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Multiple nuclear factors interact with promoter sequences of the urokinase-type plasminogen activator gene

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ABSTRACT

To characterize proteins that bind to the cyclic AMP inducible promoter of the urokinase-type plasminogen activator gene, we performed a DNAase I footprinting analysis. Within 500 nucleotides upstream of the transcription start site we found eight protected regions due to at least four different binding proteins. Among these is a single binding site for the transcription factor CTF/NF1, which is flanked on each side by two conserved binding sites for the transcription factor Sp1. A region at -380, which shares a similarity with sequences observed in the corresponding regions of other cyclic AMP regulated genes, was protected. This binding site contains a sequence of ten nucleotides which is repeated further upstream at -480 and also protected against DNAase I digestion. Comparisons of extracts from four different cell lines revealed that all DNA binding factors are present in nuclei of uPA expressing and nonexpressing cells. Mechanism underlying hormonal regulation of the gene is discussed.

INTRODUCTION

Plasminogen activators (PAs) are specific serine proteases that nick the zymogen plasminogen and convert it to plasmin which is also a serine protease but with a broad substrate specificity. There are two types of plasminogen activators, which are the products of distinct genes with different amino acid sequences: the urokinase-type (uPA) and the tissue-type (tPA) (10). PA secretion appears to be a general mechanism used by cells for inducing localized extracellular proteolysis. Plasminogen activators play important roles in fibrinolysis (1,2), inflammation (3) and tissue remodeling during development (4). PA activity has been found to be regulated in a cell type dependent fashion by many extracellular signals such as polypeptide hormones, sex or adrenocortical steroids (5,6) and tumor promoters (7). Many cancer cells express PAs at higher levels compared to their normal counterparts and a requirement for PA in metastasis has been demonstrated (8-10).

In the pig kidney epithelial cell line, LLC-PK1 (11), uPA activity is increased by the hormones calcitonin and vasopressin (11). In this cell line,

basal uPA production is very low, but may be stimulated at least 100-fold by the addition of calcitonin or vasopressin (12). cAMP is the intracellular second messenger for both hormones. This regulation has been shown to occur at the level of uPA transcription (11,13,14).

To investigate cis- and trans-acting elements involved in the basal and hormonal regulation of the uPA gene, we cloned the pig uPA gene (12,15) and developed a homologous cell free transcription system from LLC-PK1 cells (16). We demonstrated accurate initiation of transcription using a cloned fragment of the uPA gene as template, and the addition of cAMP stimulated the transcription rate up to 10-fold. Furthermore, we could demonstrate the involvement of the catalytic subunit of cAMP-dependent protein kinase (cAMP-PK) in transcriptional regulation of the uPA gene, indicating the involvement of protein phosphorylation in the uPA gene regulation by hormones. To localize the cis-acting element(s) for the cAMP regulation in vitro, we compared templates with progressive deletions at the 5'-end. Using a template containing 1300 nucleotides upstream of the transcription start site, we found that stimulation of transcription by cAMP was 5-8 fold. Further deletion to -315 and -148 still maintained the cAMP effect by 3-fold enhancement whereas deletion to -53 resulted in no correct initiation of transcription, regardless of the presence of cAMP (16).

These results suggested that in the 5'-flanking region of the uPA gene there are cis-acting sequences involved in the hormonal and basal regulation. We show here the interaction of at least four different factors with sequences localized within 500 nucleotides upstream of the transcription start site of the uPA gene. These factors are present in nuclear extracts of three other cell lines regardless of whether or not uPA gene expression is regulated by hormones in these cells.

METHODS

Plasmids and probes

The plasmid pYNg1 was constructed by isolating a 7.8 kb fragment containing 1.3 kb of the 5'-flanking region, all of the 6 kb of the transcribed region, and 0.5 kb of the 3'-flanking region from the pig genomic clone λYN4 (15), and inserting them into the EcoRI and NruI site of pBR 322. pYNg1CAT0 was constructed by replacing the XbaI fragment (nucleotides 426-5500) of pYNg1 with the bacterial Chloramphenicol acetyltansferase gene. From this plasmid a variety of Bal 31 deletions were generated, starting at the unique ClaI site (Figure 1). The SmaI-ClaI fragment was blunt ended and subcloned into the SmaI site of pUC 18. The pig actin cDNA probe was obtained as described (17).

Cell culture

Pig kidney cells LLC-PK1 (11), HT-1080 (46,47), HeLa cells and FTO-2B cells (18) were grown in monolayer cultures in Dulbecco's modified Eagle's medium (Gibco), supplemented with 10% (v/v) fetal calf serum, 0.2 mg/ml streptomycin and 50 U/ml penicillin in a humidified incubator at 37°C using a 94% air, 6% CO_2 atmosphere.

Nuclear extract

Nuclear extracts were prepared according to Dignam (19), except that with slight modifications for LLC-PK1 extract. The lysing buffer for LLC-PK1 cells contained 1.0 M NaCl. Following ammonium sulfate precipitation (45% saturation), the proteins were resuspended in a dialysis buffer containing 20 mM Hepes, pH 7.9, 100 mM KCl, 0.1 mM EDTA, 17% glycerol, 2 mM DTT, 6 mM $MgCl_2$ and 0.1 mM PMSF. After dialysis the extracts were centrifuged and clear supernatants were obtained. Extracts contained typically between 10 and 15 mg protein/ml.

Heparin agarose column fractionation of LLC-PK1 nuclear extract

Approximately 30 mg of nuclear extracts from LLC-PK1 cells were loaded on a 6 ml heparin agarose (Pharmacia) column preequilibrated with dialysis buffer (above). Bound proteins were eluted with a stepwise KCl gradient of 0.1 to 0.8 M KCl in dialysis buffer. The fraction containing footprint VII-binding activity was further characterized by gel retardation assay.

DNAase I protection assay

Sequencing reactions, 3'-labeling by E. coli DNA polymerase I, Klenow fragment, and 5'-labeling by T4-Polynucleotide Kinase were performed according to standard procedures (20,21) with (α-^{32}P)dATP or (γ-^{32}P)ATP (3000 Ci/mmol; Amersham).

Binding reactions were carried out in a total volume of 20 μl containing 20 mM Hepes, pH 7.9, 17% glycerol (v/v), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 2-4 μg poly(dI-dC) (dI-dC) (Pharmacia) and 1-5 ng o the one endlabeled fragments (10-100 fmol; 20-40 000 cpm). The assay mixture was incubated at 25°C for 30 min. Then a volume of 5 μl containing 12.5 mM $CaCl_2$, 20 mM $MgCl_2$ and depending on the amount of extract, 0.4-4.0 μg/ml DNAase I (Worthington) was added to each sample. The mixture was incubated at 25°C for 2 min and the digestion was stopped by addition of stop-buffer containing 0.25% SDS, 32 mM EGTA and 0.5 mg/ml proteinase K. After incubation at 42°C for 1 hour the DNA was immediately phenol-chloroform (1:1) extracted, ethanol precipitated and loaded onto a sequencing gel.

Gel retardation assay

The binding reaction was set up as described above. The amount of nuclear extract proteins are detailed in Figure 2. Following binding, the mixture was electrophoresed through a native 5% polyacrylamide gel (acrylamide:bisacrylamide ratio 30:1), containing 6.7 mM Tris-HCl (pH 7.5), 3.7 mM sodium acetate, and 1 mM EDTA. The gel was pre-electrophoresed for 2 h at 10 V/cm at room temperature with recirculating buffer. The gel was dried and autoradiographed.

Competition experiments with fragments and double stranded oligonucleotides

Two complementary oligonucleotides, chemically synthesized were annealed in 50 mM NaCl, 66 mM Tris-HCl, pH 7.5 and 6.6 mM MgCl by incubation for 10 min at 80°C, 10 min at 60°C, 10 min at 37°C and 10 min at room temperature. The indicated amount of double stranded oligonucleotide or restriction fragment was incubated for 10 min on ice together with poly(dI-dC) (dI-dC) and nuclear extract before the addition of the labeled fragment. Further procedures were as described for the DNAase experiments. The following specific oligonucleotides and competitors were used (see text): footprint VII oligonucleotide, 5'-AGCTTGCTAAGGGTAGAAAGGGTGAGAAAGAGCTGATTGA-3'; footprint VIII oligonucleotide, 5'-AATTCGAGTATTTTCACATTGAAAGAGACTTG-3'; competitor A, BglI-PvuII restriction fragment from SV40 containing Sp1 binding sites; competitor B, 32mer oligonucleotide 5'-ACAAGACGCTGGGCGGGGCGGATCCGGTT-3'; competitor C, 40mer unspecific oligonucleotide 5'-AGCTTAGTCGGAGATGAGTTAGACAGAAGAGTGGAAGTGA-3'.

Catalytic subunit of cAMP-PK

The catalytic subunit of cAMP-PK was a gift of Dr. B.A. Hemmings. It was isolated from rabbit skeletal muscle and purified as described by Beavo (22).

Southwestern blotting assay

Heparin agarose-fractionated nuclear proteins were resolved by 7.5% polyacrylamide-SDS gel electrophoresis (23). The proteins were electrophoretically transferred to nitrocellulose (24). Filter strips were preincubated with 5% non-fat dry milk (25) in 10 mM Hepes, pH 8.0, for 30 min at room temperature. Then, the filters were incubated with 3'-labeled oligonucleotides (0.2 X 10^6 cpm/ml) in a total volume of 5 ml binding buffer (see above) at room temperature for 3 h. The filters were washed twice in binding buffer at room temperature, air-dried and exposed to X-ray film at -80°C.

RESULTS

Ubiquitous transcription factors Sp1 and CTF/NF1 bind to the uPA promoter sequences

The immediate 5'-flanking region of the uPA gene (15) contains the putative binding sites of several transcription factors (26,27) as well as sequence motifs which are present in the corresponding regions of other cAMP regulated genes (15). To address the question whether these specific sequence motifs are recognized by factors present in LLC-PK1 cells, we performed DNase I -footprinting assays (28,29) with crude nuclear extracts. Nuclear extracts from LLC-PK1 cells were incubated with terminally labeled fragments (Figure 1) from the uPA 5'-flanking region and the mixtures subjected to DNAase I digestion. In Figure 2 (A-D) the four protected GC-boxes (indicated with GC I to IV) are clearly visible. The protected regions cover 18 to 24 bp. Although most regions were found to be equally protected on both strands, the difference for the coding (24 bp) and non-coding strand (18 bp) of GC-box II was considerable (Figure 2A and 2B).

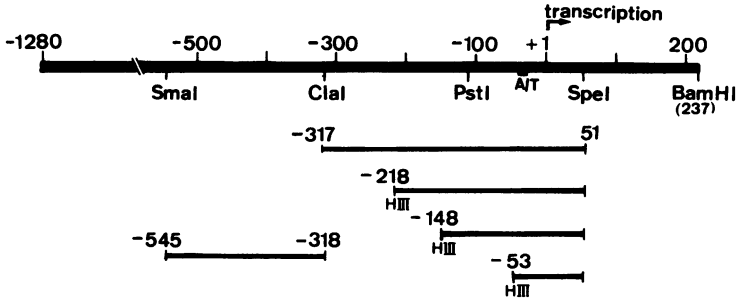
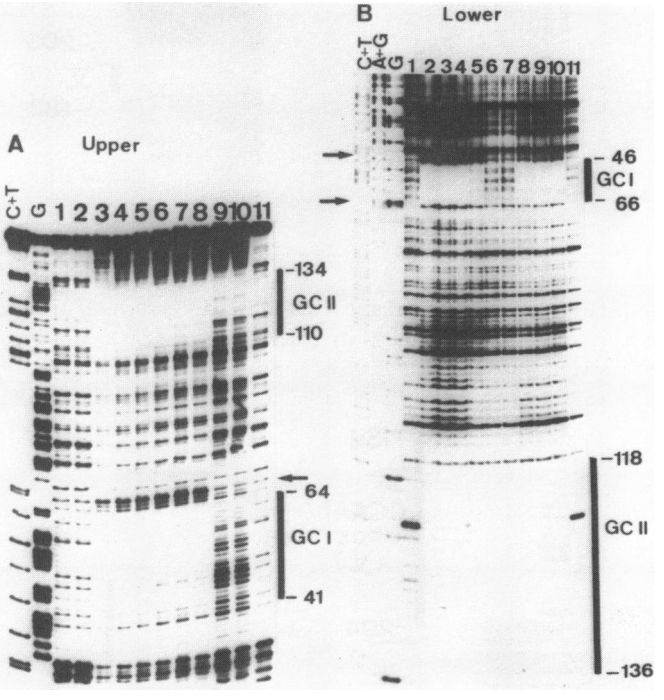
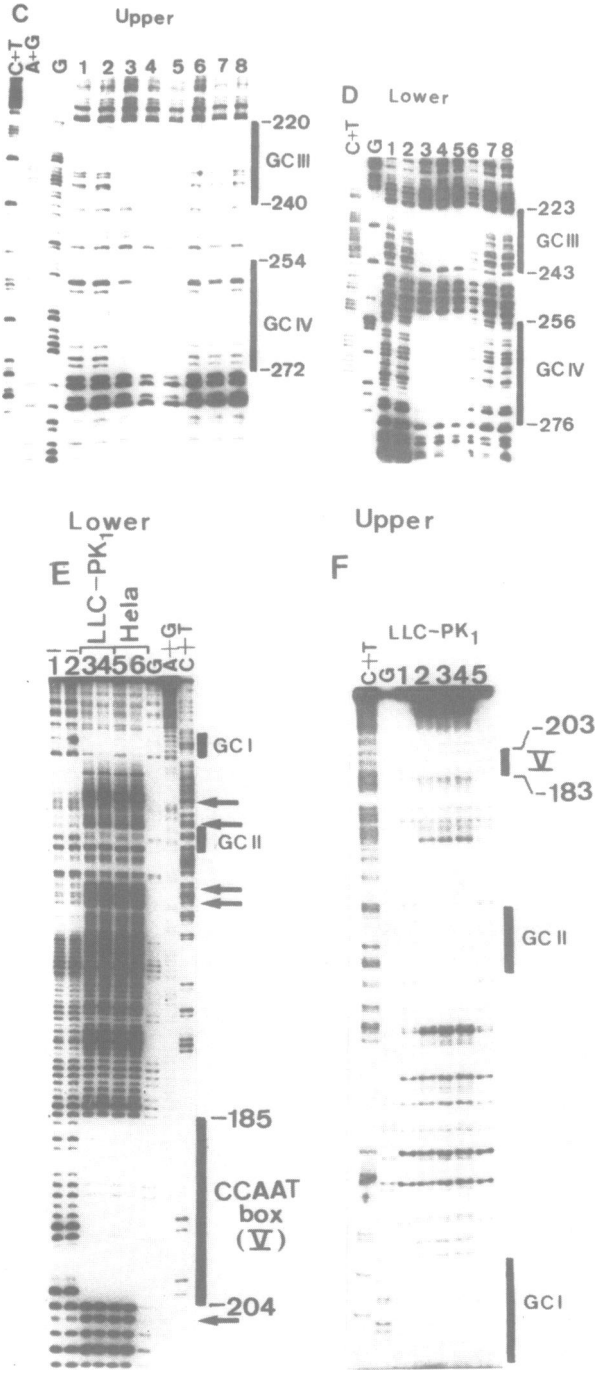


Fig. 1. The uPA promoter, flanking regions and restriction map. The plasmid pYNg1CAT0 containing the BamHI-EcoRI fragment was used for generating Bal 31 deletions at the ClaI site with a HindIII linker on the 5'-end. Fragments indicated below the restriction map were used in DNAase I protection experiments.

There is also a clear footprint centered around position -195 that contains the sequence 5'-CCAAT-3' (Figure 2E and 2F). This sequence is recognized by the transcription factor CTF/NF1, which is like the Sp1 factor a transcriptional activator. The 20 bp protected region centering on position -




C
Upper
C+T
A+G
G 1 2 3 4 5 6 7 8
-220
GC III
-240
-254
GC IV
-272
D
Lower
C+T
G 1 2 3 4 5 6 7 8
-223
GC III
-243
-256
GC IV
-276
Lower
E
LLC-PK1
Hela
1 2 3 4 5 6 G A C+T G C+T
GC I
GC II
-185
CCAAT
box
(V)
-204
Upper
F
LLC-PK1
C+T G 1 2 3 4 5
-203
V
-183
GC II
GC I

195 is similar to those found in the HSV thymidine kinase (HSV-tk) and α- and β-globin promoters (27).

To ensure that the Sp1 and CTF/NF1 transcription factors bind to the uPA promoter sequences, we competed with an excess of unlabeled double-stranded oligonucleotides derived from the HSV-tk promoter or the PvuII-BglI restriction fragment of the SV40 early promoter. The oligonucleotide and the restriction fragment fully competed for binding at the GC-box I and II and the CCAAT-box (Figure 2; CCAAT-box binding factor data not shown).

No additional factor(s) bind to the uPA promoter

In vitro transcription data suggested that there is a cis-acting element between positions -148 and -53 of the uPA gene, which is important for cAMP action (16). Recent reports on cAMP regulated genes proposed a core sequence (5'-TGACGTCA-3') for the cAMP response element (CRE) (30-32,48). A sequence that shows similarity to the CRE core sequence can be found between positions -123 and -112 of the uPA gene: 5'-TCCGAGTCA-3'. This sequence is overlapping with the protected GC-box II, centered on position -127. To know if any additional factor binds to this region when the uPA gene is induced, competing with the Sp1 transcription factor, we included agents in the assays which enhance uPA gene transcription in vitro, in conjunction with specific oligonucleotide competition. As shown in Figure 3, neither cAMP nor the catalytic subunit of cyclic AMP dependent protein kinase (C-Subunit of cAMP-PK) gave rise to new binding sites, irrespective of the presence of the competitor.

In order to search for a possible correlation between uPA gene regulation

Fig. 2. DNAase I footprinting of the uPA promoter region in LLC-PK1 nuclear extract. Endlabeled fragments were subjectd to DNAase I footprinting analysis as described in METHODS. C+T, G and A+G sequencing reactions are shown on the left side of each set. Protected areas are specified as solid bars with an identifying label on the right. Arrows indicated DNAase I hypersensitive sites. **A)** The HindIII (-148)-SpeI fragment of the uPA promoter was 3'-endlabeled at the SpeI site. **Lanes 1,2 and 11:** control reactions with 90 μg BSA. **Lanes 3-8:** binding reactions with 40,50,60,70,80 and 90 μg protein; **lanes 9 and 10:** 90 μg protein plus 125 or 250 ng of SV40 BglII-PvuII fragment as competitor. **B)** the HindIII(-148)-SpeI fragment was 3'-endlabeled at the HindIII site. **Lanes 1 and 11:** control reactions with 90 μg BSA; **lanes 2-5:** 40,50,60 and 90 μg protein; **lanes 6 and 7:** 90 μg protein plus 2 or 4 pmol of unlabeled double-stranded oligonucleotide competitor B. **Lanes 8-10:** 2,4 or 6 pmol of unlabeled, unspecified oligonucleotide competitor C. **C)** ClaI(-317)-SpeI fragment was 5'-endlabeled at the ClaI site. **Lanes 1,2 and 8:** control reactions with 60 μg BSA; **lanes 3-5:** 20,40 and 60 μg protein; **lanes 6 and 7:** 60 μg protein plus 1 or 2 pmol of competitor oligonucleotide B. **D)** ClaI-SpeI fragment was 3'-endlabeled at the ClaI site. **Lanes 1,2 and 8:** control reactions with 60 μg BSA. **Lanes 3-5:** 20,40 or 60 protein; **lanes 6 and 7:** 60 protein plus 1 or 2 pmol competitor B. **E)** HindIII(-218)-SpeI fragment was 3'-endlabeled at the HindIII site. **Lanes 1 and 2:** control reactions with 40 or 80 μg BSA; **lanes 3 and 4:** 40 or 80 μg nuclear proteins of LLC-PK1 cells; **lanes 5 and 6:** 40 or 80 μg HeLa nuclear proteins. **F)** The SpeI(+51)-HindIII fragment was 3'-endlabeled at the SpeI site. **Lanes 1 and 5:** control reactions with 90 μg BSA; **lanes 2-4:** 30, 60 or 90 μg LLC-PK1 nuclear proteins.

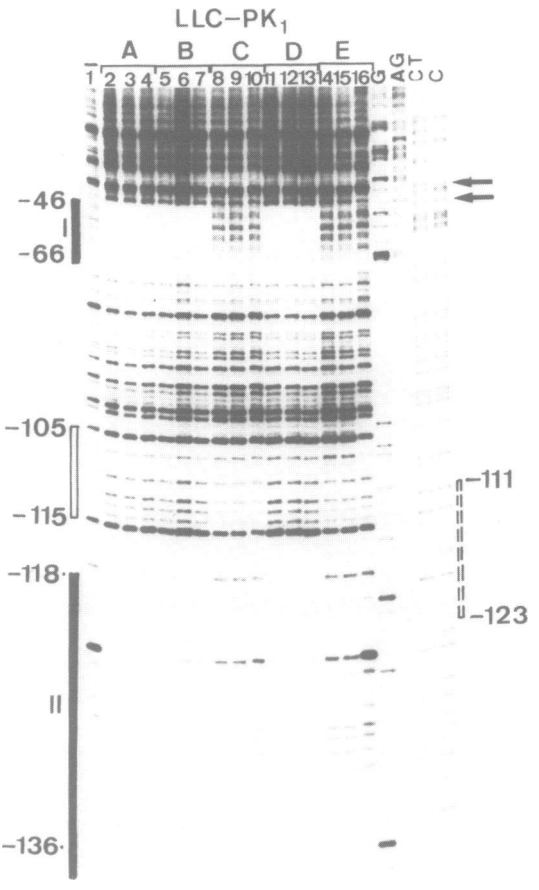


Fig. 3. In vitro modification reactions and DNAase I footprinting pattern of the uPA HindIII(-148)-SpeI fragment in LLC-PK1 nuclear extract. The fragment was 3'-endlabeled at the HindIII site. Each reaction contained 10 mM creatine phosphate, 20 5M of phosphoserine, phosphothreonine and phosphotyrosine, 10 mM NaMo and 2.5 mM ATP. The reaction mixture was subjected to DNAase I footprinting analysis as described in METHODS. To the right, sequencing reaction product. Protected areas are specified as solid bars with an identifying label on the left. The broken bar on the right side indicates the region of homology, overlapping with the protected area of GC-box II (see text). The open bar on the left side indicates the region of weak interaction in **lanes 8-10** and **14-16**. Arrows indicate DNAase I hypersensitive sites. **Lane 1:** control reaction with 50 μg BSA. **Lanes 2-15:** 50 μg LLC-PK1 nuclear protein. **A)** No addition of cAMP. **B)** Addition of 20 mM cAMP. **C)** Addition of 20 mM cAMP plus 4 pmol of oligonucleotide competitor B. **D)** Addition of catalytic subunit of cAMP-PK, 240 ng each lane. **E)** Addition of 240 ng catalytic subunit of cAMP-PK plus pmol of oligonucleotide competitor B.

and factor binding to this region (-140 to +51) we prepared nuclear extracts from three other cell lines with different uPA gene regulation: HeLa cells, in which the uPA gene expression is very low and is no longer inducible by cAMP; HT-1080 cells, in which the uPA gene is constitutively expressed and is not further induced by cAMP, and FTO-2B cells, in which the uPA gene is not expressed, but the tyrosine aminotransferase (TAT) gene is inducible by cAMP in this cell line. This region of the uPA gene promoter showed similar interaction pattern with factors present in these extracts as observed with the LLC-PK1 extract (data not shown).

Specific interaction of nuclear factors with other upstream sequences of the uPA gene

Interestingly, cell free transcription experiments showed that the extent of the cAMP induction with a longer 5'-flanking region (-1300 bp) is considerably higher than those with a shorter one (-148 bp) (16). Nagamine and Reich (33) proposed a 28 nucleotide sequence of the uPA gene as a potential cAMP regulatory element, which is very similar to corresponding regions in the cAMP regulated tyrosine aminotransferase (34) and phosphoenolpyruvate carboxykinase (PEPCK) gene (35). Results of DNAase I footprinting analysis of the 230 bp SmaI-ClaI region (-545 to -318), containing the 28 bp homologous sequence (-394 to -367) with LLC-PK1 nuclear extract is shown in Figure 4. This fragment forms a large nucleoprotein complex. Clear protection and altered DNAase I digestion patterns were obtained between nucleotides -502 and -324. There were also at least three distinct sites of protection. The 28 bp sequence was protected almost entirely by the extract (- 396/394 to -370; footprint VII). Interestingly, a 10 bp sequence in footprint VII, repeated once further upstream, was also protected (footprint VIII; -495 to -468), as shown in Figure 4.

The footprint VI (-348 to -324) contains a sequence (5'-GGGGGCAGGG-3'), which is, with the exception of one base, similar to the Sp1 consensus sequence (36). The footprint VI was competed with an excess of an oligonucleotide containing an authentic Sp1 binding sequence (data not shown).

Figure 4 shows also a partial protection centered at position -427. This "cryptic" binding site, as well as strong hypersensitive sites of DNAase I cleavage in the proximity of the protected areas were probably due to the use of crude nuclear extracts at high protein concentrations to saturate all putative binding sites. At lower protein concentrations these "cryptic" sites were not detected (data not shown).

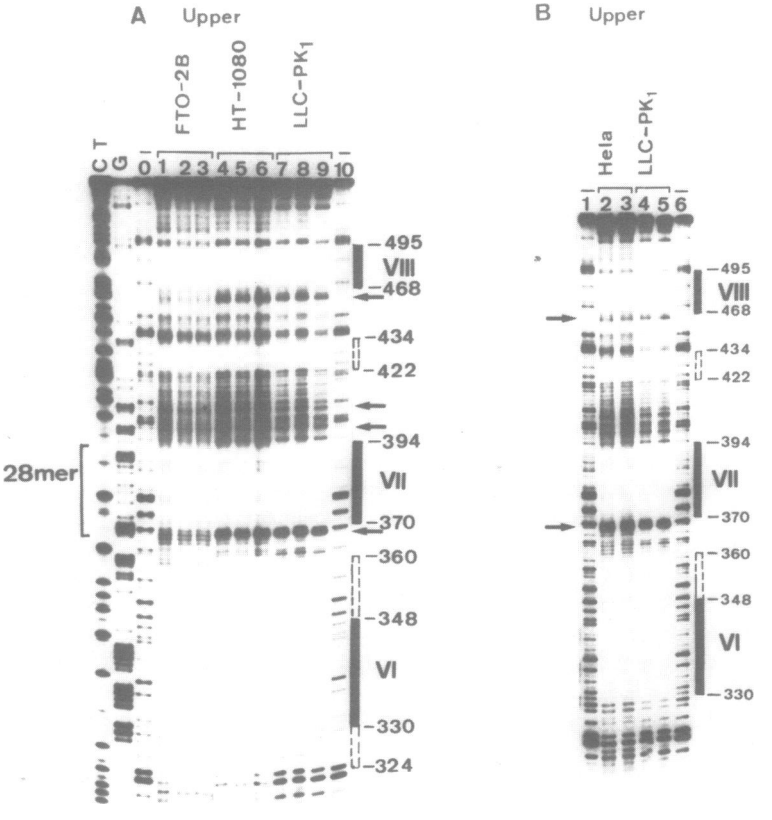
To determine whether any of these factors were specific for LLC-PK1 cells, we used nuclear extracts from cell lines described above. As shown in Figure 4, no major difference in the protected sites were obtained using nuclear extracts from various cell lines. Binding to the footprint VII region was considerably reduced in HeLa, HT-1080 and FTO-2B cell extracts (Figure 4). This is most likely due to a weaker affinity or lower abundance of the DNA-

binding proteins, or both. The digestion patterns in the neighborhood of the protected sites were also found to differ among the various extracts.

Different nuclear proteins bind to sequences of footprints VII and VIII

To answer the question whether footprints VII and VIII were generated by different proteins, we performed gel retardation experiments (37,38) with the appropriate oligonucleotides. As shown in Fig. 5, both sequences formed more than one specific complex with nuclear proteins. Competition experiments with specific and unspecific oligonucleotides revealed that in both cases (i) two shifted bands contained specific protein-DNA complexes (arrows in Fig. 5A and B), and (ii) that the sequence of footprint VII did not compete for the binding factor of footprint VIII (Fig. 5B lanes 10-12) and vice versa (Fig 5A, lanes 8-10). The results suggest that different proteins bind to these two sites.

For further characterization of the protein(s) which binds to the sequence of footprint VII we fractionated nuclear extracts by heparin agarose column and then employed the Southwestern method (39). We detected the specific binding activity in the 0.4 M KCl fraction by gel retardation assay (data not



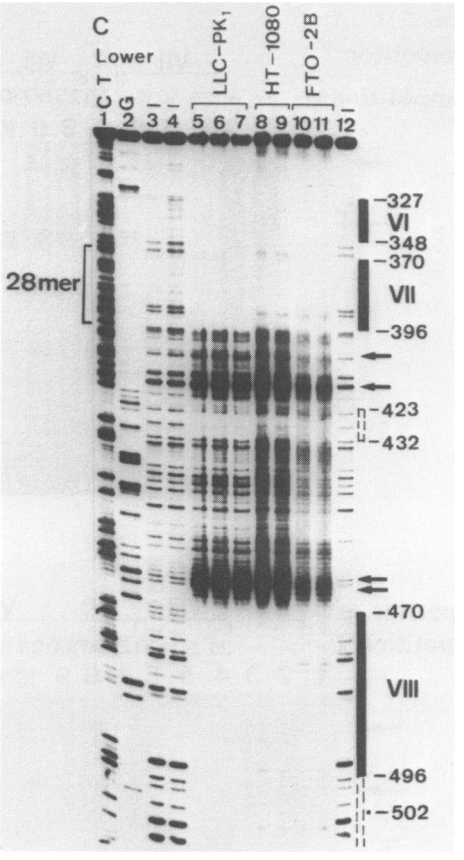


Fig. 4. Multiple protein-DNA interaction sites within the uPA promoter distal SmaI(-545)-ClaI(-318) fragment. Presence of similar DNA binding protein in various cell lines. Sequencing reactions are shown on the left. Areas of strong and weak protein-DNA interactions are specified as solid and broken bars with an identifying label on the right. The DNAase I footprints are numbered by roman numerals VI, VII and VIII. Arrows indicate DNAase I hypersensitive sites. The 28mer indicates region of homology. **A.** and **B.** Upper strand. The SmaI-ClaI fragment was labeled at the HindIII site in the polylinker region of pUC 18. Lanes **A-0, A-10, B-1** and **B-6**: control reactions with 90 μg BSA. **Lanes A-1** to **3**: 45, 75 or 105 μg FTO-2B nuclear proteins; **lanes A-4** to **7**: 60, 80 or 120 μg HT-1080 nuclear proteins; **lanes A-7** to **9**: 45, 75 or 105 μg LLC-PK1 nuclear proteins; **lanes B-2** and **B-3**: 76 or 114 μg HeLa nuclear extract protein; **lanes B-4** and **B-5**: 75 or 105 μg LLC-PK1 nuclear proteins. **C.** Lower strand. The SmaI-ClaI fragment was 3'-endlabeled at the EcoRI site in the polylinker region of pUC 18. **Lanes 3,4** and **12**: control reactions with 80 μg BSA. From left to right, binding reactions with 50, 70 or 80 μg nuclear extract proteins from LLC-PK1 (**lanes 5-7**), HT-1080 (**lanes 8** and **9**) and FTO-2B cells (**lanes 10** and **11**).

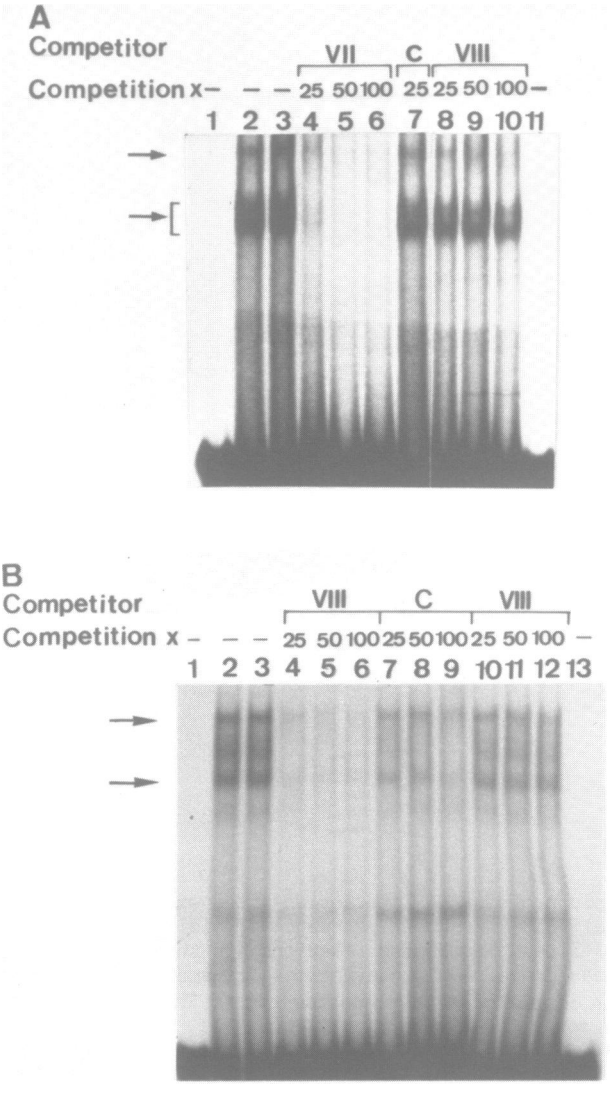


Fig. 5. Gel retardation assay with oligonucleotides containing sequences of footprint VII (A) and footprint VIII (B). The double-stranded oligonucleodites were 3' end labeled, incubated with 12 μg of nuclear extract from LLC-PK1 cells, and then subjected to gel electrophoresis. **Lanes 1, 11 and 13:** controls without nuclear extract; **lanes A-4 - A-10 and B-4 - B-12:** competition with unlabeled oligonucleotides as indicated in the figure. Arrows indicate specific DNA-protein complexes.

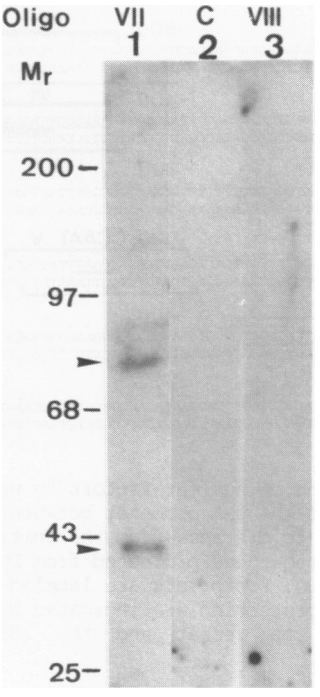


Fig. 6. Detection of DNA-binding proteins by Southwestern blot analysis. 16 μg protein of the 0.4 M KCl fraction from heparin-agarose column was fractionated by SDS-polyacrylamide gel electrophoresis, transfered electrophoretically to nitrocellulose filters, and incubated with 3' end labeled oligonucleotides containing sequences of footprint VII (**lane 1**), control (**lane 2**) and footprint VIII (**lane 3**). Arrowheads indicate the proteins which bound to footprint VII oligonucleotide.

shown). The proteins of this fraction were separated by SDS-polyacrylamide gel electrophoresis. After electroblotting of the proteins onto nitrocellulose filters and incubating with the ^{32}P-labeled oligonucleotide containing the sequence of footprint VII, we could detect two protein bands with specific DNA-binding activity (Fig. 6). We could not detect any proteins that bind to oligonucleotide of footprint VIII, which also suggest that sequences VII and VIII interact with different proteins.

DISCUSSION

Using a cell free transcription system prepared from LLC-PK1 cells, we could demonstrate (16) that the uPA promoter with a 5'-flanking sequence not longer than 148 nucleotides from the CAP site, was transcriptionally active and inducible by cAMP. Addition of the C-subunit of cAMP-PK as well as cAMP

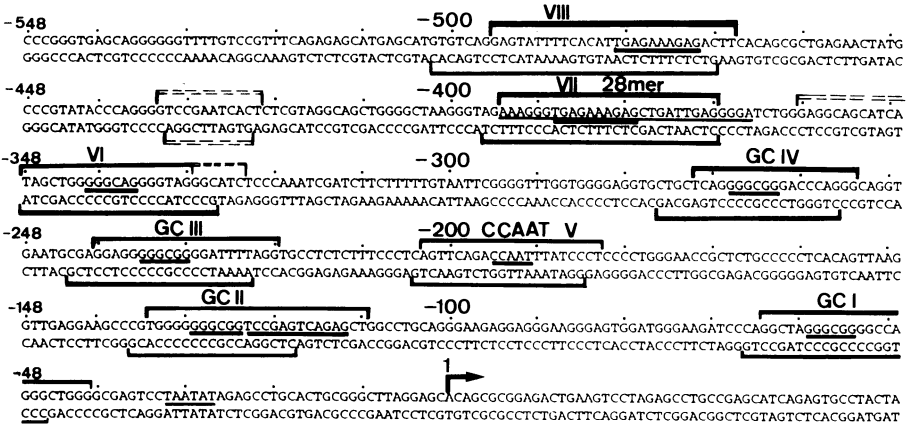


Fig. 7. Summary of binding of nuclear factors to upstream sequences of the uPA gene. The sequence of the uPA promoter between -548 and +51 (SmaI-SpeI) is shown. Numbers refer to distance from the initiation of transcription. Overlines indicate the sequences protected from DNAase I digestion after binding of nuclear factors. Footprints are labeled by roman numerals from I to VIII. Regions of weak protection are indicated by dashed lines. TATA, GC- and CAAT-boxes, the 10 bp repeat and the 28mer homology region are underlined.

enhanced the transcription in vitro, and an inhibitor of cAMP-PK suppressed this enhanced transcription. These results suggest that the effect of cAMP is mediated through the action of cAMP-PK and requires the phosphorylation of a nuclear protein.

Based on this functional study of the uPA promoter, we investigated the specific protein-DNA interactions in this region. DNAase I footprinting analysis revealed at least eight different sites within 500 nucleotides upstream of the CAP site of the uPA gene. In the region proximal to the transcription start site we found a single CTF/NF1 binding site (CAAT-box), flanked on both sides by two Sp1 binding sites (GC-boxes). An aggregate of GC-boxes with a single-CAAT-box has been observed in other eukaryotic RNA polymerase II promoters (36). A similar arrangement of this cis-elements was described for the HSV-tk promoter (40). Within a sequence of 10 kb of the uPA gene, including 3.8 kb 5'-flanking regions, GC- and CAAT-boxes appeared only in this region. It is likely that the transcriptional activators CTF/NF1 and Sp1 act in a coordinated way to modulate transcription of the uPA gene.

In addition, further upstream we detected a GC-box like sequence whose interaction with a nuclear factor was competed by an oligonucleotide containing a consensus GC-box, suggesting this sequence was also recognized by Sp1. This is in agreement with recent reports showing that there is some flexibility in the Sp1 consensus sequence (41,42).

The available experimental evidence does not allow to define the precise

role of every single GC- and CAAT-box and their corresponding trans-acting factors in the regulation of the uPA gene transcription. Analysis of templates containing a variety of 5'-flanking deletions, in a cell free transcription system, showed that transcription from a vector containing a deletion down to the GC-box I (centered at -56), but keeping the TATA-box intact, resulted in the loss of both correct initiation of the uPA gene transcription and cAMP induction, whereas transcription with a template containing GC-box I and II (centered at -126) maintained considerably high basal activity and a 3- to 5-fold induction by cAMP (16). These results and the DNAase I footprinting data suggest that the multiple Sp1 and CTF/NF1 binding sites are required for high and modulated expression of the uPA gene.

To find out how these cis-acting elements are involved in the hormonal induction of the uPA gene, we employed footprint analysis with nuclear extracts treated in vitro or nuclear extracts from hormonal treated cells. Neither extract revealed any inducible DNA binding activity. From these results we envisage a mechanism of cAMP regulation in which protein-protein interactions, rather than protein-DNA interactions play a key role. This protein-protein interaction could be modulated through phosphorylation events by cAMP-PK. However, we do not exclude the possibility, that the modification of protein factors is not reflected in the DNA binding activity in vitro. An example of the discrepancy between in vitro and in vivo protein-DNA interactions are the steroid hormone receptors. The glucocorticoid and the progesterone receptor were able to bind to DNA in vitro, in the absence of steroid hormone (43,44). In contrast, it could be shown in vivo, using the dimethylsulphate footprinting technique, that the hormone increases the affinity of the receptor for its target sequence (45). Thus, it appears that the hormone is not necessary for specific DNA binding in vitro, whereas it is in vivo.

Nagamine and Reich (33) have observed, that a 28 bp sequence beginning 367 nucleotides upstream of the uPA CAP site, is very similar to a sequence in the 5'-flanking region of the cAMP regulated rat tyrosine aminotransferase gene (34). DNAase I protection experiments revealed that this 28 bp sequence is recognized by a nuclear factor. Footprint VII overlaps well with the 28 bp sequence.

Moreover, a 10 bp sequence from footprint VII is repeated 100 nucleotides further upstream, and this was also protected, giving rise to footprint VIII. Except for the 10 bp sequence, the two sequences of footprint VII and VIII were quite different and the results of competition experiments suggested that the two binding proteins are not identical. In a Southwestern binding assay we could identify two nuclear proteins of molecular weight 40 and 80 Kda which bound specifically to the footprint VII and not to the footprint VIII sequence.

LLC-PK1 cells, stably transformed with a hybrid template, a construct containing the chloramphenicol acetyl transferase (CAT) coding region and the uPA gene promoter including 4.6 kb 5'-flanking region but lacking the SmaI-ClaI fragment (Figure 1), that contained footprints VI, VII and VIII (figure

7), showed about 50% reduction in the hormonal response after induction by calcitonin (Pearson, von der Ahe and Nagamine, unpublished results). Based on these observations we propose that transcriptional regulation of the uPA gene by cAMP is through the interplay of several cis-elements and their corresponding factors. In the case of the somatostatin gene it was reported that the cAMP responsive element was necessary but not sufficient for the full transmission of the cAMP signal (30).

To know if there is any cell- or tissue-specific factor that interacts with the uPA promoter sequences, we used nuclear extracts from different cell lines. Surprisingly, all factors that recognize the uPA gene promoter, including Sp1 and CTF/NF1 seem to be present in all cell lines tested, irrespective of whether the uPA gene in these cells is hormonally regulated, constitutively expressed or not expressed at all. This agrees with observations on the steroid inducible TAT gene, for which it was shown that cell type-specific DNA binding was detectable only in vivo but not in vitro (49). It is possible that DNA modification or changes in chromatin structure determine the binding of the factors to their DNA sequences. Alternatively, it cannot be excluded that the DNA binding factors are complexed in vivo with cell type-specific protein(s) which are the targets for posttranslational modifications, such as phosphorylation by the cAMP-PK.

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