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Identification of a New cAMP Response Element-binding Factor by Southwestern Blotting*

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We have identified in mammalian cells a novel cyclic AMP response element (CRE)-binding protein of molecular mass 47 kDa. This protein was not recognized by either the CREB-327/341 or c-Jun antisera, and its tissue distribution did not overlap with those of the CREB and Jun families. For example, hepatoma and placental tissue did not contain the 47-kDa DNA-binding protein, but did contain the CREB isoforms. On the other hand, S49 lymphoma cells contained a high level of the 47-kDa DNA-binding protein but did not contain a 47-kDa Jun-related protein which was found in normal liver and hepatoma. This new 47-kDa factor bound to the CRE in the dephosphorylated form, and phosphorylation of the protein by the catalytic subunit of protein kinase A completely abolished its DNA-binding activity. The isoforms of the CREB-327/341 family, on the other hand, bound to DNA in the phosphorylated form, and alkaline phosphatase treatment reduced significantly their interaction with CRE sequence. This reverse effect of phosphorylation/dephosphorylation on the DNA-binding property of this new 47-kDa protein in particular distinguishes it from other known CREB factors and suggests that the protein might play a unique role in the regulation of cAMP-mediated transcription.

It is well established that the molecular mechanism of transcriptional regulation of cAMP-inducible genes is based on protein factors interacting with a cis-acting DNA regulatory sequence (TGACGTCA), termed the cAMP response element (CRE)[1] (for review, see Refs. 1–3). Over the last few years a number of different protein factors have been isolated and shown to interact with the CRE. In fact, several distinct cDNA clones encoding CRE-binding proteins have been isolated so far, such as CREB-327 (δ) and CREB-341 (α) (4–9), CRE-BP1 (10, 11), CRE-BP2 (12), CREM (13), CREB-2 (14), and a family of activating transcription factors (15–17). Furthermore, a subsequent detailed analysis of cDNA and genomic clones proved that the CREB gene alone encodes multiple mRNAs coding for proteins still uncharacterized (18, 19). The complexity of transcriptional regulation is further increased by the degree of phosphorylation of individual proteins (6, 20–23) as well as their ability to form homo- and heterodimers with the structurally related members of the Fos/Jun family (12, 16, 24, 25).

Molecular cloning of the CREB proteins such as the CREB-341, CREB-327, and CRE-BP1 was followed by their detailed characterization *in vitro* particularly in terms of their structural features (4, 11, 26). However, little is known about the functional consequences of the modulation of their properties by diverse extracellular signals and the effects of such modifications on their DNA-binding activities. In our effort to better understand transcriptional competence of cells, we utilized the Southwestern blotting technique which can provide information about the DNA-binding properties of these transciption factors. This technique has been successfully used to identify proteins interacting with promoters of the c-*fos* gene (27, 28),[2] the lactate dehydrogenase A subunit gene (9, 18, 30) and the human fibronectin gene (31) among others.

In the present manuscript we have shown that the DNA-binding activities of the CREB-341/327 isoforms could be readily detected by this approach since in the phosphorylated forms these proteins retain their CRE-binding even after one- and two-dimensional SDS-PAGE separation, but lose DNA-binding activity when dephosphorylated. In addition, using this technique we have identified a unique CRE-binding factor of the molecular mass 47 kDa which did not cross-react with either the CREB-341/327 or the c-Jun polyclonal antisera. Moreover, this protein had a different tissue distribution than that of the CREB/Jun factors. Most importantly, this new 47-kDa CREB protein bound DNA in a dephosphorylated form which clearly distinguished it from other CREB isoforms, adding further to the diversity of the CREB family.

EXPERIMENTAL PROCEDURES

Preparation of Nuclear Protein Extracts—Normal liver and placental tissues (taken at the 20th day of gestation) were obtained from specific pathogen-free 200–250-g Sprague-Dawley rats bred in this laboratory. Morris hepatoma 5123tc solid tumors were propagated in 200–300-g male Buffalo rats (Harlem Sprague-Dawley Co., Indianapolis, IN) as described by MacManus (32). S49 murine T-lymphoma cells were obtained from Dr. D. Franks (University of Ottawa) and grown in suspension in a complete medium RPMI 1640 (Life Technologies, Inc.) containing 15% fetal bovine serum. Nuclei were purified following the low speed centrifugation and Triton X-100 washing procedure, using the same buffers as described previously by Sikorska and Whitfield (33). Nuclear proteins were extracted with 25 mM HEPES buffer, pH 7.9, containing 25% (v/v) glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 0.42 M NaCl as described by Dignam *et al.* (34).

Preparation of Oligonucleotide Probes—Complementary strands of oligonucleotides containing the cyclic AMP-responsive element from the somatostatin gene were prepared by the phosphoroimidate method on an Applied Biosystems 380 Synthesizer. The probes were 3′-end labeled with [α-^{32}P]dATP and [α-^{32}P]dGTP (specific activity, 800 Ci/mmol, from Du Pont-New England Nuclear) using the Klenow fragment of DNA polymerase. The labeled probes were purified through Nick columns (Pharmacia LKB Biotechnology Inc.) and used in Southwestern blotting.

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[1] The abbreviations used are: CRE, cyclic AMP response element; PAGE, polyacrylamide gel electrophoresis; SMS, somatostatin.

[2] J. Kwast-Welfeld, P. R. Walker, J. F. Whitfield, and M. Sikorska, submitted for publication.

Southwestern Blotting Procedure—The Southwestern hybridization technique was adopted from the original method of Silva *et al.* (35) as described by Kwast-Welfeld *et al.* (30). 50–200 μg of nuclear proteins were resolved on 8.5% SDS-PAGE gels which were then incubated for 2.5 h at room temperature with gentle agitation in renaturing buffer consisting of 10 mM Tris, pH 7.4, 50 mM NaCl, 2 mM EDTA, 0.5 mM dithiothreitol, and 4 M urea with three buffer changes. Following renaturation, the proteins were electrotransferred (0.1 A, 16 h) onto nitrocellulose filters (Schleicher & Schuell) and the filters were blocked for 45 min with 5% (w/v) non-fat milk powder in 10 mM Tris, pH 8.0, 2 mM $MgCl_2$, 1 mM mercaptoethanol, and 50 mM NaCl. This was followed by an additional 45-min incubation with blocking buffer containing 25 μg/ml of poly(dI-dC). After this time 0.05 μg/ml (10–12 ml total volume) of radioactive oligonucleotide probe (approximately 2–5 × 10^6 cpm/ml) was add for a further 3.5 h. After the hybridization, the filters were washed twice for periods of 10 min each with blocking buffer and once with 10 mM Tris, pH 8.0, containing 2 mM $MgCl_2$ and 1 mM mercaptoethanol. The filters were dried and autoradiographed on Kodak X-Omat film.

Preparation of Antisera, Immunoblotting, and Immunoprecipitation—The amino acid sequences of CREB-327 (7) and c-Jun (36) proteins were scanned for likely antigenic sites using the University of Wisconsin Genetics Computer Group software package and the algorithm of Jameson and Wolf (37). The peptides corresponding to residues 134–150 of CREB-327 and 301–314 of c-Jun were selected and synthesized in this laboratory using the simultaneous multiple peptide synthesis method of Houghten (38).

New Zealand White rabbits were immunized with unconjugated peptides according to the following schedule. The first intramuscular injection of 0.5 mg of peptide emulsified with complete Freund's adjuvant (1:1, v/v) was followed by two subcutaneous injections of the peptide emulsified with incomplete adjuvant at the 2nd and 3rd week. The rabbits were test bled at 7 and 14 days after last inoculation and the titer of their sera was determined by ELISA assays as described by MacManus and Brewer (39).

Nuclear proteins were resolved on 8.5% SDS-PAGE, electrotransferred onto nitrocellulose filters (Hybond C, Amersham Corp.) and immunoblotted with the appropriate sera. The antigen-antibody complexes were visualized by autoradiography on Kodak X-Omat film after being labeled with ^{125}I-protein A (Du Pont-New England Nuclear).

Immunoprecipitations were carried out in a total volume of 0.9 ml of a buffer containing 10 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 0.25 mM phenylmethylsulfonyl fluoride, 200–300 μg of nuclear proteins, and appropriate sera at a dilution 1:100 (v/v, 30–50 μg/ml). After incubation for 30 min at room temperature 0.1 ml of protein A-Sepharose CL-4B beads (Pharmacia) were added and the incubation continued at 4 °C for an additional 2 h. The beads were collected, washed three times, and heated for 5 min at 85 °C with the electrophoresis loading buffer. The precipitated proteins were resolved on 8.5% SDS-PAGE, electrotransferred, and immunoblotted with anti-CREB serum. The proteins remaining in the supernatants were freeze dried, resolved on 8.5% SDS-PAGE, electrotransferred, and analyzed for the presence of DNA-binding proteins by the Southwestern blotting.

Other Procedures—SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (40), and isoelectric focusing gels (4%) were run according to O'Farrell (41).

Autoradiographs were scanned on a Bio-Rad model 620 video densitometer, and molecular masses calculated from a polynomial standard curve drawn through the positions of ^{14}C-labeled markers (Amersham Corp.).

RESULTS

Synthetic oligonucleotides containing the CRE sequence of the rat somatostatin (SMS-CRE) gene promoter and a *Bam*HI linker for convenience of labeling, gatcCCTCCTTGGC **TGACGTCA**GAGAGAG (42) were radiolabeled and used to analyze nuclear extracts derived from rat liver for the presence of proteins able to recognize and specifically bind to this sequence using the Southwestern blotting technique. Several protein bands, ranging in molecular mass from 34 to 72 kDa, were labeled by the radioactive probe (Fig. 1). Using varying amounts of nuclear proteins from 260 to 32.5 μg (Fig. 1*A*, *lanes 1–4*) we determined that with a radioactive probe of specific activity approximately 10^8 cpm/μg DNA it was possible to detect these protein bands in as little as 100–200 μg of total nuclear proteins after a 4–5-day exposure of the autoradiograms. We have shown recently that the 34–40-kDa CRE-binding proteins are ubiquitously present in a variety of cells and tissues (28).[2] In contrast, the 72-kDa DNA-binding protein could only be detected in the liver protein extracts (Fig. 1*B*, *lane 1*), and even in liver the intensity of this labeling varied from experiment to experiment. The 47-kDa factor was present in liver and S49 cells (Fig. 1*B*, *lanes 1* and *3*), but it was not present in either the 5123tc hepatoma extract or in the placental extract (Fig. 1*B*, *lanes 2* and *4*).

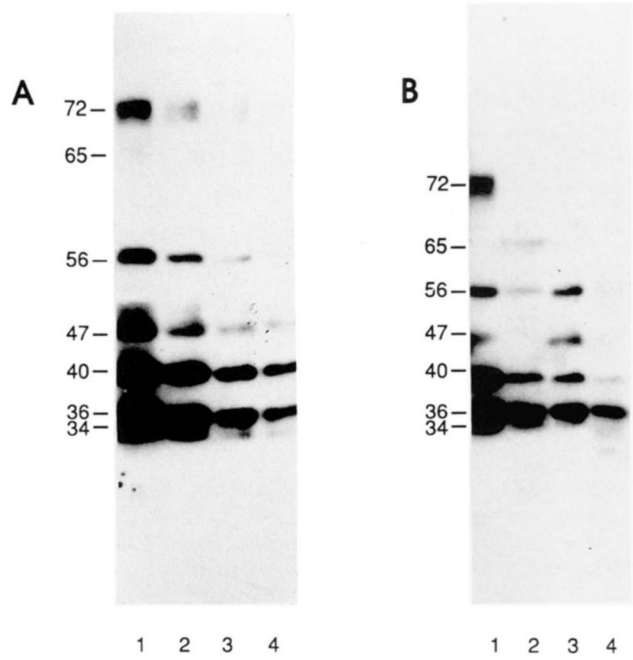

FIG. 1. **Southwestern blot of liver nuclear proteins binding to the somatostatin CRE.** *A*, varying amounts of rat liver nuclear protein (260 μg, *lane 1*; 130 μg, *lane 2*; 65 μg, *lane 3*; and 32.5 μg, *lane 4*) were separated on 8.5% SDS-PAGE, renatured, and electrotransferred onto nitrocellulose membrane. The blot was probed with ^{32}P-labeled SMS-CRE sequence. The labeled proteins were visualized by autoradiography (4 days of exposure) and the molecular masses of the DNA-binding proteins were estimated by densitometric scanning as described under "Experimental Procedures." *B*, nuclear protein extracts from rat liver (200 μg, *lane 1*), Morris hepatoma 5123tc solid tumor (200 μg, *lane 2*), S49 murine T-lymphoma cells (100 μg, *lane 3*), and rat placenta (200 μg, *lane 4*) were blotted and probed with ^{32}P-labeled the SMS-CRE. The labeled complexes were visualized by autoradiography (5 days of exposure).

To demonstrate which of the proteins identified above correspond to the proteins already known to interact with the CRE element, namely the CREB-341/327 and c-Jun proteins (25, 43), we prepared peptide-derived rabbit polyclonal antibodies against these factors and analyzed their presence by Western blotting (Fig. 2). We tested nuclear extracts from normal rat liver (*lane 1*) and S49 cells (*lane 3*) which, based on results obtained with the Southwestern blotting, contained multiple CRE-binding proteins (Fig. 1*B*, *lanes 1* and *3*) and nuclear extracts from the 5123tc Morris hepatoma (*lane 2*) and rat placenta (*lane 4*) which did not contain the 47-kDa CRE-binding protein (Fig. 1*B*, *lanes 2* and *4*).

The CREB antibody was raised against amino acids 134–150 of the CREB sequence published by Hoeffler *et al.* (7). This highly conserved region is also present in the CREB sequences identified by others (9, 16, 20). As expected, the antibody recognized a number of proteins in the nuclear

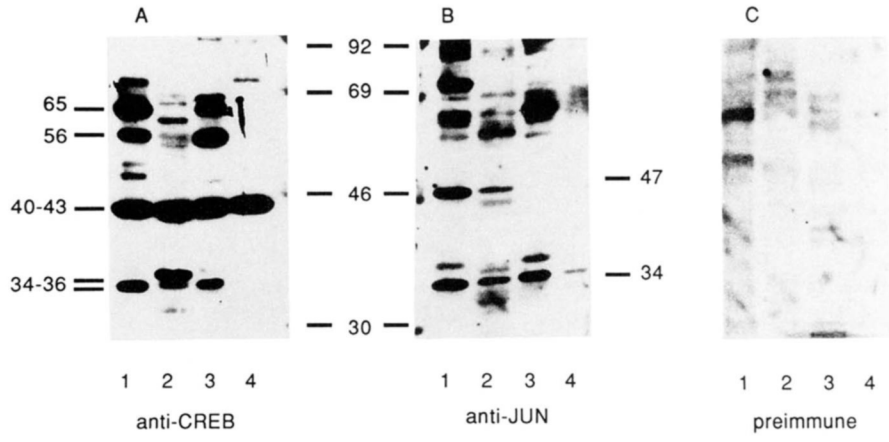

FIG. 2. **Analysis of CREB-341/327 and c-Jun proteins by Western blotting.** Approximately 200 μg of nuclear proteins from normal rat liver (*lane 1*), Morris hepatoma 5123tc (*lane 2*), S49 T-lymphoma cells (*lane 3*), and rat placenta (*lane 4*) were separated by 10% SDS-PAGE, electrotransferred, and immunoblotted with anti-CREB (*A*), anti-c-Jun (*B*), and CREB preimmune (*C*) sera diluted to a final concentration of 2–3 μg/ml. The antigen-antibody complexes were visualized by autoradiography on Kodak X-Omat film after being labeled with ^{125}I-protein A (Du Pont-New England Nuclear). The routine exposure time for the Western blots was 7 days.

extracts (Fig. 2*A*). The major CREB band, which was present in all samples, had an estimated molecular mass of approximately 40–43 kDa. In the nuclear extracts from liver (*lane 1*) and S49 cells (*lane 3*) there were two other proteins with molecular masses of 56 and 65 kDa which immunoreacted with the CREB antibody. These two samples (*lanes 1* and *3*), as well as 5123tc Morris hepatoma (*lane 2*) also contained immunoreactive proteins of molecular mass 34–36 kDa. Since none of these proteins reacted with preimmune serum (Fig. 2*C*) they are most likely different isoforms of the CREB protein. The CREB antibody did not recognize a protein equivalent to the 47-kDa band, seen on the Southwestern blots, in any of the tissues tested.

The c-Jun antibody which was raised against the leucine zipper region (amino acids 301–314) of the mouse proto-oncogene product (36) also recognized multiple nuclear proteins (Fig. 2*B*). The antibody recognized a 47-kDa Jun-related protein in liver and hepatoma tissues (*lanes 1* and *2*) but not in S49 cells or in placenta (*lanes 3* and *4*). Since the same S49 cell nuclear extract contained a high level of the 47-kDa CRE-binding protein detectable by the Southwestern blotting (Fig. 1*B*, *lane 3*) the latter protein must be different from that recognized by the Jun antibody. In addition, the anti-c-Jun polyclonal serum labeled a 34–36-kDa protein doublet in the extracts tested, but its level was hardly detectable in placenta tissue (*lane 4*). It also labeled a number of higher molecular mass proteins (60–70 kDa) which were not recognized by preimmune serum (data not shown).

To confirm that the 34–40-kDa protein bands detectable on the Southwestern blots corresponded to different isoforms of the CREB factor, we compared the pattern of liver nuclear proteins binding the SMS-CRE probe (Fig. 3*A*) to the pattern of proteins recognized by the CREB antibody (Fig. 3*B*) after two-dimensional separation. It can be clearly seen that the 40-kDa and the 34-kDa bands which were present in the one-dimensional SDS-PAGE separation (Fig. 3*A*, *left lane*) remained as single spots (indicated by the *arrows*) after the two-dimensional separation. However, the 36-kDa band was resolved into three spots, labeled *I*, *II*, and *III*, with slightly different pI values. Interestingly, following two-dimensional protein separation in this pH range (4.0–8.0) we could not detect DNA binding activity of the 47-kDa protein. This suggested that either the pI of this protein is outside the range of pH used here or that its DNA-binding ability is lost as a result of the separation of components contributing to this binding. The CRE oligonucleotide probe and the antibody both recognized the same 36–40-kDa proteins (indicated by *arrows*). On two-dimensional blots, the same as on one-dimensional blots (Fig. 2*A*), the antibody recognized the 34- and 56-kDa proteins but the spots were difficult to reproduce from the autoradiograms since the signal was much weaker as a result of the two-dimensional separation. It should be noted however, that under the experimental conditions used for the Southwestern blotting (8.5% SDS-PAGE, urea renaturation) the major CREB protein which ran on 10% SDS-PAGE gels as a 43-kDa band ran with an apparently lower molecular mass of approximately 40 kDa (Fig. 3*B*). The 36-kDa band could be resolved into three separate spots on both blots, indicating that they were different modifications of the same CREB isoform. On the other hand, the 47-kDa CRE-binding protein could not be detected on either the two-dimensional Southwestern or Western blots.

To further distinguish between the 47-kDa protein and the other CREB factors, a liver nuclear extract was treated with the CREB antibody, the immunoprecipitated proteins were then removed and the remaining proteins were resolved on SDS-PAGE and probed with the SMS-CRE (Fig. 3*C*). The antibody treatment removed all but the 47-kDa CRE-binding protein from the extracts (*lane 2*). Similar treatments with preimmune serum (*lane 3*) or with the c-Jun antibody (data not shown) did not affect the DNA-binding activity of these proteins.

It is well established that the transactivating properties of the CREB-341/327 isoforms are modified by phosphorylation (6, 20–22). However, it was not clear from these studies whether phosphorylation affected, directly, protein-DNA interactions. To address this issue we analyzed the DNA-binding activities of the liver nuclear CREB factors (Fig. 4, *lane 1*) following dephosphorylation by alkaline phosphatase (*lane 2*) and subsequent rephosphorylation by either endogenous kinases (*lane 3*) or the catalytic subunit of protein kinase A (*lane 4*). The results clearly showed that with the exception of the 47-kDa protein, the CREB factors bound to DNA in the phosphorylated form. Alkaline phosphatase treatment greatly reduced their DNA-binding properties (Fig. 4, *lane 2*) which could be fully restored upon rephosphorylation (Fig. 4, *lanes 3–4*). This experiment also showed another distinctive feature of the 47-kDa factor. It was found to bind to the CRE only in the dephosphorylated form (*lane 2*). When phosphorylated, particularly by protein kinase A (*lane 4*), the 47-kDa protein did not bind to the DNA at all. In the liver and 5123tc hepatoma extracts (Fig. 1) we could also detect a very faint band of molecular mass 65 kDa whose interaction with the CRE was phosphorylation-dependent in the same manner as the 47-kDa factor. Presently we are investigating the identity of these two CREB-like factors.

DISCUSSION

The Southwestern blotting technique, despite its apparent limitations, has proved to be a useful tool in analyzing the

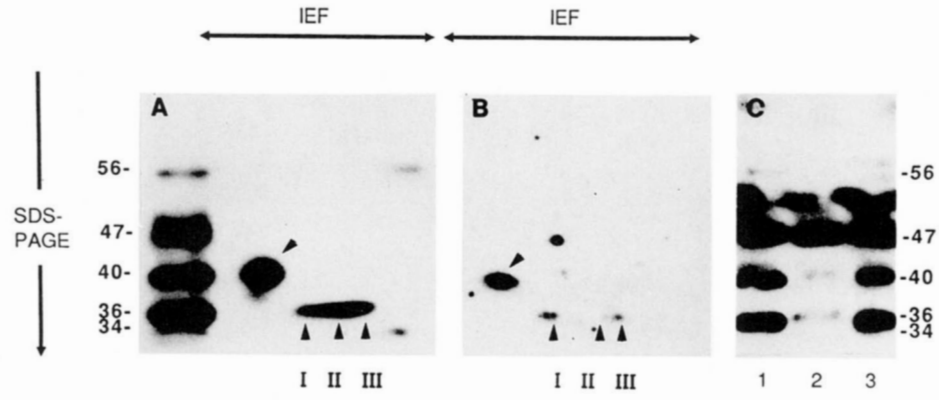

FIG. 3. **Identification of CREB-341/327 isoforms on Southwestern and Western blots.** Rat liver nuclear proteins (150 μg) were first separated by electrofocusing on 4.0% polyacrylamide gels prepared with pH 3.0–10.0 ampholines (Pharmacia) according to O'Farrell (41) and then on a 8.5% SDS-PAGE. The separated proteins were transferred onto nitrocellulose and either hybridized with oligonucleotides or immunoblotted with an antibody. *A*, Southwestern blot of nuclear proteins resolved by one- and two-dimensional electophoresis and hybridized with the SMS-CRE (exposure time, 7 days). *B*, Western blot of nuclear proteins resolved by two-dimensional electrophoresis and immunoblotted with anti-CREB serum (exposure time, 9 days). *C*, Southwestern blot of nuclear proteins left in the supernatant following the immunoprecipations with the anti-CREB serum (exposure time, 7 days). *Lane 1*, nuclear proteins treated only with the protein A-coated beads; *lane 2*, nuclear proteins after immunoprecipation with anti-CREB serum; *lane 3*, after immunoprecipitation with preimmune serum.

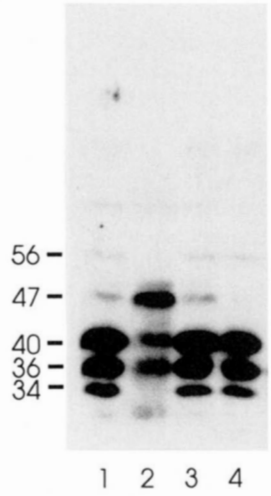

FIG. 4. **Effect of phosphorylation/dephosphorylation on the DNA-binding activity of the CREB factors.** Nuclear proteins from rat liver (200 μg) were incubated for 20 min at 37 °C in the extraction buffer (*lane 1*), with 10 units of intestinal alkaline phosphatase (Sigma) in the presence of 5 mM $MgCl_2$ (*lane 2*), with 5 mM $MgCl_2$ and 1.25 mM ATP (*lane 3*), and with 10 units of the catalytic subunit of cAMP-dependent protein kinase (Sigma) in the presence of 5 mM $MgCl_2$ and 1.25 mM ATP (*lane 4*). Following incubation the proteins were electrophoresed on 8.5% SDS-PAGE, electrotransferred onto nitrocellulose, and hybridized with the SMS-CRE. The autoradiogram was exposed for 5 days.

DNA-binding activities of transcription factors. In applying this technique, however, it is important to remember that due to the denaturation and separation processes that occur during SDS-PAGE, identification is restricted to those proteins which retain their DNA binding properties upon renaturation and can bind to regulatory elements either as monomers or possibly homodimers. Therefore, such information will permit one to assess only selected aspects of the interactions between proteins and regulatory elements. Nevertheless, we have applied this method previously to identify protein factors interacting with the c-*fos* gene promoter (27, 28) as well as to study the interaction of the CREB proteins with CREs of different genes.[2] In the present study we have shown that using a 25-base pair fragment of the somatostatin gene promoter (nucleotides −60 to −36) it was possible to label reproducibly several CRE-binding proteins of molecular mass 34, 36, 40, 47, 56, 65, and 72 kDa in as little as 100–200 μg of total nuclear nuclear proteins. These CREB proteins retained their DNA-binding properties after one- and two-dimensional electrophoretic separation, therefore, they all must be capable of binding to the DNA as monomers or homodimers. The 34-, 36-, and 40-kDa and possibly the 56-kDa protein bands were different isoforms of the CREB family since they reacted with the CREB-341/327 antibody.

The 36-kDa band actually consisted of three CRE-binding activities with slightly different pI values that could be resolved by two-dimensioanl SDS-PAGE. A group of proteins of similar molecular mass and phosphorylation-dependent DNA-binding activity has also been reported in HeLa cells (17). We have also shown previously (27, 28) and confirmed in the present study that the DNA-binding property of the 36-kDa protein band was dependent on its state of phosphorylation. Therefore, the three protein spots seen in Fig. 3 most likely represent the same CREB-327 (delta) factor phosphorylated to different degrees. The existence of multiple phosphorylated subdomains within the structure of CREB-327 protein has been demonstrated by Lee *et al.* (21).

The 40-kDa band was equivalent to CREB-341 (α) (44) by the criteria of the overlapping protein pattern on the Southwestern and Western blots as well as immunoprecipitation by the CREB antibody. Its direct DNA interaction was also phosphorylation dependent. In contrast to CREB-327, we could only identify a single form of this protein after two-dimensional separation. However, under the electrophoretic conditions used for the Southwestern blotting this protein migrated with a molecular mass slightly lower than on the Western blots. The molecular mass of CREB-341 predicted from the amino acid sequence is only 37-kDa although in some publications (possibly as a result of different electrophoretic conditions), the CREB-341 protein appears to have a molecular mass between 43 and 48 kDa on SDS-PAGE (18, 26, 44).

The 47-kDa CRE-binding protein which we previously identified in rat liver (28) appears to be a unique factor. The protein did not cross-react with either the CREB-327/341 or the c-Jun antibodies. Furthermore, its tissue distribution did not overlap with those of the CREB and Jun families. For example, liver tumors and placental tissue which did not

contain the 47-kDa DNA-binding protein (Fig. 1*B*) but, did contain a 40–43-kDa protein labeled by the CREB antibody (Fig. 2). Although the c-Jun antibody did recognize a protein of 47-kDa molecular mass in the hepatoma, the tissue did not contain a DNA-binding protein of the same molecular mass (Fig. 1*B*, *lane 2*). This Jun isoform must, therefore, bind to DNA only as a heteromimer and it cannot be detected be the Southwestern blotting technique. Conversely, a high level of the 47-kDa CRE-binding protein was detected in the S49 cell nuclear extract (Fig. 1*B*, *lane 3*), but these cell did not contain a 47-kDa c-Jun related protein (Fig. 4, *lane 3*).

An additional difference between the CREB-341/327 family and the 47-kDa factor was revealed by two-dimensional separation. The latter protein could not be detected on the two-dimensional Southwestern blots after electrophoretic separation in the pH range 4.0–8.0, suggesting that its isoelectric point was outside this range. The isoelectric points of the CREB isoforms as calculated using the University of Wisconsin GCG software package (45), are 5.18 for CREB-341, 5.03 for CREB-327, and 4.67 for CREB-2. Our experimental results (shown in Fig. 3 for both CREB-327 (the 36-kDa band) and CREB-341 (the 40-kDa band) are in agreement with these values.

Most significantly, the unique 47-kDa CRE-binding factor was capable of interaction with DNA after dephosphorylation. In fact, phosphorylation of nuclear protein extracts by the catalytic subunits of protein kinase A completely abolished the DNA-binding activity of this factor marking clearly its uniqueness. We have shown recently (28)[2] that this novel 47-kDa CREB factor binds directly to the octameric 5′-TGACGTCA-3′ sequence regardless of its flanking regions and is unable to distinguish between promoters of cAMP-inducible and non-inducible genes. Examples of such promoters which although they contain the perfect CRE octomer they do not respond to cAMP, are bovine parathyroid hormone and glucagon genes (29, 46). The 47-kDa protein bound equally well to the fragment of the SMS as to parathyroid hormone and glucagon gene promoters. On the other hand, it did not bind at all to the closely related AP-1 binding element. This specificity for the CRE suggests that it may play a very important physiological role in distinguishing between cAMP and protein kinase C responsive pathways.

The data presented in this manuscript and supported by data to be published elsewhere[2] clearly emphasize the distinct nature of the 47-kDa protein among an expanding family of CREB factors. This protein could be a constitutive repressor and therefore, we might speculate that in normal cells the protein occupies the CRE sequence and represses the genes. Upon transcriptional activation, modification of the protein by phosphorylation (possibly by protein kinase A) leads to the loss of its DNA-binding activity thus allowing other positive transcription factors, either sequence specific CREB proteins or general ones, to increase the rate of gene transcription. We are presently cloning this 47-kDa CRE-binding protein to reveal its true function.

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