Volume 16 Number 22 1988 Nucleic Acids Research

An upstream enhancer and a negative element in the 5' flanking region of the human urokinase plasminogen activator gene

Pasquale Verde[1,2], Sharon Boast[1], Annamaria Franzè[1,2], Federico Robbiati[4] and Francesco Blasi[1,2,3]*

[1]International Institute of Genetics and Biophysics, CNR, Naples, Italy, [2]Institute for Cancer Research, Columbia University, New York, NY, USA, [3]Mikrobiologisk Institut, University of Copenhagen, Denmark and [4]Lepetit SpA Research Laboratories, Gerenzano, Italy

Received June 27, 1988; Revised September 29, 1988; Accepted October 24, 1988 Accession no. X12641

ABSTRACT

The 5' flanking region of the human urokinase (uPA) gene has been fused to the reporter chloramphenicol acetyl transferase (CAT) gene and its activity assayed by transfection in two human cell lines. Progressive deletions of the uPA regulatory region from the 5' end maintain a high level of expression provided at least 1870 (in A1251 cells) or 1963 (in HFS10 cells) nucleotides of the 5' flanking region are retained. A DNA fragment from -2350 to -1824 has enhancer properties, stimulating transcription of an enhancerless SV40 early promoter independently of orientation and distance. Internal deletions that still retain the enhancer element reveal the presence of negative cis-acting sequences between -1824 and -1572. Their removal, in fact, increases uPA transcriptional activity. Differences of expression of the uPA-CAT fusion genes in the two cell lines are also observed, indicating the presence of cell-specific cis-acting sequences.

INTRODUCTION

Plasminogen activators, their inhibitors and receptors constitute a complex system which regulates extracellular proteolysis. The activation of plasminogen to plasmin allows the direct degradation of intra- and extra-vascular proteins like fibrin, fibronectin, laminin, proteoglycans, etc. as well as the activation of latent collagenases and hence collagen destruction (1). This enzymatic system has been shown to be important in cellular migration, invasiveness and metastasis of neoplastic cells (2-4) as originally proposed by Reich (5). Plasminogen activation, in addition, is likely to play a fundamental role also in a variety of physiological conditions involving cell migration and tissue involution both in embryogenesis and in the adult life (see Refs. 1,6).

The urokinase-type plasminogen activator (uPA) has been the subject of extensive studies aimed at understanding its regulation both in normal and neoplastic cells. uPA expression is induced by neoplastic transformation, and in many types of normal and transformed cells by a variety of hormonal or growth-affecting compounds, including growth factors, phorbol esters, polypeptide hormones, retinoic acid and cyclic AMP. The extensive literature is reviewed elsewhere (6). The uPA gene has been cloned (7,8)

and probes used for several regulatory studies. Briefly, uPA mRNA level is under growth and cell-cycle control (9) and can be induced by phorbol esters (10-12). These effects are exerted at the level of transcription (9,12).

Several kinds of cis-acting sequences have been shown to control the transcriptional activity of eucaryotic promoters (see Ref. 13 for a review). Transcriptional enhancers can be located far upstream from the transcriptional start site and may also contain regulatory sequences for effector molecules. Trans-acting factors binding to different elements and sequence motifs of the enhancers are becoming more and more characterized (see Ref. 14, for a rewiew). Negatively cis-acting sequences have been described in yeast (15) and in some mammalian genes, including retinol-binding protein (16), rat growth hormone (17), c-myc (18), c-fos (19) and beta-interferon (20). In these cases, the removal of the negative region results in induction of the gene. Factors binding to this region in the beta-interferon gene have been demonstrated (21-23).

In order to understand the molecular nature of the uPA gene regulation, we have now isolated and sequenced the 5'flanking region of the human uPA gene, made both 5' and internal deletions, fused them to the bacterial chloramphenicol acetyl transferase (CAT) gene and assayed the promoter activity following transfection in human cells. We report in this paper the presence of at least one positive and one negative cis-acting regulatory element involved in expression of the uPA gene.

MATERIALS AND METHODS

Materials

Restriction endonucleases, T4 DNA ligase, the Klenow fragment of DNA polymerase Bal31 exonuclease and S1 nuclease were purchased from Boehringer, Mannheim (W. Germany) with the exception of restriction enzymes OxaNI and NarI which were from New England Biolabs, Beverly, Ma. (USA). Purified chloramphenicol acetyl transferase and acetyl coenzyme A were purchased from Sigma, St. Louis, Mo. (USA).

Cell culture

All cell lines were cultured in DMEM (Dulbecco Modified Eagle Medium) supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 ug/ml). Human kidney carcinoma line A1251 (24), fibrosarcoma HT1080 (25) and the human fibroblasts transformed with the ori- mutant of SV40, HFS10 (26), have been described.

DNA sequences

The DNA sequence of the 5' flanking region of the uPA gene was determined using the dideoxynucleotide technique (27) with the reverse M13 primer on double stranded DNA templates (28).

Construction of 5' and internal deletions

All the p-uPA-CAT constructs were obtained digesting with Bal31 the BamH1-linearized DNA of plasmid p-uPA-CAT-2350-inv, containing the 2380 bp SmaI fragment of lambda-uPA-1 phage (nucleotides -2350 to +30) of the human uPA gene (8) cloned in the inverted orientation with respect to the CAT gene in the SmaI site of the polylinker of pEMBL8-CAT vector (16). The in vitro

digested fragments were gel-purified after cleavage with SmaI and re-cloned in the correct orientation, upstream of the CAT gene, using the SmaI site of pEMBL8-CAT vector. Internal deletions were generated with the appropriate restriction enzymes (see Table 2 and the simplified restriction map in Figure 2). Bal31 exonuclease and restriction enzymes were used according to the manufacturer.

DNA transfections

Cells were transfected by the calcium phosphate technique or by the electroporation technique. In both cases, cells were trypsinized 24 hrs before transfection and seeded at a density of 1-3 x 10^5 cells/60 mm dish. Calcium phosphate precipitates (29) were prepared with plasmid DNA at a concentration of 20 µg/ml. Cells were harvested, lysed and analyzed for CAT activity 48-60 hrs after the addition of DNA precipitates.

The electroporation technique was carried out according to Chu et al. (30), with a Bio-Rad Gene Pulser or a BRL Cell Porator apparatus. DNA (50-100 ug) was added to the cell suspension (10^6 cells) in 1 ml HEBS buffer (20 mM Hepes pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH2PO4, 6 mM dextrose). The cell-DNA mixture was subjected to a single electric pulse with a voltage of 230 V and a capacitance of 960 uFarad, at room temperature. Cells were analyzed for CAT activity after 72 hrs. In all cases transient expression experiments were carried out in duplicate. Multiple DNA preparations and/or different calcium-phosphate precipitates were used.

CAT activity was determined as described (31). In most cases 100-200 µg of protein extracts were used in each assay and the reaction allowed to proceed for 1 to 2 hrs. Care was taken to ensure that the assay gave a linear response both with the amount of protein and with the incubation time.

Plasmid pRSV-ß-gal, containing the E. coli beta-galactosidase gene under the control of RSV promoter (32) was used in some experiments as internal standard. In these occasions 2 to 4 µg of DNA was coprecipitated with the uPA-CAT constructs DNA. The beta-galactosidase activity was assayed as described (32).

In several experiments in which the efficiency of transfection was measured, parallel transfection with pSV2CAT or pRSV-CAT were carried out.

DNAs used for transfection were prepared by the alkaline lysis method (33) followed by a double CsCl-ethidium bromide centrifugation.

RNA preparation and S1 nuclease mapping

Total RNA was isolated 48 hours after the addition of DNA to the HT1080 cells transfected by the pUPA-2212-CAT construct, according to Gorman et al.(56). The probe for the localization of the transcription start site of the uPA-CAT hybrid mRNA molecules was a double-stranded ApaI-Eco RI fragment, 337 bp long, 5'end labeled on the Eco RI site and extending from the nucleotide -28 to the nucleotide +309. The S1 nuclease protection protocol was essentially as described by the Maniatis handbook (33). After thermal denaturation, the cellular RNA (30 ug) and the labelled probe ($2x10^5$ cpm) were hybridized for 16 hours at 39 °C or 44 °C, and subsequently subjected to digestion with 200 units of S1 nuclease, for one hour at 16 ° C. The products were analyzed on a denaturing 6% acrylamide-urea sequencing gel.

RESULTS

DNA sequence of the 5' flanking region of the human uPA gene

In order to study the function of the 5' flanking region we have constructed a plasmid in which the bacterial CAT gene is cloned downstream from 2380 bp of human uPA DNA in the pEMBL8-CAT vector (16) (see Methods). The human uPA DNA spans from nucleotides -2350 to +30 taking +1 as the transcription start site. We used exonuclease Bal31 to generate a series of 5' deletions from -2350 to -72 using plasmid p-uPA-CAT-2350 (see Methods). Each deletion mutant was sequenced to identify the end point. The nucleotide sequence of the 2350 bp uPA 5' flanking region, as obtained by the alignment of overlapping deletion endpoints, is shown in Figure 1. The sequence from +1 to -800 had been previously sequenced (8). A correction to

Figure 1. Nucleotide sequence obtained by determining the overlap of the 5' deletions of the 2350 bp of the regulatory region of the human uPA gene. The boxed area indicates the sequence homologous to the Alu consensus. Highlighted sequences are discussed in the text. The arrow shows the site of transcription initiation determined by Riccio et al. (8). Two empty spaces have been inserted at at positions -1875 and -1873, in place of two nucleotides, following to a correction of the sequence.

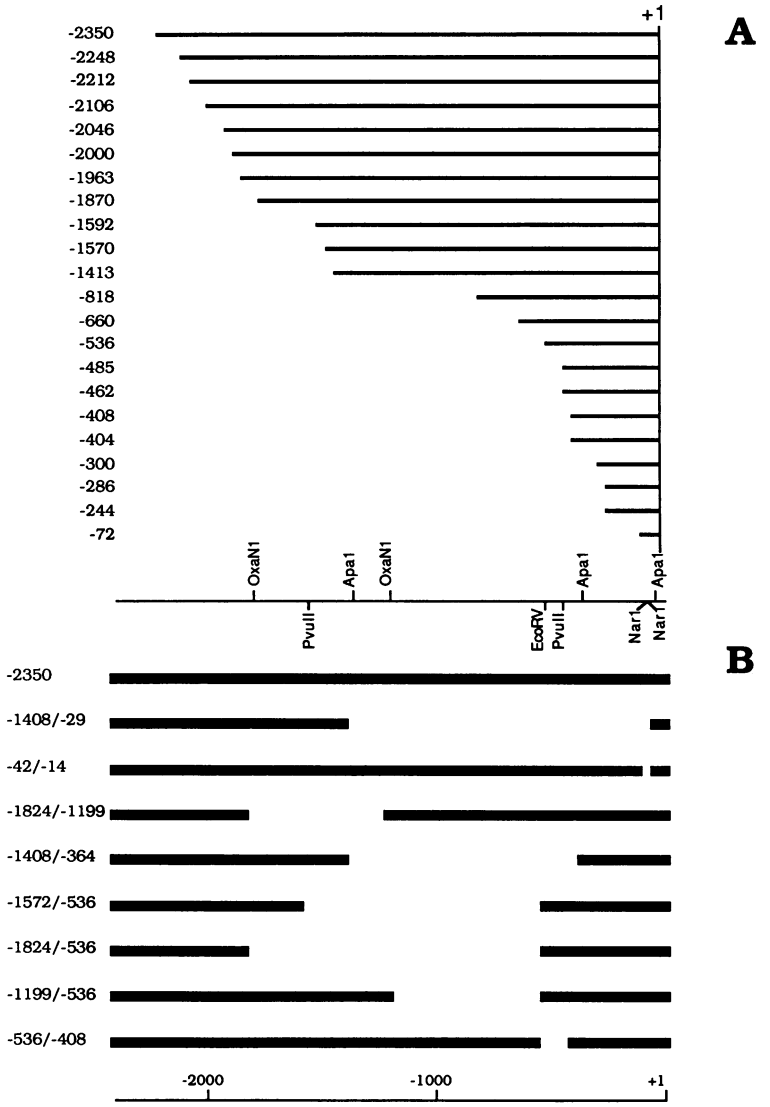


Figure 2. A schematic view of the deletion mutants used in this paper.
A: 5' deletions obtained by Bal31 exonuclease digestion of p-uPA-CAT-2350 (top line). The numbers on the left side indicate the boundary of the deleted material and the bars the length of the retained sequences.
B: internal deletions obtained with different restriction enzymes (see the restriction map on the top). The numbers on the left indicate the boundaries of the deletion. The black bars show the retained portions. The top line depicts the p-uPA-CAT-2350 plasmid constructs used to generate the internal deletions.

that sequence must be noticed, since a 24 bp-long direct repeat upstream of nucleotide -408 reported in Ref. 8 has been proved to be due to a cloning artifact. The following features of the sequence can be derived from the analysis of Figure 1.

a. A region about 75% homologous to the consensus sequence of the Alu family of interspersed repeats (34) extends from nucleotide -2350 to -2144. In particular, a dot matrix analysis (not shown) shows that nucleotides -2350 to -2275 can be aligned with the 3' part of the right monomer of the Alu dimer, while the segment from -2255 to -2156 can be matched to the 5' part.

b. The Alu-like sequence is immediately followed by a stretch of about 130 nucleotides characterized by the presence of repeated motifs (see discussion).

c. Other features include: 1. a directly repeated dodecanucleotide is present between -1870 and -1847. This sequence bears high homology to the binding octanucleotide of the adenovirus 5 EIIaE promoter (35); 2. a 26 bp-long stretch of alternating purines and pyrimidines (potential Z-DNA structure forming sequence) at -1241; 3. a group of five closely spaced putative Sp1-binding sites (three canonical GGGCGG and two atypical GGGAGG boxes) (36,37) between -74 and the TATA box at position -25. Interestingly this region is characterized by the complete absence of T residues between nucleotides -83 and -28; 4. a large number of homologies exists with binding sites for transcription factors. Some of these are discussed below.

Deletion analysis of the 5' flanking region of the human uPA gene

Three different human cell lines have been used as recipients in transfection experiments: the SV40-transformed HFS10 fibroblasts, the HT1080 fibrosarcoma and the epithelial A1251 kidney carcinoma cells. All cell lines produce uPA (38, 39 and our unpublished results).

The deletions relevant to this paper are described in Figure 2. In addition to the 5' deletions generated by Bal31 exonuclease and shown in Figure 2A, a series of internal deletions obtained by restriction endonucleases is shown in Figure 2B. Table 1 shows the results of transient expression experiments performed with the above series of deletions in the three cell lines. Transfections were always carried out at least in duplicate, by both calcium phosphate coprecipitation and electroporation methods with coinciding results. The interexperimental variations have been compensated normalizing the CAT activity data to those obtained with the longest construct, p-uPA-CAT-2212. However, constructs like p-uPA-CAT-2350 essentially behave as the latter (not shown).

The transcription start site of a uPA-CAT fusion gene carrying 2212 bp of 5' flanking sequence (puPA-CAT-2212, see below) has been determined by S1 mapping. As shown in Figure 3, an unique protected band, indicated by the arrow, is present after digestion with S1 nuclease, in addition to the 337 bp long band corresponding to the reannealed double-stranded probe. The size of the new band is in agreement with the expected lenght of 309 nucleotides, based on the 28 base pairs distance between the unlabeled end of the probe and the first nucleotide of the uPA transcript, as determined for the

Table 1. CAT activity# of uPA-CAT fusion genes containing variable portions of the 5' flanking region of the human uPA gene

	Cell line	
5' end of deletion	A1251*	HFS10**
p-uPA-CAT-2212	100	100
p-uPA-CAT-2106	89	46
p-uPA-CAT-2046	37	58
p-uPA-CAT-2000	37	24
p-uPA-CAT-1963	35	6
p-uPA-CAT-1870	12	1
p-uPA-CAT-1592	10	1
p-uPA-CAT-1570	14	ND
p-uPA-CAT-1413	11	ND
p-uPA-CAT-660	15	1
p-uPA-CAT-536	20	1.2
p-uPA-CAT-485	17	ND
p-uPA-CAT-462	17	ND
p-uPA-CAT-408	49	1.8
p-uPA-CAT-404	12	ND
p-uPA-CAT-300	4	0.9
p-uPA-CAT-286	7	1.0
p-uPA-CAT-244	12	1.0
p-uPA-CAT-72	22	1.5
p-uPA-CAT-Nar (-42 to -14)	0.6	0.3
pEMBL8-CAT	0.4	0.1

CAT activity is expressed in percent of substrate converted in 60 minutes by 100 ug extract, normalized to the activity of the longest deletion mutant, p-uPA-CAT-2212 which is arbitrarily set = 100.

* Data represent the average of at least 5 experiments carried out in duplicate, with the exception of constructs p-uPA-CAT-536, -300, -286 and -244 (average of 3 experiments), of p-uPA-CAT-2106 (2 experiments) and p-uPA-CAT-1570 and -1413 (1 experiment).

**Data represent the average of 2 experiments with a variation of 10% or less. In several other experiments in which not all constructions were used, the same pattern of expression has been consistently confirmed. ND = Not Done.

endogenous gene. Therefore, we conclude that the same transcription start site of the uPA gene is utilized in the uPA-CAT constructs.

The results of the transient expression experiments (Table 1) indicate that distal sequences are required for uPA expression in both A1251 and HFS10 cells, since their removal drastically reduces CAT activity. A more obvious quantitative difference exists between A1251 and HFS10 cells: in fact, while in A1251 cells the constructs with more distal end-points display a promoter activity 5 to 10 fold higher than the other constructions, this difference is between 50 and 100 fold in HFS10 cells

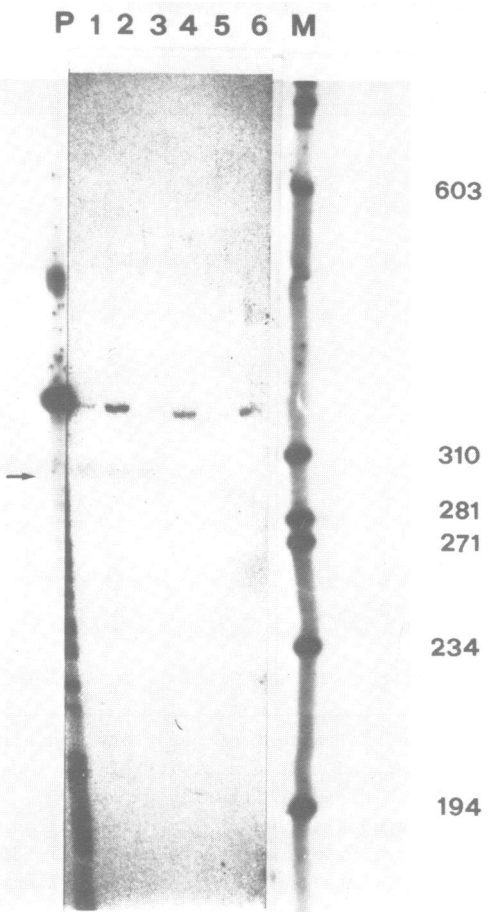


Figure 3. Identification of the transcription start site of the uPA-CAT hybrid gene by S1 nuclease mapping. Total cytoplasmic RNA (10-30 µg) was hybridized to the uPA-CAT, 337 bp long, 5'end-labelled probe extending from the nucleotide -28 to the nucleotide +309. The hybrids were treated for 1 h at 16 C.

Lane P: uPA-CAT probe without RNA and S1 nuclease digestion; lane 1: uPA-CAT probe and 30 µg of total cytoplasmic RNA from the HT1080 cells, transfected by the p-uPA-CAT-2212 construct and hybridized at 44°C; lane 2: the same amount of probe and RNA, hybridized at 39°C; lane 3: uPA-CAT probe and 10 ug of the same RNA, hybridized at 44°C; lane 4: the same amount of probe and RNA, hybridized at 39 °C; lane 5: uPA-CAT probe and 30 µg of HT1080 polyA RNA, hybridized at 44 °C; lane 6: the same amount of probe and RNA, hybridized at 39 °C; lane M: ØX 174 DNA digested with Hae III.

(compare p-uPA-CAT-2212 with p-uPA-CAT-72). Although the transfection efficiency of the two cell lines was not directly tested in the experiments of Table 1, in several other cases we have directly verified that a different efficiency of transfection does not explain this difference. Overall, A1251 and HFS10 cells can be transfected with about the same efficiency (data not shown). Since the absolute CAT activity values of the longer constructs did not vary by more than two fold in the two cell lines, the differences observed in constructions carrying 1870 bp or less of 5' flanking region likely indicate the presence of A1251-specific positive acting sequences.

In some experiments, constructs puPA-2212-CAT, puPA-408-CAT and puPA-72-CAT were cotransfected with pRSV-ß-gal in A1251 cells and the CAT activity normalized to beta-galactosidase activity. The results showed that the ratios between normalized activities of puPA-CAT constructs remained the same as in the experiment of Table 1 (data not shown).

The location of the sequences required for high level expression varies somewhat in the two cell lines. The boundary might be set upstream from -1870 in A1251 cells, or from -1963 in HFS10 cells.

Transfection of some deletions into HT1080 cells give results similar to A1251 cells rather than to HFS10 cells (not shown).

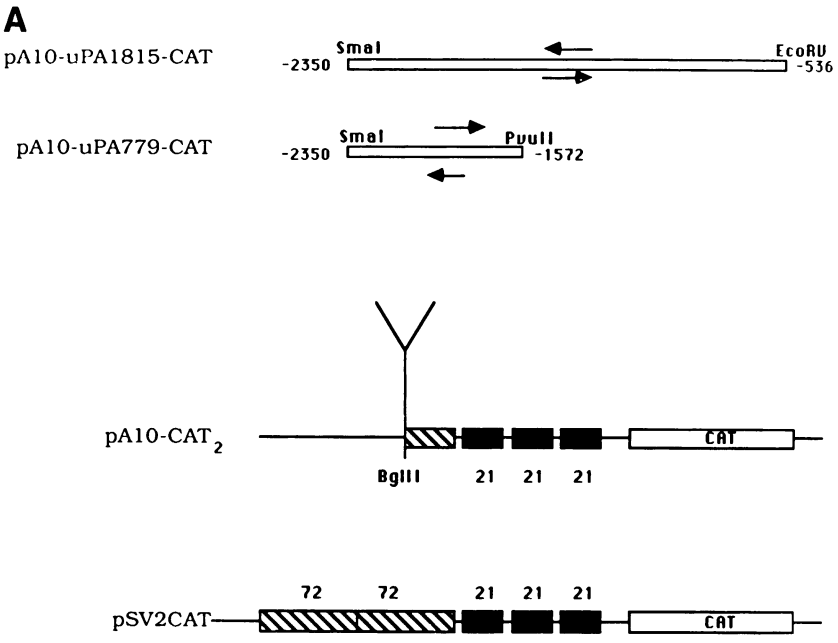
Transfection of p-uPA-CAT-Nar, an internal deletion removing from -42 to -14, and thus the TATA box, totally abolishes the activity. Thus the hybrid genes do require the physiological (8) TATA box.

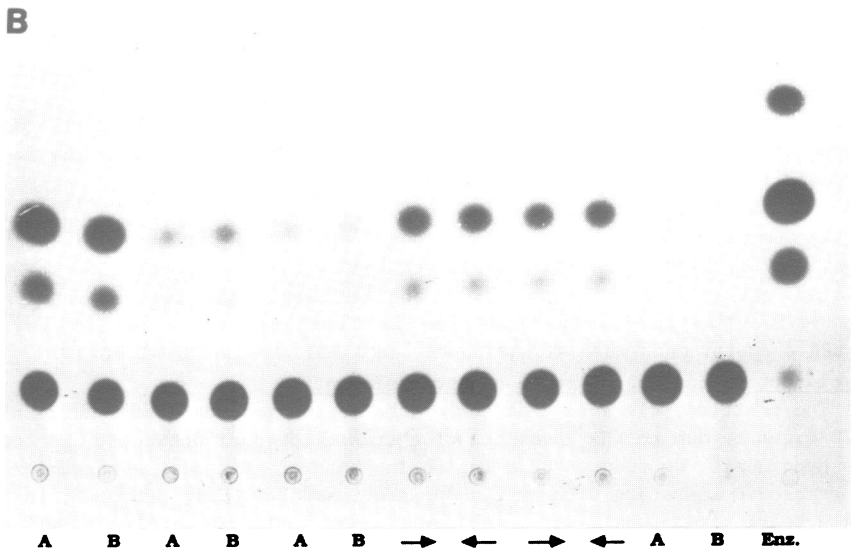
As shown in Table 1, all 5' deletions including a construct with only 72 bp upstream of the transcriptional start site, give rise to a level of CAT activity detectably above the background of the promoterless pEMBL8-CAT vector. This level does not change much in the group of constructions carrying less than 1870 bp. The major exception to this rule is the construct p-uPA-CAT-408 which shows a peak of activity which is more pronounced in A1251 than in HFS10 cells. The relative differences in these two cell lines result in a different profile of CAT activity vs. distance from the transcriptional start site.

In summary, the above experiments show that the uPA promoter is active in both A1251, HT1080 and HFS10 cells and that a cis-acting positive region (more evident in HFS10 than in HT1080 and A1251 cells) is present upstream from -1870. The region downstream from -1870 contributes to the activity of the uPA promoter much more significantly in A1251 than in HFS10 cells.

The cis-activating region is an enhancer element

The positive, cis-acting effect of the region upstream from nucleotide -1870 might be due to the presence of a transcriptional enhancer. To test this hypothesis, we cloned this region in both orientations upstream from the transcription start site of the enhancerless SV40 early promoter, in the single BglII site of pA10-CAT-2 (40) or in the SmaI site of the polylinker of pUC19-CAT (16). Two restriction fragments were used: the 779 bp SmaI-PvuII (-2350 to -1572) and the 1815 bp SmaI-EcoRV fragment (-2350 to -536) (Figure 4A). The two fragments have the same 5' end but extend for different lengths in the 3' direction.





pSV2-CAT	pSV2-CAT 1/20	pA10-CAT$_2$	pA10-CAT uPA-779		pA10-CAT uPA-1815		pEMBL8 CAT
34.5%	2.2%	1.1%	8.5%	10.5%	9.4%	9.7%	0.22%

The constructions containing the upstream activating region of the uPA gene and the control plasmids pA10-CAT2 and pSV2-CAT, which has the wild type SV40 early promoter and enhancer (41), were transfected into HFS10 cells and CAT activity assayed. As shown in Figure 4B, pSV2-CAT promoter activity is at least 20 fold higher than that of pA10-CAT2. The fragments derived from the 5' flanking region of the uPA gene stimulate about tenfold the enhancerless SV40 promoter, independently of the orientation. The extent of stimulation is about the same for the 779 and the 1815 bp fragments.

Similar data were obtained with HT1080 and A1251 cells (not shown). Thus an enhancer is present in the 5' flanking region of the human uPA gene, 5' from -1572. The results obtained with the 5' deletions (Table 1) suggest that the enhancer may be localized upstream from -1870. In a series of experiments carried out with the 525 bp SmaI-OxaNI fragment (-2350 to -1824) cloned upstream of the enhancerless SV40 promoter in pUC19-CAT, we have further defined the 3' limit of the enhancer to -1824. With this last fragment the enhancement of transcription from the SV40 promoter was about 5 fold more pronounced than with the 779 SmaI-PvuII fragment (data not shown). This difference might be compatible with the presence of cis-acting negative regulatory sequences between -1824 and -1572 (see below).

A negative cis-acting region downstream from the enhancer element

The presence of further cis-acting sequences functionally involved in the regulation of transcription from the uPA promoter was also explored using the internal deletions shown in Figure 2B. All plasmids shown in the Figure were derived from the longest uPA-CAT construction, p-uPA-CAT-2350. Several internal deletions were generated between nucleotides -1824 and -14, using the restriction sites indicated in Figure 2B, leaving intact the cis-activating region upstream of -1870.

The results of the transient expression experiments in A1251 cells are shown in Table 2. An intact TATA box is still present in all constructs of

Figure 4. Enhancer activity of fragments of the 5' flanking region of the human uPA gene. A: schematic drawing of the constructs used. pSV2CAT (38) contains both the 21 bp and 72 bp repcats of the early promoter and enhancer of SV40. Most of 72 bp repeats of the SV40 enhancer are removed in the pA10-CAT2 plasmid (37). The two top drawings indicate the fragments of human uPA 5' flanking region, with their coordinates and the restriction enzymes used, that have been cloned in the single BglII site of pA10-CAT2 and tested for enhancer function. B: CAT assay on extracts of HFS-10 cells transfected with the indicated DNA constructs by the calcium phosphate co-precipitation technique. The number in the bottom rectangles represent the relative CAT activity (in percent of converted substrate). A and B represent the activity obtained with duplicate DNA precipitates. Arrows indicate the orientation of uPA fragments cloned in pA10-CAT2. Lane labeled pSV2-CAT 1/20 represents a CAT assay carried out with 1/20 of the extract used in lane pSV2-CAT. Lane labeled pEMBL8-CAT represents the activity of a promoter-less vector DNA. Lane labeled Enz represents the CAT activity of a commercial preparation of chloramphenicol acetyl transferase.

Table 2. CAT activity of internal deletions of the 5' flanking region of the human uPA gene transfected into A1251 cells

Construct	Deletion end points	Size in bp	Relative CAT activity
p-uPA-CAT-2350	-	-	100 (5)
p-uPA-CAT-Apa	-1408/-29	1379	26 (4)
p-uPA-CAT-Nar	-42/-14	28	1 (2)
p-uPA-CAT-Oxa	-1824/-1199	625	271 (4)
p-uPA-CAT-Apa2	-1408/-364	1044	87 (4)
p-uPA-CAT-PvuRV	-1572/-536	1036	75 (4)
p-uPA-CAT-OxaRV	-1824/-536	1288	280 (3)
p-uPA-CAT-OxaRV2	-1199/-536	663	136 (4)
p-uPA-CAT-RVPvu	-536/-408	128	88 (4)

The results are expressed in percent of CAT activity of the full-size construct (p-uPA-CAT-2350) and represent the average of several experiments conducted in duplicate with less than 15% variation. The numbers in parenthesis represent the number of times the particular construct was tested. The constructs are named by the restriction enzyme used to cause the internal deletion.

this series except in p-uPA-CAT-Nar in which nucleotides -42 to -14 have been deleted. Again, this construct has essentially no activity. Deletion of the putative Sp1 binding sites in the construction p-uPA-CAT-Apa (deleted from -1408 to -29), reduces the promoter activity without abolishing it. In addition, the simple shortening of the distance between the upstream enhancer and the promoter has no significant effect, as shown by the activity of the deletions p-uPA-CAT-Apa2 (-1408 to -364) p-uPA-CAT-PvuRV (-1572 to -536), p-uPA-CAT-OxaRV2 (-1199 to -536) and p-uPA-CAT-RVPvu (-536 to -408). On the contrary, the two constructions p-uPA-CAT-Oxa and p-uPA-CAT-OxaRV, corresponding to the two overlapping deletions -1824 to -1199 and -1824 to -536, cause a 3 fold increase in uPA promoter activity. These data suggest that in these two deletions a negative cis-acting region has been removed. Since the deletion p-uPA-CAT-PvuRV (-1572 to -536) has no effect on the uPA-CAT activity, the negative cis-acting region must be located between -1824 and -1572. Similar results have been obtained in transient expression experiments with HFS10 and HT1080 cells (not shown).

DISCUSSION

The question of which cells physiologically express the uPA gene is still an open question.

Immunocytochemical studies have shown that very few cells contain uPA in intact mice and that this is a property of both fibroblast-like and epithelial-like cells (42). On the other hand, neoplastic transformation *in vivo* and *in vitro* is associated with an increase in uPA production (reviewed in Ref. 6).

We have chosen human uPA-producing transformed cells of both fibroblast and epithelial origin to dissect the DNA sequences in the 5' end of the human uPA gene. The data we report represent the first analysis of the uPA promoter.

Analysis of the nucleotide sequence of Figure 1 shows that the uPA promoter does not have a typical CAAT box and suggests that its basic elements be the TATA box and the SP1 binding sites. We have shown that the TATA box is an essential element of the uPA promoter since a 28 bp deletion (p-uPA-CAT-Nar) removing one Sp1 binding site and the TATA box, completely abolishes promoting activity (Table 2). This data also demonstrates that CAT expression from the transfected p-uPA-CAT constructs requires the physiological transcription start site, i.e. that of the endogenous uPA gene (8). S1 analysis confirmed this prediction (Figure 3).

Binding of Sp1 transcription factor to GGGCGG motifs (36) plays a relevant but not essential role in the expression of the uPA promoter. In fact, the construct p-uPA-CAT-Apa (-1408 to -29), in which all putative Sp1 binding sites have been removed, has a drastically reduced but not totally abolished CAT activity. Analysis of the DNA sequence generated by the above deletion shows that a sequence GGGCCC is generated at the junction (not shown). We cannot exclude that the residual promoter activity of the construct depends on this anomalous Sp1-like sequence.

Deletion analysis of the uPA 5' flanking region shows that about 2100 bp are required for highest expression of the uPA-CAT hybrids in both types of uPA-producing cells. At least two cis-acting regions affect basal uPA expression: an enhancer and a negatively acting region.

The upstream enhancer

The upstream activating region has the features of a transcriptional enhancer. Three different yet overlapping fragments (extending from nucleotides -2350 to -536, -2350 to -1572 or -2350 to -1824) were able to stimulate transcription from the enhancerless SV40 promoter in an orientation-independent way in HFS10 (Figure 4), HT1080 and A1251 cells (not shown). Thus at least in these cells the sequences downstream from -1824 are not required for enhancing activity. The ten fold stimulation of transcription from the SV40 promoter obtained with the two longer fragments (Figure 4B) is lower than that observed with the 525 bp fragment, or than the effect that the same DNA region exerts on its own promoter (Table 1). This is in agreement with the presence on the two longer fragments of a negative cis-acting element, located by the internal deletion analysis within -1824 and -1572 (see below).

Analysis of the DNA sequence (Figure 1) and the results obtained with the 5' deletions (Table 1) allow a broad definition of the upstream enhancer acting in HFS10 and A1251 cells. The 210 bp region matching the Alu consensus sequence is likely to play no enhancing function since its removal has little or no influence (data not shown). The enhancer may, therefore, start downstream from the Alu sequence at nucleotide -2140. The results with the 5' deletions in A1251 cells show that the -1870 and shorter constructs exhibit similar low levels of CAT activity. We therefore

tentatively position the enhancer between -2140 and -1870. Additional experiments will be needed to determine the precise boundaries. In HFS10 cells, however, the 3' limit of the enhancer appears to be around -1963.

An A1251-specific element of the enhancer, therefore, may be located between -1963 and -1870.

Many cellular and viral enhancers display a characteristic modular structure with closely spaced motifs (14). The sequence analysis of the uPA 5' flanking region has revealed a similar structural organization (Figure 1). The 80 bp region between -2133 and -2053 contains two alternatively repeated octameric motifs, box A ($^{A}/_{G}$AAGAG$^{T}/_{G}{}^{T}/_{A}$) (underlined in Figure 1) and box B (GAAGT$^{T}/_{G}$CT) (overlined in Figure 1). The latter contains the sequence (GAAGTG) which has been shown to bind the HeLa cells factor E4-TF1 (43). The spacing between corresponding nucleotides in each box A is 19, 20 and 31 nucleotides; in the case of box B, the spacing is of 30 or 18 nucleotides (see Figure 1). In addition, the sequence TGGATA, matching the core consensus of several viral and cellular enhancers (44) and which has protein binding activity (14), is present in the 14 bp stretch separating the third and fourth repeat.

Immediately downstream from this region, starting at nucleotide -2049, a $^{G}/_{A}$GGGG$^{A}/_{C}{}^{G}/_{A}$A sequence (Figure 1, highlighted by a dashed line on the top), is repeated three times. It is interesting to notice that this sequence closely resembles the AP-2 binding site (45). In A1251 cells the partial or complete removal of this entire structurally characteristic region until nucleotide -1963 (see Table 1) only reduces the transcriptional activity (Table 1), complete inactivation being reached only in the uPA-CAT-1870 construct. In HFS10 cells, however, the effect of removal of the region upstream from -1963 is much more pronounced. Thus, the region from -1963 to -1870 might have an important role in HFS10 cells.

Sub-elements of SV40 enhancer have been assigned different cell-specific functions (46). It is possible that when more cell types will have been examined, a similar and possibly higher complexity will be revealed in the uPA enhancer.

The upstream negative element

In the presence of the enhancer, removal of sequences -1824 and -1572 of the uPA 5' flanking region results in increased expression of the uPA-CAT hybrid DNA in A1251 (Table 2), HT1080 and HFS10 cells. Moreover, the enhancing activity of the region assayed with the enhancerless SV40 promoter is increased about 10 fold by the removal of the region between -1824 and -1572. These two sets of results strongly suggest the presence of a cis-acting negative region.

The increase in CAT activity is not a consequence of the shortening of the distance between the enhancer and the promoter. In fact, CAT activity of the internal deletions in which the segments between -1408 and -364, -1572 and -536, -1199 and -536, -536 and -408 were removed (Table 2), is not significantly affected.

Enhancerless 5' deletions show about the same CAT activity (with the exception of p-uPA-CAT-408 discussed below), independently of the presence of the negative region. Therefore, it seems possible that this region

reduces the efficiency of the upstream enhancer rather than block the function of the downstream promoter element.

The presence of a negative control region in the uPA gene explains previous observations that on the whole suggested the existence of one or more negative regulatory factors. First, cycloheximide treatment increases the level of uPA mRNA in several normal and transformed cells (9,12), an effect due at least in part to an increase in transcription of the uPA gene (47). Second, in LLC-PK1 cells, in which uPA production is dependent on calcitonin addition (48), recessive calcitonin-independent mutants in uPA expression have been isolated (49).

Conclusions

We have demonstrated the existence of at least two upstream regulatory regions of the human uPA gene: one enhancer and one cis-acting negative region. Both appear to affect the basal level of uPA gene expression at least in two cell lines. It is important to realize that the "basal level" of uPA gene expression may not be really basal, since it may be influenced in A1251 cells by the autocrine secretion of growth factors (50) and in HFS10 cells by the presence of SV40 T antigen (26). Therefore, the uPA regulatory region might be the target site for the action of inducing growth factors or T antigen. Further work is required to elucidate this point.

On the whole, the structure of the uPA regulatory region suggests that, like in other enhancers, regulation might involve multiple cis-acting sequences and different trans-acting factors in different cells (13).

Similarly to other enhancers, which contain both positive and negative elements (51,52), phorbol ester responsive elements (53), or other hormone responsive elements (13), the uPA enhancer might possibly extend on the 3' end well beyond nucleotide -1870 to include the negative region and more downstream sequences. In fact, we find that a long stretch of 5' flanking region, from -1572 to -72, contributes specifically to uPA-CAT expression in the kidney- derived A1251 cells, but not in the HFS10 fibroblasts. The specificity is shown by the absolute requirement of the uPA TATA box (Tables 1,2). Thus in A1251 cells the uPA gene activity depends at least in part on sequences located 3' of the negative region. Within this region the construct p-uPA-CAT-408 has a pronounced peak of activity in A1251 cells (Table 1). Since this peak is confined to one single construction, it may represent the result of a cloning artifact, in which a promoter sequence has been generated by the junction of uPA and vector sequences. An alternative possibility, however, is that this region represents a cell specific controlling element of the uPA promoter. While the available data do not yet support this conclusion (see for example the data on internal deletions of Table 2), it is worth noting that the sequence (-417 CTCCATCAGCTGCGG-403) bears a noticeable homology (underlined residues) with a cyclic AMP and phorbol esters responsive element of human enkefalin gene (-93 CTGCGTCAGCTGCAG) (54), and contains the AP-4 binding site (55). Thus, uPA-CAT-408 cuts right in the middle of a potentially significant region.

We have thus started to uncover the complex functions of the uPA promoter elements. We feel that further studies will reveal that most if not all sequences downstream from the negative element possess functions in

cell-specific expression and/or contain elements responsive to transcriptional stimulation by regulatory factors like phorbol esters, cyclic AMP, growth factors, etc.

ACKNOWLEDGEMENTS

F.B., P.V. and A.F. are particularly grateful to Robert F. Goldberger and Max Gottesman for their hospitality in the stimulating atmosphere of the Institute for Cancer Research, Columbia University, New York. FB was a recipient of the Ludwig Schaeffer award during his stay at Columbia. PV was supported by the PF Oncologia, Italy-National Cancer Institute, USA joint program. The authors are also grateful to Bruce Howard and Riccardo Cortese for providing plasmid vectors and stimulating discussions. This work has been supported by the Consiglio Nazionale delle Ricerche, Italy, PF Ingegneria Genetica and PF Oncologia; the Danish Cancer Society, the Danish Medical Research Council and the Danish Biotechnology Center for Molecular Biology of Mammalian Cells.

*To whom correspondence should be addressed at: Mikrobiologisk Institut, Oster Farimagsgade 2A, 1353 Copenhagen K, Denmark

REFERENCES

1. Blasi, F., Vassalli, J.D. and Dano, K. (1987) J. Cell Biol. 104, 801-804.
2. Ossowski, L. and Reich, E. (1983) Cell 35, 611-619.
3. Mignatti, P., Robbins, E. and Rifkin, D.B. (1986) Cell 47, 487-498.
4. Hearing, V.J., Law, L.W., Corti, A., Appella, E. and Blasi, F. (1988) Cancer Research, in press.
5. Reich, E. (1978) In Berlin, R.D., Hermann, H., Lepow, I.H. and Tanzer, J.M. (eds), Molecular Basis of Biological Degradative Processes, Academic Press, New York, pp.155-169.
6. Dano, K., Andreasen, P.A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L.S. and Skriver, L. (1985) Adv. Cancer Res. 44, 139-266.
7. Verde, P., Stoppelli, M.P., Galeffi, P., Di Nocera, P.P. and Blasi, F. (1984) Proc. Natl. Acad. Sci. USA 81, 4727-4331.
8. Riccio, A., Grimaldi, G., Verde, P., Sebastio, G., Boast, S. and Blasi, F. (1987) Nucl. Acids Res. 13, 2759-2771.
9. Grimaldi, G., Di Fiore, P., Kajtaniak, E.L., Falco, J. and Blasi, F. (1986) EMBO J. 5, 855-861.
10. Wigler, M. and Weinstein, I.B. (1976) Nature 259, 232-233.
11. Belin, J.D., Godeau, F. and Vassalli, J.D. (1984) EMBO J. 3, 1901-1906.
12. Stoppelli, M.P., Verde, P., Grimaldi, G., Locatelli, E.K. and Blasi, F. (1986) J. Cell Biol. 102, 1235-1241.
13. Maniatis, T., Goodbourn, S. and Fischer, J.A. (1987) Science 236, 1237-1245.
14. Jones, N.C., Rigby, P.W.J. and Ziff, E.B. (1988) Genes and Devel. 2, 267-281.

15. Brand, A.H., Breeden, L., Abraham, J., Sternglanz, R. and Nasmyth, K. (1985) Cell 41, 41-48.
16. Colantuoni, V., Pirozzi, A., Blance, C. and Cortese, R. (1987) EMBO J. 6, 631-636.
17. Reed-Larsen, P., Harmey, J.W. and Moore, D.D. (1986) Proc. Natl. Acad. Sci. USA 83, 8283-8287.
18. Remmers, E.F., Yang, Y.Q. and Marcu, K.B. (1986) EMBO J. 5, 899-904.
19. Sassone-Corsi, P. and Verma, I.M. (1987) Nature 326, 507-510.
20. Goodbourn, S., Burnstein, H. and Maniatis, T. (1986) Cell 45, 601-610.
21. Zinn, K. and Maniatis, T. (1986) Cell 45, 611-618.
22. Shore, D., Stillman, D.J., Brand, A.H. and Nasmyth, K. (1987) EMBO J. 6, 461-467.
23. Shore, D. and Nasmyth, K. (1987) Cell 51, 721-731.
24. Rosen, S.W., Weintraub, B.D. and Aaronson, S.A. (1980) J. Clin. Endocr. and Metab. 50, 834-841.
25. Lund, L.R., Riccio, A., Andreasen, P.A., Nielsen, L.S., Kristensen, P., Laiho, M., Saksela, O., Blasi, F. and Dano, K. (1987) EMBO J. 6, 1281-1286.
26. Boast, S., La Mantia, G., Lania, L. and Blasi, F. (1983) 2, 2327-2331.
27. Sanger, F., Nicklen, S. and Coulson, A. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
28. Hattori, M. and Sakaki, Y. (1986) Anal. Biochem. 152, 232-238.
29. Graham, F.L. and Van der Eb, A.J. (1973) Virology 52, 456-467.
30. Chu, G., Hayakawa, H. and Berg, P. (1987) Nucl. Acids Res. 15, 1311-1327.
31. Gorman, C.M., Moffat, L.T. and Howard, B.H. (1982) Molec. Cell. Biol. 2, 1044-1054.
32. Edlund, T., Walker, M.D., Barr, P.J. and Rutter, W.J. (1985) Science 230, 912-916.
33. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York.
34. Kariya, Y., Kato, K., Hayashizaki, Y., Himeno, S., Tarui, S. and Matsubara, K. (1987) Gene 53, 1-10.
35. Jalinot, P., Devaux, B. and Kedinger, C. (1987) Molec. Cell. Biol. 7, 3806-3817.
36. Kadonaga, J.T., Jones, K.A. and Tjian, R. (1986) Trends in Biochem. Sci. 11, 20-23.
37. Jones, K.A., Kadonaga, J.T., Lucius, P.A. and Tjian, R. (1986) Science 232, 755-759.
38. Ferraiuolo, R., Stoppelli, M.P., Verde, P., Bullock, S., Lazzaro, P., Blasi, F. and Pietropaolo, C.T. (1984) J. Cell Phys. 121, 368-374.
39. Medcalf, R.L., Richard, R.I., Crawford, R.J. and Hamilton, J.H. (1986) EMBO J. 5, 2217-2222.
40. Laimins, L., Gruss, P., Pozzatti, R. and Khoury, G. (1984) J. Virol. 49, 183-189.
41. Howard, B.H. and McCormick, M. (1985) In Gottesman, M. (ed), Molecular Cell Genetics, J. Wiley & Sons, New York, pp.212-233.
42. Larsson, L.I., Skriver, L., Nielsen, L.S., Grondahl-Hansen, J.,

Kristensen,P. and Dano, K. (1984) J. Cell Biol. 98, 894-903.
43. Lee, K.A.W. and Green, M.R. (1987) EMBO J. 6, 1345-1353.
44. Sassone-Corsi, P. and Borrelli, E. (1986) Trends Genet. 2, 215-219.
45. Masayoshi, I., Chiu, R. and Karin, M. (1987) Cell 51, 251-260.
46. Ondek, B., Shepard, A. and Herr, W. (1987) EMBO J. 6 1017-1025.
47. Altus, M.S., Pearson, D., Horiuchi, A. and Nagamine, Y. (1987) Biochem. J. 242, 387-392.
48. Nagamine, Y., Sudol, M. and Reich, E. (1983) Cell 32, 1181-1190.
49. Hofstetter, P., Kikinis, Z., Altus, M.S., Pearson, D. and Nagamine, Y. (1987) Molec. Cell. Biol. 7, 4535-4541.
50. Blasi, F. and Grimaldi, G. (1986) In Baserga, R., Foa, P., Metcalf, D. and Polli, E.E. (eds), Biological Regulation of Cell Proliferation, Serono Symp. Publ., Raven Press, Vol.34, pp.227-233.
51. Kadesh, T., Zera, P. and Ruezinsky, D. (1986) Nucl. Acids Res. 14, 8209-8221.
52. Wasylyk, C. and Wasylyk, B. (1986) EMBO J. 5, 553-560.
53. Imbra, R.J. and Karin, M. (1986) Nature 323, 555-558.
54. Comb, M., Birnberg, N.C., Seasholtz, A., Herbert, E. and Goodman, H.M. (1986) Nature 323, 353-356.
55. Mermod, N., Williams, T.J., and Tjian, R. (1988) Nature 332, 557-561.
56. Gorman, C.M., Merlino, G.T., Willingham, M.C., Pastan, I. and Howard, B. (1982) Proc. Natl. Acad. Sci. USA 79, 6777-6781.