

Identification of a novel mouse hepatic 52 kDa protein that interacts with the cAMP response element of the rat angiotensinogen gene

Jie WU, Qin JIANG, Xing CHEN, Xiao-Hua WU and John S. D. CHAN¹

University of Montreal, Maisonneuve-Rosemont Hospital, Research Center, 5415 Boul. de l'Assomption Montreal, Quebec, Canada H1T 2M4

To identify the nuclear protein(s) that interact with the putative cAMP response element (CRE) of the rat angiotensinogen (ANG) gene (i.e. nt 806–779 upstream of the transcriptional start site), mouse liver nuclear proteins were prepared for the present studies. The DNase I footprinting protection analysis revealed that nt –799/–788 in the 5′-flanking region of the rat ANG gene are protected by the mouse liver nuclear protein. Gel mobility-shift assays revealed that the addition of the unlabelled DNA fragment, ANG nt –806/–779 competed effectively with the binding of the labelled ANG nt –806/–779 to the mouse liver nuclear proteins but the addition of unlabelled mutants of ANG nt –806/–779 were only weakly effective in competing with the labelled ANG nt –806/–779. The addition of unlabelled CRE of the somatostatin (SOM) gene and the CRE of the tyrosine aminotransferase (TAT) gene was also ineffective in competing with the labelled ANG nt –806/–779. Southwestern blot analysis revealed that the labelled ANG nt –806/–779 inter-

acted with two mouse liver nuclear proteins with apparent molecular masses of 52 and 43 kDa, whereas the labelled SOM-CRE, TAT-CRE and the CRE of the phosphoenolpyruvate carboxykinase (PEPCK) gene interacted with one molecular species of 43 kDa. The binding of the labelled ANG nt –806/–779 to the 52 kDa protein was effectively competed for by the addition of unlabelled ANG nt –806/–779 but not by unlabelled SOM-CRE, TAT-CRE and PEPCK-CRE. Finally, Western blot analysis revealed that polyclonal antibodies against the CRE-binding protein (CREB) interacted with the mouse liver nuclear 43 kDa protein but not with the 52 kDa protein. These studies demonstrate that the CRE of the rat ANG gene (ANG nt –806/–779) interacts with the 43 kDa CREB and a novel 52 kDa protein from mouse liver. The novel 52 kDa protein is immunologically distinct from the 43 kDa CREB. These studies suggest that the 52 kDa protein might have a role in the expression of the hepatic ANG gene.

INTRODUCTION

We have previously reported on the expression of the angiotensinogen (ANG) gene in mouse hepatoma cells (Hepa 1-6) and have shown that isoprenaline or 8-Br-cAMP enhances the stimulatory effect of dexamethasone on the expression of the ANG gene in Hepa 1-6 cells [1,2]. The enhancing effect of isoprenaline is blocked by the presence of propranolol (β -adrenergic receptor blocker), ICI 118,551 (β_2 -adrenergic receptor blocker) and Rp-cAMP (an inhibitor of cAMP-dependent protein kinase AI and II), but only minimally by atenolol (β_1 -adrenergic receptor blocker). These studies demonstrate that the enhancing effect of isoprenaline is mediated predominantly via the β_2 -adrenergic receptor and the cAMP-dependent protein kinase A signal transduction pathway.

The exact molecular mechanism(s) for the enhancing effect of isoprenaline with dexamethasone on the expression of the ANG gene in Hepa 1-6 cells has not been defined. One possibility might be that the addition of isoprenaline stimulates the synthesis of intracellular cAMP, because β -adrenergic receptors are linked through a guanine nucleotide regulatory protein to adenylate cyclase on the inner part of the plasma membrane of target cells [3]. The intracellular cAMP then activates the cAMP-dependent protein kinase AI and II and subsequently phosphorylates the nuclear 43 kDa cAMP response element-binding protein (CREB) [4]. The phosphorylated 43 kDa CREB then interacts with the putative cAMP response element (CRE) (i.e. ANG nt –806/–779 containing the motif of the CRE, TGACGTAC, on nt –795/–788) in the 5′-flanking region of the rat ANG gene [5]. The phosphorylated CREB might also interact with the

activated-glucocorticoid receptor complex (GRC), which is bound to the glucocorticoid response elements in the 5′-flanking region of the rat ANG gene when stimulated by dexamethasone. Finally, the bound 43 kDa CREB/GRC unit will act synergistically with the pre-initiation complex to enhance the expression of the ANG gene. This possibility is supported by the studies of Imai et al. [6], who reported that the 43 kDa CREB interacts with the glucocorticoid receptor to stimulate the expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene. Moreover our recent studies showed that the expression of the ANG gene in opossum kidney (OK) cells is stimulated by the transfected plasmid containing the cDNA for the 43 kDa CREB [7]. The addition of isoprenaline further enhanced the stimulatory effect of the 43 kDa CREB on the expression of the ANG gene in OK cells [7].

The objective of our present study was to identify the mouse liver nuclear protein(s) that might interact with the putative CRE (i.e. ANG nt –806/–779) of the rat ANG gene. Our studies demonstrate that the CRE of the rat ANG gene interacts with the 43 kDa CREB and a novel 52 kDa nuclear protein from mouse liver. This novel 52 kDa nuclear protein is immunologically different from the 43 kDa CREB, suggesting that this 52 kDa nuclear protein might have a role in the regulation of expression of the ANG gene in the liver.

MATERIALS AND METHODS

Materials

Rabbit polyclonal antibodies against the C-terminus (residues 295–321) of the 43 kDa CREB were purchased from Santa Cruz

Abbreviations used: ANG, angiotensinogen; ATF, activating transcription factor; CRE, cAMP response element; CREB, CRE-binding protein; DTT, dithiothreitol; OK, opossum kidney; PEPCK, phosphoenolpyruvate carboxykinase; SOM, somatostatin; TAT, tyrosine aminotransferase.

¹ To whom correspondence should be addressed.

Biotechnology (Santa Cruz, CA, U.S.A.). These polyclonal antibodies are specific for the 43 kDa CREB and have no cross-reaction with other activating transcription factors (ATFs) and CREB transcription factors.

Rabbit polyclonal antibodies (Rb#8) against the residues 137–150 of the 43 kDa CREB were raised in our laboratory. Briefly, the fragments of 43 kDa CREB (residues 137–150) conjugated with keyhole limpet haemocyanin were purchased from Biosynthesis (Lewisville, TX, U.S.A.). The conjugated peptides were used to immunize New Zealand white rabbits (Charles River, St-Constant, Quebec, Canada) by the procedure described previously for ovine placental lactogen [8].

[γ -³²P]ATP (3000 Ci/mmol) was purchased from Dupont–New England Nuclear (Boston, MA, U.S.A.).

Oligonucleotides for the CRE of rat ANG gene (ANG-N-CRE) nt –806/–779 (5'-AAG AGA TTA CTT GAC GTA CTG GAT GCA A-3') [5], mutant I (MI) (5'-AAG AGA TTA CTT GAC TTA CTG GAT GCA A-3'), mutant II (MII) (5'-AAG AGA TTA CTT GAA TTA CTG GAT GCA A-3'), mutant III (MIII) (5'-AAG, AGA TTA CTT ATA TTA CTG GAT GCA A-3'), the CRE of somatostatin gene (SOM-CRE nt –59/–32, 5'-GCC TCC TTG GCT GAC GTC AGA GAG AGA G-3') [9], the CRE of the PEPCK gene (PEPCK-CRE, nt –101/–74, 5' AGG CCG GCC CCT TAG GTC AGA GGC GAG C-3') [10] and the CRE of the tyrosine aminotransferase gene (TAT-CRE, nt –3660/–3634, 5'-CTG CAG CTT CTG CGT CAG CGC CAG TAT-3') [11] were synthesized by Biosynthesis (Lewisville, TX, U.S.A.).

Restriction and modifying enzymes were purchased either from Bethesda Research Laboratories (Gibco-BRL, Burlington, Ontario, Canada), Boehringer-Mannheim (Dorval, Quebec, Canada) or Pharmacia (Baie d'Urfe, Quebec, Canada).

Other reagents were of molecular biology grade and obtained from Sigma Chemicals (St. Louis, MO, U.S.A.), Gibco-BRL, Boehringer-Mannheim or Pharmacia.

Mouse liver nuclear extract preparation

Adult mouse (CD-1) liver nuclear extract was prepared by the method of Hennighausen and Lubon [12] with slight modification. Briefly, male adult mice (aged 4–6 months) were killed under anaesthesia. The livers were removed immediately, rinsed twice in saline and cut into small pieces with scissors. The tissue fragments were homogenized with a Dounce homogenizer (2000 rev./min, three or four strokes; Electrical Stirrer, model RZR 50; Canlab Scientific Inc.) in 10 mM Hepes buffer, pH 7.6, containing 2.2 M sucrose, 5% (v/v) glycerol, 15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin and 1 μ g/ml pepstatin A. The homogenate was loaded on a 2 M sucrose cushion containing 10 mM Hepes, pH 7.6, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 0.5 M DTT, 0.5 mM 4-(2-aminoethyl)benzenesulphonyl fluoride, 2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 1 μ g/ml pepstatin A and 10% (v/v) glycerol and then centrifuged at 80000 g for 1 h at 4°C. The pellet was dissolved in a lysis buffer [10 mM Hepes (pH 7.6)/10% (v/v) glycerol/100 mM KCl/3.0 mM MgCl₂/0.1 mM EDTA/1.0 mM DTT/0.1 mM PMSF/2 μ g/ml leupeptin/2 μ g/ml aprotinin/1 μ g/ml pepstatin A]. The nuclear extract was further diluted with lysis buffer to a protein concentration of 0.5 mg/ml. The final KCl concentration was adjusted to 0.55 M. The precipitate in the nuclear extract was removed by centrifugation at 95000 g for 30 min at 4°C. Subsequently, solid (NH₄)₂SO₄ was slowly added to the nuclear extract until a concentration of 0.3 g/ml was achieved. The

extract was kept on ice for 50–60 min or kept overnight at 4°C, then centrifuged at 100000 g for 30 min. Finally the pellet was dissolved in a small volume of dialysis buffer [25 mM Hepes (pH 7.6)/10% (v/v) glycerol/40 mM KCl/1.0 mM DTT/0.1 mM PMSF] and dialysed against a large volume (i.e. 1 litre) of dialysis buffer at 4°C for 5 h with several changes of buffer. The dialysed nuclear extract was then centrifuged in an Eppendorf microcentrifuge to remove the precipitate; the supernatant (nuclear extract) was stored frozen in liquid nitrogen or at –80°C in aliquots. The protein concentration of the extract was determined by the Bio-Rad protein assay with BSA as standard.

DNase 1 footprinting protection assay

The plasmid containing nt –814/–689 of the rat ANG gene (13) was linearized with restriction enzyme *Hind*III (polylinker site of pGEM-3 plasmid) and end-labelled with T₄ polynucleotide kinase. The DNA was cleaved with a second restriction enzyme *Eco*RI (polylinker site of pGEM-3 plasmid) to release the labelled DNA fragment. The 5' end-labelled DNA was separated and isolated on a 4% (w/v) polyacrylamide gel. The labelled DNA fragment was then incubated separately on ice with either BSA (20 μ g) or mouse liver nuclear extract (0–20 μ g) in a total volume of 50 μ l of buffer containing 1 μ g of poly(dI/dC) and 10000 c.p.m. of labelled probe (final buffer concentration 20 mM Hepes/KOH, pH 7.5, containing 50 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 5 mM MgCl₂, 1 mM CaCl₂ and 10% (v/v) glycerol). After a 30 min incubation at room temperature, 0.2 unit of DNase 1 (Pharmacia) was added to the reaction mixture and incubated for a further 5 min at room temperature. The reaction was stopped by the addition of 100 μ l of 100 mM Tris/HCl, pH 7.6, containing 100 mM NaCl, 15 mM EDTA, 0.375% SDS, 150 μ g/ml proteinase K and 100 μ g/ml tRNA. The reaction mixture was then incubated at 37°C for 20 s followed by an additional incubation at 90°C for 2 min. The reaction mixture was extracted once with phenol/chloroform (1:1, v/v) and the DNA digest was separated in an 8% (w/v) urea sequencing gel and exposed overnight for autoradiography. The nucleotide position and DNA sequence were determined by parallel running of Maxam–Gilbert sequencing ladders [13].

Gel mobility-shift assay

The DNA fragments, ANG nt –806/–779, were 5' end-labelled with [γ -³²P]ATP by using T₄ polynucleotide kinase. Mouse liver nuclear proteins (10 μ g) or BSA (10 μ g) in the presence of 0.3 units of poly(dI/dC) in 20 mM Hepes (pH 7.6)/1 mM EDTA/50 mM KCl/2 mM spermidine/1 mM DTT/0.5 mM PMSF/10% (v/v) glycerol were incubated for 30 min at room temperature. Then the 5'-labelled probe (0.1 pmol) was added and further incubated for 30 min at room temperature. After being chilled on ice, the mixture was run on an 8% (w/v) non-denaturing polyacrylamide gel and exposed for autoradiography.

In competition assays, a 100–500-fold excess (or more) of unlabelled DNA fragments was added to the reaction mixture and incubated for 30 min at room temperature before incubation with the labelled probe.

Southwestern blot

Southwestern blot analysis was performed in accordance with the procedures found in [14,15], with slight modifications. Briefly, mouse liver nuclear proteins (50–200 μ g) were resolved by SDS/PAGE [4–20% (w/v) gradient gel] and then electro-

transferred to a nitrocellulose membrane (0.45 μm pore size) (Schleicher & Schuell, Keene, NH, U.S.A.). The membrane was incubated with 10% (w/v) non-fat milk proteins in a binding buffer containing 10 mM Hepes, pH 7.0, 10 mM MgCl_2 , 50 mM NaCl, 0.25 mM EDTA and 2.5% (v/v) glycerol for 1 h at 4 $^\circ\text{C}$. The membrane was then washed at least twice with the binding buffer containing 0.25% non-fat milk proteins. Subsequently the membrane was hybridized with ^{32}P -labelled double-stranded oligonucleotides (approx. 1.0–2.0 pmol; 10^6 c.p.m./ml) in binding buffer containing 0.25% non-fat milk proteins and 300 $\mu\text{g}/\text{ml}$ non-denatured herring sperm DNA at 4 $^\circ\text{C}$ overnight. The membrane was washed, air-dried and exposed for autoradiography.

In competition assays, a 50–100-fold excess of unlabelled DNA fragments was incubated with the membrane overnight before blotting with the radioactive ANG nt –806/–779.

Western blot

Western blot analysis was performed to analyse the mouse liver nuclear proteins by employing rabbit polyclonal antibodies against the C-terminus (residues 295–321) of the 43 kDa CREB, or employing rabbit polyclonal antibodies (Rb#8) against residues 135–150 of the 43 kDa CREB, Bio-Rad's anti-rabbit horseradish peroxidase conjugates and the avidin–horseradish peroxidase conjugates, in accordance with the protocol of the supplier (Bio-Rad, Richmond, CA, U.S.A.).

RESULTS

DNase 1 footprinting protection assay

Figure 1 shows that the nucleotides of ANG nt –799/–788 were protected by the mouse liver nuclear proteins. No protected region, however, was observed with BSA.

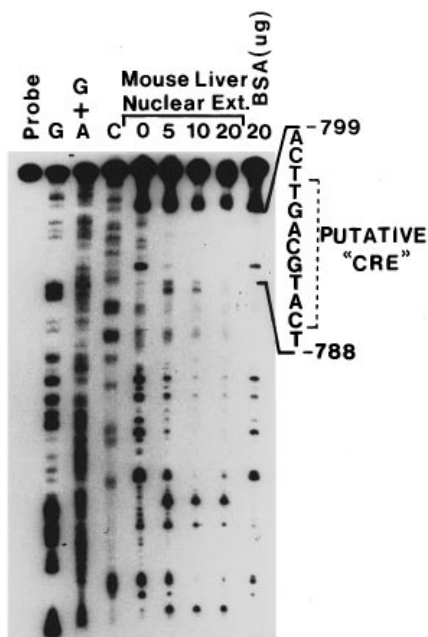


Figure 1 DNase 1 footprinting analysis of the DNA fragment, ANG nt –814/–689, of the ANG gene

The DNA was 5' end-labelled and incubated with BSA (20 μg) or with mouse liver nuclear extract (0–20 μg of protein), as described in the Materials and methods section. The protected DNA sequence was ANG nt –799/–788 as indicated. Abbreviation: μg .

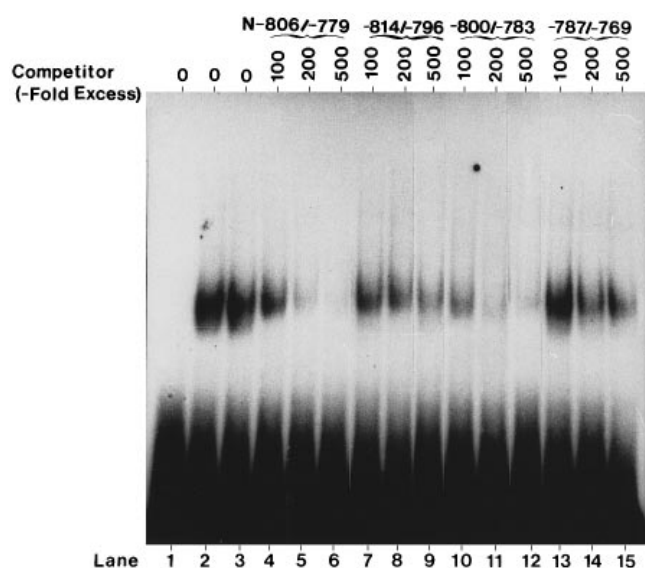


Figure 2 Gel mobility-shift assay of the radioactively labelled DNA fragment ANG nt –806/–779 with the mouse liver nuclear proteins

The labelled DNA probe (0.1 pmol) was incubated with BSA (10 μg) (lane 1) or mouse liver nuclear proteins (10 μg) (lanes 2–15) in the presence of 0.3 i.u. of poly(dI/dC). Competitions with various amounts of unlabelled ANG nt –806/–779, ANG nt –814/–796, ANG nt –800/–783 and ANG nt –787/–769 are shown in lanes 4–6, lanes 7–9, lanes 10–12 and lanes 13–15 respectively. Similar results were obtained in another experiment.

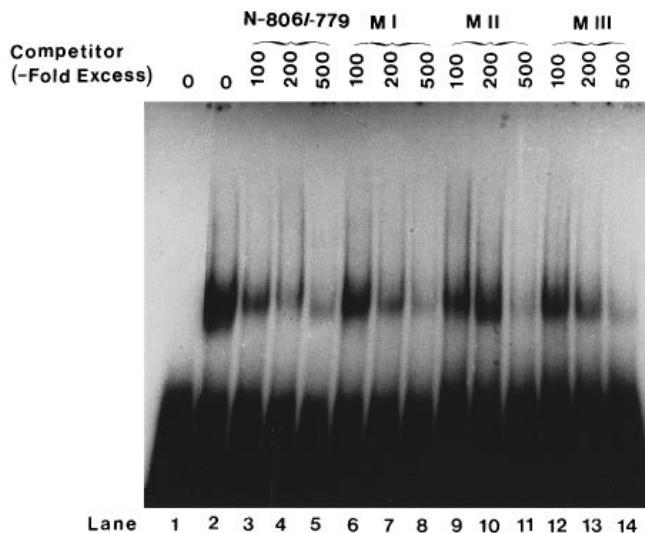


Figure 3 Autoradiography of the gel mobility-shift assay of the radioactively labelled DNA fragment ANG nt –806/–779 with the mouse liver nuclear proteins

The labelled DNA probe (0.1 pmol) was incubated with BSA (10 μg) (lane 1) or mouse liver nuclear proteins (10 μg) (lanes 2–14) in the presence of 0.3 i.u. of poly(dI/dC). Competitions with various amounts of unlabelled ANG nt –806/–779, mutant I, mutant II and mutant III are shown in lanes 3–5, lanes 6–8, lanes 9–11 and lanes 12–14 respectively. Similar results were obtained in two other experiments.

Gel mobility-shift assays

The interaction of the CRE (ANG nt –809/–779) of the rat ANG gene with the mouse liver nuclear proteins was analysed by

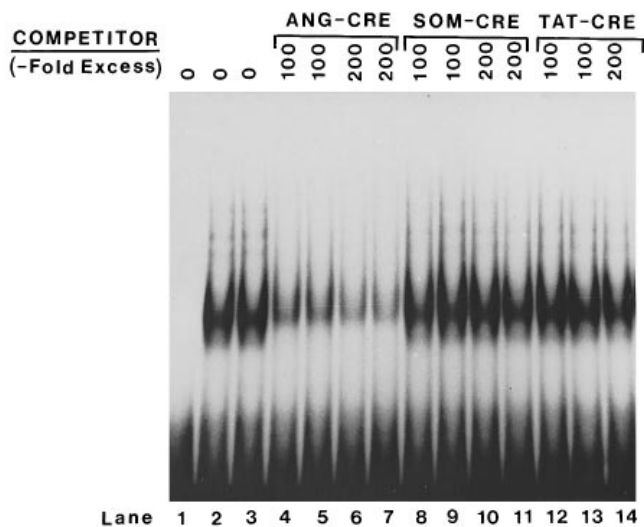


Figure 4 Gel mobility-shift assay of the radioactively labelled DNA fragment ANG nt $-806/-779$ with the mouse liver nuclear proteins

The labelled DNA probe (0.1 pmol) was incubated with BSA (10 μ g) (lane 1) or mouse liver nuclear proteins (10 μ g) (lanes 2–15) in the presence of 0.3 i.u. of poly(dI/dC). Competitions with various amounts of unlabelled ANG nt $-806/-779$, SOM-CRE and TAT-CRE are shown in lanes 4–7, lanes 8–11 and lanes 12–14 respectively. Similar results were observed in three other experiments.

a gel mobility shift assay as shown in Figure 2. When the labelled DNA fragment nt $-806/-779$ was incubated with the mouse liver nuclear proteins, one major band appeared with retarded mobility. No slowly migrating band was observed when the

labelled DNA was incubated with BSA. The addition of an unlabelled DNA fragment, ANG nt $-806/-779$ or ANG nt $-800/-783$, was effective in competing with the binding of labelled ANG nt $-806/-779$ to the nuclear protein(s) (i.e. at 100–200-fold excess of unlabelled DNA fragment) but the unlabelled DNA fragments representing ANG nt $-814/-796$ and ANG nt $-787/-769$ were only weakly effective (Figure 2). Similarly, the addition of the unlabelled mutants of ANG nt $-806/-779$ (i.e. M1, M2 and M3) was not as effective as the unlabelled ANG nt $-806/-779$ in competing for the binding of labelled ANG nt $-806/-779$ to the nuclear protein(s) (i.e. at 100-fold and 200-fold excesses of unlabelled DNA fragments) (Figure 3). These results indicate that the CRE motif is localized within nt $-795/-788$ (i.e. TGACGTAC) and is important for binding to the mouse liver nuclear proteins.

Figure 4 displays the effectiveness of the DNA fragments representing the CRE of the SOM gene (SOM-CRE) and the CRE of the TAT gene (TAT-CRE) in competing with the labelled ANG nt $-806/-779$ for the mouse liver nuclear protein(s). The addition of unlabelled SOM-CRE and TAT-CRE was not effective in competing with the binding of labelled ANG nt $-806/-779$ to the nuclear protein(s).

Southwestern blot analysis

The interaction of the ANG-CRE (ANG nt $-806/-779$) with nuclear proteins was examined by Southwestern blot analysis as shown in Figure 5(A). The labelled ANG nt $-806/-779$ interacted with one major and one minor protein band. The apparent molecular masses of the major and minor proteins were 52 and 43 kDa respectively. In contrast, Figure 5(B) shows that a single protein band with an apparent molecular mass of 43 kDa interacted with the 32 P-labelled SOM-CRE, the labelled PEPCK-CRE and the labelled TAT-CRE.

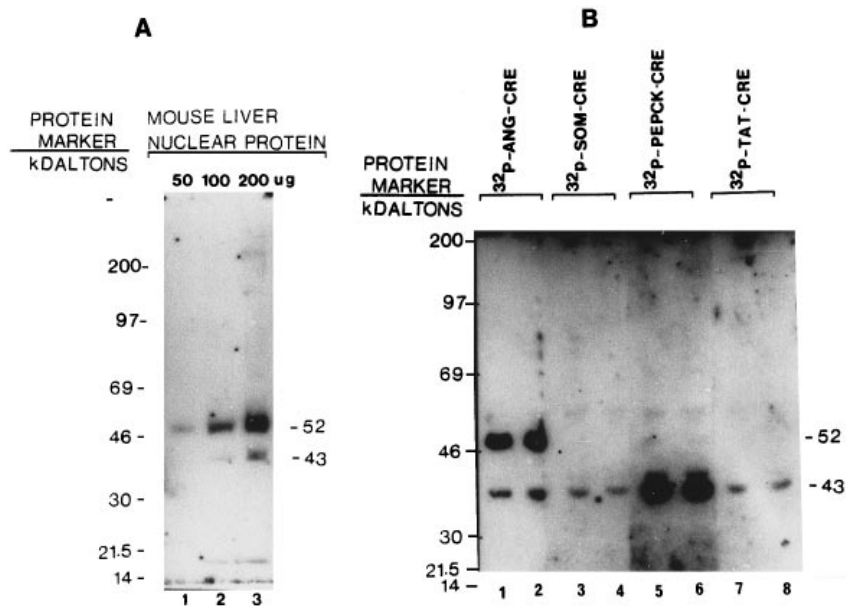


Figure 5 Southwestern analysis with the labelled DNA probes

(A) Mouse liver nuclear extracts were resolved by SDS/PAGE [4–20% (w/v) gradient gel], transferred to a nitrocellulose membrane, hybridized with radioactive ANG-CRE (ANG nt $-806/-779$), washed and finally subjected to autoradiography. Rainbow protein markers were used as molecular mass markers. (B) Comparison of the membrane hybridized with labelled ANG nt $-806/-779$ (lanes 1 and 2), labelled SOM-CRE (lanes 3 and 4), labelled PEPCK-CRE (lanes 5 and 6) or labelled TAT-CRE (lanes 7 and 8) (200 μ g of nuclear proteins was loaded per well).

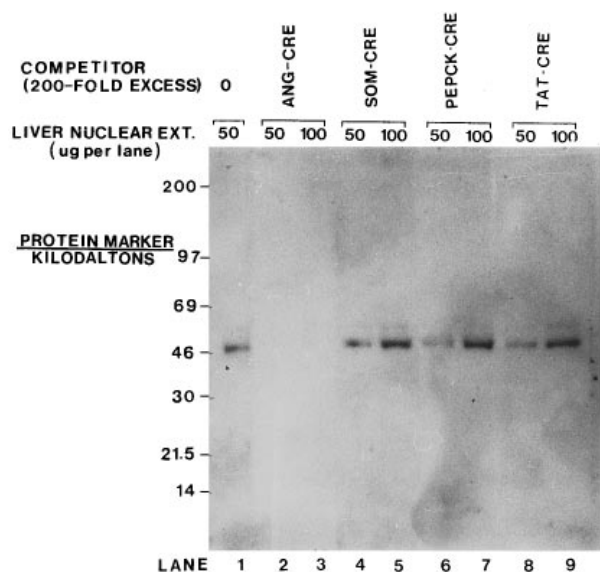


Figure 6 Effect of addition of the competitor DNA in the Southwestern analysis with labelled ANG nt $-806/-779$

Mouse liver nuclear extracts (50 or 100 μg per lane) were resolved by SDS/PAGE [4–20% (w/v) gradient gel], transferred to a nitrocellulose membrane, hybridized overnight with 200-fold excess of unlabelled ANG-CRE (ANG nt $-809/-779$) (lanes 2 and 3), SOM-CRE (lanes 4 and 5), PEPCK-CRE (lanes 6 and 7) or TAT-CRE (lanes 8 and 9) at 4 $^{\circ}\text{C}$. Then the membrane was hybridized with radioactively labelled ANG nt $-806/-779$, washed and subjected to autoradiography.

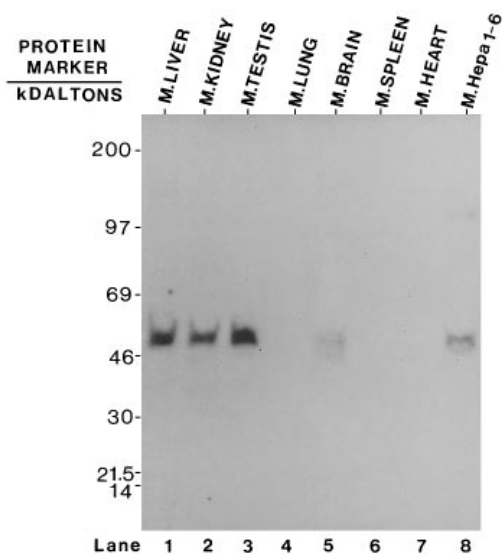


Figure 7 Distribution of the 52 kDa protein in various mouse tissues as analysed by Southwestern blot analysis

Nuclear extract from various mouse tissues or mouse hepatoma (Hepa 1-6) cells (100 μg per lane) were resolved by SDS/PAGE [4–20% (w/v) gradient gel], transferred to a nitrocellulose membrane, hybridized with radioactive ANG nt $-806/-779$, washed and subjected to autoradiography. Similar results were obtained in another experiment.

Figure 6 shows that the addition of a 200-fold excess of unlabelled DNA fragment ANG nt $-806/-779$ competed effectively for the binding of labelled ANG nt $-806/-779$ to the 52 kDa protein but not of the unlabelled DNA fragment of SOM-CRE, PEPCK-CRE or TAT-CRE.

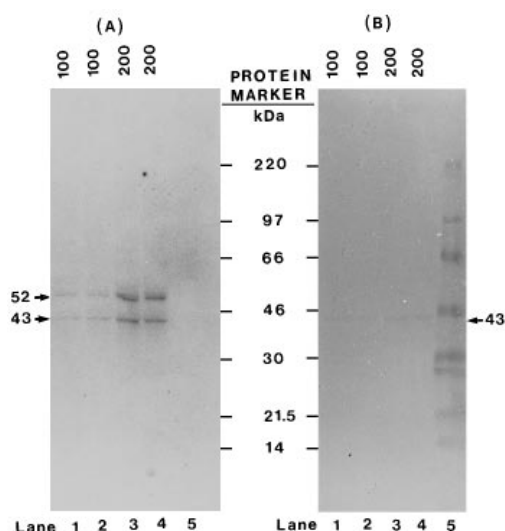


Figure 8 Southwestern and Western blot analysis of immunoreactive 43 kDa CREB from the mouse liver nuclear extract

(A) Southwestern blot analysis: different amounts (100 or 200 μg) of mouse liver nuclear extract were resolved by SDS/PAGE [4–20% (w/v) gradient gel], transferred to a nitrocellulose membrane, hybridized with radioactive ANG nt $-806/-779$, washed and subjected to autoradiography. (B) Western blot analysis: after Southwestern blot analysis, the same nitrocellulose membrane was blotted with rabbit polyclonal antibodies against the C-terminal portion of the 43 kDa CREB. Rainbow protein markers were used as molecular mass markers. Similar results were obtained in another experiment.

Figure 7 illustrates the tissue distribution of the 52 kDa protein in various mouse tissues. It is apparent that the 52 kDa protein is present in the liver, kidney, testis and brain, as well as in mouse hepatoma (Hepa 1-6) cells. The 52 kDa protein was not detectable in the lung, heart or spleen.

Western blot analysis

Figure 8(A) shows the Southwestern blot analysis of the mouse liver nuclear extract by employing the labelled ANG nt $-806/-779$. After Southwestern blot analysis, the same membrane was blotted with the polyclonal antibodies against the C-terminus (residues 295–321) of the 43 kDa CREB (Figure 8B). The polyclonal antibodies against the 43 kDa CREB interacted with one species of the 43 kDa CREB-like protein in the mouse liver nuclear extract. The antibodies did not interact with a 52 kDa nuclear protein.

Similarly, Figure 9(A) shows the Southwestern blot analysis of the mouse liver nuclear extract by employing the labelled ANG nt $-806/-779$. After Southwestern blot analysis, the same membrane was blotted with the polyclonal antibodies (Rb#8) against residues 135–150 of the 43 kDa CREB (Figure 9B). Again, the polyclonal antibodies interacted with the 43 kDa species but not with the 52 kDa species in the mouse nuclear extract. These studies demonstrate that the 52 kDa nuclear protein is immunologically distinct from the 43 kDa CREB.

DISCUSSION

We have previously demonstrated that the transfected pRSV/CREB stimulates the expression of the ANG gene in OK cells in a dose-dependent manner [7]. The addition of isoprenaline further enhances the stimulatory effect of pRSV/CREB [7]. We have also demonstrated that the CREB binds to the CRE (ANG nt $-806/-779$) of the rat ANG gene [16]. These studies support

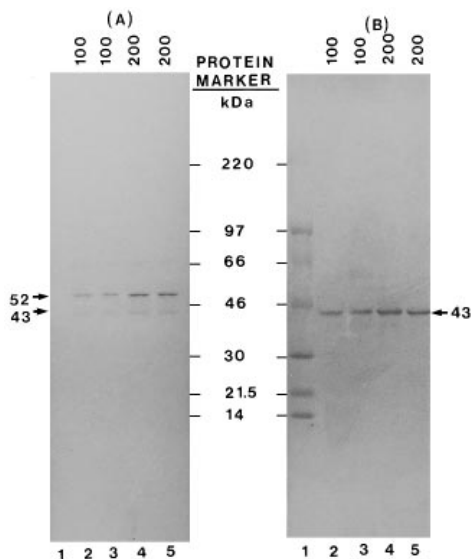


Figure 9 Southwestern and Western blot analysis of 43 kDa CREB from the mouse liver nuclear extract

(A) Southwestern blot analysis: 100 or 200 μg of mouse liver nuclear extract were resolved by SDS/PAGE [4–20% (w/v) gradient gel], transferred to a nitrocellulose membrane, hybridized with radioactive ANG nt $-806/-779$, washed and subjected to autoradiography. (B) Western blot analysis: after Southwestern blot analysis, the same nitrocellulose membrane was blotted with rabbit polyclonal antibodies (Rb#8) against the mid-region (residues 137–150) of the 43 kDa CREB. Rainbow protein markers were used as molecular mass markers. Similar results were obtained in another experiment.

the hypothesis that the nuclear 43 kDa CREB stimulates the expression of the ANG gene via its interaction with the putative CRE (ANG nt $-806/-779$) in the 5'-flanking region of the rat ANG gene.

To investigate whether ANG nt $-806/-779$ interacts with endogenous CREB or other protein(s) in mouse liver extracts, we performed DNase 1 footprinting protection and gel mobility-shift assays. Our DNase 1 footprinting protection assay revealed that the nucleotide sequence ANG nt $-799/-788$ is protected by the mouse liver nuclear extract (Figure 1). These studies provide strong evidence that the DNA fragment, ANG nt $-806/-779$, contains the putative CRE.

Our gel mobility-shift assays showed that one major retarded band is observed with the labelled ANG nt $-806/-779$. The addition of unlabelled ANG nt $-806/-779$ and ANG nt $-800/-783$ competed effectively with the binding of labelled ANG nt $-806/-779$ to the nuclear protein(s) (Figure 2). In contrast, the addition of unlabelled ANG nt $-814/-796$ and ANG nt $-787/-768$ was only weakly effective in competing with the labelled ANG nt $-806/-779$ (Figure 2). These studies suggest that ANG nt $-800/-783$ is the core CRE and that the DNA fragment ANG nt $-806/-779$ is interacting with mouse liver nuclear protein(s).

Furthermore our studies showed that the mutants of ANG nt $-806/-779$ (i.e. mutations in nt $-795/-788$) were less effective in competing with the binding of the labelled ANG nt $-806/-779$ to the mouse liver nuclear proteins than the unlabelled ANG nt $-806/-779$ (Figure 3). These studies further demonstrate that nt $-795/-788$ (TGACGTAC) represent the CRE motif, which is essential for the binding to the mouse liver nuclear proteins.

In contrast, the addition of competitors, SOM-CRE and TAT-CRE, was only weakly effective in competing with the labelled ANG-CRE for the binding to the mouse liver nuclear proteins

compared with the unlabelled ANG-CRE (Figure 4). These results suggest that nuclear protein(s) other than the 43 kDa CREB might interact with the ANG-CRE.

Indeed, our Southwestern blot experiments showed that the labelled ANG nt $-806/-779$ binds to the two mouse liver nuclear proteins with apparent molecular masses of 52 and 43 kDa (Figures 5A and 5B), whereas labelled SOM-CRE, PEPCK-CRE or TAT-CRE interact only with one molecular species of 43 kDa (Figure 5B). These experiments suggest that ANG nt $-806/-779$ interacts with a novel 52 kDa nuclear protein and a putative 43 kDa CREB. Furthermore the addition of unlabelled ANG nt $-806/-779$ competed effectively for the binding of the labelled ANG nt $-806/-779$ with the 52 kDa nuclear protein but not the unlabelled SOM-CRE, PEPCK-CRE and TAT-CRE (Figure 6). These studies indicate that the 52 kDa nuclear protein might have a higher binding affinity for the labelled ANG nt $-806/-779$ than for SOM-CRE, PEPCK-CRE and TAT-CRE. The 43 kDa species was not apparent in Figure 6. This might be explained by the small amounts of nuclear proteins (i.e. 50 or 100 μg) that were loaded into the well.

Interestingly, our tissue distribution analysis revealed that the 52 kDa protein is detectable in the nuclear extracts of mouse liver, kidney, testis and brain but not in the heart, lung and spleen (Figure 7). Mouse liver, kidney, testis and brain are known to express ANG mRNA [17]. These observations raise the possibility that the expression of the 52 kDa protein might have a role in the expression of the ANG mRNA in these tissues. Again, the 43 kDa species was not observed in Figure 7. This might be explained by the small amount of nuclear protein (i.e. 100 μg) that was loaded per well and by the over-washing of the membrane. The 43 kDa species was visible in mouse liver, kidney, testis and brain when 200 μg of nuclear proteins were loaded per well (J. Wu and J. S. D. Chan, unpublished work).

Our Western blot analysis of the mouse liver nuclear proteins revealed that the polyclonal antibodies against the C-terminus (residues 295–321) and the mid-region (residues 137–150) of the 43 kDa CREB did not interact with the 52 kDa molecular species, but interacted with the 43 kDa molecular species (Figures 8B and 9B). These studies demonstrate unequivocally that the 52 kDa nuclear protein is immunologically distinct from the 43 kDa CREB.

At present the exact molecular structure of the 52 kDa nuclear protein is not known. The apparent molecular mass of this nuclear protein is not similar to CRE-BP2, ATF-1 or CREM-related proteins (reviewed in [18]). Moreover we have observed that the antibodies against ATF-1 did not interfere with the binding of the labelled ANG nt $-806/-779$ to the 52 kDa protein as analysed by Southwestern blot (J. Wu and J. S. D. Chan, unpublished work). These studies suggest that the 52 kDa protein might be a novel CREB-like protein. The physiological role(s) of this 52 kDa nuclear protein is unknown. Experiments such as cloning and expression of the 52 kDa proteins are definitely warranted, to demonstrate the biological activity of the 52 kDa protein.

In summary we provide evidence that ANG nt $-799/-788$ is protected from the DNase 1 digestion by the mouse liver nuclear extract. Our studies demonstrate that ANG nt $-806/-779$ interacts with two mouse liver nuclear proteins with apparent molecular masses of 52 and 43 kDa. It seems that the 43 kDa molecular species is immunologically similar to the 43 kDa CREB reported by Gonzalez et al. [4], whereas the 52 kDa nuclear protein is immunologically distinct from the 43 kDa CREB. Our studies raise the possibility that the novel 52 kDa nuclear protein might have a role in modulating the expression of the ANG gene in the liver.

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