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Purification and cDNA cloning of a transcription factor which functionally cooperates within a cAMP regulatory unit in the porcine uPA gene

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ABSTRACT

One of cAMP-regulatory sites in the porcine urokinase-type plasminogen activator (uPA) gene resides 3.4 kb upstream of the transcription initiation site and is composed of three protein binding domains, FPA, FPB and FPC. Whereas FPA and FPB contain a CRE-like sequence, the FPC sequence is not related to any known protein recognition sequences, yet all three domains are required to mediate cAMP action on a heterologous promoter. To study the functional cooperation among these three domains we purified and cloned a FPC-binding protein (FPCB) from porcine kidney derived LLC-PK$_1$ cells. Sequence comparisons showed that FPCB is homologous to mouse LFB3 and rat vHNF1. LFB3/vHNF1 is related to a liver specific transcription factor HNF1, it recognizes the same sequence as HNF1 and is highly expressed in kidney cells. FPCB and HNF1 recognition sequences are dissimilar, nevertheless both sequences are recognized by in vitro-translated LFB3 and FPCB, indicating that binding to the two different sequences is an intrinsic character of FPCB/LFB3/vHNF1. In HeLa cells, this cAMP-responsive site was inactive whether FPCB was overexpressed or not, suggesting a requirement for an additional cell-specific factor. These results may suggest a mechanism by which hormonal control is integrated into cell-specific gene regulation.

INTRODUCTION

In eukaryotes, the expression of many genes is temporally regulated by transcription factors interacting with specific regulatory elements in the gene. The protein level or activity of a transcription factor is programmed within the cell or by extracellular signals. For instance, homeobox proteins, which are transcription factors and mostly involved in embryonic pattern formation by activating specific genes, are expressed only at very well defined period of time during development (1–3). Other transcription factors are activated when cells are induced by growth-factors, hormones or mitogens (4,5). Various signal transduction pathways have been identified which convey extracellular signals to activate specific transcription factors (6). The expression of the gene is also spatially determined. Many genes are expressed only in a limited types of tissues, and this is again realized through tissue-specific transcription factors (7,8). Accordingly, various genes have been shown to be under the control of both spatial and temporal regulation (9,10). To study the coordination between these two aspects of gene regulation, we investigated cAMP-dependent regulation of the urokinase-type plasminogen activator (uPA) gene in porcine kidney cells.

Several transcription factors have been shown to mediate cAMP-dependent induction (for reviews see (11–13)). Cyclic-AMP responsive element binding protein (CREB) (14) and a number of other proteins related to CREB (15–17) recognize the same or closely related CRE sequences. While the interaction of CREB with CRE is sufficient for cAMP-dependent induction for some genes, there are cases where the gene regulation through the cAMP-dependent signaling pathway or CRE sequence is also modulated by the association and interaction of CREB or a CREB-like protein (14,16–18) with other transcription factors and regulatory proteins (15,19–21).

Urokinase-type plasminogen activator (uPA) gene is regulated by a variety of extracellular signals depending on cell-type (22). We have shown that uPA gene expression is induced through the cAMP-dependent signaling pathway in LLC-PK1 cells (23–25), a cell line derived from porcine renal epithelial cells (26). cAMP-dependent induction of the uPA gene is cell-specific, because cAMP is unable to induce the uPA gene in U937 cells (27), HeLa and COS cells (D.Pearson, unpublished). This observation suggests that cAMP-dependent regulation of the uPA gene is also under the influence of a cell-specific regulatory mechanism.

The porcine uPA gene has a cAMP inducible enhancer 3.4 kb upstream of the transcription start site (25,28). This region, 73 bp in length, is comprised of three protein binding domains, termed FPA, FPB and FPC according to footprinting analyses. FPA and FPB contain a CRE-like element, whereas the sequence of FPC is not homologous with any known protein recognition sequences. The binding activity of nuclear proteins to FPA and FPB is increased by the addition of the catalytic subunit of the cAMP dependent protein kinase as determined by gel retardation studies (25). As previously shown (21,25), the binding of nuclear proteins to FPA and FPB can be efficiently competed by the

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somatostatin CRE, suggesting that CREB or a related protein is interacting with CRE-like sequences in FPA and FPB in LLC-PK_1 cells. Interestingly, however, FPA and FPB alone are unable to fully convey cAMP responsiveness on a heterologous promoter. Full activation is observed only when the FPC is present although FPC by itself produces no effect. Competitive gel retardation analyses suggested a protein-protein interaction between FPC-binding protein (FPCB) and CREB or CRE-like protein (25). Therefore, we have focused our effort on purification and characterization of the FPC-binding protein to understand the mechanism underlying this apparent functional cooperativity between these proteins in cAMP-dependent uPA gene regulation.

MATERIALS AND METHODS

Purification of the FPC-binding protein

All the following procedures were carried out at 4°C. Nuclear extracts were prepared according to Schreiber et al., (29) with some modifications. LLC-PK_1 cells (10^9 cells) were lysed directly on plates with buffer L (10 mM Tris·HCl, pH 8.0, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 0.6% NP-40, 0.5 mM PMSF and 1 μg/ml leupeptin). Lysate was collected and centrifuged at 3000×g for 10 min and the nuclear pellet was rinsed once with buffer L. Nuclei were resuspended in 10 ml of the extraction buffer E (25 mM Tris−HCl, pH 8.0, 450 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 5 mM spermidine, 1 mM PMSF, 1 μg/ml leupeptin and 25% glycerol) and stirred gently with a magnetic stirrer for 30−45 min. After centrifugation at 12 000×g for 10 min, the supernatant of crude nuclear extract was dialyzed against buffer A (20 mM Tris·HCl pH 8.0, 20 mM NaCl, 0.5 mM EDTA, 1 mM DTT and 10% glycerol). The dialysate was then applied to a 10 ml DEAE-sepharose column equilibrated with the same buffer. The FPCB was eluted with a linear gradient of 20 to 500 mM NaCl. The elution of FPC-binding activity was monitored by gel retardation assay, and the active fractions eluting between 150−200 mM NaCl were pooled and dialyzed against buffer A supplemented with 0.1% NP-40. To reduce non-specific binding, 5 μg/ml of calf thymus DNA and 10 μg/ml of poly(dI-dC) (Pharmacia) were added to the sample prior to chromatography on the affinity column. The affinity column was prepared according to Eisenberg et al., (30) and made of Sepharose 4B-CNBr (Pharmacia) to which DNA fragments of FPC 16-mer (head-to-tail) were covalently coupled. The bound fraction was eluted stepwise with 100−800 mM NaCl. The FPC-binding fractions were eluted with 350−400 mM NaCl and concentrated by ultrafiltration (Amicon). A part of the concentrated sample was subjected to Southwestern analysis (see below) and the rest was electrophoresed on 10% SDS-polyacrylamide gel (31) and electroblotted onto a polyvinylidene difluoride membrane (PVDF; Biorad). The amidoblack stained 66 kDa FPC band was excised and digested with trypsin (Boerhinger-Mannheim) for 16 hours at 37°C. Peptides were eluted from the membrane with 80% formic acid and fractionated by reverse phase HPLC C18 Vydac column, followed by gas-phase amino acid sequence analysis (32).

Isolation of FPCB cDNA

Oligonucleotides of degenerated sequences based on two peptide sequences (Table 1) were prepared. Polymerase chain reactions (PCR) were performed using these oligonucleotides and oligo(dT)-primed cDNA synthesized from total poly(A)$^+$RNA from LLC-PK_1 cells. PCR for one orientation (Table 1) produced a fragment of 200 bp. This 200 bp fragment was subcloned into pBluescript KS^- vector (Stratagene) and sequenced. The same fragment was further used to screen a λ-gt11 cDNA library prepared from LLC-PK_1 cells. Inserts from positive phage clones were subcloned in pBluescript KS^- vector and sequenced on both strands by the chain termination method (33) using successive primers. Amino acid and nucleic acid sequences were compared with Swissprot and EMBL/Genbank databases using FASTA program (34). Nucleic acid and deduced amino acid sequences have been deposited in EMBL data library under the accession number X69675.

Gel retardation assays

Fractions containing FPCB (0.05−5 μg protein) were incubated in 20 μl binding reaction mixtures (10% glycerol, 20 mM Tris pH 8.0, 0.5 mM EDTA, 50 mM NaCl, 0.07−0.1 μg/μl of double strand poly(dI-dC), 0.05 μg/μl single-strand DNA and 30 fmoles of radioactive probe) for 15 min at room temperature. Five μl aliquot of the binding reaction were electrophoresed on a 5% native polyacrylamide gel (acrylamide:bisacrylamide = 30:1) in 1×TAE buffer (0.04 M Tris−Acetate, 0.001 M EDTA, pH 8.0). The gels were dried on DE81 paper (Whatman) and exposed to Xomat AR film (Kodak) with an intensifying screen at −70°C. The radioactive probe was prepared by 3′ filling reaction of double stranded oligonucleotides with 5′ protruding ends using ^{32}P-dATP and Klenow fragment.

Southwestern blotting

Samples containing FPC-binding activity were electrophoresed on 10% SDS-polyacrylamide gel and electroblotted on nitrocellulose-membrane BA81 (Schleicher & Schuell). Proteins were denatured by incubating the membrane in 6 M guanidine-HCl, 100 mM KCl and 50 mM Tris·HCl, pH 8.0, for 30 min and then renatured by incubation in 10% glycerol, 25 mM Tris·HCl pH 8.0, 1 mM DTT, 0.2 mM EDTA, 50 mM NaCl, 0.1% NP-40 and 5% non-fat powdered milk for 1 h. Binding reaction was carried out in the renaturation buffer supplemented

Table 1. Sequences of tryptic peptides of purified FPCB and deduced degenerated nucleotide sequences

A.	W	G	P	A	S	Q	Q	I	L	Y	Q	A	Y	D		
					5′-	CAR	CAR	ATH	CTN	TAY	CAR	GCN	-3′			
B.	V	Y	N	W	F	A	N	R								
	3′-CAD	ATR	TTR	ACC	AAR	CGN	CTR	KC-5′								
C.	V	D	V	T	G	L	N	Q	S	H	L	S	Q	H	L	N
D.	A	L	V	E	E	C	N	R	A	E	C	L	Q			

The following degenerate codes were used in the nucleotide sequences: D= A,G or T; H= A,C or T; K= G or T; N= A,G,T or C; R= A or G; Y= C or T.

with 0.25% non-fat powdered milk and 10^6cpm/ml of ^{32}P-labelled FPC-oligonucleotide for 1 h. The membrane was washed with several changes of renaturation buffer minus the milk for 15 min, air dried and autoradiographed as above.

Oligonucleotides and plasmid constructions

Only upper strand sequences of double stranded oligonucleotides used are provided. Synthetic oligonucleotides corresponding to FPA (CTGTGCCTGACGCACAGGAG), FPB (GCCCATGA-CGAACACTGG), FPC (GTGAATGAATAAAGGAATAAA-TGAATGATTTCACA) or combinations of these were inserted into polylinker sites of pGL2-promoter (Genelight plasmids, Promega). In the pGL2-promoter vector the luciferase gene is linked to the SV40 early gene promoter. The RSV-B3FL expression vector (kindly provided by R.Cortese) contained the full length cDNA of LFB3. To derive a FPCB expression vector (pSV-FPCB), coding sequence of FPCB was amplified with Taq DNA polymerase (Perkin-Elmer, Cetus) using 5′-CGAGAAT-TCATGGTGTCCAAGCTCACGTC-3′ and 5′-CTCGGATCC-GGGTCCTTGTTGCTGTGCAC-3′ as primers and the PCR product was subcloned, after digestion with *Eco*RI and *Bam*HI, into the polylinker site of pSG5 (35). Plasmid pT7-CREB (kindly provided by M.R. Montminy) containing the rat CREB cDNA coding sequence, was digested with *Pst*I and subcloned into the *Pst*I site of pSG5 (35), resulting in pSV-CREB. For in vitro translation experiments, coding sequences of both FPCB and LFB3 were amplified by PCR using 5′-CGAGAATTCATGG-TGTCCAAGCTCACG-3′ and 5′-CTCGGATCCGGGTCCTT-GTTGCTGTGCAC-3′ as primers. After digestion with *Eco*RI and *Bam*HI, the PCR products were subcloned into pBGO, a plasmid derived from Bluescript KS$^-$ containing the β-globin leader sequence (kindly provided by P.Matthias).

As probes for gel shift assays and Southwestern analysis several double-stranded oligonucleotides were prepared: FPC (see above); the sequence encompassing rat albumin promoter HNF1 recognition sequence (36), 5′-TCGAGTGTGGTTAATGAT-CTACAGTTA-3′; FPI which is a composite cis-elements for Ets and AP1 at 2 kb upstream of the uPA gene, AATTCGTCCAA-GAGGAAATGAGGAGATCCTG; 36 bp oligonucleotide as a non-specific competitor, 5′-AATTCTCAAGATCCGATTA-GGCAATCCATCG-3′. All these oligonucleotides were flanked with *Eco*RI or *Xho*I linker and labeled using DNA polymerase I-Klenow fragment.

Transfections and luciferase assays

Eighteen hours prior to transfection, 0.8×10^6 LLC-PK_1 cells were seeded in 36 mm plastic dishes with 5 ml of DMEM containing 10% FCS. Aliquot of 1 μg of various luciferase constructs were cotransfected with 1 μg of pSV-βGal (Promega, a vector expressing the β-galactosidase) and 1 μg of pBluescriptKS$^-$ to adjust the total amount of DNA to 3 μg. In some experiments, 0.5 μg of pRS-B3FL, pSV-FPCB or pSV-CREB were cotransfected with luciferase gene constructs and pSV-βGal. All transfection were carried out using the DEAE-dextran method (37). Eighteen hours after transfection, cells were treated for 8 hr with or without 1 mM 8-bromo-cAMP, and cell extracts were prepared by lysing cells with 300 μl of lysing buffer (1% Triton X-100, 25 mM Glycylglycine pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA and 1 mM DTT). Fifty to 100 μg of protein were used for the assay, and the luciferase activity of each sample was normalized against β-galactosidase activity. To measure luciferase activity, each sample was suspended in 300 μl of solution containing 25 mM glycylglycine buffer pH 7.8, 5 mM ATP and 0.2 mM luciferin and the activity was quantitated in a luminometer (Autolumat LB 953, Berthold). The β-galactosidase activity was measured according to Jain et al. (38).

Northern blotting

FPCB and HeLa cells mRNA were analyzed by Northern blot hybridization according to Ziegler et al. (39). Five micrograms of total RNA of both cell lines were resolved on a 1% formaldehyde-agarose gel and transferred onto a charged-nylon membrane. The blot was then probed with ^{32}P-labelled FPCB-DNA probe.

RESULTS

Cooperation among three protein binding domains in a cAMP regulatory site in the uPA gene.

We have previously shown that one of cAMP regulatory sites in the uPA gene promoter resides 3.4 kb upstream of the transcription initiation site (25). This site is composed of three

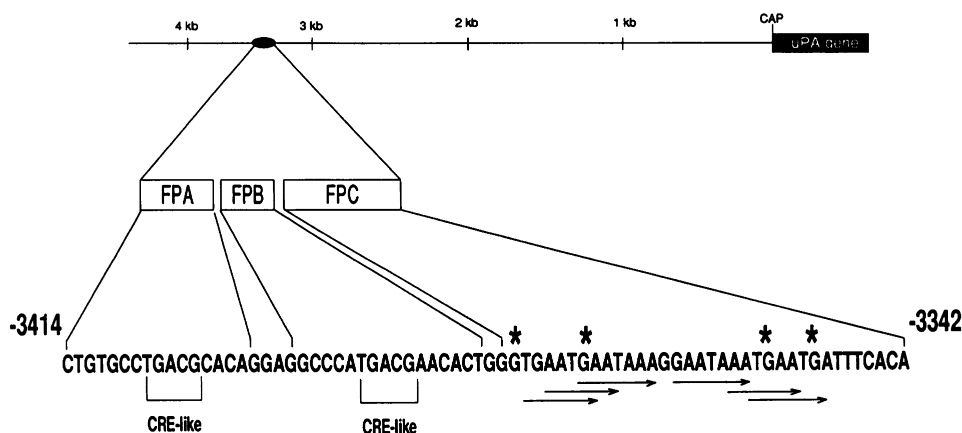

Figure 1. Sequence of −3.5 kb cAMP-responsive site in the uPA gene promoter. A, B, and C denote footprints, FPA, FPB and FPC, respectively (25). Arrows under FPC show three different sets of repeats. Asterisks on the FPC domain shows protein interacting G residues.

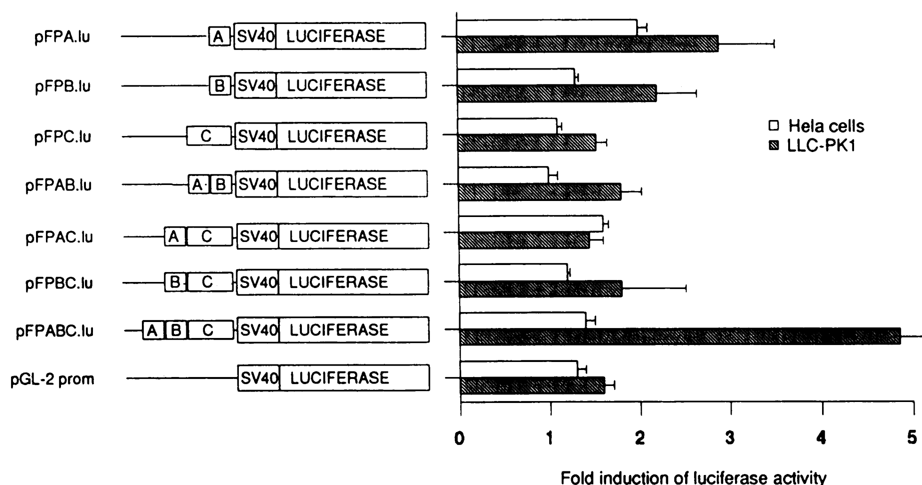

Figure 2. Cooperation between three footprint sequences in cAMP-induced gene expression. LLC-PK_1 or HeLa cells were transiently transfected with different plasmids containing FPA, FPB or FPC in various combinations upstream of the promoter (left panel) and induced with 1 mM 8-Br-cAMP for 8 hours. The induction is relative to luciferase activity detected in untreated transfected cells. Experiments were repeated three times, each in duplicate, with similar results. A typical result is shown here.

protein binding domains, termed FPA, FPB and FPC, of which two contiguous domains contain a CRE-like sequence but the third domain is not homologous with any known protein binding sequences (Figure 1). Functional analysis of these domains by stable transfection of various CAT gene-linked constructs showed cooperation among three domains (25). By transient transfection experiments with luciferase gene-linked constructs we obtained essentially the same results using LLC-PK_1 cells; full cAMP-dependent inducibility was observed only when FPC was associated with both FPA and FPB, and FPC by itself does not convey cAMP responsiveness (Figure 2). Interestingly, when these constructs were tested in HeLa cells, FPC did not show such cooperativity to FP-AB (=FPA+FPB), suggesting that the functional cooperation among three domains is cell specific (Figure 2). We observed the same lack of cAMP-mediating activity for FP-ABC in COS, HT1080 and F9 cells. This suggests that cAMP-dependent induction via FP-ABC requires cell specific transcription factors. To elucidate the mechanism underlying this cell-specific cooperation among transcription factors involved in cAMP-dependent gene regulation, we focused our effort on purification and characterization of FP-C-binding protein. Due to the homology between FPA + FPB to CRE consensus sequence, we expected the protein binding to these sites to belong to the CREB/ATF family (12,16,17,20).

Purification of Footprint C binding protein

Gel retardation analysis using crude nuclear extracts from LLC-PK_1 cells and FPC oligonucleotide as a probe, we observed a single distinct band (Figure 3, lane 1) which was competed with molar excess of the identical unlabelled oligonucleotide (lane 2) but not with a nonspecific oligonucleotide (lane 3). The band was not competed with FPI nor with FPA and FPB oligonucleotides (not shown). These data indicate that a specific protein interacts with FPC.

Starting from crude nuclear extracts, FPCB was purified by DEAE-Sepharose column chromatography followed by two cycles of FPC-specific affinity column chromatography (for details see Methods). Approximately 10 μg of FPCB were recovered from 30 mg (protein) of crude nuclear extracts. The FPCB protein was determined to have an apparent molecular weight of 66 kD by SDS−PAGE (Figure 4, lane 2) and was confirmed to be the FPC-binding protein by Southwestern analysis (Figure 4, lane 3), and by gel elution-renaturation and gel shift analysis (data not shown). When the binding reaction was carried out using purified FPCB fractions in non-saturating conditions, i.e. in the absence of non-specific oligonucleotide or double stranded poly(dI-dC) as competitor, we observed an additional band migrating slower than that obtained using crude nuclear extracts (Figure 3, lane 4−8). This slow migrating band, which is less stable than the fast migrating band, may be a

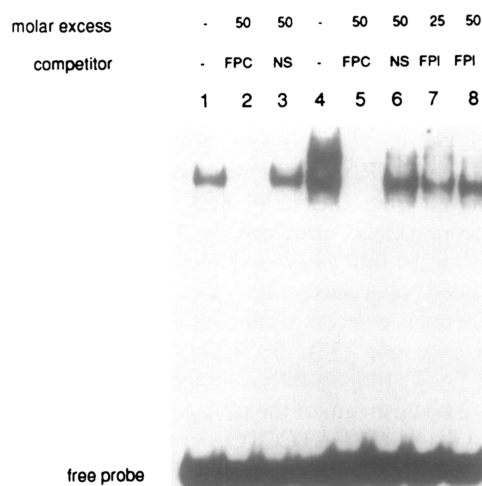

Figure 3. Presence of specific FPC-binding protein. FPC binding activity was tested by gel-shift assay using crude nuclear extracts (lanes 1−3) from LLC-PK_1 cells and purified FPCB protein (lanes 4−8). Binding was competed with molar excess of different oligonucleotides shown on top of the lane. Double stranded poly(dI-dC) was omitted in lanes 4−8.

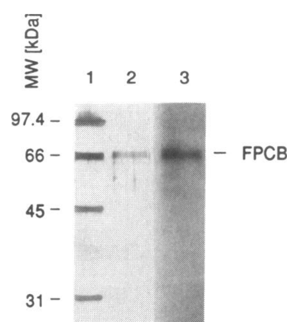

Figure 4. SDS–PAGE of purified FPCB protein and corresponding southwestern blot analysis. Purified FPCB protein was fractionated by SDS polyacrylamide gel electrophoresis and silver stained (lane 2) or processed further by Southwestern analysis for FPC-binding activity (lane 3). Lane 1, Low molecular weight markers (Biorad).

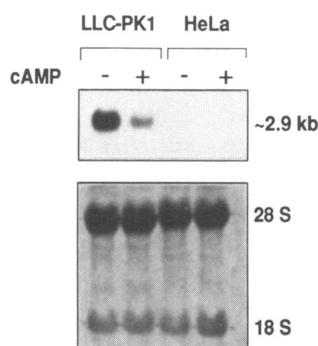

Figure 5. Cell-specific expression of FPCB mRNA and the effect of Br-cAMP on its level. Five μg of total RNA were analyzed by Northern blot analysis. Total RNA was prepared from LLC-PK1 cells (lanes 1 and 2) and HeLa cells (lanes 3 and 4) with (lanes 2 and 4) or without (1 and 3) 1 mM Br-cAMP pretreatment for 2 h. Lower panel shows the staining of the filter with methylene blue after blotting.

multimer of FPCB, because this upper band appeared only when protein concentration was high and the purity of the FPCB preparation was >90%, estimated by silver staining of an SDS polyacrylamide gel electrophoresis of the sample (Figure 4, lane 2).

cDNA cloning of FPC-binding protein

Approximately 7 μg of purified FPCB was digested with trypsin as described in Methods. The amino acid sequence of the four tryptic peptides (Table 1) shared a high homology (88–100%) with LFB3, a recently described member of the HNF1-family (40). This protein is the mouse homologue of the rat vHNF1 or HNF1β (41,42). From amino acid sequences of tryptic peptides A and B, two sequence-degenerated oligonucleotide primers were prepared, assuming that peptide B was downstream of peptide A in comparison with the amino acid sequence of LFB3. The PCR amplification of cDNA prepared from polyA$^+$RNA of LLC-PK_1 cells produced a 200 bp fragment, which was subcloned and sequenced. The nucleotide sequence analysis of this fragment confirmed the homology of FPCB with LFB3 and vHNF1. Using this 200 bp fragment as a probe, a λgt11 cDNA library of LLC-PK1 cells was screened. Fourteen clones were obtained and the one containing the longest insert (2.9 kb) was subcloned into pBluescript, and both strands were sequenced. It was confirmed to encode FPCB by the presence of sequences of the tryptic peptides C and D. Four other clones were partially sequenced and all of which were contained in the longest clone. Sequence homologies of nucleotide and derived amino acid sequences with LFB3 and vHNF1 were 91% and 93%, respectively.

FPCB cDNA sequence and mRNA expression

The strong similarity of FPCB with LFB3 and vHNF1 in nucleic acid sequences and in amino acid sequences (94% and 95%, respectively), highly suggests that FPCB is the pig counterpart of the mouse LFB3. FPCB has an additional glutamic acid residue at position 306 but misses the histidyl residue at position 425 (not shown; the sequence is deposited in EMBL databank) in comparison to LFB3 and vHNF1. This additional amino acid residue is located within the homeodomain with respect to vHNF1. FPCB has an additional glutamic acid residue at position 404 compared to vHNF1. Furthermore, a stretch of amino acids (424–431) of the FPCB is missing in LFB3, but still present in vHNV1. It remains to be determined whether these differences have any biological significance. Another interesting feature is the absence of the classical polyadenylation signal AATAAA (43,44). The length of this cDNA clone (2.9 kbp) corresponds well with that deduced from Northern blot hybridization (Figure 5) and probably represents the full length cDNA. Absence or different putative polyadenylation signals have been reported in HNF-1 and vHNF-1 cDNA (36,41).

Reflecting the cell-specific cAMP regulation, FPCB mRNA is constitutively expressed in LLC-PK1 cells but not in HeLa cells (Figure 5). Interestingly, the level of FPCB mRNA was drastically decreased after 2 h of Br-cAMP treatment in LLC-PK_1 cells, suggesting a mechanism of negative feedback of cAMP-dependent uPA gene induction. Negative feedback had been suggested by the time course of uPA mRNA induction, in which the accumulation of uPA mRNA leveled off after 2 h of Br-cAMP treatment (23).

FPCB and LFB3 recognize both HNF1-binding and FPC DNA sequences

LFB3/vHNF1 recognizes the HNF1-recognition sequence, GGTTAATNATTAACA (8). The fact that the amino acid sequences of both FPCB and LFB3/vHNF1 are almost identical, suggested that they could recognize both HNF1-recognition and FPC sequences. To test this possibility, coding sequences of the FPCB and LFB3 proteins were inserted into pBGO and in vitro transcription–translation reactions were performed. Gel retardation experiments were then performed using one μl of the reaction products and a radioactive oligonucleotide of FPC or HNF1 recognition sequence as a probe. The two proteins recognized both probes (Figure 6, lanes 2 and 5, 9 and 12) and binding to each probe was efficiently competed by a molar excess of unlabelled oligonucleotide of either sequence but not by a non-specific 36 bp competitor (Figure 6, lanes 3–4, 6–7, 10–11 adn 13–14), indicating that they specifically recognize the FPC and HNF1 recognition sequences. When either FPC or HNF1 oligonucleotide was competed with the non-specific oligonucleotide, the binding increased, which might be the result of competition for non-specific binding protein (Figure 6, lanes

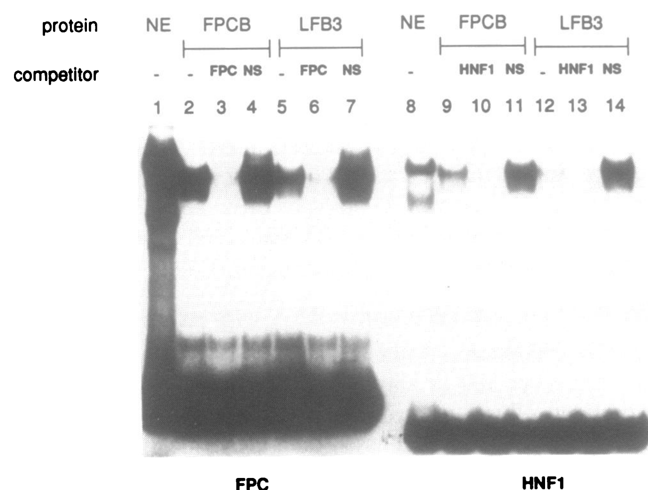

Figure 6. FPCB and LFB3 specifically recognize both FPC and HNF-1 recognition sequences. Gel shift analysis was performed using crude nuclear extracts (NE: lanes 1 and 8), in vitro translated FPCB (lanes 2–4 and 9–11) or in vitro translated LFB3 (lanes 5–7 and 12–14) in the presence of molar excess (50-fold) of various competitors as shown on top of the lane. Probes used are indicated at the bottom of each panel.

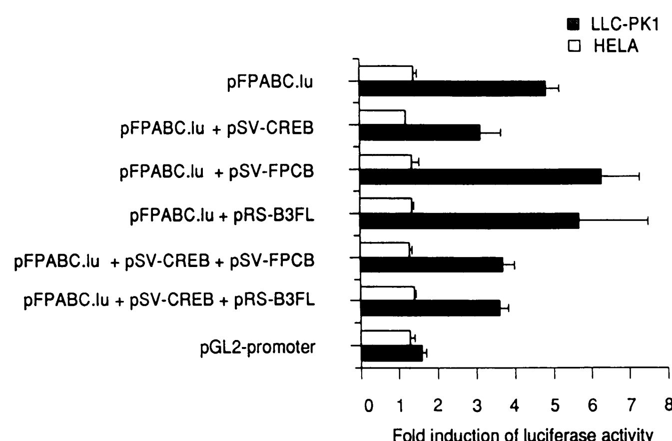

Figure 7. Effects of the overexpression of CREB, FPCB and LFB3 on cAMP-dependent induction of pABC.lu. LLC-PK_1 and HeLa cells were transiently cotransfected with pABC.lu and CREB and/or LFB3 expression vectors. Results are mean values of five independent experiments.

4 and 7, 11 and 14). Interestingly, the retarded bands of in vitro translated protein migrated slightly faster than the band obtained using the LLC-PK_1 cells nuclear extracts (Figure 6, lanes 1–2 and 8–9). This might be due to in vivo posttranlational modifications, such as glycolsylation, which has been reported for HNF-1 (45) and vHNF1 (41).

There are three sets of repeated sequences in FPC (Figure 1). We have shown by methylation interference experiments that four guanosine residues in FPC are involved in DNA–protein interactions (25), suggesting that FPCB recognizes the second set of repeat to bind FPC. The protein was unable to recognize any single unit of these repeats (not shown).

Neither CREB nor ATF2 is interacting with FPCB

We have shown that all three DNA sequences (FPA, FPB and FPC) must be present to convey the full response to cAMP. Assuming that FPA and FPB-binding proteins are CREB-related proteins, we expected that overexpression of CREB in LLC-PK_1 cells would increase the luciferase activity. However, when the CREB expression vector (pSV-CREB) wascotransfected with the luciferase reporter gene containing the FP-ABC sequences (pABC.lu), the luciferase activity was decreased by 35% (Figure 7). Similar decreasing effects were also observed using other expression vectors for CREB-related proteins such as ATF2 and CELF (16,46) (data not shown). Furthermore, no effect of these vectors was observed on the luciferase construct containing only FP-AB. In contrast, when either the FPCB or LFB3 expression vector (pSV-FPCB or pRSV-B3FL, respectively) was cotransfected with pABC.lu, the luciferase activity increased by 23% of the activity obtained with pABC.lu alone. This inhibitory effect of CREB was slightly overcome by coexpressing FPCB or LFB3, but the induction did not reach the basal level produced by pFPABC.lu alone. The overexpressed CREB was confirmed to be active by cotransfecting –71CAT, a CAT gene construct containing somatostatin gene CRE (47), and pSV-CREB into LLC-PK_1 cells. Overexpression of CREB increased cAMP inducibility from 6 to 17-fold (not shown). These results suggest that there is a cooperative binding among the three different footprint-binding proteins and that the FPA and FPB binding proteins are probably different from CREB. Moreover, CREB mRNA and protein were not detected, either by Northern or by Western blot analysis (data not shown).

DISCUSSION

One of the cAMP responsive sites in the uPA gene is located 3.4 kb upstream of the transcription initiation site and is composed of three protein binding domains, FPA, FPB and FPC. All three domains are necessary to confer cAMP responsiveness on a heterologous promoter in a cell-specific manner, suggesting functional and cell-specific cooperation among three domains. FPA and FPB contain a CRE-like sequence and the binding of nuclear proteins to these sites is enhanced by a catalytic subunit of protein kinase A and is efficiently competed by somatostatin CRE (25). Interestingly, the FPC sequence does not contain a sequence related to any known protein binding motifs. Expecting that the protein binding to FPA and FPB is one of CREB/ATF family, we directed our effort towards the identification of the FPC-binding protein (FPCB) to further investigate the functional interaction between these three domains. The purified FPCB we obtained was partially sequenced. Based on some of these sequences we obtained a cDNA clone for FPCB mRNA. Deduced amino acid sequence of the cDNA clone contains all other peptide sequences not utilized in screening for FPCB clones. That the clone encoded FPCB was further confirmed by the binding of in vitro transcription–translation product to FPC. The sequence analysis showed that FPCB is the porcine equivalent of mouse LFB3 or rat vHNF1.

LFB3/vHNF1 is highly related to liver specific transcription factor HNF1 (or HNF1α) and recognizes the same sequence as HNF1. Accordingly, we found that both FPCB and LFB3 recognize the FPC and HNF1-recognition sequences. The consensus HNF1-recognition sequence is a dyad symmetrical sequence (8), whereas FPC is comprised of three overlapping sets of direct repeats, raising an interesting possibility that LFB3/vHNF1 can bind two different sequences and take up different configurations of dimerization on DNA. Depending on

the mode of dimerization, LFB3/vHNF1 may exhibit different functions: when it dimerizes with two-fold symmetry it may act as a transcription factor in its own right, such as shown in albumin gene regulation in liver (40,41); when it dimerizes head-to-tail, it may act as a modulator of other transcription factors, such as shown in this work. In this context it should be mentioned that binding of LFB3/vHNF1 to two different sequences is not due to the fact that there is a heterogeneity of the these proteins, because bacterially expressed recombinant FPCB and LFB3 also bound to both sequences.

LFB3/vHNF1 (FPCB) is highly expressed in kidney cells (40,42), suggesting that it plays a role in kidney-specific gene expression. Through functional interaction, LFB3/vHNF1 would confine the regulation by general transcription factors in kidney cells. During induction of the uPA gene by a peptide hormone calcitonin or cAMP in LLC-PK_1 cells (23,24), FPCB might provide a converging point for tissue- and hormone-specific regulation. Thus, a tissue-specific manifestation of hormone action would be ensured not only by a tissue-specific receptor for the hormone but also by a tissue-specific modulator of a transcription factor. Although uPA is so far the only gene which is regulated by FPCB in kidney cells, the high level of FPCB mRNA and proteins found in these cells suggests that there may be target genes for FPCB other than uPA. It is interesting to note that the mouse uPA gene does not contain a pig-equivalent cAMP responsive enhancer at the corresponding region of the promoter (48). It may reflect the fact that in mouse kidney, unlike pig kidney, uPA mRNA level is constitutively high, suggesting that the mouse uPA gene is not under temporal regulation and therefore does not need to respond to hormonal stimuli (P.-A. M. and F. Botteri, unpublished).

Cotransfection experiments suggested that tissue-specific hormonal regulation by the cAMP responsive site at 3.4 kb upstream of the uPA gene promoter is not solely dependent on the availability of FPCB. Involvement of FPCB in cAMP responsiveness of this far upstream enhancer was confirmed by cotransfecting an expression vector for FPCB or LFB3 with the pABC.lu reporter gene. In LLC-PK_1 cells, luciferase gene expression was augmented by overexpressing FPCB or LFB3. In this experiment the extent of enhancement was less than two-fold. Since FPCB mRNA is constitutively expressed in high amounts in these cells, FPCB may not be a limiting factor. On the contrary, in HeLa cells, luciferase gene was not at all induced by cAMP whether or not FPCB or LFB3 were overexpressed. Surprisingly, overexpression of CREB, ATF2 or CELF did not enhance luciferase gene induction by cAMP with or without FPCB overexpression in both cells. In fact, it slightly but reproducibly reduced the expression of luciferase gene. CREB was functional as a cAMP responsive transcription factor, as overexpression of CREB enhanced somatostatin-CRE-mediated reporter gene expression in LLC-PK1 cells. This reporter gene was refractory to cAMP induction in HeLa cells even with overexpression of CREB. Apparently it is not CREB nor ATF2 that interacts with CRE-like sequences in FPA and FPB and mediates cAMP action in LLC-PK_1 cells. It may be argued that the reason why FPCB overexpression does not restore cAMP responsiveness in HeLa cells is that FPA and FPB binding proteins are also tissue specific. We do not know how specifically FPA- and FPB-binding proteins are distributed among different tissues. We cannot exclude the possibility that a tissue-specific cofactor is required to increase the affinity of FPCB to DNA that interact with FPCB or mediates the interaction between FPCB and FPA- and FPB-binding proteins. This possibility is worthwhile to consider in the light of the fact that FPCB-DNA complex migrates differently in gel-shift assays depending on whether the source of FPCB was crude nuclear extracts or purified fractions. Crude extracts produced a slower migrating complex than purified fractions, suggesting that crude extracts contain a protein that can associate with FPCB when it is bound to DNA. From the analogy of DCoH (49), which interacts with HNF1, and nuclear factor 1-like protein, which interacts with CREB (50), it would be interesting to see if there is a related protein interacting with FPCB.

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