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Phosphorylation of CREB affects its binding to high and low affinity sites: implications for cAMP induced gene transcription

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Cyclic AMP treatment of hepatoma cells leads to increased protein binding at the cyclic AMP response element (CRE) of the tyrosine aminotransferase (TAT) gene *in vivo*, as revealed by genomic footprinting, whereas no increase is observed at the CRE of the phosphoenolpyruvate carboxykinase (PEPCK) gene. Several criteria establish that the 43 kDa CREB protein is interacting with both of these sites. Two classes of CRE with different affinity for CREB are described. One class, including the TATCRE, is characterized by asymmetric and weak binding sites (CGTCA), whereas the second class containing symmetrical TGACGTCA sites shows a much higher binding affinity for CREB. Both classes show an increase in binding after phosphorylation of CREB by protein kinase A (PKA). An *in vivo* phosphorylation-dependent change in binding of CREB increases the occupancy of weak binding sites used for transactivation, such as the TATCRE, while high affinity sites may have constitutive binding of transcriptionally active and inactive CREB dimers, as demonstrated by *in vivo* footprinting at the PEPCK CRE. Thus, lower basal level and higher relative stimulation of transcription by cyclic AMP through low affinity CREs should result, allowing finely tuned control of gene activation.

Key words: cAMP response elements/CREB/DNA binding/*in vivo* footprinting/PKA protein phosphorylation

Introduction

Cells respond to changes in their environment by transmitting external signals through several internal pathways, eliciting immediate alterations by changes in enzyme activities or later responses by reprogramming gene expression. One well characterized signal transduction pathway acts through protein kinase A (PKA). An increase in the level of the second messenger cyclic AMP (cAMP) activates PKA, resulting in phosphorylation of numerous protein substrates (reviewed in Edelman *et al.*, 1987), leading in some cases to changes in expression of target genes (reviewed in Roesler *et al.*, 1988; Montminy *et al.*, 1990). The study of genes whose transcription is cAMP responsive revealed a common cAMP response element (CRE) TGACGTCA (Deutsch *et al.*, 1988a,b). A 43 kDa protein termed CREB binds to this sequence (Montminy and Bilezikjian, 1987) and leads to increased activation of transcription after phosphorylation by PKA (Yamamoto *et al.*, 1988; Gonzalez and Montminy, 1989). A number of proteins have been identified which are related to CREB in their DNA binding domain and which recognize identical or closely related DNA sequences (Hardy and Shenk, 1988; Hai *et al.*, 1988, 1989; Gaire *et al.*, 1990; Hurst *et al.*, 1990 and references therein). All appear to be leucine zipper proteins which bind DNA as dimers (Landschulz *et al.*, 1988) and CREB has been shown to be present as a dimer in solution (Dwarki *et al.*, 1990). Many of the leucine zipper proteins have been shown to heterodimerize with various other members of their class (Benbrook and Jones, 1990; Hai and Curran, 1991), however, CREB is relatively selective in that it has been reported to heterodimerize only with ATF-1 (Hurst *et al.*, 1991) and CREM (Foulkes *et al.*, 1991). CREB and CREMτ are the only members of the CREB/ATF family which have been shown directly to mediate cAMP induction of transcription (Gonzalez and Montminy, 1989; Hurst *et al.*, 1991; Foulkes *et al.*, 1992).

The tyrosine aminotransferase (TAT) gene contains a functional CRE 3.6 kb upstream of the transcription start site as an essential component of a liver-specific enhancer (Boshart *et al.*, 1990; Weih *et al.*, 1990). Protein binding to this site depends on the state of phosphorylation as revealed by genomic footprinting: increased intracellular cAMP concentration led to an increase in protein binding (Weih *et al.*, 1990), and inhibition of PKA by overexpression of the regulatory subunit RIα results in a virtual disappearence of this DNA binding activity (Boshart *et al.*, 1991).

We have focused on purification and characterization of the cAMP-induced DNA binding protein from rat liver which interacts with the CRE of the TAT gene (TATCRE) *in vivo*. Since PKA was reported not to stimulate binding of CREB to the somatostatin CRE (Montminy and Bilezikjian, 1987; Yamamoto *et al.*, 1988), we anticipated that a different member of the CREB/ATF family would recognize the TATCRE in a phosphorylation-dependent manner. However, we show that phosphorylation of CREB by the catalytic subunit of PKA increases binding to several CREs, including the TATCRE. In hepatoma cells, genomic footprinting revealed that binding at the TATCRE, a weak CREB binding site, increased after cAMP stimulation, whereas for the PEPCK gene which is also cAMP responsive, the high-affinity CREB binding site showed no further increased binding after cAMP stimulation. Hence, the basal and stimulated PKA activity levels within the cell are very important in determining the degree to which CRE enhancers are bound by CREB and activate transcription.

Results

Two separable binding activities interact with the TATCRE

Genomic footprinting studies showed that binding activity at the TATCRE *in vivo* correlates with PKA activity (Weih

et al., 1990; Boshart *et al.*, 1991). To characterize this binding activity, rat liver extracts were fractionated into three specific binding activities, A, B and C (Figure 1A), as characterized earlier in bandshift assays using crude nuclear extracts of hepatoma cells (Weih *et al.*, 1990). The B and C complexes are very closely related by all criteria of analysis and are referred to as BC. Methylation interference and bandshift analyses have shown that both A and BC proteins bind to the TATCRE, but not to the mutant version (MUTCRE), which has three substitutions at nucleotides contacted by protein *in vivo* (Weih *et al.*, 1990). The three MUTCRE sequence changes were also shown to be sufficient to inactivate the enhancer *in vivo* (Boshart *et al.*, 1990; Weih *et al.*, 1990). Since the BC complex was more abundant in cells following cAMP induction, in contrast to the A complex (Weih *et al.*, 1990), we anticipated that this binding activity may correspond to the cAMP-inducible binding activity observed *in vivo*. The BC activity recognizes both the CRE of the TAT and somatostatin genes while the protein forming the A complex recognizes only the TATCRE (data not shown).

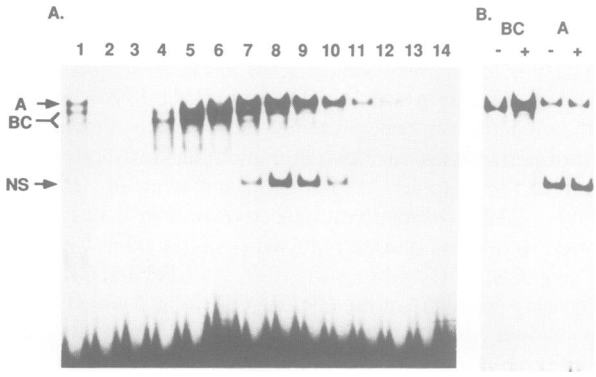

Fig. 1. Two separable factors interact with the TATCRE. Rat liver nuclear extracts were applied to DEAE-sepharose and proteins were eluted with a 50–500 mM KCl gradient. Aliquots of fractions were assayed for binding activities in bandshift assays with the TATCRE oligodeoxynucleotide (**A**). The BC activity elutes at ~100 mM KCl from DEAE-sepharose (lanes 4–6). The A activity elutes at ~200 mM KCl (lanes 7–11). Lane 1 shows the starting material activities. A non-specific binding protein complex is marked by NS and binds equally well to the TATCRE, SOMCRE and MUTCRE DNAs (not shown). In (**B**) fractions from the DEAE-sepharose, containing primarily the BC or the A activity, were treated with the catalytic subunit of protein kinase A (+) prior to the bandshift assays.

BC binding to the TATCRE can be increased by treatment with the catalytic subunit of protein kinase A

We tested directly the effects of the catalytic subunit of PKA on the bandshift activities, after fractionating rat liver nuclear extracts using DEAE-sepharose or S-sepharose. We found that the shifted band due to binding of BC to the TATCRE was altered in mobility and intensity after PKA treatment, whereas the A binding activity was not affected by PKA (Figure 1B). This shows a direct link between PKA and the BC binding activity, which is not apparent for the A protein(s). Hence, BC is most likely to be the binding activity responsible for the cAMP-inducible footprint *in vivo*. Each of the two separable activities (A and BC, Figure 1) was purified independently using native DNA cellulose and specific TATCRE oligodeoxynucleotide chromatography.

CREB and the BC proteins are both recognized by antibodies to a short peptide of CREB

In order to define the relationship between the BC activity from liver and the CREB factor (Yamamoto *et al.*, 1988), we also purified CREB from rat brain. To determine the antigenic relatedness of CREB and the BC protein from liver, polyclonal antibodies against CREB were generated using a peptide representing amino acids 137–150 of rat brain CREB as antigen (Gonzalez *et al.*, 1989). In Western blot analyses, the anti-CREB antiserum reacted with a single

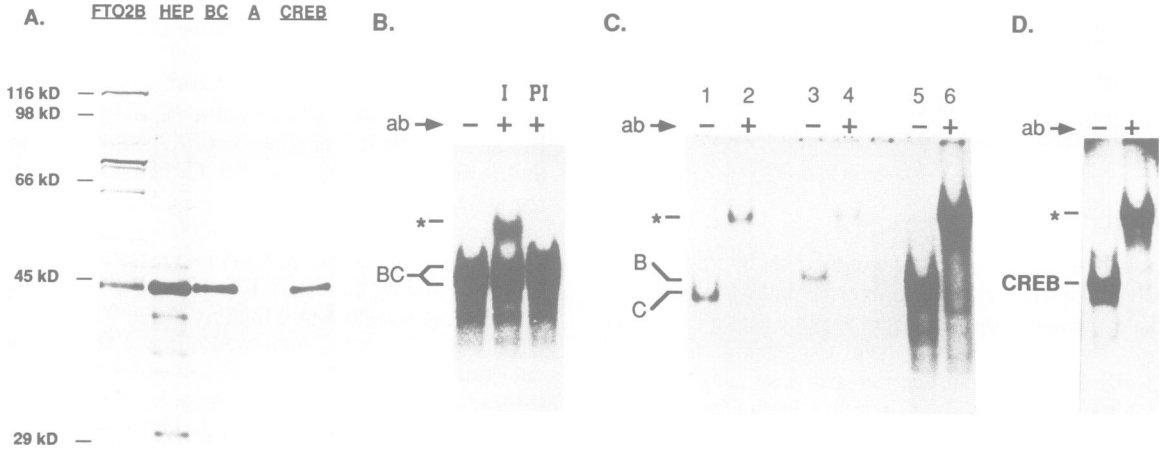

Fig. 2. Anti-CREB antibodies react with a 43 kDa protein in the BC fraction from liver which comigrates with CREB from brain. In (**A**) proteins from liver and brain of rats were included in Western blot analysis using anti-CREB antibodies generated to a short peptide sequence from CREB. Samples include 150 μg protein from FTO2B extract, 50 μg of a partially purified heparin-sepharose fraction (HEP) and samples of oligodeoxynucleotide affinity purified A, BC and brain CREB fractions (~1, 1 and 0.2 μg protein, respectively). Positions of marker proteins from Sigma are shown at the left. CREB is 43 kDa relative to egg albumin (45 kDa). In (**B**) affinity purified BC of liver was preincubated with pre-immune serum (PI) or antiserum (I) to CREB, prior to the addition of labelled DNA in a standard bandshift reaction. The CREB–antibody–DNA complex is marked by (*). Using concentrated antibodies (ab) (**C**), separated B and C forms of the BC fraction were completely immunoshifted when bound to the TATCRE (lanes 1–4). The BC fraction bound to the consensus SOMCRE was immunoshifted (lanes 5–6), as was the purified brain CREB bound to the SOMCRE (**D**).

protein migrating at 43 kDa in both the BC and CREB fractions (Figure 2A). This protein was also detected as the most abundant specific immunoreactive protein in nuclear extract from FTO2B cells (a hepatoma cell line that expresses TAT) and a partially purified BC fraction from liver. The antibodies did not recognize protein from the purified A fraction. Therefore, BC and CREB share specific antigenic determinants corresponding to amino acids 137–150 of CREB.

In addition, bandshifts performed with purified BC from liver in the presence of antiserum show a specific immunocomplex, denoted by *, most likely corresponding to a BC–antibody–DNA complex, which is not present in bandshift assays containing the pre-immune serum (Figure 2B). Hence, the antibodies recognize native BC protein bound to DNA. To test the relationship between complexes B and C, two fractions that were almost devoid of the other form were tested in immuno-bandshift assays using concentrated anti-CREB antibodies (see Materials and methods). A complex of slower mobility with proteins comprising either the B or C complex from liver (showing relatedness of the two forms; Figure 2C), as well as the purified CREB from rat brain (Figure 2D) was obtained, indicating that the proteins each share the CREB epitope. The shift in mobility by the affinity-purified antibodies (Figure 2C and D) appears to be quantitatively complete as DNA binding is not inhibited. These results were observed with the BC fraction bound to the TATCRE or to the consensus CRE of the somatostatin gene (SOMCRE). As also shown in Western blot analysis (Figure 2A), the anti-CREB antibodies do not recognize the A protein(s) in immuno-bandshift assays (data not shown). Thus, BC from liver and CREB may be related or identical, while the A protein does not appear to be related to CREB.

Phosphorylation of CREB purified from rat brain increases binding to the TATCRE

To test whether the increased binding seen in partially purified fractions from liver after PKA treatment is brought about by CREB homodimers, we tested highly purified CREB protein from brain, consisting predominantly of a 43 kDa band (Figure 3A), for phosphorylation-induced binding to the TATCRE. We analysed binding activity of brain CREB which had been treated with either the catalytic subunit of PKA to fully phosphorylate the PKA site(s), or with protein phosphatase 2A (PP2A) to remove phosphates. CREB treated with PP2A showed little binding to the TATCRE as compared with the PKA-treated sample (Figure 3B). The bandshift activity after PP2A treatment could be recovered when the protein was rephosphorylated by PKA in the presence of okadaic acid, which inhibits PP2A. Therefore, as for the BC activity from rat liver, it appears that the phosphorylation state of CREB influences DNA binding to the TATCRE.

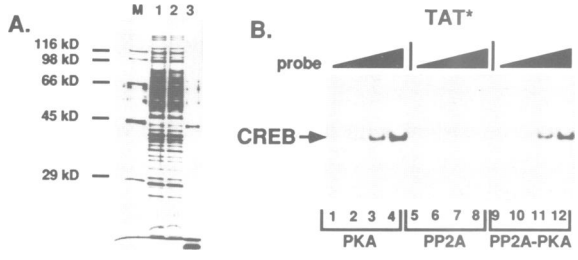

Fig. 3. CREB binding to the TATCRE is dependent on phosphorylation. In (**A**) proteins from the oligodeoxynucleotide affinity purification of rat brain CREB were analysed by SDS–PAGE and silver staining. Aliquots of the starting material (lane 1), the flow-through (lane 2) and the retained protein (lane 3) are shown. The marker proteins are shown at the left (lane M). Bandshift assays with PKA- or PP2A-treated brain CREB are shown in (**B**). Equivalent amounts of purified CREB were incubated with PKA alone, PP2A alone, or PP2A followed by PKA in the presence of okadaic acid to inhibit PP2A activity. All lanes in Figure 3B contain the same amount of purified CREB protein. Each of the three samples of treated CREB was mixed with 0.5, 2.5, 5 or 10 fmol (lanes 1–4, 5–8 and 9–12, respectively) of labelled TATCRE and incubated for 1 h on ice. The enzyme treatment of CREB is shown at the bottom of the lanes.

A 43 kDa protein present in the CREB and BC fractions is the major substrate for the catalytic subunit of PKA in vitro

The most probable explanation for the increased binding to the TATCRE with the liver BC and the brain CREB fractions after incubation with PKA is that phosphorylation of a protein present in both of those fractions mediates DNA binding. To identify the substrates for PKA, the purified BC and CREB fractions were phosphorylated *in vitro* using purified catalytic subunit of PKA and [γ-^{32}P]ATP. The reaction products were resolved by SDS–PAGE and reveal that the only major substrate for PKA in each of these fractions was a 43 kDa protein (Figure 4A). This phosphoprotein comigrates with the immunoreactive protein shown in Western blot analysis (Figure 2A) and the purified protein in the silver stained SDS gel (Figure 3A), suggesting that they are identical. Additional phosphoproteins detected in the liver fraction were not consistently present in this assay and may be degradation products of the 43 kDa protein. When the A fraction was used as substrate for PKA, no phosphoproteins were seen, confirming that it is not a direct target of PKA (not shown).

Phosphorylation of Ser133 by PKA increases binding to the TATCRE

Phosphoamino acid analysis of the major 43 kDa phosphoprotein confirmed that both BC and CREB were labelled only at serine residues upon phosphorylation with PKA *in vitro* (not shown). To determine the site(s) of phosphorylation, we performed tryptic digests and two-dimensional peptide analyses of the 43 kDa phosphoproteins from the BC and CREB fractions. They each showed the same single tryptic phosphopeptide, following phosphorylation by the catalytic subunit of PKA *in vitro*, whose identity was confirmed by mixing experiments including a synthetic marker peptide predicted from the CREB sequence (Figure 4B). Thus, a single site (Ser133) on CREB from rat brain and BC from liver is phosphorylated by PKA *in vitro*. An otherwise identical marker peptide containing Ala in place of Ser at position 133 could not be phosphorylated by PKA, confirming the phosphorylation site (data not shown).

To compare the residue(s) modified *in vitro* and *in vivo*, phosphorylation of the 43 kDa protein in response to forskolin was examined in FTO2B hepatoma cells. Following phosphorylation *in vivo*, only a 43 kDa phosphoprotein was immunoprecipitated from soluble cellular proteins by the CREB antibodies and peptide analysis was performed. The same tryptic phosphopeptide was observed after phosphorylation *in vivo* as compared with

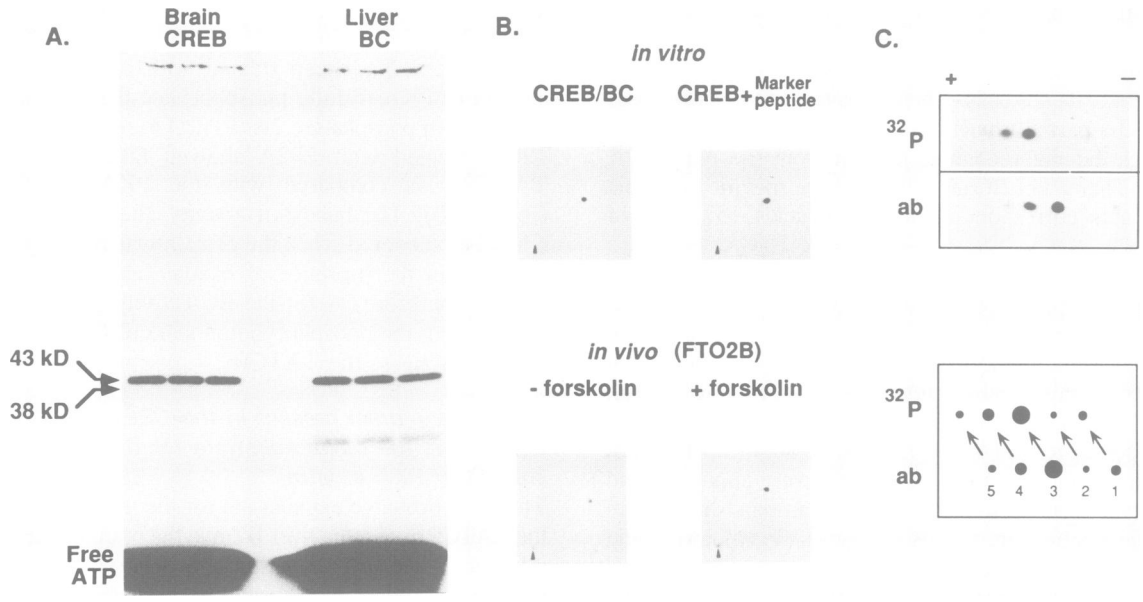

Fig. 4. The major substrate of PKA in the purified BC and brain CREB fractions is a 43 kDa protein, and two-dimensional tryptic peptide mapping of the 43 kDa phosphoprotein identifies Ser133 as the PKA-dependent phosphorylation site. Aliquots of either brain CREB or liver BC fractions were phosphorylated by PKA using [γ-^{32}P]ATP and reaction products were run in triplicate on a 10% SDS−polyacrylamide gel (**A**). The 43 kDa phosphoprotein from BC or CREB fractions was digested by trypsin following phosphorylation with PKA *in vitro*. Trypsin digests were subjected to phosphopeptide mapping as described in Materials and methods. Peptides were resolved in the horizontal dimension by electrophoresis and in the vertical dimension by ascending chromatography (**B**). An arrow marks the origin. A single phosphopeptide is observed with BC or CREB, phosphorylated *in vitro* by PKA and it migrates at an identical position as the phosphorylated marker peptide when mixed (CREB + Marker peptide). Phospholabelled CREB was immunoprecipitated from equal amounts of extracts of FTO2B cells which had been incubated for 4 h with $^{32}P_i$, then treated with forskolin (or solvent control) and subjected to phosphopeptide mapping (B). The single phosphopeptide observed migrated identically with the marker peptide in mixing experiments (not shown). CREB from liver is a multiply modified protein (**C**). An aliquot of the BC fraction from liver was labelled using PKA and [γ-^{32}P]ATP, mixed with a 100-fold excess of untreated BC fraction and resolved by two-dimensional electrophoresis. The resulting gel was further analysed by Western blotting and the immunostained forms are shown in the panel labelled ab. The autoradiograph of the blotted proteins is shown in the panel labelled ^{32}P. Immunostaining with anti-CREB antibodies reveals five forms migrating at 43 kDa. The ^{32}P signal identifies those proteins which have been labelled *in vitro* by PKA. The CREB proteins shift by only one charge interval (as determined by isoelectric focusing gel markers) after PKA phosphorylation and are summarized schematically in the lower half of the figure. The cathode (+) and anode (−) for the isoelectric focusing gel are noted at the top of the figure.

purified CREB or marker peptide phosphorylated by PKA *in vitro*. The amount of phosphate at the Ser133 site increased after forskolin treatment of the cells (Figure 4B and Boshart *et al.*, 1991). Therefore, the collective evidence from bandshift assays, antibody analysis, SDS gel migration, PKA phosphorylation and phosphopeptide mapping shows that the BC protein from liver is the same as CREB from brain.

Multiple modified forms of CREB exist in liver

To see whether multiple phosphorylated forms exist *in vivo*, we analysed CREB from liver by two-dimensional protein gel electrophoresis, followed by Western blot analyses. We found that there are five protein species of M_r 43 000 (labelled 1−5), differing by net charge increments of one and centred at pI = ~5.8, which are recognized by the CREB antibodies (Figure 4C). After phosphorylation with PKA of purified CREB from liver, all five species (1−5) quantitatively moved one charge interval toward the cathode (+), consistent with increased negative charge from one added phosphate. This indicates that only one PKA site is present in CREB, in agreement with the tryptic peptide analysis (Figure 4B). Since all CREB forms (1−5) are substrates for the PKA phosphorylation, prior modification appears not to be required for phosphorylation at Ser133. Two forms of CREB (α and Δ) have been identified which differ only by an alternatively spliced α-exon, encoding 14 amino acids (Yamamoto *et al.*, 1990; Ruppert *et al.*, 1992). The α-specific amino acids contribute a net charge of +2, so CREBα forms should migrate two charge units toward the anode (−), relative to the CREBΔ forms. This was confirmed using the α and Δ forms of CREB protein expressed in *Escherichia coli* (data not shown). With this in mind, forms 3−5 correspond to CREBΔ while forms 1−3 correspond to CREBα (Figure 4C). Hence, CREBΔ would comprise 80−90% of the CREB protein found in both liver and brain (proteins from both sources show identical 2-D patterns), consistent with mRNA ratios in liver (Ruppert *et al.*, 1992) and brain (Yamamoto *et al.*, 1990).

Phosphorylation consensus sites for additional protein kinases are present N-terminal to the PKA site in CREB (Lee *et al.*, 1990) and are not resolved in tryptic peptide analysis as the tryptic fragment containing these sites is larger than 100 amino acids. Surprisingly, the migration of the CREB forms in the 2-D gel analysis (Figure 4C) are not changed by extensive treatment with several phosphatases, arguing that these forms differ by something other than phosphates, or that the phosphates are extremely resistant to these phosphatases. However, phosphatases PP2A and PP1 are capable of completely reversing the migration change caused by PKA treatment *in vitro* (not shown). These modifications may include glycosylation as can be predicted from the protein sequence. In any case PKA phosphorylation at Ser133 does not require protein modification at other sites.

In vivo footprinting reveals enhanced binding at the TATCRE but not at the PEPCK CRE after forskolin treatment

We have seen previously in hepatoma cells that binding to the TATCRE was increased by PKA stimulation (Weih *et al.*, 1990). We wished to extend our analysis to another CRE that is active in hepatoma cells and therefore analysed protein binding *in vivo* at the CRE of the PEPCK gene, with and without stimulation of PKA by forskolin. The CRE of the PEPCK gene is well characterized (Quinn *et al.*, 1988) and is a representative of the symmetric CRE type (see below). As well, PEPCK gene transcription is induced by increased intracellular cAMP levels in hepatoma cells (Wynshaw-Boris *et al.*, 1984; Stewart and Schütz, 1987; Sasaki and Granner, 1988). As shown in Figure 5, the TAT and PEPCK CREs clearly show a footprint in FTO2B hepatoma cells, relative to the DMS protection pattern in the fibrosarcoma cell line XC, which does not express TAT or PEPCK and does not show a footprint even after forskolin treatment (not shown). The DMS protection patterns at the PEPCK CRE in uninduced (un) and forskolin induced (cAMP) hepatoma cells appear to be identical. This is in contrast to the increased occupancy at the TATCRE after forskolin induction (Figure 5A; Weih *et al.*, 1990). We also examined protein binding at the PEPCK CRE in FTO2B hepatoma cell derivatives (clones #17 and #21) which have a strongly repressed level of PKA activity, resulting from overexpression of the PKA regulatory subunit RIα (Boshart *et al.*, 1991). In these cells, the footprint at the PEPCK CRE and the TATCRE nearly disappears, confirming that binding to both CREs is sensitive to PKA activity, and correlating with the strongly reduced expression of the PEPCK and TAT genes in these cell lines (Boshart *et al.*, 1991).

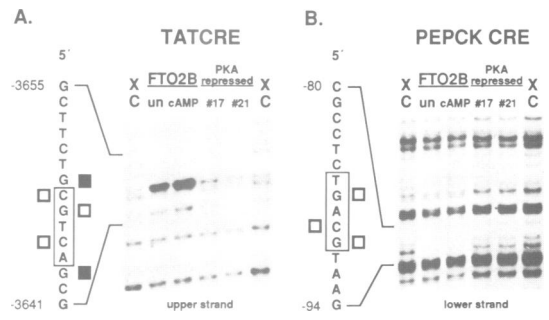

Fig. 5. The *in vivo* footprints at the TATCRE and the PEPCK CRE are regulated by PKA activity. Wild-type FTO2B cells and those overexpressing the RIα regulatory subunit of PKA under the control of the mouse metallothionein I promoter (clones #17 and #21; Boshart *et al.*, 1991) were grown and induced as described in Materials and methods. The FTO2B cell samples were either uninduced (un) or stimulated with forskolin (cAMP) to activate PKA. Cells were incubated with DMS and prepared for genomic footprinting as outlined in Becker *et al.* (1992). A TAT and PEPCK non-expressing fibrosarcoma cell line (XC) was included as a control. Altered DMS reactivity of guanosines is marked with closed squares for enhancements and open squares for protections, for both strands. Numbers indicate the positions of base pairs relative to the start site of transcription. The conserved CRE core is boxed and shows an identical protection pattern for both genes.

High and low affinity CREs show increased binding by CREB after PKA phosphorylation

The observation that binding of CREB to the TATCRE is enhanced after PKA activation *in vivo* or *in vitro* prompted us to examine other CREs in bandshift assays, particularly as the PEPCK CRE did not show increased binding *in vivo* when hepatoma cells were stimulated with forskolin (Figure 5). Since the most striking difference between the TATCRE and consensus CREs such as the somatostatin CRE is that the former is asymmetric (ctgCGTCA) and the latter symmetric (TGACGTCA), we analysed additional well characterized CREs from these two classes (Figure 6). Two CREs upstream of the urokinase-type plasminogen activator gene (uPA-A and uPA-B; von der Ahe *et al.*, 1990) and a CRE from the proenkephalin gene (ENK; Comb *et al.*, 1988) are in the asymmetric CRE category, while the somatostatin (SOM; Montminy and Bilezikjian, 1987), α-chorionic gonadotropin (αCG; Delegeane *et al.*, 1987) and the fibronectin (FIB; Dean *et al.*, 1989) genes have symmetric CREs. We also designed a mutant with three nucleotide changes in the symmetric SOMCRE which changed it to the asymmetric class (SOM 5/8). This collection of nine sequences was then tested in bandshift assays with CREBα protein from an *E.coli* expression clone, thereby assuring homodimer CREB protein (Figure 6). Similar results were found using protein encoded by the CREBΔ cDNA in either *E.coli* or baculovirus expression systems (data not shown). Clearly, a difference in binding affinity is apparent for the two CRE classes. The asymmetric CREs are much weaker binding sites relative to the symmetric sites (Figure 6A). Additionally, when the CREB protein was treated with PKA *in vitro* (+), binding to the asymmetric CREs was increased relative to the PP2A-treated (−) samples, whereas binding to the symmetric CREs was unaffected under these conditions (Figure 6A). Increased binding to the symmetric CREs was only observed when assayed with sufficient excess DNA fragment (Figure 6B; four times more DNA fragment than in Figure 6A). Thus, it appears that there are two types of CRE elements. Symmetric CREs bind CREB tightly, whereas asymmetric CREs are relatively weakly bound. Binding to both classes is increased by phosphorylation of CREB, though as shown at the PEPCK CRE in hepatoma cells, high affinity sites *in vivo* may be already bound by CREB at basal level PKA activity.

To determine the difference between the symmetric SOMCRE and asymmetric TATCRE binding affinities for the CREB protein, as well as the magnitude of phosphorylation-induced increase in this binding, titration experiments with CREBα expressed in *E.coli* were performed. Using a constant amount of protein and labelled DNA, increasing amounts of the same unlabelled DNA fragment were added to the bandshift reactions (Figure 7). As seen in Figure 6, the increased binding at the symmetric consensus CRE of the somatostatin gene after PKA treatment was observed only at higher DNA concentrations (lanes 4−7), where there was a sufficient excess of DNA. CREB affinity for the TATCRE was measured to be ~10-fold lower than that for the SOMCRE under our binding conditions (Kd_{TAT} of ~20 nM and Kd_{SOM} of ~1.5 nM), and the phosphorylation at Ser133 increased binding to the CREs by ~3-fold. CREB−DNA complexes on the SOMCRE were also stable at significantly higher KCl concentrations in bandshift reactions than complexes with the TATCRE (300 versus 150 mM, data not shown), again

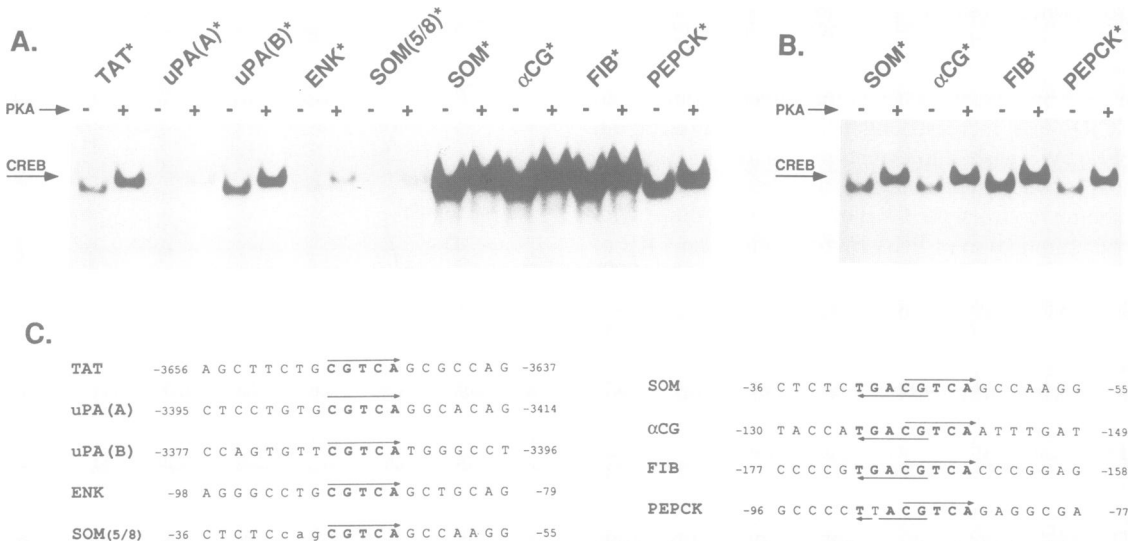

Fig. 6. Phosphorylation of CREB affects its binding to two sets of CREs. *E.coli* expressed CREBα was analysed in bandshift assays using the DNA sequences from two different classes of CREs, asymmetric and symmetric, shown in (C) (left and right columns, respectively). The nucleotide positions of the CRE relative to the start site of transcription is given at the ends of each sequence. The resulting bandshifts with asymmetric or symmetric CREs are shown in panel A and were performed with CREB protein pre-incubated with either PP2A (− lanes) or PKA (+ lanes). Each lane contains 50 fmol of double-stranded oligodeoxynucleotide probe. The CREB−DNA complexes are indicated on the left. The bandshift assays with the symmetric CREs (SOM, αCG, FIB and PEPCK), shown in Figure 6B, contain four times more DNA probe (200 fmol per reaction; 20 nM) and also show phosphorylation-enhanced binding for the high affinity CREs.

reflecting inherent binding affinity differences at physiological ionic strength.

Discussion

CREB binding is affected by PKA activity

In accordance with increased *in vivo* protein binding at the TATCRE after activation of PKA by forskolin, we show here that treatment of a purified liver nuclear protein with PKA *in vitro* results in increased binding to the TATCRE sequence. This protein is shown by direct comparison to be the 43 kDa CREB protein, using antibody recognition in Western blot analyses, bandshift experiments and by mapping of the site phosphorylated by PKA *in vivo* and *in vitro*. Specific DNA binding is reduced by PP2A and can be restored by PKA treatment *in vitro*. The magnitude of the increased binding of CREB to the TATCRE in response to phosphorylation at Ser133 is comparable to the increased footprint *in vivo*. Furthermore, the *in vivo* occupancy of the TATCRE exactly reflects the PKA activity state (repressed, uninduced, induced) and the transcription rate of the TAT gene in hepatoma cells (Weih *et al.*, 1990; Boshart *et al.*, 1991). In uninduced FTO2B hepatoma cells, CREB is already bound to the high affinity CRE of the PEPCK gene and this binding is not increased further after forskolin treatment, even though PEPCK transcription is increased. When basal PKA activity is strongly repressed by overexpressing its regulatory subunit RIα, the PEPCK CRE binds less protein, presumably resulting from lower affinity of the non-phosphorylated form of CREB. This corresponds to a strongly reduced expression of PEPCK under these conditions (Boshart *et al.*, 1991) and confirms that binding to both the TATCRE and the PEPCK CRE are responsive to *in vivo* PKA activity. One possible explanation is that when both subunits of CREB dimers are in the dephosphorylated form at Ser133, as in the PKA repressed cells, no binding

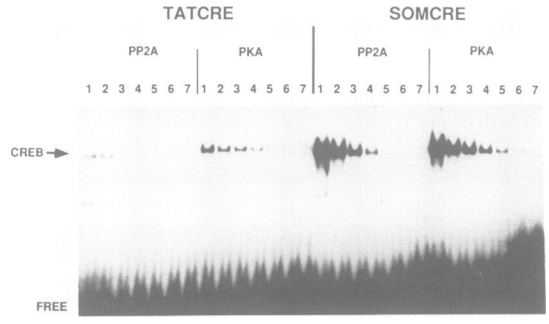

Fig. 7. Competition binding assays for CREB with the TATCRE and SOMCRE. To determine the relative affinities of CREB for the TATCRE and SOMCRE, bandshift reactions with increasing amounts of DNA fragment were performed. A constant amount of labelled DNA fragment was added in each lane (20 fmol) while unlabelled DNA (same sequence as the probe) was increased from 0 to 640 fmol as competitor (for lanes 1−7: 0×, 1×, 2×, 4×, 8×, 16×, 32×, molar excess unlabelled DNA, respectively). A constant amount of *E.coli* expressed CREBα protein (~10 fmol), treated with either PP2A or PKA, was present in each reaction.

in vivo is observed at the endogenous CREs. At the basal, uninduced level of PKA activity, a mixture of CREB dimers with one or two Ser133 phosphates leads to full occupancy of the PEPCK CRE, but at an intermediate transcription level. Upon PKA stimulation, CREB dimers with both Ser133 phosphates may show greater transactivation function through the PEPCK CRE, as it remains fully bound. CREBα homodimer protein from an expression clone in *E.coli* showed inducible binding to CREs. We have also made similar observations with CREBΔ expressed in *E.coli*, baculovirus, or rabbit reticulocyte lysate systems (not shown). In addition, *in vitro* translated ATF-1, a closely related protein to CREB, can heterodimerize with CREB, but in contrast to the heterodimers, binding of ATF-1 homodimers was unaffected by PKA (W.Schmid,

unpublished data). This agrees with poor transcriptional transactivation by ATF-1 in response to cAMP, as demonstrated by Hurst *et al.* (1991). Recently, one CREM isoform (CREMτ) which is highly expressed in testis and is also very similar to CREB has been described. CREMτ can act as a PKA-dependent transcription activator, in contrast to the other CREM forms which act as repressors (Foulkes *et al.*, 1992), but it is unclear whether DNA binding is affected by PKA treatment. It is possible that a CREB–CREM repressor form is replaced by a PKA phosphorylated CREB homodimer upon forskolin treatment of FTO2B cells, explaining the constitutive footprint at the PEPCK CRE. However, this would not explain the absence of a footprint in PKA repressed cells and we have not observed significant CREM protein in FTO2B or liver. Phosphorylation of a number of other transcription factors has been shown to lead to changes in the overall transcription profile, resulting in some cases from altering DNA binding affinity. The c-Myb protein may activate a different set of genes after its phosphorylation by CKII (Lüscher *et al.*, 1990). Phosphorylation of transcription factors can thereby link signal transduction to differential gene expression (for review see Bohmann, 1990).

Two classes of CREs exist which differ by CREB affinity

Most characterized CRE sequences fall into two categories. The palindromic TGACGTCA CRE is composed of two of the functional CGTCA units which overlap in opposite DNA strands to form a symmetrical CRE. The asymmetric CREs contain only one CGTCA unit. The presence of two CGTCA units, not the presence of a palindromic sequence *per se* (Fink *et al.*, 1988), stabilizes binding of the homodimeric CREB complex. The scissors-grip model for leucine zipper DNA binding proteins (Vinson *et al.*, 1989) would predict that for asymmetrical CREs, only one of the two subunits of a CREB homodimer has an optimal CGTCA binding site, resulting in a lower net affinity, as demonstrated in bandshift assays above (Figures 6 and 7) and in Southwestern experiments using symmetric or asymmetric CRE probes (M.Nichols and B.Luckow, unpublished data). For low affinity, asymmetric CREs, phosphorylation-dependent transcriptional activation results in part from increased CREB binding to the CRE. This is also consistent with the model that phosphorylation at Ser133 leads to further transactivation through protein–protein interactions with the RNA polymerase II complex (Lee *et al.*, 1990; Yamamoto *et al.*, 1990). These observations imply that two distinct properties, DNA binding and transcription transactivation, can be modulated by one and the same phosphorylation at Ser133 of CREB.

What is the physiological significance of this differential binding? Phosphorylation at Ser133 is required for transcriptional activation by CREB (Gonzalez and Montminy, 1989). Since high affinity CREs bind CREB more efficiently than low affinity sites, more of the phosphorylated (active) CREB will be bound to symmetric CREs, exhibiting a higher basal level expression for those genes. On the other hand, high affinity sites should also bind more non- or hemi-phosphorylated (inactive) CREB dimers than low affinity sites, leading to potential inhibition or blocking of those sites. Consequently, an increase in CREB phosphorylation after cAMP stimulation should result in a higher fold induction in transcription of genes with a low affinity CRE for two reasons: unphosphorylated (transcriptionally inactive) CREB dimers are less likely to bind to asymmetric sites and the basal level expression is lower initially. Concordantly, CREB has been shown to bind the AP-1/CRE site of the c-*jun* gene and block transactivation by AP-1 through DNA binding at that site (Lamph *et al.*, 1990). However, if the PKA activity in the cells was increased by cotransfecting the catalytic subunit gene, phosphorylation of CREB led to increased reporter gene expression alone, or in addition to AP-1 transactivation by TPA. A CREB mutant which contained Ala133 only acted as a repressor which was not relieved by increased PKA activity, and a CREB mutant which does not bind DNA neither represses nor transactivates (Lamph *et al.*, 1990). Hence, CREB can be a repressor or activator at the same DNA site, depending on its state of phosphorylation. CREB may bind high affinity symmetric CREs in an analogous way. For the PEPCK CRE in FTO2B hepatoma cells, phosphorylation of CREB by PKA, resulting in increased transcription, must either occur directly on CREB dimers already bound to the CRE or be dependent on the turnover rate of inactive CREB dimers bound at the CRE. The TATCRE is free to bind CREB dimers after they are phosphorylated by PKA, increasing the occupancy at the TATCRE as seen in genomic footprinting assays, and hence the transactivation. This also predicts that asymmetric CREs show a lower basal level transcription but a higher fold of induction by cAMP than the symmetric CREs. Consistent with that, the *TAT* gene does show lower basal level and higher fold induction by cAMP than the PEPCK gene in newborn mouse livers (Ruppert *et al.*, 1990) and in FTO2B hepatoma cells (Stewart and Schütz, 1987; Boshart *et al.*, 1991), cells in which both genes are normally expressed. The dual role of CREB in activation or repression also explains the requirement for cotransfection of the PKA catalytic subunit gene with the CREB gene to see transactivation at CREs (Gonzalez and Montminy, 1989; Lamph *et al.*, 1990).

Do conformational changes of CREB due to phosphorylation increase binding to the TATCRE? The phosphate at Ser133 most likely does not contact the DNA binding site directly but results in a different conformation of CREB. This is suggested experimentally by the altered mobility of the PKA-treated CREB bandshifts in native PAGE (e.g. Figures 6 and 7) and is consistent with previous proposals (Yamamoto *et al.*, 1990; Gonzalez *et al.*, 1991). This conformational change facilitates binding to the CREs. Thus, as cAMP levels increase and PKA is activated above basal level, the low affinity CREs are more often in the bound state, broadening the overall cAMP response. Similarly, phosphorylation of c-Myb by CKII selectively inhibits binding to the weaker Myb binding sites whereas binding to the stronger Myb elements is relatively unaffected (Lüscher *et al.*, 1990). Therefore the set of target genes for activation by CREB and c-Myb may change after increased phosphorylation. For other transcription factors, it has been shown that protein conformation can influence and be influenced by DNA binding. The yeast protein PRTF has alternative conformations when bound to enhancer sequences of α-specific versus **a**-specific yeast genes, differing in transactivation (Tan and Richmond, 1990). The AP-1 factor changes conformation upon DNA binding (Patel *et al.*, 1990), as does the GCN4 DNA binding domain (Weiss *et al.*, 1990). Similarly, phosphorylation of CREB may lead

to conformational changes which increase DNA binding as well as subsequent transcription activation of target genes.

CREB exhibits multiple forms in vivo

Two-dimensional electrophoresis of CREB purified from liver indicates that CREB exists in multiple forms *in vivo* (Figure 4C). The fact that all forms of CREB (forms 3–5 for CREBΔ, forms 1–3 for CREBα) can be a substrate for PKA implies that phosphorylation by PKA is independent of previous modifications. However, the nature of some modifications may include glycosylation, as PP2A and PP1 phosphatases were unable even partially to change the 2-D pattern. It is unknown whether Ser133 phosphorylation is a prerequisite for phosphorylation at other sites. Phosphorylation at sites other than Ser133 may occur as the result of activation of other signal transduction pathways which alter the activity of CREB toward possible subsets of binding sites, leading to transactivation or transrepression. Sites on CREB for protein kinase C (Yamamoto *et al.*, 1988) and CKII have been shown to be phosphorylated *in vitro* by those enzymes, though with no clear functional consequence, and still other phosphorylation sites have been proposed (Lee *et al.*, 1990). Ca^{2+}/calmodulin-dependent kinases I and II (CaM kinase), mediating calcium and depolarization signals in neuronal PC12 cells, phosphorylate CREB *in vitro* at Ser133, correlating to increased transcription of c-*fos* through a CRE (Dash *et al.*, 1991; Sheng *et al.*, 1991). Other CREB/ATF proteins may also modulate CRE enhancer function by binding CREs independently of CREB or as heterodimers with CREB. Clearly, the DNA binding and transactivation functions of CREB could be controlled by many converging pathways, resulting in a complex balance of transcriptional control.

Materials and methods

Purification of TATCRE binding activities from rat liver

After preparation of nuclei from rat liver (Gorski *et al.*, 1986), nuclear extract was prepared as described by Dignam *et al.* (1983). A 10–60% ammonium sulfate fractionation of the extract was used as starting material for chromatography. The extract was desalted using a G25 column or dialysis into a buffer containing 10 mM HEPES (pH 7.9), 1 mM DTT, 1 mM EDTA, 5 mM $MgCl_2$, 10% glycerol and 50 mM KCl and chromatographed on DEAE-sepharose (Pharmacia). The BC activity (CREB) eluted from DEAE-sepharose at ~100 mM KCl while the A activity eluted at ~200 mM KCl. The BC and A activities were pooled separately, dialysed against 10 mM HEPES (pH 7.9), 5 mM DTT, 10 mM EDTA, 5 mM $MgCl_2$, 20% glycerol and 200 mM KCl and chromatographed on DNA cellulose (Pharmacia) to enrich for DNA binding proteins. Finally, a DNA affinity column made with oligodeoxynucleotides containing the TATCRE sequence (CTGCGTCA) was employed to isolate the CRE binding proteins (Kadonaga and Tjian, 1986). Ten μg/ml calf thymus DNA was added to the starting material prior to chromatography on the TATCRE DNA column to reduce non-specific protein binding.

Preparation of CREB from rat brain or overexpressing cDNA clones

Extracts from rat brain were fractionated on DEAE-sepharose, DNA cellulose and then on an oligodeoxynucleotide affinity matrix containing the palindromic somatostatin CRE, as described by Zhu *et al.* (1989). The preparation of the 43 kDa CREB protein was used in comparative analyses with the liver factor(s) which bind to TATCRE.

Full length cDNA clones of CREBα and CREBΔ from mouse (Ruppert *et al.*, 1992) were cloned into either *E.coli* expression vectors (Rosenberg *et al.*, 1987) or baculovirus (Invitrogen). Baculovirus expressed CREB was purified by the same procedure as CREB from rat brain, while isolation of *E.coli* expressed CREBα and CREBΔ was performed as described in Müller *et al.* (1989). The *E.coli* expressed protein used in bandshift assays was a 30–60% ammonium sulfate fraction from the crude extract, followed by dialysis.

Bandshift assays

Oligodeoxynucleotides were synthesized which included the TATCRE sequence, CTGCGTCA, centred within 26 nucleotides of wild-type TAT sequence. This DNA was cloned (Weih *et al.*, 1990) and then used for bandshift assays, or synthetic double-stranded oligodeoxynucleotides were used. A mutant oligodeoxynucleotide (MUTCRE), with three base changes in the core sequence (CTGtaTgA) at the three contact sites known from *in vivo* footprinting (Weih *et al.*, 1990), was used as negative control. Differential competition and binding affinities between the wild-type and mutant DNAs were the criteria for potentially interesting DNA–protein complexes. The SOMCRE oligodeoxynucleotide contains the palindrome TGACGTCA from the −50 bp region of the somatostatin gene. Bandshift oligodeoxynucleotides were labelled at the 3′-ends with Klenow polymerase and gel-purified. The conditions for the bandshift reactions are described in Weih *et al.* (1990). In experiments with purified or expressed proteins, no non-specific DNA was added to the reactions and 0.5 mg/ml of carrier protein was included (BSA or insulin). The incubation of DNA fragment with protein factor was for 30 min on ice, unless otherwise stated. Reactions to determine binding affinity were performed at 24°C with 130 mM KCl, 10 mM DTT, 5 mM HEPES (pH 7.9), 0.5 mg/ml BSA for 60 min, before native PAGE in 8% gels containing 0.3×TBE. Immune or pre-immune antisera were incubated with protein for 15 min before addition of labelled DNA, in those reactions where immuno-bandshifts were analysed.

Protein kinase A (PKA catalytic subunit) and protein phosphatase 2A (PP2A) assays

Protein kinase A assays were performed by treating purified liver, brain, or recombinant factors with 0.1 μg (30 ng) of purified catalytic subunit of protein kinase A provided by U.Walter and S.Lohmann (Würzburg). The reaction buffer contained 10 mM HEPES (pH 7.9), 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, 10% glycerol and 20–100 mM KCl. Incubation was at 30°C for 20–60 min. Phosphorylation reactions intended to generate radioactive substrate proteins contained 0.2 μM [γ-^{32}P]ATP (no unlabelled ATP added). Purified PP2A from rabbit muscle was provided by B.Hemmings (Basel) and used in a buffer containing 10 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM EDTA, 10 mM DTT, 10% glycerol and 20 mM KCl. Incubation was at 30°C for 1 h, a sufficient time to remove 95% of the *in vitro* incorporated radioactive phosphate on protein (data not shown). For samples which were treated with PKA after PP2A, okadaic acid was added to 100 nM to inhibit the PP2A activity, prior to addition of PKA and ATP.

Western analysis

Rabbit polyclonal antibodies recognizing CREB were generated using a synthesized peptide from the published CREB sequence (amino acids 137–150, Gonzalez *et al.*, 1989) as antigen. We further affinity-purified the antibodies using the synthetic CREB oligopeptide bound to a column, as described in Harlow and Lane (1988), to increase the specific antibody titre. Proteins were resolved on SDS gels, electroblotted onto nitrocellulose membranes and treated in Western analysis as described by Harlow and Lane (1988). Signals arise from the activity of alkaline phosphatase, linked via Extravidin to biotinylated goat anti-rabbit IgG (Sigma).

Two-dimensional protein gel analysis

These experiments were performed as outlined in Ausubel *et al.* (1988). Samples were loaded on the isoelectric focusing gels with gradients set up by 3 parts pH 3–10 ampholyte and 5 parts pH 4–6.5 ampholyte (Pharmacia), and the IEF marker used was 30 kDa carbonic anhydrase (pI 4.8–6.7; Pharmacia). Second dimension SDS–PAGE was followed by electroblotting on to nitrocellulose membranes and Western analysis (see above).

Phosphopeptide mapping analysis

To map the sites of phosphorylation of the liver and brain CREB proteins, the purified factors were labelled *in vitro* with the catalytic subunit of PKA as described above and then isolated from 10% SDS gels. The only major and consistently labelled band migrated at 43 kDa (see Figure 4A). Phosphoamino acid analysis was performed by digesting the labelled protein gel piece in 6 N HCl at 110°C for 5 h and then running high voltage electrophoresis (including phosphoserine, phosphothreonine, phosphotyrosine and P_i as markers) to resolve the products. Tryptic peptide analysis was performed as described in Boshart *et al.* (1991). A marker peptide was synthesized with the sequences of amino acids 124–136 of CREB (Gonzalez

et al., 1989). Phosphorylation by PKA and trypsin digestion of the 13 amino acid peptide generated the tryptic marker phosphopeptide, containing [^{32}P]Ser133 of CREB.

Tryptic mapping of *in vivo* labelled protein was performed similarly (see Boshart *et al.*, 1991, for more details). Briefly, FTO2B cells were labelled with 1 mCi $^{32}P_i$ in P_i-free medium for 4 h and then treated with forskolin (10 μM), or 0.1% ethanol for 10 min. Equal amounts of protein in extracts from the ^{32}P-labelled cells were incubated with the anti-CREB antibodies described above to normalize the subsequent phosphopeptide mapping. CREB was immunoprecipitated, using sheep anti-rabbit IgG coupled to paramagnetic beads (Dynal, Hamburg) and purified on a 10% SDS gel.

Genomic footprinting and probe synthesis

Cell lines were cultured as described earlier (Boshart *et al.*, 1991). FTO2B cells were either induced with 20 μM forskolin for 30 min or treated with 0.1% ethanol as solvent control. The cell lines clones #17 and #21 were treated for 18 h in serum-free medium with 25 μM $ZnCl_2$ to induce the mouse metallothionein I promoter driving a mutated version of the mouse RIα cDNA (Boshart *et al.*, 1991). As control, FTO2B cells were also treated with 25 μM $ZnCl_2$. *In vivo* methylation of cells, purification and piperidine cleavage of genomic DNA were carried out as described (Becker *et al.*, 1992). DMS-treated DNAs were subjected to a linear amplification reaction using *Taq* polymerase followed by gel electrophoresis, transfer onto a nylon membrane and hybridization as outlined in Reik *et al.* (1991) with modifications. Briefly, the amplification reaction (in 100 μl) contained: 15 μg DNA, 30 ng of a 27mer primer corresponding to the TAT sequence −3457 to −3430 or to the PEPCK sequence −380 to −354, 500 μM of each dNTP, 40 mM NaCl, 10 mM Tris−HCl (pH 8.9), 5 mM $MgCl_2$, 0.17 mg/ml BSA and 5 U *Taq* polymerase (Perkin Elmer). Twenty five cycles at 94°C for 1 min, 64°C for 1 min and 72°C for 1.6 min were performed. The amplified DNA was further processed and analysed on sequencing gels as described (Reik *et al.*, 1991; Becker *et al.*, 1992). Genomic filters were hybridized at 68°C with a cDNA probe of high specific activity synthesized from an RNA template (Weih *et al.*, 1988). The probe used for the TAT filter (HS127) recognized positions −3643 (*Hha*I) to −3516 (*Sty*I); the probe used for the PEPCK filter (NS158) recognized positions −363 (*Nde*I) to −205 (*Stu*I).

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