Activation of cAMP-dependent protein kinase alters the chromatin structure of the urokinase-type plasminogen activator gene promoter

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ABSTRACT

In LLC-PK₁ cells, the urokinase-type plasminogen activator (uPA) gene is induced by two of the major signal transduction pathways, the protein kinase C (PKC) and the cAMP-dependent protein kinase (PKA) pathways. We have analyzed the chromatin structure of 26 kb of the uPA gene locus and have shown that PKA activation but not PKC activation induces major chromatin structural alterations in the uPA gene promoter. In uninduced cells, several DNase I hypersensitive (HS) sites were detected in the 5' and 3' flanking regions but not in the transcribed region. Two of the sites correspond to previously characterized regulatory sites: a cAMP responsive site at nucleotide position -3500 with respect to the initiation site, and the PEA3/AP1 site at -2100 that mediates PKC activation. After the activation of PKA but not PKC, a strong HS site was induced at -2600. Functional analysis of this region revealed cAMP responsive activity. Chromatin structural alterations again brought about specifically by PKA but not by PKC were also detected in the upstream of the promoter by topoisomerase I cleavage site analysis, with two prominent sites appearing at -2800 and -3300. These results suggest that the strong cAMP induction of the uPA gene requires structural alterations that permit cooperative interactions between the multiple cAMP responsive sites.

INTRODUCTION

That chromatin structure influences gene transcription is well established. It is thought to affect the accessibility of the underlying sequences to transcription factors and to facilitate interactions between different regulatory elements (1,2). Active genes and their flanking regions have a less compact chromatin structure and consequently are less protected and approximately 10-fold more sensitive to DNase I digestion than inactive genes. The region of increased DNase I sensitivity surrounding a gene is thought to encompass the sequences that can influence that

gene. Within this domain, short stretches of sequence either 5' or 3' of transcription units are more hypersensitive to DNase I digestion by about two orders of magnitude compared to bulk chromatin (3). DNase I hypersensitive (HS) sites often indicate the location of regulatory elements, where the chromatin is in a state that permits those elements to be functional. It is not known how HS sites are formed, but for developmentally regulated genes the appearance of DNase I hypersensitive sites is tissue- and stage specific and precedes the initiation of transcription (4–6). Apart from steroid hormones (7,8), little is known about transcriptional stimuli that cause the formation of HS sites. The alteration of promoter chromatin structures by the activation of cAMP-dependent protein kinase (PKA) has not been reported.

Topoisomerase (Topo) I is an important regulator of DNA topology (for review, see 9,10). *In vitro*, its principal enzymatic activity is the relaxation of negative and positive supercoils in topologically restrained DNA. *In vivo*, Topo I is physically associated with the transcription unit of transcriptionally active genes and is thought to play a role in transcriptional elongation by relieving torsional stress induced on the template (11,12). Topo I sites have also been mapped to promoter regions (11), although their appearance has not been correlated with transcription induction.

The urokinase-type plasminogen activator (uPA) gene has a complex regulation, being expressed in a developmental, tissue-specific and inducible manner (13). We are studying the regulation of the uPA gene in LLC-PK₁ cells, a nontransformed cell line derived from nontransformed porcine renal epithelia (14). In these cells the uPA gene is regulated independently through several signal transduction pathways involving PKA (15,16), protein kinase C (PKC) (16), cytoskeletal reorganization (17,18), and okadaic acid-sensitive protein phosphatase (19,20). Responsive elements involved in uPA gene regulation have been identified. It was found that an AP-1-like site at -2 kb is essential and sufficient to mediate uPA gene induction by PKC, cytoskeletal reorganization and okadaic acid (18,20). In contrast, there are multiple, weakly cAMP-responsive sites (21), and only one of them has been studied in detail (21,22). Interestingly, while the endogenous uPA gene is induced 60-fold by 8-bromo-cAMP

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(Br-cAMP) (16), a chimeric CAT hybrid gene containing 4.6 kb of the 5' flanking region of the uPA gene was less inducible (at most six-fold) in transient transfection assays (18, and B.Rajput, unpublished), suggesting either that there are additional regulatory elements not present in the chimeric genes or that chromatin structure plays a role. To investigate these aspects, we analyzed the chromatin structure of the uPA gene promoter by HS site analysis. Several changes in the HS site pattern were observed following PKA activation, suggesting that PKA activation causes major structural alterations of the uPA gene promoter. Chromatin structural alterations in the uPA gene promoter following PKA activation were also detected by Topo I cleavage site analysis. We discuss the role of chromatin structural alterations in the context of cAMP-dependent uPA gene induction and the relationship between HS sites and Topo I sites in the uPA gene promoter.

MATERIAL AND METHODS

Reagents

DNase I was purchased from Worthington, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) from Pharmacia, and 8-bromo-cAMP (Br-cAMP) and camptothecin from Sigma Chemical Co.

Cell culture

LLC-PK₁ cells (14) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Diagnostics) containing 5%–10% (v/v) fetal calf serum, 0.2 mg/ml streptomycin and 50 U/ml penicillin, in a humidified atmosphere at 37°C with 5% CO₂.

Nuclear preparation, DNase I hypersensitive site and Topo I cleavage site analyses

Nuclei from tissues were prepared by taking tissue samples (a gift from P-A. Menoud and F. Botteri) that had been placed on dry ice immediately after sacrificing the pig. All procedures were performed at 4°C. A sample of 1–2 g of tissue was finely chopped in 10 ml of buffer A (0.3 M sucrose, 15 mM Tris-HCl, pH 7.5, 25 mM KCl, 0.5 mM spermidine, 0.15 mM spermine, 1 mM EDTA, and 0.1 mM EGTA), passed through a stainless steel sieve (pore size 0.5 mm) and homogenized in a dounce homogenizer (10 strokes). After addition of Triton X-100 to 0.3% (v/v), the homogenate was again homogenized (5 strokes) and mixed with 20 ml of buffer B (40% glycerol, 50 mM Tris-HCl,

pH 8.3, 1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT). The nuclei were pelleted at 2000 rpm for 10 min, resuspended in buffer B, and stored at –70°C. Nuclei from LLC-PK₁ cells were isolated as described previously (23). For DNase I HS site analysis, nuclei containing 50 µg of DNA were suspended in DNase I digestion buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 6 mM MgCl₂, 0.2 mM CaCl₂, 0.1 mM EDTA, and 1 mM β-mercaptoethanol) and incubated with a range of concentrations of DNase I at 37°C for 20 min. The reaction was quenched by the addition of an equal volume of STOP solution (20 mM Tris-HCl, pH 7.4, 0.6 M NaCl, 10 mM EDTA, 1% SDS, and 1 mg/ml proteinase K) and incubated at 37°C for 2 h. The DNA was extracted by phenol and chloroform: isoamylalcohol (24:1) and ethanol precipitated. DNA samples were then digested with appropriate restriction enzymes, resolved on a 0.9% agarose gel electrophoresis and transferred to nitrocellulose filters as described (24). The filters were prehybridized, and hybridized as described (24), using radioactive probes (5×10⁶ cpm/ml) prepared by the random priming method (25). After washing with 0.1 SSC and 0.1×0.1% SDS at 65°C, filters were exposed to Kodak X-OMAT AR films with two intensifying screens at –70°C.

Topo I cleavage sites were visualized according to Stewart and Schütz (26) with some modifications. LLC-PK₁ cells (0.5×10⁶) were plated on 35-mm dishes with 2 ml medium, and treated with camptothecin and Br-cAMP 24 h later for the times and at the concentrations indicated in the figure legends. Cells were harvested by the addition of 500 µl of STOP solution. The mixtures were incubated at 37°C for 2 h, and the DNA was extracted by phenol chloroform:isoamylalcohol (24:1) and ethanol precipitated. Genomic DNA was digested with appropriate restriction enzymes and resolved on 1.0–0.8% alkaline agarose gel electrophoresis as described (24). After neutralizing the gels by incubating in 0.5 M Tris-HCl, pH 8.0, and 1.5 M NaCl for 2 h with several changes, the DNA was blotted to nitrocellulose.

Probes

PCR reactions were performed to obtain the following fragments: P1, from –430 to +230 with respect to the transcription initiation site; P2, from +62 to +750; P3, from +750 to +1429 (Fig. 1). The PCR products were resolved on an agarose gel and bands of the correct molecular weight were isolated, the fragments blunt-ended using DNA polymerase I Klenow fragment and then

Figure 1. Genomic map showing the DNase I hypersensitive sites in 26 kb of the uPA gene locus, before and after activation of protein kinase A with Br-cAMP. Topo I cleavage sites induced by 8-bromo-cAMP in the 5' flanking region are marked. The probes, P1, P2 and P3 used for indirect-end labeling are shown. B = BamHI; restriction sites are marked for the 5' flanking region, whereas they are too numerous in the transcription unit to mark, and in the 3' flanking region they have not been mapped. H = HindIII; all sites are marked. Sequence is available from 4.6 kb 5' of the transcription initiation site to 500 bp 3' of the poly(A) addition site.

ligated into the *Sma*I site of pGEM1. Inserts were verified by sequencing. Radioactive probes for Southern blots were prepared by the random oligo-primed reaction (25) using purified inserts.

Constructs

Fragments of the uPA gene promoter covering the regions of interest were isolated and blunt-ended by a Klenow fill-in reaction and subcloned into the *Bgl*III site of a CAT reporter gene containing only the minimal promoter and no enhancer sequences (pCATP, Promega). The fragments used were from -3473 to -3324 and from -2530 to -2350. The other constructs have been described previously (21).

Transient transfection assays

Cells (0.7×10^6) were plated in 35-mm plastic dishes with 3 ml medium, and transfected 24 h later by the DEAE-dextran method (27). Briefly, prior to transfection the cells were washed twice with phosphate-buffered saline (PBS) then 2 ml of DMEM containing 10% NuSerum (Collaborative Research Inc.). After addition of 4 μ g of plasmid DNA and 420 μ g DEAE/dextran (prewarmed to 37°C), the incubation continued at 37°C for 2–3 h. The medium was removed and the cells were treated with 1 ml of 10% DMSO in PBS at room temperature for 1 min, washed with PBS and incubated with 3 ml DMEM containing 10% fetal calf serum for 2–3 h. The cells were then treated with the inducers at the times indicated and harvested by trypsin/EDTA treatment, washed twice with PBS and resuspended in 100 μ l of 0.25M Tris-HCl, pH 8.0. Extracts were prepared by three cycles of freezing and thawing, followed by heat treatment at 65°C for 10 min and centrifugation for 5 min in an Eppendorf centrifuge. CAT activities were measured according to Gorman *et al.* (28).

RESULTS

Localization of DNase I hypersensitive sites in the 5'- and 3' flanking regions of the uPA gene—effect of PKA and PKC activation

A restriction map of 26 kb of the porcine uPA gene locus showing the region analyzed for HS sites and Topo I sites is given in Figure 1. The locations of the probes used are indicated: P1 recognizes a *Bam*HI restriction fragment from +230 to -9 kb with respect to the transcription initiation site, P2 recognizes a *Hind*III restriction fragment from +750 to -11 kb, and P3 recognizes a *Hind*III restriction fragment from +750 to about 10 kb 3' of the poly(A) addition site.

Figure 2A shows a typical HS site analysis of nuclei from uninduced and cAMP-induced LLC-PK₁ cells using P1. Uninduced cells display four major HS sites at -1.8 kb, -2.1 kb, -3.5 kb and -4.0 kb, and two minor HS sites at -1.3 kb and -2.6 kb. The HS sites at -2.1 kb and -3.5 kb are adjacent to known regulatory elements that mediate the action of PKC (18) and part of the PKA response (22), respectively. A strong increase in the intensity of the HS site at -2.6 kb was observed following cAMP induction, and additionally the HS pattern was subtly altered in the region from -1.3 kb to -4.4 kb with respect to intensity and position (Fig. 2A, compare lanes 3 and 4 with 7 and 8). We used P2 to confirm the HS site pattern obtained with P1; this gave an HS site pattern similar to that obtained with P1 but with an additional major HS site adjacent to the transcription initiation site at -0.1 kb (compare Figs. 2A and 2B). The -0.1 kb HS site is too close to the *Bam*HI site at +230

to be detected with P1. The same cAMP-dependent changes in the HS pattern were also observed with P2 (data not shown). PKC activation with TPA resulted in an HS pattern essentially identical to that of uninduced cells (Fig. 2C).

We subsequently analyzed 5N4, a mutant LLC-PK₁ cell line which has a lesion in the cAMP signaling pathway downstream of PKA (L.Catanzariti and B.A.Hemmings, in preparation). In 5N4, PKA can be activated normally but PKA-dependent uPA gene induction is impaired. On the other hand, PKC-dependent uPA gene expression and the gene structure is the same as the wild type, suggesting a lesion in the PKA signaling pathway between PKA and the uPA gene. In 5N4 the cAMP-inducible HS site at -2.6 kb is not induced following cAMP treatment (Fig. 2B, compare lanes 1–5 and 6–10). Thus, the appearance of the HS site at -2.6 kb correlates with uPA gene induction by PKA and is not due to nonspecific cellular changes resulting from PKA activation.

P3 was used to look for HS sites in the coding region and 10 kb of 3' flanking region. In uninduced cells there is one major HS site at 1.9 kb 3' of the poly(A) addition site (+7.6 kb from the CAP site); its intensity decreases following cAMP induction and some minor bands appear at 500 bp and 150 bp 3', and 350 bp 5' of the poly(A) addition site (Fig. 3). Some minor bands observed could correspond to transcription stop sites. The faint band of 11 kb is the remnant of a previous probing of the blot with probe 2 (see Fig. 2C). Essentially identical changes in the HS site pattern were observed in the 3' flanking region following PKC activation (data not shown). As the HS site at 1.9 kb 3' of the poly(A) addition site has properties suggestive of a silencer, we looked for its presence in liver, a tissue which expresses low levels of uPA mRNA, and in kidney, a tissue which expresses high levels of uPA mRNA. Figure 3B shows that the HS at 1.9 kb 3' of the poly(A) addition site is relatively strong in liver (about equivalent to uninduced LLC-PK₁ cells) and weak in kidney. This correlation supports the notion that the site has a silencer function or that its prominent appearance is a marker of a silent uPA gene.

Topo I cleavage sites are induced in the uPA gene promoter by PKA activation

So far the results show a correlation between chromatin structural alterations in the uPA gene promoter and uPA gene induction by cAMP. To further investigate this aspect, Topo I cleavage sites were mapped in the uPA gene promoter following PKA and PKC activation. Camptothecin stabilizes the covalent Topo I-DNA complex, leaving the DNA with a single-stranded nick at the site of Topo I activity (11). The position of the nick is then determined by indirect-end labeling using a procedure similar to that of HS site analysis, except that the DNA is resolved in a single-stranded form. Figure 4A shows the results of Topo I cleavage site analysis of the uPA gene promoter from -11 kb to +750. Camptothecin alone did not induce single-stranded nicks, indicating that there is no Topo I activity on the uninduced promoter. Upon cAMP treatment, Topo I activity appeared on the uPA gene promoter. The prominent site was at -2.8 kb, and it remained for at least 4 h following cAMP treatment. Other minor Topo I cleavage sites were apparent, including a cluster at -0.95 kb, -1.2 kb and -1.5 kb. Overexposure of the filter to X-ray film did not reveal additional sites. PKC activation resulted in a low level of Topo I activity at -2.8 kb (Fig. 4B, compare lanes 5 and 6). A more detailed examination of the site at -2.8 kb revealed that it was a doublet at positions -3.3 kb

Figure 2. DNase I hypersensitive sites in the 5' flanking region of the uPA gene in LLC-PK₁ cells; effect of PKA and PKC. (A) Nuclei were prepared from LLC-PK₁ cells grown in the presence or absence of 1 mM 8-bromo-cAMP for 1 h, and subjected to HS site analysis using P1 on a *Bam*HI restriction digest (see Materials and Methods and Figure 1). Amount of DNase I: lanes 1 and 5, 0 ng/ml; lanes 2 and 6, 1 ng/ml; lanes 3 and 7, 10 ng/ml; lanes 4 and 8, 20 ng/ml. (B) As for A, but using a mutant LLC-PK cell line (5N4) defective in the cAMP-response. Amount of DNase I: lanes 1 and 6, 0 ng/ml; lanes 2 and 7, 1 ng/ml; lanes 3 and 8, 5 ng/ml; lanes 4 and 9, 10 ng/ml; lanes 5 and 10, 20 ng/ml. (C) and (D) as for A but nuclei from uninduced and TPA-induced (100 ng/ml) cells were analyzed with P2 on a *Hin*DIII restriction digest.

and -2.8 kb (Fig. 4D, lane 5). This was achieved by using P1, which is superior to P2 in terms of signal and intensity, and by using a longer and lower percentage agarose gel. The cluster at -0.95 kb, -1.2 kb and -1.5 kb detected with P2 was also detected with P1 but was overshadowed by smearing.

Topo I cleavage sites appear throughout the transcription unit of several genes following induction, presumably to relieve torsional stress (11). To see if the situation is similar for the uPA gene, we mapped Topo I activity on the transcription unit of the uPA gene. The same filter shown in Figure 4A was stripped of P2 and reprobed with P3 to analyze a region of about 15 kb covering almost all of the transcription unit, apart from the first 750 bp of 6.6 kb. Figure 4C demonstrates that transcriptional induction results in the appearance of Topo I cleavage sites throughout the transcribed region.

Multiple cAMP-responsive regions are present in the uPA gene promoter, with a strong site at -2350 to -2530

Since specific regions of the chromatin were altered by cAMP, we wanted to determine if sequences in those regions could mediate a response to cAMP. Previously, the cAMP-responsive sequences in the uPA gene promoter in LLC-PK₁ cells were analyzed by stable transfection assays using constructs containing progressive deletions from -4.6 kb of the uPA gene promoter (21). The same constructs as well as several fragments from the region around -2.6 kb, corresponding to the region where PKA activation induced a HS site, were analyzed using transient transfection assays. Progressive 5' deletions of the uPA gene promoter, -4.6 kb, -3.4 kb, -2.6 kb and -1.7 kb, gave progressively weaker cAMP responses. In contrast, the TPA

Figure 3. DNase I hypersensitive sites in the 3' flanking region of the uPA gene in stimulated and unstimulated LLC-PK₁ cells, liver and kidney. (A) As described in Figure 2, nuclei from cAMP-induced and uninduced LLC-PK₁ cells were analyzed for HS sites, but here we used P3 on a HinDIII restriction digest. (B) As for A but using nuclei from liver and kidney.

response was lost between -2.6 kb and -1.7 kb. Similar cAMP-responsive regions were identified in a previous study in our laboratory using stable transfection assays (21). Both the transient and stable transfection assays suggest that there are multiple cAMP-responsive elements in the uPA gene promoter.

To analyze cAMP-responsive sequences in the -2.6 kb region, a 929-bp *Xba*I restriction fragment from -2.7 kb to -1.7 kb was dissected into five overlapping fragments. Each was ligated to an enhancer-less SV40 promoter linked to a CAT reporter gene (pCATP, Promega) and its responsiveness to cAMP analyzed. cAMP-responsive sequences were localized at -2530 to -2350 (180 bp). The region analyzed, from -2693 to -1768 , contains constitutive silencer and enhancer activity. The presence of many regulatory elements within this region is consistent with it being highly conserved between the porcine and murine uPA gene promoters (29).

Analysis of overlapping fragments in the highly conserved region from -2.7 kb to -1.7 kb shows a complex pattern of *cis*-activity (Fig. 5). Fragment *a* has constitutive silencer activity, which may explain why it does not mediate a response to cAMP, whilst smaller fragments contained within this fragment (*c*, *e*) do induce a response to cAMP. Fragment *d* overlaps with the putative silencer region of fragment *a* but it does not have silencer activity. The 3' region of fragment *d* also overlaps with fragment *b*, which has a higher constitutive enhancer activity than fragment *d*. Thus, the activity of fragment *d* may reflect the interplay of silencer and enhancer elements. The results of previous studies with stable transfection assays also indicate constitutive silencer activity in the region -2693 to -1768 (21) which, in the context examined, may silence the enhancer and cAMP-responsive sequences. In the present study, we localized a silencer to the region -2073 to -2371 and a constitutive enhancer to the region -1768 to -2073 . We have shown that the footprinted region -2009 to -1984 , containing contiguous AP-1-like and PEA3 elements, has basal enhancer activity of two- to three-fold (18) which may account for the enhancer activity observed.

The cAMP responsive region -2530 to -2350 we have identified is adjacent to a cAMP-inducible HS site and is shown in Figure 5. It is about two-fold more responsive to cAMP than a previously characterized region from -3473 to -3324 (21,22) (Fig. 5, compare fragments *e* and *f*). The sequence has several elements similar but not identical to the CRE, and is also characterized by stretches of As and Ts.

DISCUSSION

To identify potential regulatory sites involved in uPA gene expression, we have mapped DNase I hypersensitive sites and Topo I cleavage sites over 26 kb of the uPA gene region, from uninduced, PKA-induced and PKC-induced LLC-PK₁ cells. We found several HS sites 5' of the transcription initiation site and 3' of the poly(A) addition site in both uninduced and induced cells. In contrast, HS sites were conspicuously absent in the transcribed region. Upon cAMP induction, the faint HS site at -2.6 kb became very prominent, the strong site at 1.9 kb downstream of the poly(A) addition site became much weaker, and few new sites appeared within 500 bp 5' of the poly(A) site. PKC induction did not affect the -2.6 kb site, but the 3' sites were modified in a way similar to that following cAMP-induction. As expected, Topo I sites were induced by both inducers throughout the entire transcribed region. In addition, cAMP induction but not PKC induction gave rise to two strong Topo I sites at -2.8 kb and -3.3 kb. Interestingly, these two Topo I sites are very close to the HS sites that we showed by functional analysis to contain cAMP-responsive elements. One of the two HS sites, at -3.5 kb, is adjacent to a cluster of three protein-binding domains, from -3414 to -3342 , that cooperatively confer cAMP inducibility on a heterologous promoter (21,29). The other HS site, at -2.6 kb, which is strongly enhanced by cAMP induction, is adjacent to a cAMP-responsive region, from -2530 to -2350 (Fig. 5). Close association of the Topo I sites and HS sites suggests that they functionally cooperate in cAMP-dependent uPA gene regulation. It also points to a possible role for Topo I in the formation of HS sites or the release of torsional stress following the formation of HS sites.

The HS site at 1.9 kb 3' of the poly(A) addition site has properties suggestive of a cell-specific silencer. The site appears to be more prominent in liver, a tissue that expresses low levels

Figure 4. Topoisomerase I cleavage sites induced by PKA in the 5' and 3' flanking regions and uPA transcription unit. (A) Cells were treated with 0.2% DMSO, 1 mM 8-br-cAMP, 20 mM camptothecin or left untreated for 60 min (lanes 1–4, respectively). Alternatively, the cells were treated with Br-cAMP and then 10 mM or 20 mM camptothecin was added in the last hour of treatment (lanes 5–14). The DNA was extracted from the cells (see Material and Methods), digested with *Hind*III and in single-stranded form was resolved on an alkaline agarose gel and probed with P2 (see Materials and Methods, Figure 1). (B) As for A, but only one time point (1 h); in addition the effect of TPA at 100 ng/ml was analyzed. (C) The filter of experiment A was stripped of P2 and reprobed with P3. The open arrow indicates the poly A addition site. (D) As for A, but P1 was used on a *Bam*HI digest using a longer gel of 0.8%.

template				basal activity*	fold induction*	
uPA gene	5' flanking DNA	promoter			8-bromo-cAMP**	TPA**
-4660	-3473	-2693	-1768	1.7 ± 0.3	5.3 ± 0.4	13.1 ± 3.4
				3.0 ± 0.4	4.4 ± 0.3	11.0 ± 2.2
				3.2 ± 0.6	3.4 ± 0.2	12.1 ± 2.1
				1.2 ± 0.3	2.2 ± 0.3	1.6 ± 0.3
H	R	H		0.4 ± 0.2	1.3 ± 0.2	1.2 ± 0.2
557 bp	b	305 bp		4.1 ± 0.4	2.7 ± 0.5	12.9 ± 2.3
325 bp	c	604 bp		1.2 ± 0.2	8.0 ± 2.3	2.0 ± 0.4
180 bp	e			2.2 ± 0.3	2.4 ± 0.7	14.0 ± 2.0
				1.4 ± 0.3	13.7 ± 2.1	0.7 ± 0.3
		-3473	-3324	1.4 ± 0.2	7.4 ± 1.3	not determined
		none		1.5 ± 0.2	2.7 ± 0.8	1.3 ± 0.2

* nmols × 10⁻² chloramphenicol/mg protein/hour

** 1 mM 8-bromo-cAMP; 100 ng/ml TPA

sequence of fragment e

-2530
 5' - CTGGCTTTGACCTCATCAACTATGTATCTTCACTCCACCTCATTTTTCTCATCAGCAAAACGGATATAATGATTCCCAGCTAGCTCATAA
 3' - GACCGAACTGGAGTAGTTGATACATAGAGTGAGGTGGAGTAAAGAGTAGTCGTTTTCCTATATTACTAAGGGTCAGTGCAGTATT
 -2350
 GGCAGTTGTGAGTTTAAAGAGGAGTGTTTGTATCAGAGTGCTTTTGGCAAAAGATGAAATGAGGTCAGTACCATATTTTATGGCAAGG
 CCGTCAACACTCAAATTTCTCTCACCAACATAGTCTCACGAAACCGTTTCTACTTTTACTCCAGTCATGGTATAAAATACCGTTCC

Figure 5. Analysis of cAMP-responsive sequences in the uPA gene promoter using transient transfection assays. The assays and the plasmids used are described in Materials and Methods. Briefly, the plasmids contained the uPA gene promoter with various deletions linked to a CAT reporter gene; the plasmids with the indicated lengths of 5' flanking region were 4660pCATP, 3473pCATP, 2693pCAT and 1768pCAT. Induction was for 8 h. For a more detailed analysis of the region -2693 to -1768, overlapping fragments within this region were ligated to an enhancer-less SV40 promoter linked to a CAT reporter gene; the induction time was 12–13 h. The sequence of a cAMP-responsive region in the uPA gene promoter between -2350 and -2530 is shown at the bottom of the figure. The data shown are the average of four independent experiments.

of uPA mRNA, and less prominent in kidney, a tissue that expresses high levels of uPA mRNA. Furthermore its intensity decreased following induction of the PKA or PKC pathways, both of which induce the uPA gene. The question of whether this site has silencer function remains to be answered. We have not yet assessed the role of this region by transfection studies. In addition, PKA or PKC induction resulted in the appearance of minor sites at 500 bp 3', 150 bp 3', and 350 bp 5' of the poly(A) addition site. Although the functional significance of these sequences remains to be determined, functional analysis of CAT reporter gene constructs with and without this region suggest that these sequences may be important for high level expression of the uPA gene (Lee and Nagamine, unpublished).

Besides the two cAMP sites at -3.3 kb and -2.5 kb, there are several other cAMP-responsive sequences in the uPA gene promoter of LLC-PK₁ cells; at least five sites were indicated by functional analysis of 4.6 kb of the uPA gene promoter in this and other studies (21,29,30). The levels of cAMP-dependent induction driven either by the entire uPA gene promoter or by fragments containing putative cAMP-regulatory sites were low (at most 10-fold) when tested in either stable or transient transfection assays (21,29,30). In contrast, the endogenous gene was induced by cAMP 60-fold above basal level after 1 h, and remained at 20-fold for at least 24 h (16). In this context, it is noteworthy that while cAMP was a more efficient inducer of the endogenous uPA gene than TPA (16,23,31), both were similarly effective, or TPA was more potent, in the induction of transfected uPA gene constructs (Fig. 5; J.S.L. and D. Pearson, unpublished). Because TPA activates the uPA gene in LLC-PK₁ cells via a single AP-1-like site located at -2.0 kb (18), it may be that TPA-dependent uPA gene induction does not require the extensive chromatin structural alterations observed with cAMP that utilizes multiple elements. The fact that stably transfected uPA gene promoters do not respond to cAMP induction as strongly as the endogenous uPA gene might indicate that transfected genes and the endogenous uPA gene take up different structures.

We do not know whether chromatin structural alterations are an intrinsic requirement for cAMP-dependent gene regulation. Such changes have not been reported for cAMP-induction of other genes. The majority of cAMP-inducible genes studied to date contain only a single cAMP-responsive element or region close to the transcription initiation site (32) and the elicitation of significant structural alteration may not be necessary for the gene to be activated by cAMP. Following cAMP induction, many genes are rapidly repressed whereas the uPA gene is not; the formation of a complex chromatin configuration in the promoter could exclude repressor molecules and prevent the down-regulation of the uPA gene.

Taken together, the following three observations of uPA gene regulation suggest a model whereby cAMP-dependent uPA gene induction is highly dependent on chromatin structural alterations: (i) cAMP-dependent chromatin alterations in the uPA gene promoter, (ii) the presence of multiple cAMP-responsive sites in the uPA gene promoter, and (iii) low levels of responsiveness of these elements out of the context of their chromatin structure. Cooperative interplay between multiple elements spaced several kilobases apart may be required for strong cAMP induction, and the mobilization of all potential regulatory elements may need precise DNA looping or bending, which is possible only when the gene is associated with a host of proteins in a specific configuration.

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