell-type specificity of this silencing activity, using in addition to CV1 cells, also human HeLa and PC3 prostate-adenocarcinoma cells. As shown in Figure 3, the silencing activity of the −660/−86 fragment while clearly visible in the CV1 and HeLa cells, is totally absent in the PC3 cells. Interestingly, while CV1 and HeLa cells do not produce urokinase, PC3 adenocarcinoma is a high producer of uPA (24; our unpublished data). In HeLa cells the wild type construct p-2212-βG, which contains the entire 5′ flanking sequence up to −2212, is expressed at a much lower level than p-73-βG-EN, and at a slightly higher level than p-660-βG-EN. This may be due to the presence of the silencer. However the action of other negative and positive regulatory sequences spread between −1824 and −660, missing in the p-660-βG-EN, is not excluded.

We have further delimited the location of the silencer on the −660/−86 fragment by deletion analysis dividing the fragment in three parts with restriction endonucleases (see Figure 1A). Removal of the leftmost (5′ end) one third (deletion Δ_1), results

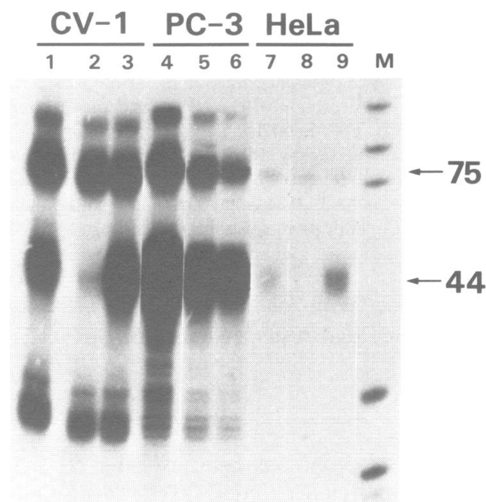

Figure 4. Silencing activity of the −660/−85 region on the heterologous β-globin promoter/SV40 enhancer. In this experiment the p-85-βG-EN template (15 μg DNA/plate) was used as internal reference in all cases in co-transfection experiments with OVEC-REF (2μg DNA/plate) (lanes **3**, **6** and **9**), OVEC-SIL [1 μg DNA/plate] (Lanes **2**, **5** and **8**) or OVEC-SIL [5 μg DNA/plate] (Lanes **1**, **4** and **7**). The cells used for transfection are indicated on top.

in loss of the silencing activity in CV1 cells (compare lanes 3 and 7, figure 2). Removal of the middle one third (deletion Δ_2), on the other hand, results in loss of enhancer-dependence of the silencer effect: in fact, the presence of the silencer is now felt not only on the constructions containing the uPA enhancer, but also on those missing the enhancer (compare lanes 3 vs. 5 and 8 vs.12, Figure 2). This effect is reproduced also if the middle one third is not simply removed, but is substituted by a 178 bp fragment taken from pUC18 showing that the loss of enhancer-dependence is not due to the change of distance from the transcription start site (data not shown) . Finally, removal of the 3′ one third (deletion Δ_3) has no effect neither in the presence

nor in the absence of the enhancer (compare lanes 3 with 6 and 9 with 12, Figure 2).

Silencing activity of −660/−85 on heterologous promoter

We have then tested the effect of the −660/−85 fragment on the SV40 enhancer-β-globin promoter; for this experiment, we have introduced the −660/−85 fragment at the Kpn1 site, i.e. at the 3′ end of the β-globin gene in the OVEC-REF construction producing OVEC-Sil. For this experiment, the p-85-βG-EN construct is used as internal control in the OVEC-Sil transfection, utilizing again the same S1 mapping strategy. In this case, naturally, the 44 nucleotides band represents the sample band, while the 75 nucleotides band represents the internal control. As shown in Figure 4, the −660/−85 fragment has a strong silencing activity both in CV1 and HeLa cells (compare lanes 2 vs. 3 and 8 vs. 9); again, as in the case of the uPA promoter/enhancer, no effect was observed in the PC3 cells (compare lane 6 vs. 5). The silencing effect in CV1 and HeLa cells can be partly titrated out by increasing the amount of transfected OVEC-Sil DNA by five fold (lanes 1 and 7); in PC3 cells, however, increasing the amount of OVEC-Sil results in an even higher signal (lane 4). Thus the silencing effect of the −660/−85 fragment is not only enhancer-dependent and cell-type specific, but can be also reproduced with a different enhancer-promoter system.

DISCUSSION

The role of uPA in such delicate phenomena requiring extracellular proteolysis like cell migration and tissue remodeling, justifies a very complex regulation of the transcriptional activity of the uPA gene. In the intact organism very few uPA-producing cells are observed, with the tubular portion of the kidney and the vas deferens as the highest producers, while in other organs, like intestine and lung, uPA appears to be present in sparse fibroblastic-like cells (13). In culture, however, a large number of cells lines are able to synthesize or to be stimulated to synthesize uPA. This may be matched by the observation that uPA production is increased in many tumors and tumor cell lines. In *in vivo* tumors, it is not always clear whether the increase in uPA synthesis is due to the cancer cells themselves or to stromal cells or even invading macrophages (6,26; unpublished data). This information, on the whole, suggests a certain degree of cell-type specificity of uPA synthesis and an important role for inducing factors. Thus regulatory differences may exist within different cell types as well as cell combinations.

Previous studies on the regulation of the endogenous uPA gene in both human, porcine and mouse cells, have shown that this gene is inducible by a variety of agents, including phorbol esters, growth factors and hormones (18−21) (see refs. 6 and 27 for a more comprehensive reference list). In all cases tested, induction of uPA mRNA was not blocked by inhibitors of protein synthesis; cycloheximide alone rather caused an increase of uPA mRNA while in combination with an inducer it led to a superinduction. In many cases it has been established that induction of uPA mRNA is due to a transcriptional activation of the gene (18, 19), and in at least one case it has been shown that cycloheximide effect was also due to an increase in transcription (20). These data suggest therefore that uPA synthesis may be regulated at least in part by negatively acting regulatory proteins, and that at least in some cells these proteins may be short lived.

Preliminary studies showed that the 5′ flanking region of the human urokinase gene contains regulatory elements with both enhancing and silencing activities (16). These elements are located more than 1500 bp upstream of the transcription start site. Recent data (C. Nerlov, P. Rørth, F. Blasi & M. Johnsen, to be published) show that both these elements display cell type specificity.

The data reported in this paper uncover the existence of another regulatory region with silencing activity. Sequences between −660 and −86 have the capacity to strongly decrease transcription from both the uPA and β-globin promoters in the presence of the uPA and SV40 enhancers. The effect is orientation independent, enhancer dependent and can be titrated out by increasing the concentration of transfected DNA.

The silencer effect of the −660/−86 region is cell type specific. It is observed in fact with the simian CV1 and the human HeLa but not with the human PC3 cells. It is noteworthy that CV1 and HeLa cells are among the few cell lines that produce no or very low levels of uPA ((9) and our unpublished results), while the prostatic adenocarcinoma PC3 produces high levels of uPA (28; our unpublished data). The −660/−86 region has been further subdivided into three regions by deletion analysis: the silencing effect is contained within the −660/−536, while the enhancer-dependence is located within the −536/−303 fragment.

The relationship between the proximal silencer and the enhancer requires to be clarified. In both human and mouse uPA the activity of the enhancer is dependent upon the combined action of at least the PEA3 and AP1 complexes (17); Nerlov, Rørth, Blasi and Johnsen to be published). It is interesting to note that in the silencer containing fragment, the sequence −549/−538 contains a 9/10 identity to the TGF-β1-responsive element of the transin/stromelysin gene (TIE element). This sequence, in TGF-β1 stimulated cells, is known to bind a nuclear protein complex which includes the product of the c-*fos* oncogene (29). It will be interesting to investigate whether the TIE-like sequence in human uPA is an essential part of the silencer and whether it also mediates a TGF-β1 regulation. Cycloheximide induction of uPA mRNA synthesis, indicating a possible negative role for a short-lived protein, agrees with a possible role of c-*fos* , a short-lived protein (30), in repression. C-*fos* participates in AP-1 complexes which are required for activity of the uPA enhancer (17; Nerlov, Rørth, Blasi and Johnsen, unpublished results). Its ability to bind to the AP1-like and the TIE-like elements might be related to the enhancer-dependence of the silencer activity. However, the separation of the enhancer dependence from the proper silencing effect possibly excludes this possibility. This separation implies that different proteins are involved in the silencing effect in the presence and absence of the enhancer. A more detailed mapping of both silencing and enhancer-dependence is required in order to positively identify the involved proteins. In any case,the absence of c-*fos* mRNA in human PC3 cells (31) might be in agreement with the cell-type specificity of the uPA silencer.

The presence of negative regulators in eukaryotic gene expression is becoming more and more acknowledged (32, 33). In addition to inducible silencers like the TIE element of transin/stromelysin (29), constitutive silencers have been demonstrated in several genes including c-*myc* (34), c-*fos* (35), vimentin (36) collagenase type IV (37) and lysozyme (38). The c-*fos* promoter itself is the target of the *fos* activity; in this case the responsible sequence appears to be different from the one that recognizes AP-1 (35). Also the type IV collagenase gene silencer is enhancer dependent and cell type specific (37), which

is interesting in view of the similarity with uPA in the nature of the gene products and in their functional role in cell migration and tissue remodeling.

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