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# INTERACTIONS OF NUCLEAR PROTEIN FROM CULTURED RAT HEPATOCYTES WITH THE CYCLIC AMP RESPONSIVE ELEMENTS AND THE NF1-CTF SITE IN THE PROMOTOR OF THE RAT PHOSPHOENOLPYRUVATE CARBOXYKINASE GENE

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Nuclear extracts from cultured rat hepatocytes were analyzed by gel mobility shift assay for protein binding to the cyclic AMP responsive elements CRE1 (-96/-77) and CRE2 (-152/-132) and the NF1-CTF binding site (-121/-99) of the phosphoenolpyruvate carboxykinase (PCK) promotor. Binding was very weak to the CRE2 and CRE1. The NF1-CTF site formed two complexes with nuclear protein. Protein binding was increased, when the NF1-CTF site was coupled to the CRE1, and further, when it was coupled to both the CRE1 and the CRE2. Complex formation was not altered by treatment of the hepatocytes with glucagon or with glucagon and insulin. Thus, protein binding was most efficient when all three elements were in context, which might be necessary for full transcriptional activation of the PCK gene. 

DNaseI footprinting analysis has revealed that the PCK promotor contains two cAMP regulatory elements CRE1 and CRE2, the first at -96/-77 and the second at -152/-132, as well as a nuclear factor 1-CAAT transcription factor (NF1-CTF) binding site at -121/-99 (1). Transfection of gene constructs between the 5'-deleted PCK promotor and the neomycin phosphotransferase gene (PCK-neo) into FTO-2B rat hepatoma cells has shown that the basal and the cAMP-stimulated promotor activities of the region -174/+73, containing the CRE2, NF1-CTF and CRE1, were 4-fold and 10-fold, respectively, higher than those of the region -109/+73, containing only part of the NF1-CTF and the CRE1 (2). Transfection of gene constructs between the PCK promotor, internally mutated in 10 bp sequences flanking the 5'- and 3'- end of the CRE1, and the chloramphenicol acetyltransferase gene (PCK-CAT) into CV1 cells has demonstrated that the regions flanking the CRE1 on either side were important both for basal and cAMP-activated gene expression (3). Gel mobility shift assay using synthetic wild type and mutant oligodeoxynucleotide probes with 4

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contextual bases at the 5'- and 6 bases at the 3'-end of the CRE1 core sequence TTACGTCA clarified the structural requirements of the CRE1 for nuclear protein binding (4). The interactions between the NF1-CTF site, the CRE1 and the CRE2 in nuclear protein binding have not been studied so far.

Therefore the present work employed gel mobility shift assays with nuclear extracts from cultured rat hepatocytes, which have not been used so far, to investigate interactions between the NF1-CTF site and the CRE1 and CRE2. It was found that for efficient protein binding the three elements have to be in context, which might be a prerequisite for full activation of PCK gene transcription, and that protein binding was not affected by treatment of the hepatocytes with glucagon or glucagon in combination with insulin.

## MATERIALS AND METHODS

Chemicals were of analytical grade and from commercial sources. Collagenase was from Worthington/Biochrom (Berlin). Culture medium M199 and fetal calf serum were from Boehringer (Mannheim). Molecular biology products were either from Boehringer (Mannheim) or from Life Technologies (Eggenstein). Amersham/Buchler delivered [$\alpha$-$^{32}$P]deoxycytidine 5'-triphosphate (110 TBq [3000 Ci]/mmol). Nick columns$^{TM}$ were purchased from Pharmacia/LKB (Freiburg).

Nuclei from cultured hepatocytes were prepared by a modification of a procedure described previously (5). Cells were isolated from male Wistar rats and cultured on Nunc culture dishes (140 mm diam.). After 24 h of culture cells from 3 dishes were washed twice with cold phosphate-buffered saline and scraped off in 1.6 ml Tris-HCl buffer (10 mM, pH 8.0, 0.32 M sucrose, 3 mM $CaCl_2$, 2 mM $Mg(acetate)_2$, 0.1 mM ethylenedinitrilo tetraacetate (EDTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 % (v/v) trasylol, 0.15 mM spermine, 0.5 mM spermidine, 1 mM dithioerythritol (DTE)). Cells were homogenized 20 times with a motor-driven glass/teflon homogenizer at maximal speed. The homogenate was loaded onto a 4.8 ml cushion of 10 mM Tris-buffer (pH 8.0, 2 M sucrose, 5 mM $Mg(acetate)_2$, 0.1 mM EDTA, 1 mM DTE, 0.1 mM PMSF, 0.15 mM spermine, 0.5 mM spermidine, 1 % trasylol) and centrifuged for 1 h at 50.000 *g* in a Kontron TST rotor. The nuclear pellet was resuspended in 450 µl 15 mM Hepes buffer (pH 7.6, 0.12 M KCl, 5 mM $MgCl_2$, 1 mM DTE, 0.1 mM EDTA).

Nuclear extracts were prepared as described (6). 50 µl 4 M $(NH_4)_2SO_4$ (pH 7.9) were added stepwise, the suspension mixed after each addition and afterwards shaken for 30 min in the cold. The precipitate was pelleted at 100.000 *g* in a Kontron TFT rotor. To the supernatant 165 mg solid $(NH_4)_2SO_4$ was slowly added with intermittent shaking. Proteins were precipitated for 1 h in the cold and pelleted at 100.000 *g* as described above. The pellet was dissolved in 50 µl 25 mM Hepes (pH 7.6, 40 mM KCl, 1 mM EDTA, 1 mM DTE, 10 % glycerol (v/v), 40 µg/ml bestatin, 0.5 µg/ml leupeptin, 0.14 µg/ml pepstatin) and dialyzed for 6 h against 2 x 250 ml of the same buffer. Protein concentration was determined by the Bradford method and yielded

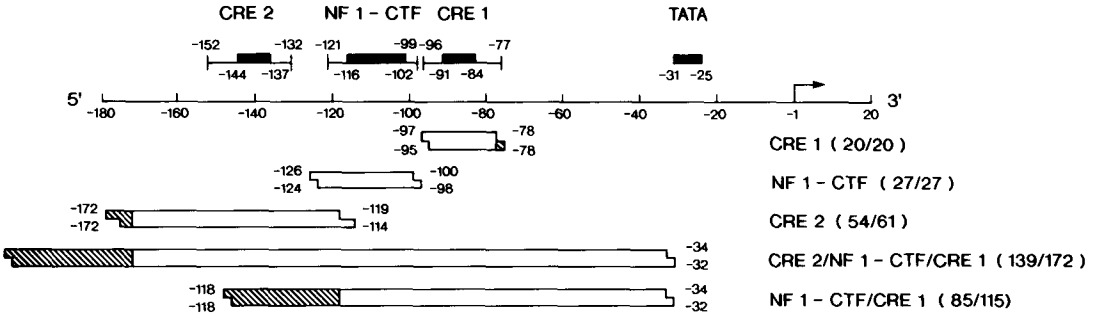


*Fig. 1.* **Oligonucleotides and DNA fragments from the rat PCK gene promotor used in gel mobility shift assays.** The location in the promotor of the TATA-box, CRE1, NF1-CTF site and CRE2 is shown with the solid boxes indicating the core sequences. The open boxes show the oligonucleotides and DNA fragments, which were employed in the assays. Hatched areas represent non-genuine PCK sequences, which were derived from synthesis or cloning procedures. The numbers indicate the positions of the fragments relative to the transcriptional start site of the PCK gene (arrow). The first number in parentheses indicates the length of the DNA fragment containing a genuine PCK sequence, the second number shows the total length of the fragments.

0.5-1.0 µg/µl nuclear protein. Nuclear extracts from HepG2 or HeLa cells were prepared essentially as described (7).

Two oligonucleotides and three DNA fragments were used in the gel mobility shift assay (Fig. 1). Oligonucleotides were chemically synthesized with a 392 DNA/RNA synthesizer (Applied Biosystems). The CRE1 of the PCK gene (4) was synthesized from position -97 to - 78: 5'-GGCCCCTTACGTCAGAGGCG-3' on the one strand and from position -95 to -78: 5'-ggCGCCTCTGACGTAAGGGG-3' on the other strand (core sequence underlined). The two "g" were attached to the 5' end of the second strand for the purpose of labelling by filling in radioactive deoxycytidine 5'-monophosphate. The NF1-CTF site (3) was synthesized from position -126 to -100: 5'-GTGCTGACCATGGCTATGatccaaaGG-3' on the one strand and from position -124 to -98: 5'-GGCCtttggatCATAGCCATGGTCAGC-3' on the other strand (NF1 site underlined, CTF site in small letters). The DNA fragments were cut from a promotor fragment of the PCK gene ranging from position -172 to +73 (2). This fragment was further cloned into the EcoRI and BamHI sites of the Bluescript vector. The CRE2 was gained as a 61 bp EcoRI/NcoI fragment, the combined CRE2/NF1-CTF/CRE1 as a 172 bp fragment by cutting with AccI at an internal site and a site within the Bluescript polylinker region. NF1-CTF/CRE1 was obtained as a 115 bp fragment by also cutting with AccI after removal of the CRE2 and religation of the remaining vector. The two latter fragments carried a 33 bp and a 30 bp DNA segment, respectively, which is not a genuine PCK sequence but derived from the Bluescript polylinker sequence. DNA fragments were purified by phenol extraction after agarose gel electrophoresis.

Oligonucleotides and DNA fragments were labeled to a specific radioactivity of 1000 cpm/fmol by filling the 5'-protruding ends with Klenow enzyme in the presence of [α-$^{32}$P]deoxycytidine 5'-triphosphate (8). Routinely, for gel mobility shift assay 3 µg nuclear protein was incubated in a total volume of 20 µl 15 mM Hepes buffer (pH 7.9, 15% glycerol, 120 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.75 mM DTE, 4 mM spermidine 2 µg BSA, 2 µg poly-

deoxyinosinic-deoxycytidylic acid) with 30-50 fmol labeled DNA for 20 min at room temperature. In competition experiments a molar excess of unlabeled DNA was incubated with nuclear protein for 10 min at room temperature before adding labeled DNA. Samples were loaded onto a 5% native polyacrylamide gel, which was run in 45 mM Tris-borate buffer (pH 8.3). Therafter gels were dried and exposed to Kodak X-Omat AR films at -70°C.

## RESULTS

*Increased protein binding to CRE2/NF1-CTF/CRE1 compared with CRE2 or NF1-CTF/CRE1 and to NF1-CTF/CRE1 compared with CRE1 or NF1-CTF*

To show interactions between the two CREs and the NF1-CTF site of the rat PCK promotor three series of gel mobility shift assays were performed. In the first series nuclear protein binding to the CRE2 and the NF1-CTF/CRE1 DNA fragments was compared with binding to a combination of the two, i.e. CRE2/NF1-CTF/CRE1 (Fig. 2, left). The CRE2 did not bind nuclear protein from cultured rat hepatocytes. With the NF1-CTF/CRE1 fragment two protein/DNA complexes were formed. When the combined CRE2/NF1-CTF/CRE1 fragment was employed, protein binding was strongly increased indicating a cooperative effect of the CRE2 in protein binding to the CRE2/NF1-CTF/CRE1.

In the second series nuclear protein binding to synthetic oligonucleotides of the CRE1 and the NF1-CTF site was compared with binding to a PCK promotor fragment containing the combined NF1-CTF/CRE1 (Fig. 2, middle). The CRE1 bound nuclear protein only weakly. The NF1-CTF site formed two protein/DNA complexes. The combined NF1-CTF/CRE1 bound protein very strongly compared with the NF1-CTF site alone, indicating a cooperative effect of the CRE1 on protein binding to the NF1-CTF site.

The present finding of weak protein/CRE1 complexes with nuclear extracts from cultured rat hepatocytes (Fig. 2, middle) is in contrast to one major and an additional weak protein/CRE1 complex with nuclear extracts from rat liver observed before (4, 9). To confirm the reliability of the method nuclear extracts from HeLa and HepG2 carcinoma cells were assayed in a third series for protein binding to the rat CRE1 (Fig. 2, right). With both extracts strong complexes were observed. It can be supposed that either the content or the affinity of the binding protein differs between cultured rat hepatocytes, intact liver and hepatoma cells.

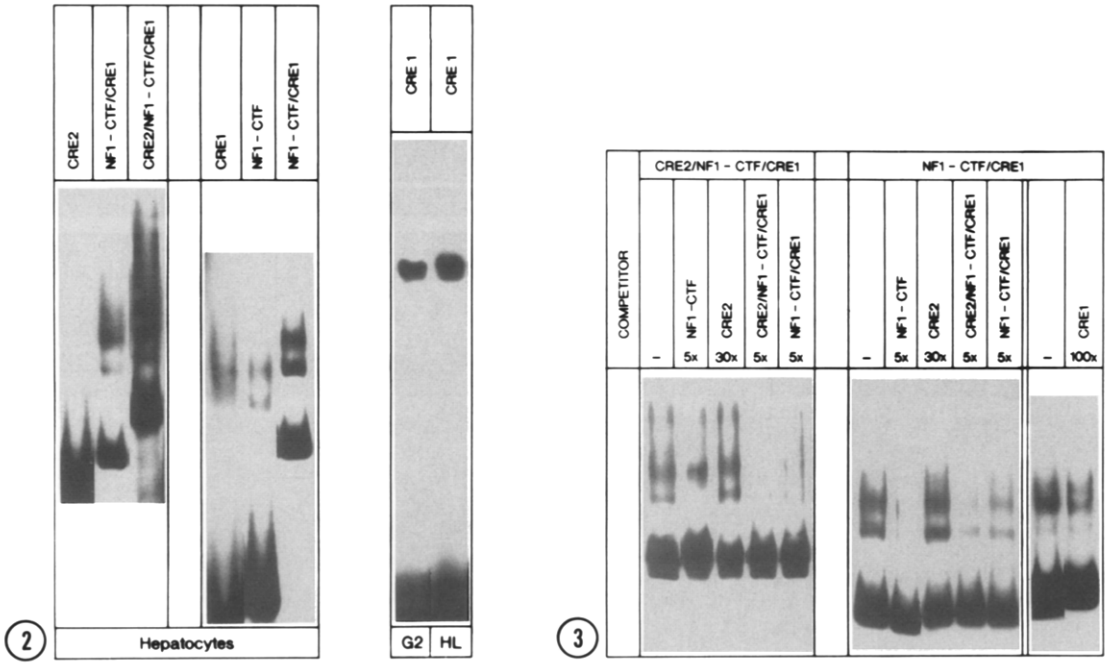


*Fig. 2.* **Gel mobility shift assay of oligonucleotides and DNA fragments of the rat PCK gene promotor with nuclear extracts.** 50 fmol of labeled DNA as indicated was incubated in three different series with 3 µg of nuclear extracts from cultured rat hepatocytes, HeLa (HL) or HepG2 (G2) carcinoma cells. The first series (left) was exposed longer than normal so that weaker binding to the CRE2 and NF1-CTF/CRE1 could be detected; this led to "overexposure" of the CRE2/NF1-CTF/CRE1 fragment. The different size of the DNA fragments analyzed (cf. Fig. 1) explains their different mobility.

*Fig. 3.* **Gel mobility shift assay of DNA fragments of the rat PCK gene promotor with hepatocyte nuclear extracts. Competition experiments.** 3 µg of nuclear extracts were incubated with 5-, 30- and 100-fold molar excess, respectively, of competitor DNA as indicated 10 min prior to the addition of 50 fmol labeled DNA fragments. Competition of NF1-CTF/CRE1 with the CRE1 was performed in a separate experiment. The different size of the DNA fragments analyzed explains their different mobility.

*Specificity of protein binding to CRE2/NF1-CTF/CRE1 and NF1-CTF/CRE1*

The specificity of nuclear protein binding to the PCK promotor DNA fragments CRE2/NF1-CTF/CRE1 and NF1-CTF/CRE1 was assayed with competition experiments (Fig. 3). Protein binding to the CRE2/NF1-CTF/CRE1 could be competed with the NF1-CTF oligonucleotide. Competition was more effective with the combined NF1-CTF/CRE1 and most effective with the CRE2/NF1-CTF/CRE1. Apparently, protein binding to the CRE2/NF1-CTF/CRE1 fragment was specific. Moreover, the experiments showed that the most effective protein binding was obtained with fragments con-

taining the CRE2. Yet, the CRE2 alone was not able to compete for protein binding even with a 30-fold molar excess (Fig. 3, left).

Protein binding to the NF1-CTF/CRE1 fragment could be competed effectively with the NF1-CTF oligonucleotide and with the combined CRE2/NF1-CTF/CRE1; competition was less pronounced with the NF1-CTF/CRE1. Protein binding to the NF1-CTF/CRE1 was also specific. Again, the CRE2 was cooperative for protein binding, although it alone was unable to compete for protein binding. Also the CRE1 was not able to compete for protein binding, even at a 100-fold molar excess (Fig. 3, right).

*Protein/DNA complex formation with nuclear extracts from hormone-treated hepatocytes*

The transcriptional activation of the PCK gene by glucagon via cyclic AMP could be elicited by protein binding to the CRE2/NF1-CTF/CRE1 promotor region as a result of increased affinity of proteins for their target DNA sequence. This should result in an enhancement of complex formation in the gel mobility shift assay. The glucagon antagonist insulin could then be expected to diminish complex formation. Thus hepatocyte cultures were treated with 0.1 - 10 nM glucagon, nuclear extracts were prepared at 0.5, 1, and 2 h sufficient to activate the gene (5, 10) and gel mobility shift assays were performed. No significant changes in complex formation could be observed under all conditions with the synthesized CRE1 and NF1-CTF and the combined CRE2/NF1-CTF/CRE1 and the NF1-CTF/CRE1 (not shown). Similarly, no change was seen with nuclear extracts from hepatocytes, which were treated simultaneously with 0.1 nM glucagon and 10 nM insulin, concentrations found previously to be antagonistic (5).

## DISCUSSION

Binding of nuclear protein from cultured rat hepatocytes to the CRE2/NF1-CTF/CRE1 promotor fragment at -152/-77 of the rat PCK gene was augmented through the influence of the CRE2 at -144/-137 located 5' and the CRE1 at -91/-84 located 3' of the NF1-CTF site. This indicates interactions of the three elements in protein binding, which might be necessary for efficient transcriptional activation of the PCK gene.

*Promotor protein binding - promotor activity relation*

For an understanding of the mechanism of gene activation protein binding to the promotor as revealed by DNaseI footprint analyses and gel mobility shift assays has to be correlated with transcriptional activity of the promotor as observed in transfection studies and *in vitro* transcription experiments.

In DNaseI footprinting experiments of the PCK promotor binding of nuclear protein from rat liver to the reciprocally mutated NF1-CTF and the CRE1 sites occurred independently from one another indicating that the two elements acted non-cooperatively in protein binding (1, 3). Yet, transfection of CV1 cells with the chloramphenicol acetyltransferase gene linked to the PCK promotor with a mutated CTF site adjacent to the CRE1 resulted in a 50 % reduction in relative promotor activity without changing the inducibility by cAMP (3). Transfection of FTO-2B rat hepatoma cells with the neomycin phosphotransferase gene under the control of the deleted PCK promotor showed that the CRE1 was essential and sufficient for basal and cAMP-induced gene activation and that both activities were strongly enhanced by a promotor fragment containing the CRE1 plus the NF1-CTF site (2). Enhancement of basal promotor activity by the NF1-CTF site compared to the CRE1 alone was also obtained in *in vitro* transcription assays using rat liver nuclear extracts (11). The stronger protein binding to the NF1-CTF/CRE1 than to the CRE1 alone observed in the present gel shift assays (Fig. 2) is in line with these studies. Thus the functional activity of a promotor is determined by its full environment, i.e. proper sequences surrounding each regulatory element and proper context of adequately spaced elements, which might induce DNA structural changes leading to functional cooperation of various elements after a hormonal signal (12).

*Increase in promotor protein binding or in promotor activity by cAMP-dependent phosphorylations*

Cyclic AMP-regulated expression of genes including the PCK gene is mediated by the catalytic subunit of cyclic AMP-dependent protein kinase (PK-A) (11, 13, 14). Binding of nuclear protein from rat liver nuclear extracts to the PCK CRE1 was not changed by incubation with the catalytic subunit of PK-A or with phosphatase indicating that the affinity of the binding protein was not altered by phosphorylation/dephosphorylation (9). Similarly, in the present gel shift assays protein binding to the

various regulatory elements was not enhanced, when nuclear extracts from cultured rat hepatocytes treated by 0.1-10 mM glucagon, which efficiently activated the PCK gene (5, 10), were used. Yet, increased protein binding was shown with liver nuclear extracts from cyclic AMP-treated rats (15).

The prevailing evidence then appears to indicate that PK-A phosphorylates the CRE-binding protein (CREB) in preexisting CREB/CRE complexes and thereby induces an increase of transcriptional efficacy (16, 17).

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