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Macromolecular interaction on a cAMP responsive region in the urokinase-type plasminogen activator gene: a role of protein phosphorylation

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ABSTRACT

We have studied the regulation of urokinase-type plasminogen activator gene expression by cAMP in LLC-PK$_1$ cells. We found a cAMP responsive region 3.4 kb upstream of the transcription initiation site, which comprised three protein-binding domains designated A, B, and C. Domains A and B both contain a sequence, TGACG, homologous to a consensus cAMP response element (CRE; TGACGTCA). Effective cAMP-mediated induction was achieved when these two domains were linked with domain C, which by itself did not confer cAMP responsiveness to a heterologous promoter nor contained CRE-like sequence, suggesting a functional cooperation among these domains. Results of competition studies using gel retardation and DNase I footprinting assays suggest that there is a protein-protein interaction between a CRE binding protein and a domain C binding protein. In gel retardation assays, binding of a nuclear protein to domains A and B was strongly augmented by addition of the catalytic subunit of cAMP-dependent protein kinase, whereas the protein binding to domain C was slightly inhibited, suggesting that protein phosphorylation is involved in the regulation of protein-DNA interaction.

INTRODUCTION

Cultures of LLC-PK$_1$ pig kidney epithelial cells express increased levels of urokinase-type plasminogen activator (uPA) mRNA after treatment with the cAMP elevating hormone, calcitonin. The induction of mRNA is mainly due to the transcriptional activation of the gene (1, 2), and does not require de novo protein synthesis (1, 3), suggesting that the factors mediating the response are present before induction. To elucidate the molecular mechanism of cAMP-mediated regulation of uPA gene expression it is necessary to understand both the signal transduction pathway for this system and the biochemical characteristics of the factors involved.

A palindromic consensus sequence for the cAMP response element (CRE; TGACGTCA) has been previously identified in many genes whose expression is modulated by cAMP (4–10). The corresponding binding protein (CREB) has been purified (11) and the cDNA encoding the CREB has been cloned (12, 13). It is unclear if this 8 base pair (bp) sequence alone is sufficient for cAMP-mediated regulation of gene transcription, as there are contradictory reports that support both arguments (4, 7). It is also unclear how CREB mediates the cAMP response (14), although cAMP-dependent protein phosphorylation seems to be crucial in cAMP-dependent gene regulation (15–21). In contrast to other cAMP regulated genes, the uPA gene contains multiple cAMP responsive regions located over several kilobases of 5′ flanking region. In this report we show that one of these cAMP responsive sites, located at 3.4 kb upstream of transcription initiation site, is composed of three protein-binding domains. Protein-protein interaction on these domains seems to play an important role in mediating cAMP action on uPA gene induction.

MATERIALS AND METHODS

Cell culture and nuclear extract preparation

LLC-PK$_1$ cells were derived from pig kidney epithelia (22) and cultured in Dulbecco modified Eagle medium (DMEM; Gibco Diagnostics) supplemented with 10% (vol/vol) fetal calf serum (FCS), 0.2 mg of streptomycin per ml and 50 U of penicillin per ml in a humidified incubator at 37°C with 6% CO_2. Nuclear extracts (NE) were prepared according to Dignam et al. (23) and modified for LLC-PK$_1$ extract as described previously (24).

Materials

The catalytic subunit (C-SU) of cAMP-dependent protein kinase (cAMP-PK) was a gift of Dr. D. Walsh (University of California, Davis, USA). It was isolated from bovine heart and purified as described (25). DNA restriction and modification enzymes were purchased from New England BioLabs, Genofit, and Biofinex. DNase I was from Sigma Chemical Co., RNase T1 was from

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Boehringer Mannheim, and RNase A was from Sigma Chemical Co. The plasmids pSP64 and SP6 RNA polymerase were from Promega Biotec. G418 was purchased from GIBCO and Hygromycin B was from Boehringer Mannheim. Synthetic salmon calcitonin was a gift of S. Guttman (Sandoz AG, Basel), 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) was purchased from Pharmacia and 8-bromo-cAMP (Br-cAMP) was from Sigma Chemical Co. [α-^{32}P]-dCTP and [α-^{32}P]-ddATP were from New England Nuclear and [α-^{32}P]-rGTP was from Amersham.

Clones

The parental plasmid CAT4660 was constructed by the following procedures. The *Eco*RI fragment of a pig uPA genomic phage clone, λYN4 (26), which contains entire coding region and 4.66 kb and 0.5 kb of 5′ and 3′ flanking regions, respectively, was subcloned into the *Eco*RI site of pBR322. Then, the protein coding region that begins in the second exon and ends in the eleventh exon was removed by *Xba*I digestion and replaced with the chloramphenicol acetyltransferase (CAT) gene prepared from pCM-7 (Pharmacia). This resulted in the uPA-CAT hybrid gene denoted as CAT4660. To obtain 5′ flanking deletion templates, we removed a portion of CAT4660 by double digestion with *Nde*I, which cuts pBR322 at the site near the 5′ end of the hybrid gene, and a relevant enzyme which cuts the hybrid gene in the 5′ flanking region. The digests were blunt-ended with Klenow fragment, ligated to *Hin*dIII linkers and recircularized under dilute conditions. The vector pSV3*gpt* was constructed by digesting pSV2*gpt* (27), in which xanthine-guanine phosphoribosyltransferase (XGPRT) gene (*gpt*) expression is driven by the SV40 early gene promoter, with *Pvu*II and *Nsi*I, which removed the 72 base pair repeats. The plasmid was then recircularized using a *Pvu*II-*Nsi*I adapter. The pSV3*gpt-Xho/Nsi* series were constructed by subcloning the 149 bp *Xho*I-*Nsi*I fragment (position −3473 to −3320) of the pig uPA gene into the *Xho*I-*Pst*I sites of a derivative of pUC 18 plasmid. The fragment was then excised by *Eco*RI and *Hin*dIII digestion. The fragment was blunt-ended with Klenow and inserted into either the *Pvu*II or *Bam*HI site of pSV3*gpt*, which had been blunt-ended with Klenow polymerase.

Stable Transfection

Eighteen hours prior to transfection, 10^6 LLC-PK$_1$ cells were seeded in 100-mm plastic dishes with 10 ml of DMEM containing 10% FCS. The next day, 6−10 μg uPA-CAT or uPA-*gpt* chimeric plasmids that had been linearized with *Pvu*I were co-transfected with 0.5 μg of either pSV2*neo* (28) or pX343, a vector expressing hygromycin phosphotransferase, by the calcium phosphate precipitation method (29). Transfections were normalized to 10 μg total DNA with pBR322, After 24 h, cells were replated at a density of 2×10^5 per 100-mm dish in 10 ml of selection medium. After approximately 14 days of drug selection, several thousand colonies were pooled and expanded.

RNA Isolation and measurement of specific mRNAs

1×10^6 transfected cells were plated on 60 mm dishes and were allowed to grow for 2 days. The medium was then changed to DMEM (plus 10% FCS) containing 100 ng/ml salmon calcitonin (SCT) or 1mM 8-Br-cAMP and incubation was continued for the times indicated. Total RNA was then isolated according to the method of Chomczynski and Sacchi (30). RNAase start mapping (31) was used to quantitate correctly initiated transcripts. The vectors providing RNA probes for start mapping were prepared by inserting appropriate gene fragments containing transcription initiation sites into the polylinker site of either pSP64 or pGEM2 plasmid. These plasmids were linearized, and uniformly labeled RNA probes were prepared by transcribing with SP6 RNA polymerase in the presence of a radioactive precursor. Total RNA (25 μg) was hybridized at 45°C with 50,000 cpm of SP6 generated RNA probe in 10 μl hybridization buffer (80% formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl and 1 mM EDTA). After 16 h, the solution was mixed with 300 μl RNase digestion buffer (0.3 M sodium acetate, pH 7.0, 1mM EDTA, 10 μg/ml RNase A, and 1 μg/ml RNase T1) and incubated at 37°C for 1 h. The digest was extracted with phenol-chloroform (1:1), ethanol precipitated and subjected to electrophoresis in a 5% acrylamide sequencing gel. The gels were transferred to Whatman 3 MM paper, dried and exposed to X-Omat AR film (Kodak) at −70°C with an intensifying screen. Quantitation of specific transcription was done by scanning the autoradiogram by a Shimadzu CS-930 densitometer.

Probes for gel retardation and footprinting assays

A 780 bp *Xho*I-*Xba*I fragment of the uPA 5′ flanking region was subcloned into pUC18. The plasmid was digested with *Xho*I, labeled (3′-end) with Klenow fragment, recut with *Xba*I and the fragment then purified by gel electrophoresis. The subcloned (pUC18) 149 bp *Xho*I-*Nsi*I fragment was cut with *Eco*RI or *Hin*dIII, 3′ endlabeled with Klenow fragment and recut with *Hin*dIII or *Eco*RI. Cloning of plasmids, 3′ endlabeling and fragment isolation procedures were performed following standard techniques (32).

Double stranded DNA oligonucleotides were obtained by annealing the chemically synthesized complementary strands as previously described (24). The following oligonucleotides were used (only the upper strand): 24mer oligo A, 5′-AATTCTGTGCCTGACGCACAGGAG-3′ harboring footprint A (−3414 to −3395); 26mer oligo B, 5′AATTCGGAGG-CCCATGACGAACACTG-3′ harboring footprint B (−3398 to −3380); 45mer oligo C, 5′-AATTCCTGGGTGAATGAAT-AAAGGAATAAATGAATGATTTCACAG-3′ harboring footprint C (−3379 to −3342); 42mer oligo A+B, 5′-AATTCT-GTGCCTGACGCACAGGAGGCCCATGACGAACACTGG-3′ harboring footprint A+B (−3414 to −3378); 33mer oligo Som-CRE, 5′-AATTCGCCTCCTTGGCTGACGTCAGAGA-GAGAG-3′ harboring the Somatostatin gene promoter region −60 to −32 (25); 42mer oligo hCG-CRE, 5′-AATTCAAATT-GACGTCATGGTAAAAATTGACGTCATGGTAAG-3′ harboring the human α-subunit chorionic gonadotropin gene promoter region −145 to −110 (7); 28mer oligo uPA-Sp1, 5′-GATCCAGGCTAGGGCGGGGCCAGGGCTG-3′ harboring the uPA gene promoter proximal Sp1 site (−67 to −43); 40mer oligo, 5′AGCTTCACTTCCACTCTTCTGTCTAACTCATCT-CCGACTA-3′ random sequence.

DNAase I footprinting

DNase I protection experiments were performed according to von der Ahe et al.(24).

Methylation interference assay

Single stranded oligonucleotides were 3′ endlabeled with terminal transferase and [α-^{32}P]-ddATP, and annealed to a complementary oligonucleotide. The resulting double stranded DNA was purified by electrophoresis on 20% polyacrylamide gels. Labeled probes (oligos A and B: 32 ng; 20 pmol; oligos

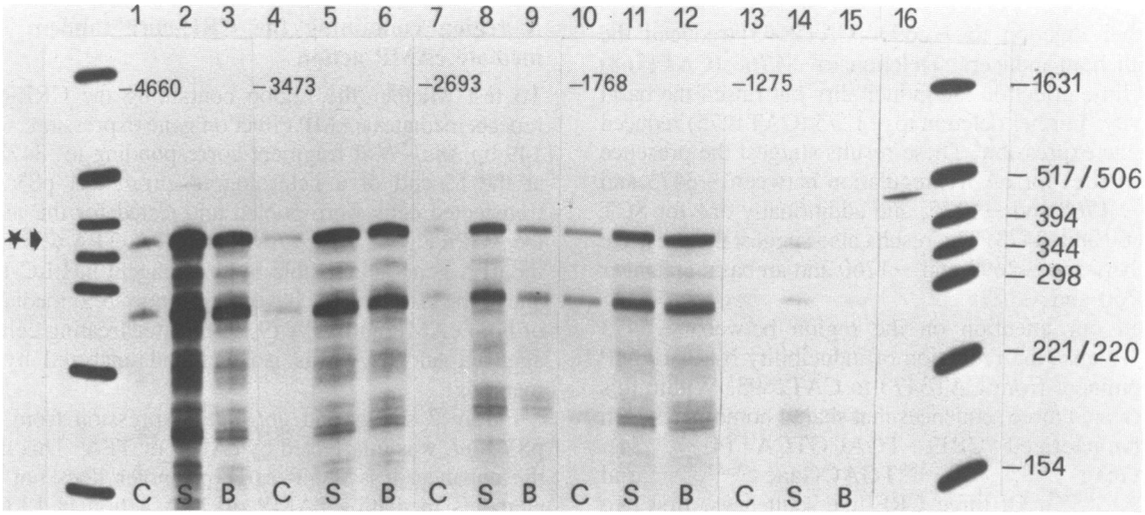

Fig. 1. Effects of 5′ deletion of the uPA gene on cAMP-mediated induction. Cells were treated with 100 ng/ml SCT or 1 mM 8-Br-cAMP for two hours and total RNA then isolated. 25 μg of total RNA was analyzed for specific transcripts by RNA start mapping as described in MATERIALS AND METHODS. Total RNA was prepared from untreated cells (C), or cells treated with 100 ng/ml SCT (S), or 1 mM 8-Br-cAMP (B). The 5′ end of each hybrid gene is indicated at the top of each group of three lanes. Molecular weight markers (*Hin*fI digests of pBR322) were run in parallel. The star indicates the expected size of accurately initiated transcripts.

(A+B) and C: 60 ng; 20 pmol) were methylated by dimethylsulfate for 6 min at 23°(33) and then incubated with 30 μg of LLC-PK$_1$ nuclear extract (NE) in a large scale binding reaction for 25 min at 25°C. Samples were then loaded onto a preparative DNA-binding gel (see gel retardation assay). After 2 h autoradiography, the bands corresponding unbound and protein-bound forms were separately recovered, cleaved with piperidine and analyzed on sequencing gels.

Gel retardation assay

Gel retardation experiments were performed according to von der Ahe et al. (24), with some modifications. Radiolabeled probe (100 fmol; 20,000 cpm) was incubated with various amounts of LLC-PK$_1$ NE (1−12 μg). Following binding, the DNA-protein complexes were resolved on a 6% native polyacrylamide gel (acrylamide to bisacrylamide weight ratio of 30:1) in 3-fold diluted TBE buffer (1×TBE: 89 mM Tris-HCl, 89 mM H_3BO_3, 2.5 mM EDTA, pH 8.3). Gels were pre-run in TBE buffer for 30−60 min at 7 V/cm and run at 12 V/cm at room temperature. The gels were transferred to Whatman 3 MM paper, dried and autoradiographed.

RESULTS

Several cAMP responsive sites are present in the 5′-flanking region of the uPA gene

To localize *cis*-acting elements mediating cAMP action, uPA-CAT hybrid genes containing different lengths of 5′ flanking region of the uPA gene were co-transfected to LLC-PK$_1$ cells with pSV2*neo*. Stably transfected cells were treated with Br-cAMP or SCT for two hours, total RNA was then isolated and specific transcripts were measured by RNA start mapping. Correct initiation and splicing were predicted to give rise to a band of 343 nucleotides. Correctly initiated RNA without splicing would give rise to a band of 260 nucleotides, a consequence of inefficient splicing. We compared only the bands reflecting the mature mRNA.

Cells transfected with CAT 4660 produced a 8-fold and 5-fold induction of CAT mRNA over basal level after treatment with SCT and Br-cAMP, respectively (Fig. 1). Deletion to −3473 (CAT3473) reduced the induction with SCT but not with Br-

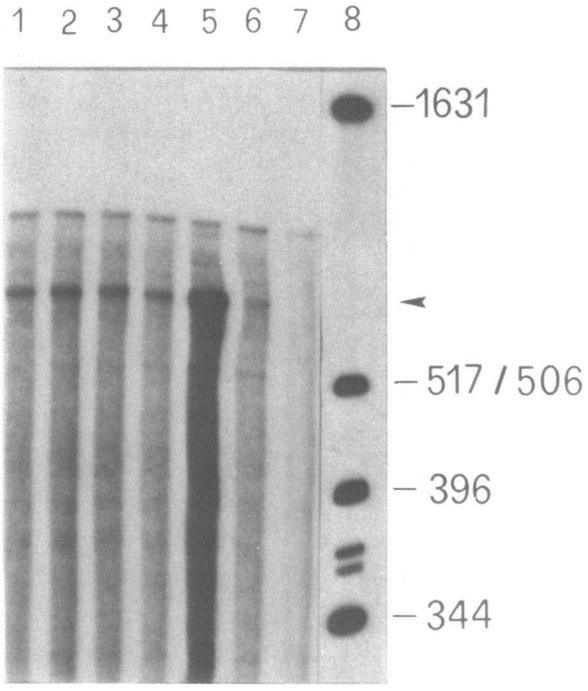

Fig. 2. cAMP responsiveness of a sequence in the far upstream region of uPA gene. Cells stably transfected with either a vector alone (pSV3*gpt*) or the pSV3*gpt-Xho*I/*Nsi*, which contains the dimerized *Xho*I-*Nsi*I fragment at the 5′ position (Fig. 5, lanes 7 and 8), were treated with either 1 mM 8-Br-cAMP or 100 ng/ml TPA for four or two hours, respectively. Total RNA was isolated from cells transfected with pSV3*gpt* (lanes 1 to 3) or pSV3*gpt-Xho*I/*Nsi* (lanes 4 to 6) and analyzed for specific transcripts by RNase start mapping. Lanes: 1 and 4, untreated; 2 and 5, treated with 1mM 8-Br-cAMP, and 3 and 6, treated with 100 ng/ml TPA; 7, RNA from cells transfected with pX343 (Hygromycin selectable marker) alone; 8, molecular weight markers. The arrow indicates the size of accurately initiated transcripts.

cAMP. Further deletion to −2693 (CAT2693) reduced the induction with both inducers. Deletion to −1768 (CAT1768) showed very little effect on the inducibility but raised the basal template activity. Further deletion to −1275 (CAT1275) reduced the overall gene expression. These results suggest the presence of at least two sites for cAMP regulation between −3473 and −2693, and −1768 and −1275, and additionally one for SCT between −4660 and -3473. The results also suggest the presence of a silencer between −2693 and −1760 and an basal enhancer between −1760 and −1275.

We focused our attention on the region between −3473 and −2693, because the reduction of inducibility by Br-cAMP was most prominent from CAT3473 to CAT2693. Within this region, we detected three sequences that shared homologies with the well characterized CRE, TGACGTCA (4−7, 34): $^{-3407}$TGACGcag^{-3400}, $^{-3389}$TGACGaac^{-3382}, and $^{-2842}$TGACGctc^{-2871}. Of three CRE-like sequences, first two were arranged in a direct tandem repeat separated by 18 nucleotides.

A region containing the CRE-core tandem repeats can mediate cAMP action

To test whether the region containing the CRE-core tandem repeats mediates cAMP effect on gene expression, we placed the 149 bp *Xho*I- *Nsi*I fragment corresponding to -3473 to −3320, at the 5′ end of a heterologous promoter, pSV3*gpt*. Stably transfected cells were pooled and tested for the inducibility of the XGPRT gene by Br-cAMP and TPA. TPA was tested because the uPA gene is inducible by this reagent in LLC-PK$_1$ cells (2) and some *cis*-elements have been reported to mediate the action of both cAMP and TPA (9, 35). After treating cells with these agents total RNA was isolated and analyzed by RNA start mapping.

Figure 2 shows that *gpt* gene expression from the control, pSV3*gpt*, was unaffected by cAMP or TPA. This indicates that the enhancer-less SV40 early promoter does not contain *cis*-elements mediating cAMP or TPA action in LLC-PK$_1$ cells. The insertion of the dimerized 149 bp *Xho*I-*Nsi*I fragment from the uPA gene at the site immediately upstream of the 21 bp repeats

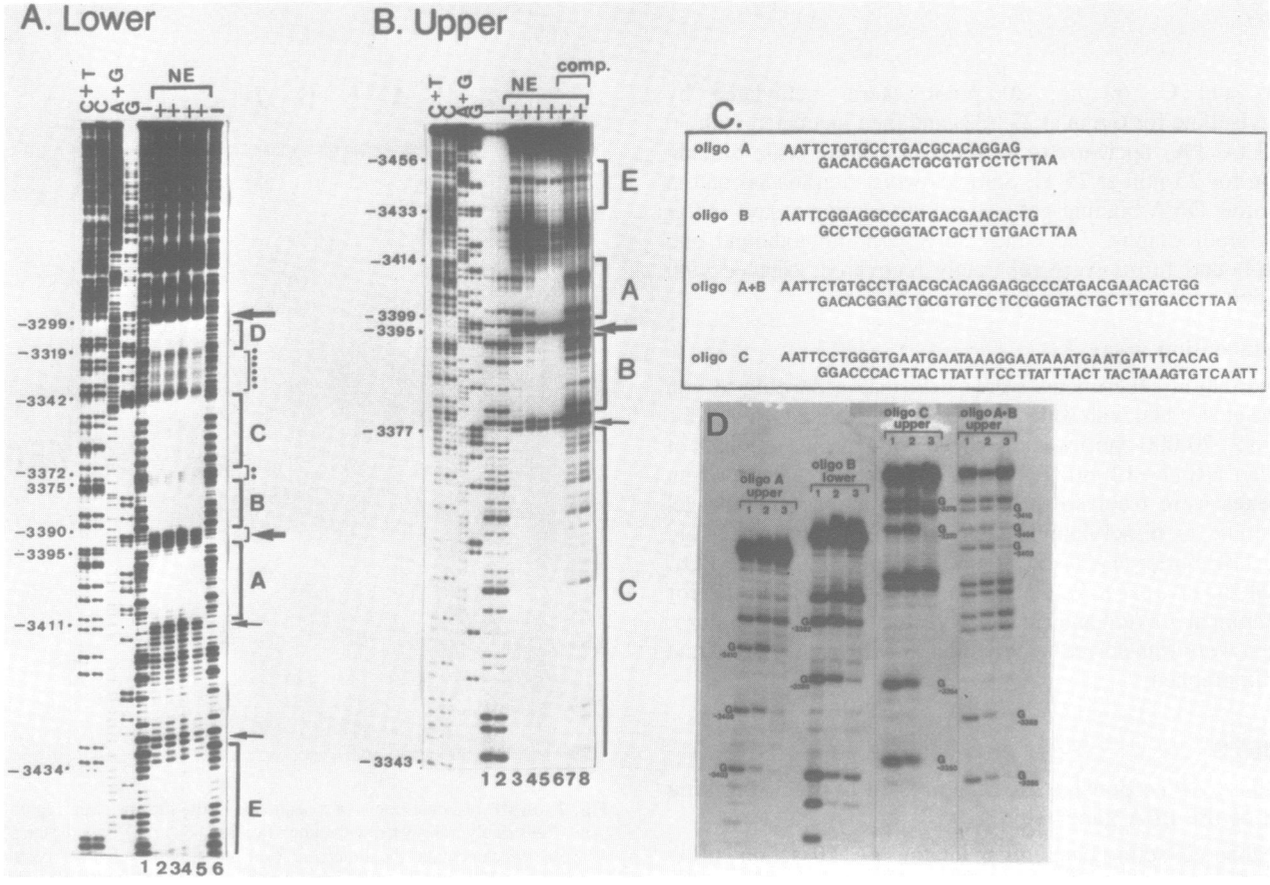

Fig. 3. DNAase I footprinting and methylation interference analyses of the cAMP inducible enhancer in the uPA gene. **Panel A**. Binding reactions were carried out with 2 fmol (30,000 cpm) of the *Xho*I (−3473)-*Xba*I (−2694) fragment, 3′ end-labeled at the *Xba*I site. Reactions were without nuclear extract (NE) (lanes 1 and 6), or with 40 μg (lanes 2 and 3) and 60 μg (lanes 4 and 5) of LLC-PK$_1$ NE. **Panel B**. Binding reactions were carried out with 2 fmol (40,000 c.p.m.) of the *Nsi*I (−3324)-*Xho*I (−3473) fragment, 3′ end-labeled at the *Nsi*I. Reactions were without NE (lanes 1 and 2), or with various concentrations of NE (lanes 3 to 8: 30, 35, 40, 45, 45 and 45 μg, respectively) in the absence (lanes 3 to 6) or presence (lanes 7 and 8) of 15-fold molar excess of *Nsi*I-*Xho*I fragment. Protected areas are specified with brackets and the margins are indicated on the left. Arrows indicate hypersensitive sites. Dots indicate sites of weak protection. Maxam and Gilbert sequencing reactions(C+T, C, A+G, G) are shown on the left side of each set. **Panel C**. Oligonucleotides derived from Footprints A, B and C. **Panel D**. Determination of essential contact residues by methylation interference experiments. Binding reactions were carried out with partially methylated oligo probes (oligos A, B, C and A+B as indicated) in the presence of LLC-PK$_1$ NE (30 μg). Lanes: 1, standard Maxam and Gilbert sequencing reaction; 2, unbound oligo probe; and 3, bound oligo probe. Protected G residues and their position in the recognition sequences are indicated and summarized in Fig. 4.

of SV40 early promoter rendered this gene strongly inducible by cAMP but not by TPA. This indicates that the 149 bp DNA fragment contains element(s) selectively responsive to cAMP.

DNase I footprinting analysis (36) was performed to localize protein-binding regions in the uPA gene promoter. Five footprints protein binding sites were detected spanning from −3456 to −3299, designated as FP-A, -B, -C, -D, and -E (Fig. 3A and B, and summarized in Fig. 4). Three of them, FP-A, -B, and -C, were closely associated but clearly separated by DNAase I hypersensitive or weakly protected sites. The FP-A and -B both contain a CRE-like sequence, TGACG, in the same orientation. Methylation interference assays showed that guanine nucleotides in TGACG sequence in FP-A and -B are critical for protein-DNA interaction, suggesting that TGACG sequence is the binding motif in FP-A and -B (Fig. 4). FP-D and -E were not studied further because they did not appear to be involved in cAMP responsive control of the uPA gene (data not shown).

Cooperativity among FP-A, -B and -C for cAMP induction

To assess the role of each footprint in cAMP responsiveness, the 149 bp *Xho*I-*Nsi*I fragment or a synthetic oligonucleotide corresponding to these footprints was inserted to either the *Nsi*I or *Pvu*II site (5′ side) or the *Bam*HI site (3′ side) of the *gpt* gene in the pSV3*gpt* vector. Constructs were then stably transfected into LLC-PK_1 cells and analyzed in the same manner as described in the legend to Fig. 2.

The results showed that the 149 bp *Xho*I-*Nsi*I fragment (−3473 to −3320) conferred cAMP inducibility (8-fold) on the SV40 early promoter (Fig. 5, lane 2 vs lane 1). The cAMP responsiveness was manifested regardless of the orientation or the location of the fragment with respect to the *gpt* gene (Fig. 5, lanes 1 to 10). This fragment conforms to the characteristics of an inducible enhancer as described by Serfling et al. (37). Dimerization of the fragment in the correct orientation did not enhance the transcription (lane 6 vs lane 5), while dimerization in the opposite orientation enhanced both basal and inducible expression (lane 8 vs lane 7).

The *Xho*I-*Nsi*I fragment contains, in addition to FP-A, -B and -C, another footprint (−3456 to −3423) part of which is seen in Fig. 4. To exclude the possibility that this additional protected region is essential for the cAMP regulation, we made several constructs using synthetic oligonucleotides which span only the three footprints, FP-A+B+C, and tested them for cAMP inducibility. With these constructs we observed a 6-fold induction (Fig. 5, lane 12 vs lane 11), and like the *Xho*I-*Nsi*I fragment, dimerization of FP-A+B+C did not enhance the effect (lanes 13, 14 vs lanes 11,12). We then tested DNA sequences corresponding to each of the individual footprints: FP-A sequence produced little induction (2-fold; lane 18 vs lane 17) but multiplication enhanced cAMP inducibility (lanes 19 to 24); FP-B sequence showed no induction (for correct orientation; lane 26 vs lane 25) or weak induction (for opposite orientation; lane 28 vs lane 27) but dimerization enhanced the inducibility (lane 30 vs lane 29); FP-C sequence showed no inducibility, although basal expression increased significantly (lane 32 vs lane 31). The combination of footprints: FP-A+B sequences showed slight

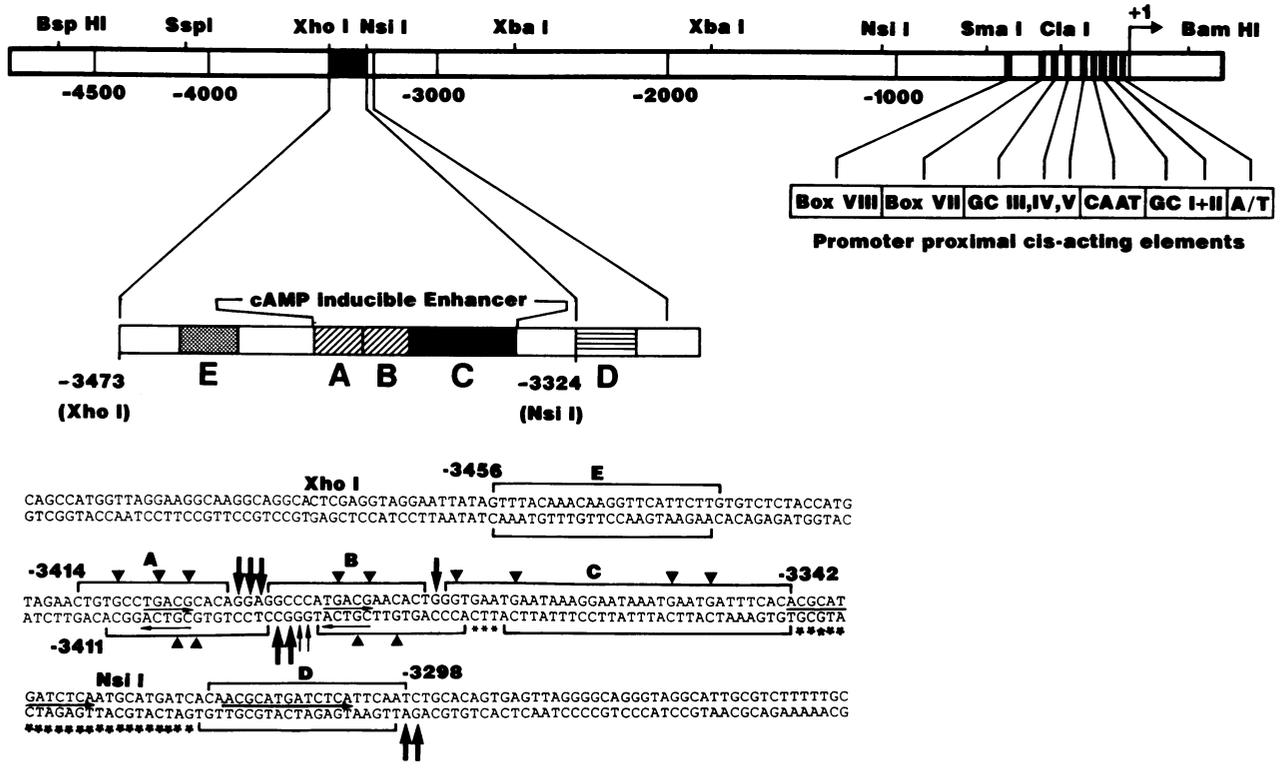

Fig. 4. Sequence around a cAMP responsive site 3.4 kb upstream of the transcription initiation site. Promoter proximal *cis*-acting elements are described elsewhere (24). The restriction map of 4.6 kb of 5′-flanking region and the organization of the enhancer is shown on the top of the figure. Footprints are labeled as A, B, C, D and E. The sequence of the enhancer that contains *Xho*I-*Nsi*I fragment is shown on the bottom. Numbers refer to the distance from the initiation of transcription. Brackets indicate DNA sequences protected from DNAase I by nuclear factors. Regions of weak protection are indicated by stars. The positions of apparent protein contacts as determined by the methylation interference assay are indicated by closed triangles.

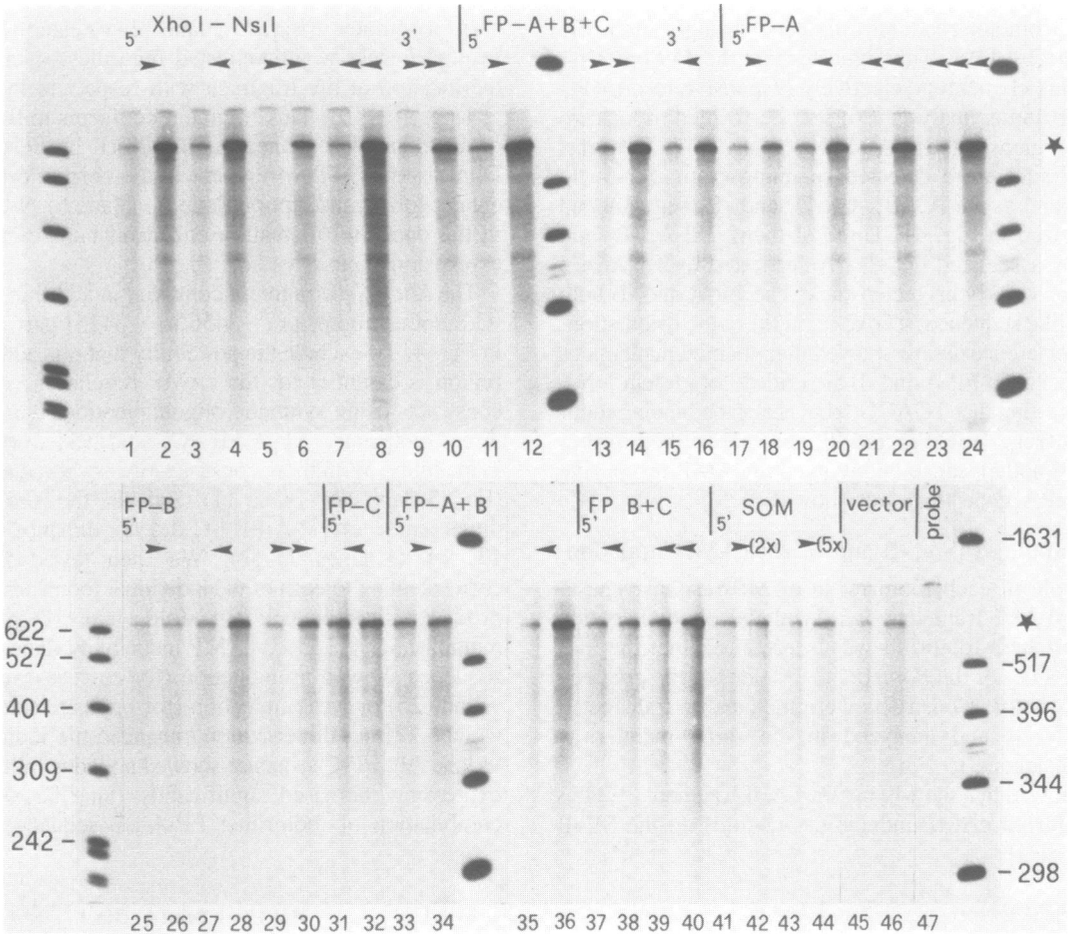

Fig. 5. Roles of FP-A, -B, and -C in cAMP regulation. Total RNA was prepared from cells stably transfected with various hybrid genes and analyzed as described in Fig. 2. The protected band migrating at 643 nucleotides (star) is the expected length for the proper initiation of transcription driven by SV40 early promoter. The types of DNA fragments inserted into the pSV3*gpt* vector are indicated on the top of the gel. Their orientation and multiplicity are indicated by the direction and number of arrow-heads on top of the lanes. Except for lanes 9, 10, 15 and 16 they are were inserted at 5′ side of the vector. SOM, somatostatin gene CRE. The lanes of odd numbers represent untreated cells and even numbers represents cells treated with 1 mM 8-Br-cAMP. Lanes 45 and 46 represent cells transfected with the vector pSV3*gpt*. Lane 47 is the RNA probe used in the analysis.

inducibility (lanes 33−36), while FP-B+C sequences showed no inducibility (lanes 37 to 40). As a control we tried the well characterized rat somatostatin gene-derived CRE sequence, but it did not confer cAMP inducibility to the early promoter (lanes 41 to 44) (see discussion). The vector alone was unaffected by cAMP treatment (lane 46 vs lane 45).

The sequences corresponding to FP-A, FP-B and the combination of both, showed a significant difference in conferring inducibility depending on the orientation. A possible reason is the lack of preservation of the eight base palindrome sequence in the CRE. The polarity implies a need for specific directional alignment to interact efficiently with the transcriptional machinery.

Competition analysis of the three protein binding domains

We performed a series of gel retardation competition experiments to examine the correlation of the domains, A, B, and C, with respect to the binding of nuclear proteins. The binding of nuclear proteins to labeled oligonucleotides (oligos) were competed by various unlabeled homologous and heterologous ones containing the CREs of somatostatin (4) and chorionic gonadotropin α-subunit (6) genes (Fig. 6). The results are shown in Fig. 6A, B, C and summarized in Fig. 6D: (1) oligos A and B were competed by each other as well as by themselves (Fig. 6A, lanes 5−8; and 6B, lanes 5 and 6) but not by non-related oligos Sp1 and 40mer (Fig. 6A, lanes 13 and 14; and 6B, lanes 4 and 10); (2) oligos A and B were more efficiently competed by Som-CRE and hCG-CRE than by themselves (Fig. 6A, lanes 11 and 12; and 6B, lanes 8 and 9); (3) oligos A and B were equally well competed by oligo C as by homologous competitors (Fig. 6A, lanes 9 and 10, and 6B, lane 7); (4) competition of oligo B by oligo A gave rise to a new complex with a higher mobility, referred to as complex B_2 (Fig. 6B, lane 6); (5) oligo C provided several forms of complexes—one major, C_1 and three minor, C_2, C_3, and C_4—and they were all competed by oligo C (Fig. 6C, lanes 2,3 and 5); (6) complex C_1 was also competed by heterologous oligo hCG-CRE but not by other heterologous oligos, and minor complexes C_2, C_3 and C_4 were enhanced by oligos A, B, hCG-CRE and Som-CRE (Fig. 6C, lanes 7−11). The most striking observation was that oligos A and B were competed not only by themselves but also by oligo C, although oligo C shares no sequence homology with oligo A and B. These results suggest a protein-protein interaction between CREB (or CREB-like proteins) and an oligo C-binding protein. Conversely, binding of oligo C was not competed by oligos A and B, possibly because the binding of CREB to the imperfect CRE in oligos

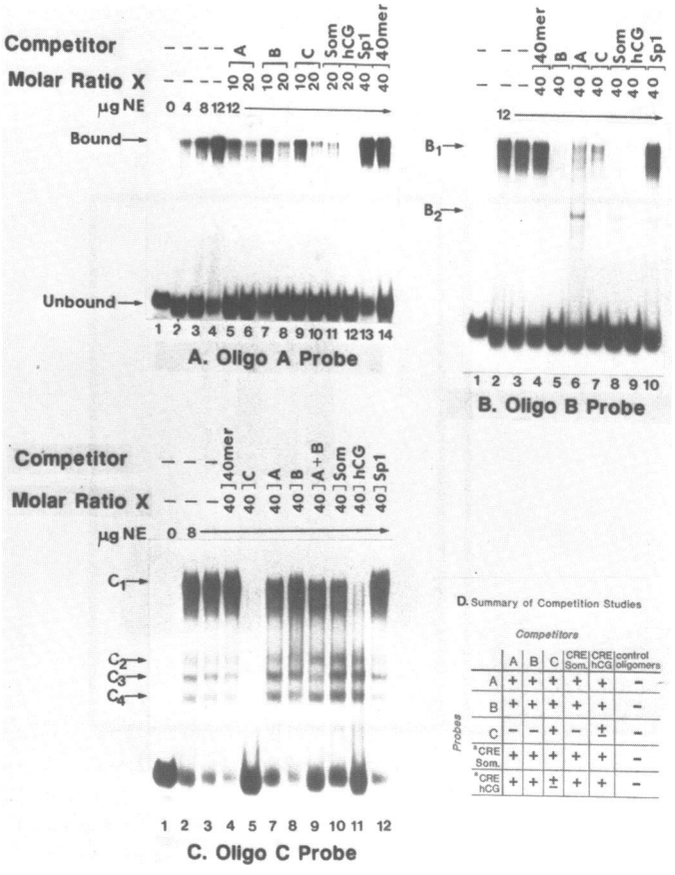

Fig. 6. Gel retardation competition analysis. Oligonucleotides were 3′ end-labeled by Klenow (100 fmol; 20,000 cpm) and incubated with amounts of LLC-PK_1 NE as indicated. The molar excess of unlabeled competitors is given on top. A complete description of oligo probes, competitors and their preparation is given in Materials and Methods. A, oligo A; B, oligo B; C, oligo C. Som, Somatostatin gene CRE; hCG, human Chorionic Gonadotropin α-subunit gene CRE; Sp1, Sp1 binding site of the uPA gene (39); 40 mer, a random sequence. [a] data with radiolabeled Som. CRE and hCG CRE are not shown.

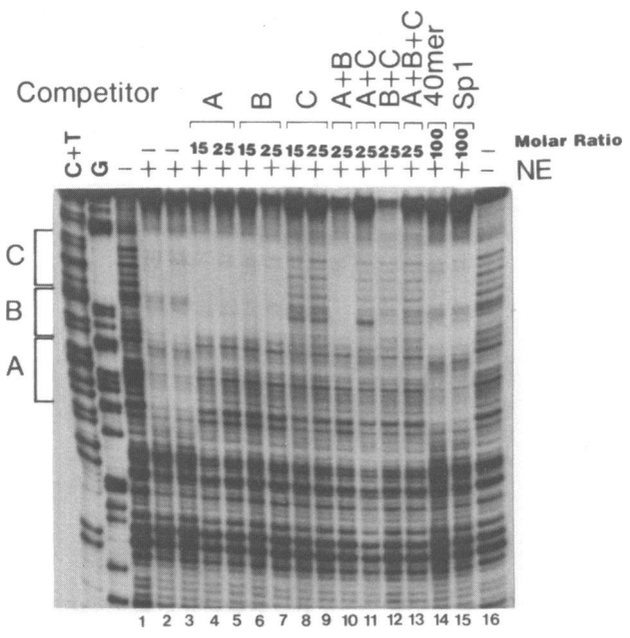

Fig. 7. DNAase I Footprinting competition analysis. The subcloned *Xho*I (−3473) -*Nsi*I (−3324) fragment was 3′ end-labeled at the *Eco*RI site of the pUC18 polylinker region (lower strand). 10 fmol (40,000 cpm) of probe was incubated with 45 μg of LLC-PK_1 NE and an excess of the appropriate competitor as indicated above each lane. The reaction products were analyzed on a 6% sequencing gel. C+T and G sequencing reactions are shown on the left.

A and B might be weaker than the interaction of CREB with an oligo-C binding protein.

To verify the competition seen in the gel retardation assay we did another competition study using DNAase I footprinting assay. With moderate concentrations of competitors (15 to 25 fold molar excess) oligos A and B competed the binding of nuclear protein(s) to domain A but not to domain B. In contrast, oligo C competed strongly for all three domains (Fig. 7, lanes 8 and 9). These results suggest that there is a tight interaction between the complexes C and B. Otherwise the excess of oligos A and B should compete for protein binding to the domain B as well. A 50-fold molar excess of oligos was also insufficient to compete for protein binding (data not shown).

Catalytic subunit of cAMP-PK stimulates DNA-protein complex formation

The results of preceding experiments and functional analysis of the 5′ flanking region suggested that CRE-like (FP-A and -B) and non-CRE (FP-C) sequences constitute a cAMP inducible enhancer complex in the uPA gene. How does cAMP then induce gene activation? We examined the effects of protein phosphorylation on DNA-protein interactions on FP-A, -B , and -C. In gel retardation analyses using oligos A and B as probes, pretreatment of nuclear extracts with a purified C-SU preparation increased the amount, without changing the mobility, of protein bound forms of oligos, while C-SU itself did not show any binding activity (Fig. 8A and B). For both oligos A and B, the increase was 2.5 fold as measured by counting the radioactivity of the gel corresponding to protein bound forms. In contrast, C-SU caused different effects on oligo C: a slight decrease, if at all, of a major complex C_1, and marked decrease of minor complexes C_2, C_3, and C_4 (Fig. 8C).

Unlike the *in vitro* effect of the purified C-SU on the amount of DNA-protein complexes formed we could not detect differences between nuclear extracts prepared from hormonally treated and untreated LLC-PK_1 cells (data not shown). A possible reason for the absence of *in vivo* effect is that under physiological conditions the half-life of the activated CREB is very short or activated state is easily converted to basal state during the preparation of nuclear extracts.

DISCUSSION

All of the functional CREs so far identified are located within the first 300 bp of the 5′ flanking region of their respective genes (38). In our previous attempt to locate the cAMP response element(s) in the uPA gene using homologous cell-free transcription system, we observed cAMP-mediated induction with a template containing only 148 bp of the 5′ flanking region (19). The result implied the presence of a promoter proximal cAMP responsive sequence in the uPA gene. However, in this cell-free system the upstream region from −1.3 kb to −4.6 kb of the uPA gene was not investigated in detail. The experiments using stably transfected cells showed additional multiple cAMP regulatory sites located over several kilobases of the 5′ flanking region; one of them residing 3.4 kb upstream of the transcription initiation site and conforming to the characteristics of an inducible

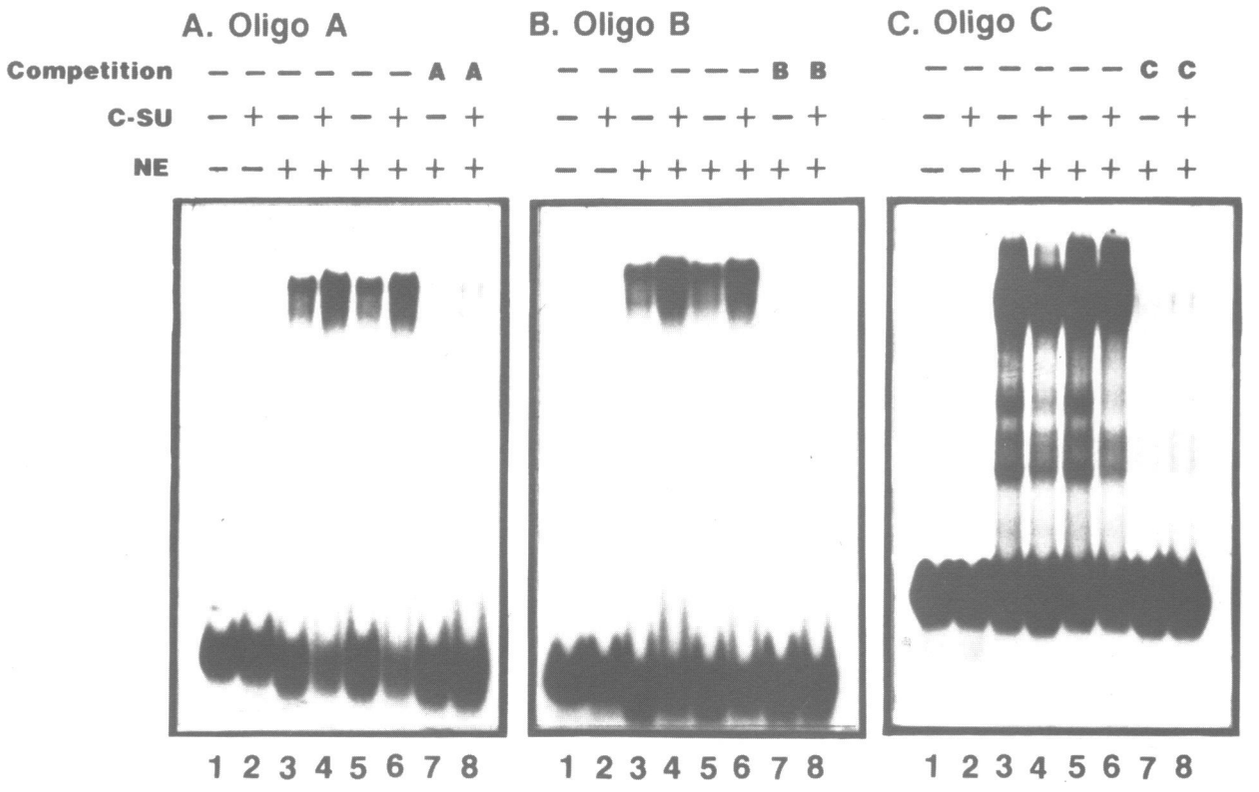

Fig. 8. Effect of catalytic subunit of cAMP-dependent protein kinase on DNA-protein complex formation on the sequences of FP-A, -B, and -C. Nuclear extract (2 μg) was pre-incubated with 0.36 μg of the C-SU of cAMP-PK at 15 min (lane 4 in each panel) and 30 min (lanes 6 and 8 in each panel). After addition of the 3′ endlabeled oligo probe (100 fmol; 20,000 cpm) and 15 min further incubation, the reaction mixtures were electrophoresed on a native 6% polyacrylamide gel as described in Materials and Methods. Competitors were added in 50-fold molar excess (lanes 7 and 8 in each panel). Radioactive probes oligos A, B and C were used in panels A, B and C, respectively.

enhancer as described by Serfling et al. (37). This region is composed of three closely spaced protein binding domains, FP-A, -B, and -C. FP-A and -B contain a cAMP response element core sequence, (CRE-core; TGACG), however, they only moderately conferred cAMP responsiveness on a heterologous promoter. Effective cAMP-mediated induction was achieved when these two core sequences were flanked by a third sequence, FP-C, which did not contain a CRE-core sequence and by itself was unresponsive, suggesting the cooperation among three domains. In this context it should be noted that the somatostatin CRE sequence alone could not confer cAMP inducibility on SV40 promoter (Fig. 5), although it has a high affinity to CREB and produces a gel-shift (Fig. 6). It may be that even for perfect palindromic CREs to exert cAMP mediating activity, additional cis- and trans-acting elements are necessary in LLC-PK_1 cells, or that surrounding sequences affect the mode of DNA-protein interaction and consequently affect the biological activity of CREB (or CREB-like protein).

Recently the cooperation of CRE with adjacent *cis*-acting elements in several cAMP regulated genes was reported. Cooperation was observed with a CAAT box in the phosphoenolpyruvate carboxykinase gene (39); with AP1, AP2 and AP4 binding sequences in proenkephalin gene (40, 41); and with a tissue specific *cis*-acting element in proopiomelanocortin gene (5). From these and our observations it seems that a variety of *cis*-acting elements and *trans*-acting factors can cooperate with the CRE element. The CREB appears to exert its function in multiple DNA-protein complexes.

We have shown that three domains of the cAMP inducible enhancer, FP-A, -B and -C, are independently recognized *in vitro* by nuclear proteins of LLC-PK_1 cells. Results of gel retardation competition assays suggest a physical interaction among them; the sequence of FP-C can interfere with the protein-DNA complex formation on FP-A and -B. Interestingly, the sequences of FP-A and -B do not interfere with complex formation on FP-C. The observed nonreciprocal competition argues against the idea that the same protein recognizes two different sequence motifs. This is also confirmed by the different responses of these binding proteins to *in vitro* phosphorylation (see below). It is likely that a FP-C binding protein has two binding sites—one for the DNA sequence in the FP-C and another for a CREB (or CREB-like) protein which binds to FP-A and -B—and that protein-DNA interaction is much stronger in FP-C than in FP-A or -B; therefore, excess of FP-C complexes should compete for proteins binding to FP-A and -B. Unlike other CRE containing sequences, the hCG-CRE competes for proteins binding to FP-C, which might be due to stronger interaction between a CREB and the CRE sequence in hCG-CRE because the hCG-CRE contains two CRE sequences. We do not know the exact role of the FP-C binding protein(s), but it may serve to stabilize DNA-protein interactions in the adjacent CRE-CREB complexes to permit efficient cAMP responsiveness in LLC-PK_1 cells.

The key question in the study of a cAMP inducible enhancer is how cAMP mediates gene regulation. Experimental evidence available supports the notion that the activation of cAMP-dependent protein kinase is the essential step in cAMP-mediated regulation of gene expression (4, 16–18, 21). We have previously shown in a cell free transcription system that the C-

SU could directly stimulate the transcription of the uPA gene (19). These results suggests that phosphorylation events are central in the underlying mechanism. However, it is still not clear how protein phosphorylation modulates gene transcription.

In the gel retardation assays the addition of C-SU of cAMP-PK increased the amount of CREB bound to CRE-like sequences without inducing the dimerization of the protein (Fig. 8), suggesting that the role of CREB phosphorylation by cAMP-dependent protein kinase is either to increase its affinity to CRE sequence or to increase the amount of CREB available for DNA binding. The latter possibility is analogous to NF-$\varkappa$B regulation in pre-B cells in which the NF-$\varkappa$B transcription factor is complexed with a specific inhibitor in the cytoplasm. Upon phosphorylation either by protein kinase C or by cAMP-PK, NF-$\varkappa$B is released from the inactive complexed and translocated to nuclei (42, 43). Since the enhancement of protein-binding to the CRE site is found after the addition of catalytic subunit to nuclear extracts, it seems that CREB activation in the cell takes place in the nuclei, possibly as a consequence of the translocation of free catalytic subunit to the nuclei. Elucidation of exact role of CREB phosphorylation by cAMP-PK awaits further investigation.

In contrast to FP-A and -B, the binding of nuclear proteins to FP-C was not enhanced by the C-SU of cAMP-PK; in fact, the formation of minor complexes C_2, C_3 and C_4 was inhibited, while the major C_1 complex was not affected. Therefore, it is possible that phosphorylation of FP-C binding protein(s) in these minor complexes is also biologically relevant. At the moment we do not know whether phosphorylation of these proteins modifies the interaction between FP-C complex and FP-A and -B complexes.

Results of our work suggest that the interaction between CREB (or CREB-like protein(s)) and the far upstream enhancer of the uPA gene is regulated at least by two different mechanisms. Firstly, a sequence, FP-C, flanking to the CRE-core sequences, FP-A and -B, increases the affinity of the CREB for the CRE through protein-protein interaction(s). Secondly, protein phosphorylation by cAMP-PK increases the affinity of CREB for the CRE or the availability of CREB for CRE-binding. However, the direct proof of these mechanisms should await the isolation of these binding proteins or corresponding cDNA clones from LLC-PK_1 cells.

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