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Cloning And Characterization Of A New cAMP Responsive Element Binding Protein On Rat Angiotensinogen Gene

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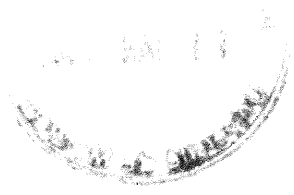

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The thesis title

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Presented by

Jie Wu

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Abstract

Angiotensinogen (ANG) is a single peptide glycoprotein that contains 452 amino acids in human (453 amino acids in rodents). ANG releases a 10-amino acids peptide known as angiotensin I (Ang I) from it's N-terminal when acted by renin, a acidic protease. Ang I can be further converted into angiotensin II (Ang II) by angiotensin converting enzyme (ACE). Ang II is one of the most potent vasoconstrictors known. Ang II also acts on the brain to increase blood pressure. Ang II acts on the adrenal cortex to increase the secretion of aldosterone. The levels of ANG in plasma or local tissues directly contribute to the levels of Ang II. Therefore the studies of regulation of ANG gene expression is important to understand the molecular mechanisms of some related diseases, such as hypertension.

Previous studies in which the transfection of the fusion genes that were generated with various lengths of 5'-flanking region of the rat ANG gene linked to a bacterial chloramphenicol acetyl transferase (CAT) gene as a reporter into mouse hepatoma (Hepa 1-6) cells and opossum kidney (OK) cells, identified a putative cyclic AMP responsive element (CRE) in the rat ANG gene 5'-regulatory region (N-806/-779). Compared with palindromic CRE octamer (TGACGTCA), the putative CRE of the ANG gene (ANG-CRE) is almost identical except the last two bases were in reversed order (TGACGTAC).

In the present study, we isolated a full-length cDNA of 1345 base pairs from mouse liver cDNA library, which encodes a nuclear DNA-binding protein consisting of 436 amino acids with an apparent molecular weight of 52 kilodalton (kDa). This protein binds to the ANG-CRE, and was designated as 52-kDa protein. Southwestern blot revealed that this 52 kDa protein was present in the following tissues, such as liver, kidney, testis, brain, but not in spleen.

Analysis of the deduced amino acid sequence shows no apparent basic region-leucine zipper (bZIP) structure, indicating ANG-CREB is structurally distinct from bZIP family members. The antiserum against 43-kDa-CREB or ATF-2 can not interact with this 52-kDa protein, further supporting that the 52-kDa protein is immunologically different

from the bZIP family. The 52-kDa protein may represent a new group of CRE-binding proteins.

Competitive gel mobility shift assays and Southwestern blot analysis revealed that the ANG-CREB binds to ANG-CRE, and this binding is not displaceable by excess amounts of CREs from somatostatin (SOM), phosphoenopyruvate carboxyl kinase PEPCK), and tyrosine amino transferase (TAT) genes *in vitro*. These data suggest that the 52-kDa protein binds specifically to ANG-CRE and may regulate the ANG gene expression.

The biological function of the 52-kDa protein is not known at present. The primary data from transient gene transfection assays showed that this protein has repressor activity on the ANG gene promoter when ANG gene was stimulated by isoproterenol, but showed no effect on the ANG basal level (without any stimulation). Further experiments will be needed to study the biological function of the 52-kDa protein.

Résumé

L'angiotensinogène (ANG) est une glycoprotéine formée de 452 acide aminés chez l'humain (453 acide aminés chez les rongeurs). Une protéase acide, la rénine, clive l'ANG de son côté N-terminal en libérant un peptide de 10 acide aminés connu sous le nom de l'angiotensine I (AngI). Ce dernier, peut être converti en angiotensine II (AngII) sous l'action d'une enzyme de conversion dite ACE. L'Ang II est un puissant vasoconsticteur. Il agit au niveau du cerveau en augmentant la pression sanguine et au niveau du cortex rénal en augmentant la sécrétion de l'aldostérone. Les niveaux de l'ANG dans le plasma ou dans les tissus locaux sont directement proportionnels aux niveaux de Ang II. Ainsi les études de régulation de l'expression du gène d'Ang II sont importantes pour comprendre les mécanismes moléculaires de certaines maladies, comme l'hypertension. Des études antérieures par transfection en utilisant des gènes de fusion générés avec différentes longueurs de région flanquées en 5' du gène ANG de rat lié au gène rapporteur de bactérie, le chloramphénicol acetyl transférase (CAT), dans les cellules hépatome (Hepa 1-6) de souris et dans les cellules rénales de sarigue, ont permis d'identifier un présumé élément réponse (CRE) d'AMP cyclique dans la région régulatrice en 5' du gène ANG de rat (N-806/-779). En comparant l'octamère palindromique de CRE (TGACGTCA) avec le présumé CRE du gène ANG (ANG-CRE), il semble qu'ils sont identiques à l'exception des deux dernières bases qui sont dans un ordre inversé (TGACGTAC). Dans cette étude, nous avons isolé à partir d'une banque de cDNA du foie de souris, une cDNA de 1345 paire de bases qui code pour une protéine de liaison à l'ADN nucléaire. Cette protéine est formée de 436 acide aminés avec un poids moléculaire apparent de 52 kilodalton (kDa). En se liant à ANG-CER, cette protéine est désignée comme la 52-kDa protéine. Le "Southwestern blot" a révélé que la 52-kDa protéine de 52 kDa est présente dans les tissus suivants: foie, rein, testicule et cerveau; mais absente dans la rate. L'analyse de cette séquence déduite d'acide aminés ne montre pas du structure apparent à la région basique de la ferméture éclaire à leucine, bzip (basic region-leucine zipper), indiquant que la 52-kDa protéine est

structurellement distincte des membres de la famille bzip. L'antisérum dérigé contre le 43-kDa-CREB ou contre ATF-2 n'intéragit pas avec la 52-kDa protéine confermant ainsi que la 52-kDa protéine est immunologiquement différente des membres de famille bzip. La 52-kDa protéine représente donc, un nouveau groupe des protéines de liaison à CRE. Les essais sur gel à mobilité compétitive et les analyses de "southwestern blot" ont révélé que la 52-kDa protéine lié ANG-CRE et que cette liaison n'était pas déplaçable par un excès du gène CRE de somatostatin (SOM), phosphoenopyruvate carboxy kinase (PEPCK) et tyrosine amino transférase (TAT) in vitro, suggérant que la 52-kDa protéine se lié spésifiquement à ANG-CRE et qu'il peut réguler l'expression du gène ANG. Les fonctions biologiques de la 52-kDa protéine ne sont pas encore connus. Les premiers résultats obtenus à partir des essais de transfections des gènes transitoirs montrent que cette protéine a une activité represseur sur le promoteur du gène d'ANG quand ce dernier est stimulé par l'isoprotérénol, et que, sans stimulation, elle a aucun effet sur le niveau basal d'ANG. Les expériences futures seront nécessaires pour étudier les

fonctions biologiques d'AND.

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List of Abbreviations

ACE:	angiotensin converting enzyme
AI:	angiotensin I
AII:	angiotensin II
AIII:	angiotensin III
AMP:	adenosine monophosphate
ANG:	angiotensinogen
AP-1:	activator protein 1
ARE:	AU-rich element
AT1:	angiotensin receptor type I
AT2:	angiotensin receptor type II
ATF:	activating transcription factor
ATP:	adenosine triphosphate
AVP:	arginine vesopressin
bp:	base pair(s)
8-Bromo-cAMP:	8-Bromo adenosine 3',5'-cyclic monophosphate
BSA:	bovine serum albumin
bZIP:	basic region-leucine zipper (domain)
cAMP:	adenosine 3',5'-cyclic monophosphate
CAT:	chloramphenicol acetyl transferase
CBP:	CREB-binding protein
C/EBP:	CCAAT box / enhancer binding protein
cGMP:	cyclic guanosine monophosphate
CNS:	central nervous system
CRE:	cAMP responsive element
CREB:	cAMP responsive element binding protein
CREM:	cAMP responsive modulator protein
DEX:	dexamethasone
dH2O	distilled water
DMEM:	Dulbecco's modified eagle's medium
DNA:	deoxyribonucleic acid
DTT:	dithiothreitol
ER:	reticulum
FBS:	fetal bovine serum
G protein:	GTP-binding protein
β-gal:	β-galactosidase

GH:	growth hormone
GRE:	glucocorticoid responsive element
GRU:	glucocorticoid responsive unit
GTP:	guanosine triphosphate
hCG:	human chorionic gonadotrophin
HEPES:	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMrA:	high-molecular-weight angiotensinogen
hr:	hour(s)
HRE:	hormone response element
hsp:	heat shock protein
IPTG:	isopropyl-β-D-thiogalactopyranoside
kDa:	kilodalton
KID:	kinase inducible domain
LmrA :	low-molecular-weight angiotensinogen
min:	minute(s)
OK:	opossum kidney
ORF:	open reading frame
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PEPCK:	phosphoenopyruvate carboxykinase
pfu:	plaque form unit
PKA:	protein kinase A
PKC:	protein kinase C
PMSF:	phenymethy-sulfony fluorid
Pol I:	DNA-dependent RNA polymerase I
Pol II:	DNA-dependent RNA polymerase II
Pol III:	DNA-dependent RNA polymerase III
PT:	proximal tubule
RAS:	renin-angiotensin system
RNA:	ribonucleic acid
RT:	room temperature
SDS:	sodium dodecyl sulfate
sec:	second(s)
SMG:	submandibular gland
SOM:	somatostatin
SV40:	simian virus 40

T3:	3, 3' 5'-triiodo-L-thyronine
TAT:	tyrosine amino transferase
TBP:	TATA box binding protein
TFIIA:	transcription factor IIA
TFIIB:	transcription factor IIB
TFIID:	transcription factor IID
TFIIE:	transcription factor IIE
TFIIF:	transcription factor IIF
TFIIH:	transcription factor IIH
TFIIJ:	transcription factor IIJ
TK:	thymidine kinase
TRE:	thyroid hormone responsive element

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1. Chapter 1. Introduction

1.1 RENIN-ANGIOTENSIN SYSTEM (RAS)

1.1.1 Renin-angiotensin System (RAS):

Renin-angiotensin system (RAS) is a regulatory system, which plays an important role in body electrolyte homeostasis, vascular tone and cardiovascular remodeling. RAS comprises three components: renin, angiotensins and angiotensin-converting enzyme (ACE).

1.1.1.1 Renin

Renin is a single peptide glycoprotein with a molecular weight of 43 kDa in human. There are two potential sites for N-linked glycosylation (Asn-X-Ser/Thr) located at amino acid position 5-7 and 75-77 respectively. The carbohydrate content accounts for about 5% of the total glycoprotein (Galen *et al.,* 1979). The renin protein molecule is made up of 2 lobes, between which the activation sites are located in a deep cleft. Two aspartic acid residues, one at the position 32, and another at position 215, are juxtaposed at the mouth of the cleft and are essential for renin activity (Sielecki *et al.,* 1989).

Although the circulating renin is thought to be synthesized and secreted by juxtaglomerular cells of the renal afferent arteriole, in situ hybridization studies of renin mRNA revealed that renin could be synthesized by many other cells, such as, testicular leydig cells (Samani *et al.,* 1988), adrenal zonal glomerulosa cells (Samani *et al.,* 1988), pituitary gonadotrops (Kettani *et al.,* 1991), ovarian mature follicle (Shaw *et al.,* 1989), and corpus lutetium (Duncan *et al.,* 1989), as well as in granular duct cells of mouse submandibular gland (SMG). PCR technique has detected renin mRNA in the following approximate relative concentration: kidney, 100%; adrenal 10%; testis, 1%; ovaries and liver, 0.1%; brain, spleen, lung and thymus, 0.01% (Paul *et al.,* 1993).

The renin gene is present in single copy in human (Hobart *et al.,* 1984), rat (Burnham *et al.,* 1987) and some strains of mouse with low levels of (SMG) renin, such as C57/BL10 (Mullins *et al.,* 1982). By contrast, mouse strains with high levels of SMG renin, such as DBA/2J, carry two copy renin genes (Ren1, 2d; Mullins *et al.,* 1982). The single renin gene is approximately 11,000-12,000 bases in length and consists of nine exons and eight introns. The amino acid sequence predicated from the cDNA sequence indicates that the rat renin precursor consists of 402 amino acid residues, and the human renin precursor consists of 403 amino acids, which include mature renin and 66-residue amino-terminal prepropeptide (Morris, 1986). The homology of renin gene between rat and mouse is 85%, whereas between rat and human is 68% (Kriger and Dzau, 1991).

There are two TATAA box sequences in the promoter region of the human renin gene, located at -29 to -23 (promoter 1) and -77 to –71 (promoter 2) relative to the transcription start site (Fukamizu *et al.,* 1986). In human kidney only promoter 1 is used, but in the mouse kidney, a minor proportion of transcripts are initiated at promoter 2 (Hobart *et al.,* 1984; Burnham *et al.,* 1987; Mullins *et al.,* 1982). In two renin gene mice extrarenal tissues use promoter 1 (Fukamizu *et al.,* 1986), and SMG uses promoter 2 as well. Associated with the TATAA boxes in the human renin gene are two CAAT boxes at position -51 and -92 (Fukamizu *et al.,* 1986) in human's renin gene. Several regulatory

sequences in the 5'-flanking region of human renin gene have been found. The hexanucleotide sequence TGTCCT, TGTTCT, and AGTCCT proposed as the motifs of glucocorticoide-responsive element (GRE) are located at -185 / -190, -461 / -465, and +233 / +238 (first intron) (Fukamizu *et al.,* 1986). There are two DNA sequences at -981 to -999 (5'ATCTCCTTGGGGGTTAGTT-3') and -300 to -318 (5'-GTCCCAGTTTTGA-3') with different degrees of similarity in the consensus sequence of the progesterone-responsive element (PRE) (Fukamizu *et al.,* 1986). The DNA sequence (5'-GGAGCTGGAAA-3') has been proposed as an estrogen-responsive element (ERE) (Fukamizu *et al.,* 1986). The sequence at -117 to -145 (5'-CCCTTCACCCACCTA-GCTCTGTCCCGCAG-3') has been proposed as a cAMP responsive element (CRE) (Fukamizu *et al.,* 1986)

Human renin is synthesized as a large preprorenin, consisting of 406 amino acid residues. After removal of a leader sequence of 23 amino acid residues from the N-terminal, prorenin is released. The prorenin has relatively little biological activity, but following the removal of the pro-sequence from the N-terminal of the prorenin, the active renin that contains 340 amino acid residues is released (Morris, 1986). Renin in mouse salivary glands undergoes an additional cleavage near the C-terminal end to produce 2 separate peptide chains connected by disulfide bond. The half-life of human renin in the circulation is 30-80 minutes (Konrads *et al.,* 1981). Only a small amount of prorenin is converted to renin in kidney. The remaining prorenin is released into circulation. Prorenin is the major circulating form of renin, accounting for as much as 90% in human plasma (Sealey *et al.,* 1986; Glorioso *et al.,* 1986). Prorenin can be considered the transport form of renin for delivery to the tissues, where it is converted to active renin by the tissue kallikrein, an enzyme that generates kinin by hydrolyzing kininogen. There is very little conversion to active renin in the circulation (Morris, 1986).

Renin functions as acidic protease. Once released into the circulation, renin acts on angiotensinogen (ANG), and cleaves the first 10 amino acid residues from the N-terminal of ANG, known as angiotensin I (Ang I). The remainder of the ANG molecule is called des-

Ang I-ANG. Renin has species specificity. The human ANG can be cleaved only by primate renin. Human renin may cleave more efficiently other mammalian ANG, but other mammalian renin can't cleave human ANG (Burton and Quinn, 1988).

1.1.1.2 Angiotensinogen (ANG)

Angiotensinogen (ANG) is a single peptide glycoprotein and represents a unique substrate of renin,. Although it was first recognized in 1939 that renin was a proteolytic enzyme that released a hypertensive factor from a plasma protein (Munoz *et al.,* 1939), and many attempts were made to purify this plasma protein, definite information about its structure was not obtained until 1957. A tetradecapeptide, that, now, is known as angiotensin I (Ang I) plus four additional amino acid residues, was purified from a tryptic digest of an ammonium sulfate fraction of horse plasma. The first pure preparation of ANG protein was obtained from hog plasma in 1963 (Skeggs *et al.,* 1963). Subsequently, the rabbit ANG (Ryan and McKenzic, 1968), human ANG (Printz *et al.,* 1977; Tewksbury *et al.,* 1979), rat ANG (Hilgenfeldt *et al.,* 1980), ovine ANG (Fernley *et al.,* 1986), and dog ANG (Moffett, 1987) were purified between 1970 and 1980. In the period 1983-1984, the entire amino acid sequence of rat (Ohkubo *et al.,* 1983), mouse and human ANG (Kageyama *et al.,* 1984) was deduced from the nucleotide sequence of their respective cDNAs. The homology of ANG protein between rat and mouse is 87%, whereas between rat and human is only 60% and between mouse and human is 64% (Lynch and Peach, 1991). In all species, Ang I is invariant. The amino acid sequence immediately following this decapeptide is dissimilar in various species. In humans, renin must cleave a Leu-Val bond to release Ang I, but in all other species, the renin must cleave Leu-Leu bond. Human ANG has histidine at position 13, whereas rat, dog, and horse have tyrosine at that position. It was suggested that these substitutions might contribute to the known species specificity exhibited by renin (Tewksbury, 1983).

ANG is secreted by a variety of cells, most prominently by hepatocytes, adipocytes, and astrocytes. Liver is the major organ which secrets the plasma ANG. Following the

synthesis of ANG, it is secreted into the plasma. The ANG protein is synthesized at the polyribonucleosome in the granular endoplasmic reticulum, processed and glycosylated in the Golgi apparatus. The ANG protein is synthesized as a pre-ANG (477 amino acids in the rat and mouse; 485 amino acids in the human) (Ohkubo *et al.,* 1983; Kageyama *et al.,* 1984). There is a signal peptide in the N-terminal of pre-ANG, which contains 25-34 amino acid residues and is subsequently removed as the ANG is entered into the rough endoplasmic reticulum (ER). The mature ANG protein is 453 amino acids in rodents, and 452 amino acids in human. There are three potential sites for N-linked glycosylation (Asn-X-Ser/Thr) in rat ANG. They are located at position 47-49, 259-297, and 319-321. Four glycosylation sites are present in human ANG. They are found at positions 14-16, 137-139, 271-273, and 295-297. The carbohydrate accounts for about 14% of the total ANG protein molecular weight. The different glycosylation patterns are apparently responsible for the different isoelectric points and sizes of circulating ANG. In addition to the well-characterized predominant form of plasma ANG, which is termed low-molecular-weight angiotensinogen (LMrA), ANG with a larger apparent molecular weight has been identified and is referred to as high-molecular-weight angiotensinogen (HMrA) (MW 450-500 kDa). The HmrA has been found in pregnant women (Tewksbury, 1986) and is predominant form of ANG in placenta and amniotic fluid (Tewksbury, 1996). Two different subtypes of HMrA have been found, HMrA-1 and HMrA-2. HMr-1 is composed of different subunits of which at least one is very similar to low-molecular-weight angiotensinogen (LMrA). HMrA-2 is composed by the same subunit that is very similar to LMrA. The subunits are bound together by disulfide bonds and hydrophobic interaction (Tewksbury, 1986).

ANG is a ubiquitous, moderately abundant macroglycoprotein with plasma and cerebrospinal fluid concentrations of approximately 10^{-6} M (55-65 μg/ml) and 0.2 X 10^{-6} M (11-13 μg/ml), respectively (Genuine *et al.,* 1984). In plasma from men and menstruating women, only 37% of the total ANG was HMrA. But in plasma from pregnant women or women taking oral contraceptive containing estrogen, the HMrA is increased to 16-61% (Tewksbury, 1986). The significance of these HMrA is not yet known. As a member of the

serpin gene super-family, ANG is faintly, but significantly, related to α-antitrypsin, antithrombin and ovalbumin.

1.1.1.3 Angiotensin converting enzyme (ACE)

Angiotensin converting enzyme (ACE) is a single peptide protein consisting of two isozymes.The ACE protein of 170 kDa, is produced by a variety of tissue types including vascular endothelium, renal tubular epithelium, ciliate gut epithelium, and macrophages and is known as somatic ACE. The other which is known as testicular ACE has molecular mass of 90 kDa and is produced only in the testis by developing germ cells. The complete amino acid sequence deduced from the somatic ACE cDNA contains 1306 residues, beginning with a signal peptide of 29 amino acid residues. ACE is an acidic glycoprotein that contains a high percentage of acidic residues (21-22%). There is a molar equivalent of zinc in the single, large polypeptide chain of ACE molecule. The removal of zinc abolishes all activity, and the activity can be restored by addition of various divalent metal ions (Bunning and Riordan, 1981; Cushman and Cheung, 1971). There is considerable interspecies homology at the N-terminus. The C-terminal differences may be due to species variation. A highly hydrophobic sequence located near the C-terminal extremity of the molecule most likely constitutes the anchor to the plasma membrane. A hypothesis has been proposed that ACE is anchored through its C-terminus, either by a glycolipid moity containing the covalently attached phosphatidylinositol or by a hydrophobic C-terminal amino acid sequence (Hooper *et al.,* 1987). The sequence of ACE reveals a degree of internal homology between two large domains, suggesting that the molecule resulted from gene duplication. Each of these two domains contains short amino acid sequence identical to those located around critical residues of the active site of other metallopeptidases (thermolysin, collagenase) and therefore bears a putative active site (Hurbert *et al.,* 1991). Early studies have elucidated several most important properties of ACE, including its chloride dependence, metalloprotein nature, and specificity for the removal of intact dipeptide units from the C-terminal of peptide substrates.

Only a single ACE gene exists within the mammalian genome. It is 21-Kb in length and consists of 26 exons separated by 25 introns (Hurbert *et al.,* 1991). It has been demonstrated that a single ACE gene codes both somatic ACE and testicular ACE with different promoters. The somatic ACE promoter contains typical sequences (TATAA box and several SP1 binding sites) identified in the 5' flanking region (Hurbert *et al.,* 1991). The somatic ACE mRNA (4.3-Kb) is transcribed from exons 1-26, excluding exon 13 by differential splicing. In contrast, transcription of the germinal ACE mRNA (3-Kb) is initiated within the gene at exon 13 and proceeds through exon 14 to 26 (Hurbert *et al.,* 1991).

1.1.2 Angiotensin I, II, III (Ang I, II, III)

1.1.2.1 Angiotensin I (Ang I)

Ang I is a decapeptide which is released from the N-terminal of ANG by renin. The amino acids sequence of Ang I in various species is identical. Ang I appears to function only as the precursor of Ang II and does not have any other established function. The remainder of ANG, after removal of Ang I, is des (Ang I)-ANG. Its physiological function is not clear. It has been suggested that des (Ang I)-ANG is renin inhibitor (Poulsen and Jcobsen, 1986) but this has not been confirmed experimentally.

1.1.2.2 Angiotensin II (Ang II)

Ang II is generated by ACE, following the cleavage of histidyl-leucine from the physiologically inactive Ang I. The conversion of Ang I to Ang II is slow in isolated plasma but fast *in vivo* (Coldwell *et al.,* 1976), indicating that most of the hydrolysis occurs by the action of ACE in the endothelial cells, predominantly in the pulmonary capillaries. Ang II is destroyed rapidly; its half-life in humans is 1-2 minutes. The enzymes that destroy Ang II are grouped together under the term angiotensinase (Peach, 1977).

1.1.2.3 Angiotensin III (Ang III)

Ang III is derived from Ang II by the removing of the Asp residue from the N-terminal of Ang II by angiotensinase or aminopeptidase. Ang III has about 40% of the repressor activity of Ang II but 100% of the aldosterone-stimulating activity. It has been suggested that Ang III is the natural aldosterone-stimulating peptide, whereas Ang II is the blood-pressure-regulating peptide (Peach, 1977).

The relationship of the components of RAS is summarized in Fig.1-1.

1.1.3 Ang II receptors

Ang II elicits cellular responses in all target tissues by binding to the specific high-affinity cell surface receptors. By measuring the binding of ^{125}I-Ang II to liver cell membranes (Gunther, 1984), two types of receptors were identified: one of high affinity (dissociation constant Kd, 0.35 nM) and the other with a low affinity Kd, 3.1 nM. Only the high affinity site was inactivated by treatment with reducing agent dithiothreitol (DTT). Further more, the availability of pure Ang II antagonists has clearly established that there are at least two types of angiotensin receptors. They are now referred to as AT1 subtype (blocked by Losartan, Dup753) and AT2 subtype (blocked by PD 123177). For the rat AT1 receptor, two subtypes have been cloned and sequenced. The rat vascular and rat kidney AT1 receptors have been designated AT1a subtype, and rat adrenal gland receptors have been designated AT1b subtype. There is 96% amino acid homology between the two subtypes (Iwai and Inagami, 1992).

The AT1 and AT2 receptor subtypes are not uniformly distributed in all somatic tissues (Chang and Lotti, 1991). Some tissues have a nearly homogeneous population of AT1 or AT2 receptors, while others are characterized by co-existence of both receptor subtypes. Tissues such as liver, lung, kidney, placenta, urinary bladder, gastrointestinal tract, and aortic smooth muscle cells express only AT1 receptors. In contrast, other tissues such as pancreas and ovarian granulosa express mainly AT2 receptors. Both receptor subtypes are expressed in the adrenal, heart, renal arteries, and uterus (Chang and Lotti, 1991). In

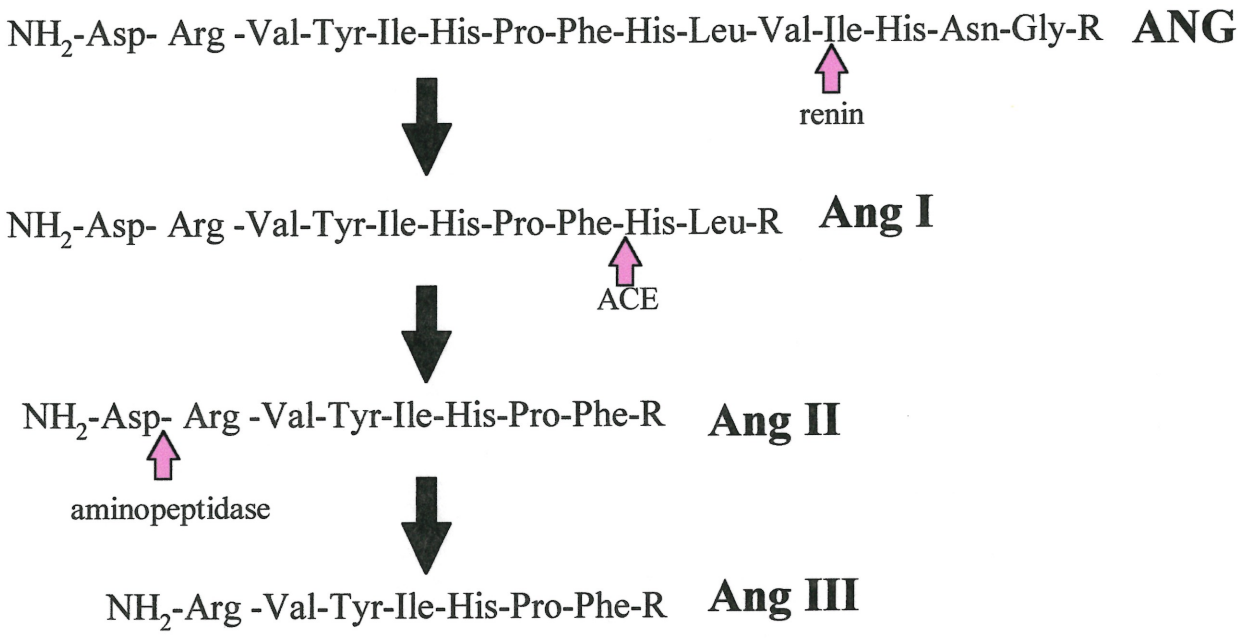


Fig. 1-1 Schematic relationship of the components of renin-angiotensin system.

brain, the regions expressing a high concentration of Ang II receptors have been known for several years (Mendelsohn, 1985; Under *et al.,* 1988). Brain regions that mainly or exclusively express the AT2 receptor are the inferior and superior colliculus. In the midbrain, brainstem, and hypothalamus, both receptor subtypes are present in an approximately equal quantity; the AT1 receptor slightly dominates in the brainstem and hypothalamus, while the AT2 receptor is mainly expressed in the midbrain. Several of the brain regions that mainly or exclusively express the AT1 receptor are known to be responsible for the central actions of Ang II in the regulation of the blood pressure, water and electrolyte balance, indicating that these well-known actions of Ang II may be mediated by the AT1 receptor (Steckelings *et al.,* 1992).

Although the traditional view considers that the actions of Ang II are mediated by AT1 receptor, recent studies have demonstrated that AT2 receptor also mediated some important physiological functions. The central AT2 receptor has been found to inhibit the release of vessopressin (Hohle *et al.,* 1995). The AT2 receptor has been also shown to inhibite the T-type calcium current in non-differentiated NG 108-15 cells (Buisson *et al.,* 1995), cell proliferation of both coronary endothelial cells (Stoll *et al.,* 1995) and N1E-115 neuroblastoma cells (Mahmias *et al.,* 1995). The AT2 receptor in a similar fashion to the AT1 receptor was coupled to the contraction of portal vein smooth muscles (Pelet *et al.,* 1995).

Analysis of the cloned rat AT1 receptor cDNA indicates the receptor consists of 359 amino acids with a predicated molecular weight of 40.9 kDa, containing seven transmembrane domains (Sasaki *et al.,* 1991). Analysis of the genomic DNA sequence of the rat AT1 receptor reveals the presence of three exons. The first and second exons encode the 5' untranslated region while the third exon encodes a small portion of the 5' untranslated region, the entire coding region, and the entire 3' untranslated region. Further analysis of the genomic sequence 5' to the start site demonstrates typical sequence motifs found in many eukaryotic promoters. Theses include a potential SP1 binding site, a putative cap signal

(GCAGTT) immediately 5' to the start site, TATAA box (CTATAAATA) 36 nucleotides 5' to the start, and a GC box (AGGGCGGGGCGGGG) 60 nucleotides upstream of the TATAA box. The coding strand from positions 274 to 417 is composed of virtually all pyrimidines. Such DNA structure has been reported to bind abnormally to histones in the formation of nucleosomes. Whether this region influences the transcriptional activity of the AT1 gene is not known. A second important feature is the sequence from positions 2116 to 2147, which is composed of 16 repeats of the nucleotide AG. This sequence probably will show length polymorphism between different strains of rats; such minisatellite DNA has been used as markers for linkage analysis.

The human AT1 receptor gene has been cloned recently (Guo *et al.,* 1994; Su *et al.,* 1994), and is comprised of at least four exons that are greater than 60 kb in length. The introns are 8 to 10 kb or longer (Guo *et al*, 1994; Su *et al.,* 1994).

The putative human AT2 receptor is comprised of 363 amino acid residues with an estimated molecular weight of 41 kDa. Hydropathy analysis of the deduced amino acid sequence revealed the presence of seven putative transmembrane domains (Tsuzuki *et al.,* 1994; Koi *et al.,* 1994). Sequence analysis showed that the human AT2 receptor gene is composed of three exons and spans at least 5 Kb. Exon 1 and 2 encode for 5'-untranslated mRNA sequence and exon 3 encodes the entire uninterrupted open reading frame of the human AT2 receptor. The open reading frame of the human AT2 receptor gene is highly homologous to the coding regions of cDNAs for the rat (90%) (Mukoyama *et al.,* 1993) and mouse (90%) (Nakajima *et al.,* 1993). Sequence analysis of the 5'-flanking region of the human AT2 receptors revealed that it contains the typical sequence motifs found in many eukaryotic promoters. There is a TATAA box (at -33 bp), a putative C/EBP (-1434) (Akira *et al.,* 1990), SP1, AP-3, NF-1 and GRE site. This promoter region also includes an interferon consensus sequence binding protein site (ICSBP) and a putative embryonal, long terminals repeat binding protein (ELP) site. The presence of these novel putative

transcription factor binding sites suggest that this gene may be regulated by transcription factors known to play a role in embryogenesis.

Evidence has demonstrated that in addition to the different types of Ang II receptors on plasma membrane, some cytosol (Hagiwara *et al.*, 1993) or nucleus (Re *et al.*, 1981) proteins can also bind to Ang II. The cytosolic Ang II binding protein is not a classical AT1 or AT2 receptor since no affinity for either losartan or PD123177 was found (Bumpus *et al.*, 1991). The nuclear Ang II binding protein in liver nuclei, in term of molecular weight and binding affinity, has similarities to, but also some difference from, Ang II receptors present in the plasma membrane. The roles of the cytosolic and nuclear Ang II binding proteins are unclear at the present time.

1.1.4 Local renin-angiotensin system (RAS)

Traditionally, Ang II has been viewed as a hormone synthesized solely within plasma by the pathway described above. The discovery of the renin-angiotensin system in many tissues of the body has led to the hypothesis that, in addition to a plasma hormone, Ang II may be formed locally and therefore possesses additional autocrine or paracrine actions. The existence of an intrarenal RAS has now been generally accepted (Dzau *et al.*, 1987).

1.1.4.1 Intrarenal RAS:

By employing the immunohistochemical and biochemical techniques, renin mRNA has been located in the juxtaglomerular (JG) cells (Dzau *et al.*, 1984). A small number of renin-containing cells are also present in the glomerulus and afferent arteriole. ANG mRNA has been located predominantly in the proximal tubule (PT), with lesser quantities in glomeruli, distal tubules, and intrarenal vessels (Dzau *et al.*, 1987). Rat kidney ANG mRNA is estimated to be 5% of the rat liver ANG mRNA (Dzau *et al.*, 1987). In mice the amount is 20% of liver ANG mRNA (Dzau *et al.*, 1987). ACE has been located in the proximal tubule (Lindpainter and Ganten, 1991). Both brush border and basolateral membranes contain ACE (Danilov *et al.*, 1987). The highest concentration is found on brush border

(Defendini *et al.,* 1983). In adult kidney, AT1 is the predominant receptor, except in large preglomerular vessels where AT2 receptor is present. In contrast, in fetal kidney, the AT2 receptor appears predominant. Studies for the whole animal have shown the intrarenal formation of Ang II occurs after perfusion of the renal mass with renin substrate (Mendelsohn, 1979). The levels of luminal Ang II in the rat renal proximal tubular are as high as 10^{-9} M, whereas the levels of plasma Ang II are less than 10^{-12} M (Seikaly *et al.,* 1990). These studies indicate that the intrarenal RAS may play an important role in the regulation of renal function. In addition, evidence has indicated that there may be a renin-angiotensin system within the renal proximal tubular cells. ANG mRNA as well as renin mRNA (Burns *et al.,* 1993) and ACE mRNA have been found in proximal tubule cells. The presence of these components of the RAS in proximal tubule (PT) suggests that locally produced Ang II could modulate PT function. The regulation of these components in both physiological and abnormal states such as hypertension awaits further study.

The local renal RAS may play an important role in the renal development, as the renin mRNA, ACE mRNA, and ANG mRNA have been detectable in the fetal kidney. In the fetal kidney the AT2 receptor is predominant, whereas in the adult kidney AT1 is the major receptor (Grady *et al.,* 1991).

1.1.4.2 RAS in brain:

Components of RAS have been detected in the brain, as the local production of angiotensin peptides has been observed (Schelling *et al.,* 1980). The peptide fragment Ang II 1-7 may be important mediator of the action of Ang II in the brain. Current data suggest the existence of a complete intracellular RAS in gonadotropes and a separate extracellular system that utilizes the high concentration of angiotensinogen from perisinusoidal cells (Sernia *et al.,* 1997). It is postulated that gonadotrope Ang II serves mainly reproductive functions, while the proximity of angiotensinogen-secreting cells to folliculostellate cells, and their access to the intercellular sinusoidal and follicular spaces, places the extracellular RAS in a strategic position to affect pituitary growth and the mediation of acute-phase

immune response. In the rat brain, angiotensinogen is expressed by the 16-18th day of fetal life and by areas generally concerned with vasopressor, electrolyte, and fluid homeostasis. Antisence deoxyoligonucleotides to angiotensinogen mRNA lower blood pressure in hypertensive rats and inhibited *in vitro* growth of neuroblastoma cells, indicating a significant role for Angiotensinogen in mitogenic and homeostatic functions (Sernia *et al.*, 1997).

1.1.4.3 Local RAS in other tissues:

The synthesis of renin, ANG, and angiotensin peptides has been observed in both cardiac (Lindpainter and Ganten, 1991) and vascular myocytes and cultured vascular smooth muscle cells (Schelling *et al.*, 1991) as well as in the eye (Sramek *et al.*, 1988) and the ovary (Speth and Husain, 1988). Current evidence would suggest that in some cell types, such as Leydig (Pandey *et al.*, 1984) and neuroblastoma cells (Pandey *et al.*, 1984). Ang II is synthesized intracellularly and secreted into the extracellular environment (Pandey *et al.*, 1984). However, at the organ level, it is possible that different cell types secrete different components of the RAS, and these cells combine to produce the extracellular formation of Ang II. The locally generated Ang II may play a role in the function of these organs and tissues.

1.2 RENIN-ANGIOTENSIN SYSTEM & HYPERTENSION

The most important biological function of renin-angiotensin system (RAS) is the regulation of blood pressure by affecting electrolyte homeostasis, vascular tone via Ang II. Jeunemaitre et al. (Jeunemaitre *et al.*, 1992) has provided Genetic linkage between human ANG gene variants and essential hypertension. In his study, among the 15 molecular variants of the ANG gene identified thus far, significant association with hypertension was observed for two distinct amino acid substitutions, Met 235 Thr and Thr 174 Met. These two variants were encoded by two distinct nucleotide substitutions T 704 C (thymidine -

cytidine) and C 521 T (cytidine-thymidine, + 521). Both of the nucleotide substitutions were located in Exon 2 (Jeunemaitre *et al.,* 1992, 1995). Animal experiments demonstrated that the development of hypertension in a number of rat strains, for example, in both Dahl rat (Rappet et al, 1989) and spontaneously hypertensive rat (SHR) (Kuetz *et al.,* 1990), is associated with renin gene variants. Also the genetic locus of ACE gene has been linked to blood pressure (BP) variation in the SHR-stroke prone (Hilbert *et al.,* 1991; Jacob *et al.,* 1991). On the other hand, the angiotensinogen-deficient substrains of the SHR, as well as in the Dahl rat, have a sustained decrease in blood pressure (Tanimoto *et al.,* 1994; Deng and Rapp, 1992). Further more, the transgenic mice carrying both human renin and angiotensinogen genes have a sustained increase in blood pressure (Fukamizu *et al.,* 1993).

The clinical research of the RAS began as early as 1971. The studies involving the blockade of the RAS system provided the recognition of "angiotensinogenic" hypertension (Streeten *et al.,* 1975). Numerous clinical studies have shown that the inhibitors of the components of RAS are effective in treating essential hypertension. For example, saralasin (a competitive inhibitor of the Ang II receptor) (Pals *et al.,* 1971), renin specific antibody (Dzau *et al.,* 1972), ES-8891 (a specific inhibitor of human renin), and captopril (an inhibitor of angiotensin converting enzyme) (Gavras *et al.,* 1978) are effective for the treatment of essential hypertension (Ondetti and Cushman, 1982; Sutton 1986).

1.3 THE BIOGICAL FUNCTIONS OF Ang II

The actions of Ang II are mediated by specific surface receptors on various target organs such as the heart, blood vessels, adrenal cortex, kidney, brain, noradrenergic nerve endings, and gastrointestinal tract etc.

1.3.1 Heart:

Ang II acts upon the heart during the plateau phase of the action potential in atrial and ventricular myocytes. During this period, Ang II increases the entry of calcium (Ca^{++})

through the voltage-sensitive channels, prolongs the action potential and thereby increases the force of contraction of these cells (Peach, 1986).

1.3.2 Blood vessels:

Ang II plays an important role in the regulation of blood pressure by directly and potently constricting the vascular smooth muscle (VSM) (Morgan, 1987), which result in an increase of vascular resistance. Ang II stimulates the arteriolar constriction and increases the systolic and diastolic blood pressure. Peripheral arteriolar or precapillary vasoconstriction is the most rapid effect of Ang II, regulating in a rapid and sustained increase in arterial pressure.

1.3.3 Adrenal cortex:

Ang II acts upon glomerulosa cells of the adrenal cortex to increase both the synthesis and release of aldosterone (Aguilera and Marusic, 1971). Ang II also acts on the inner zone of the adrenal cortex, where it induces cortisone/corticorsterone biosynthesis. The stimulation of aldosterone release by Ang II occurs at very low doses as compared to those required to influence the blood pressure directly (Peach, 1977).

1.3.4 Kidney:

Ang II exerts a direct effect on the proximal tubular (PT) sodium transport (Harris and Young, 1977) through binding to its receptors on the peritubular basolateral membrane (BLM). The direct application of Ang II to either the basolateral or brush-border side of the isolated proximal convoluted tubules alters Na^+ absorption in a dose dependent manner. Ang II stimulates Na^+ absorption at low doses (10^{-12}- 10^{-10} M) and inhibits absorption at higher doses (10^{-7}-10^{-5} M) (Harris and Young, 1977; Schuster *et al.*, 1984).

The renal vasculature is highly sensitive to Ang II, as the intrarenal infusion of low-dose Ang II elicits an increase in renal vascular resistance and consequently leads to a decrease in renal plasma flow (RPF) and to a lesser degree, in glomerular filtration rate

(GFR) (Fagard *et al.,* 1976; Lohmeier *et al.,* 1977). Ang II differentially constricts the postglomerular efferent arterioles more than the pre-glomerular afferent arterioles (Hall, 1986a). In normal circumstances, the efferent arterioles are more sensitive than the afferent arterioles to the constrictive effect of Ang II. It leads to an increase in glomerular filtration pressure and a rise in intraglomerular pressure. Thus Ang II reduces renal blood flow with proportionally less reduction in the glomerular filtration rate (GFR) (Earley and Friedler, 1966; Laragh *et al.,* 1963). Increased intraglomerular pressure helps to constrict glomerular mesangial cells.

1.3.5 Brain:

Ang II stimulates the release of vasopressin from the pituitary following injection of the peptide both into the brain and intravenously. The effect of Ang II injected intravenously on vasopressin release is potentiated by dehydration (Reid, 1984). While peripheral administration leads to renal re-absorption of Na^+, Ang II injected i.c.v. administered at doses that do not enter the peripheral circulation, induces a dose-related natriuresis (Unger *et al.,* 1988). The mechanism is unclear at present. At peripherally administered physiologically doses Ang II are antinatriuretic and blockade of endogeneous Ang II produced natriuresis. These observations point to a primary antinatriuretic action of Ang II.

Ang II enhances the release of adrenocorticotrophic hormone (ACTH), prolactin, and oxytocine from the pituitary, although the physiological role of Ang II on the release of these hormones is unclear at the present time (Peach, 1977; Reid, 1984).

1.3.6 Autonomic Nervous system:

Ang II interacts with the autonomic nervous system in three ways: (a) at sympathetic nerve endings, (b) in the adrenal medulla, and (c) in the brain. At physiological levels, Ang II binds to specific receptors present upon sympathetic nerve terminals to enhance the release of norepinephrine (NE) per neural impulse (Malic and Nasiletti, 1976). At high doses, Ang II also directly causes the release of NE from sympathetic nerve terminal and appears to

enhance the sensitivity of the postsynaptic membrane to the effects of released NE (Palaic and Khang Irullah, 1967). Ang II depolarizes adrenal chromaffin cells, leading to release of both NE and epinephrine (Peach, 1977; Bickerton and Duckley, 1961). Ang II acts in the brain via the structures outside the blood-brain barrier to increase central sympathetic outflow as well as the release of catecholamines (Unger *et al.*, 1988)

1.3.7 Gastrointestinal tract:

Ang II has been shown to alter the absorption of Na^+ and water, mainly from the small intestine, in a dose-dependent manner (Levens *et al.*, 1981 a,b). At low doses (10^{-12}-10^{-10} M), Ang II stimulates sodium and water absorption from the lumen of the intestine, while Na^+ and water absorption is inhibited at higher doses (Levens *et al.*, 1981 a,b). The inhibition of absorption in response to high dose of Ang II appears to be mediated by the local production of prostaglandin (Levens *et al.*, 1981a,b). Both the increased and inhibited transport effects of Ang II appears to be acting directly upon the small intestinal epithelia since they occur also in isolated preparations of intestine that are devoid of a blood supply (Crocker and Munday, 1970).

1.3.8 Trophic effects of Ang II:

The possible growth-promoting role of Ang II is an area of intensive research (Dzau *et al.*, 1991; Phillips *et al.*, 1991; Schelling *et al.*, 1991). The trophic effects of this peptide have been studied *in vitro* on primary and established cell lines, as well as *in vivo*. The mitogenic properties of Ang II have been demonstrated 3T3 fibroblasts (Schelling *et al.*, 1979); human (Cambell and Robertson, 1981) and rat vascular smooth muscle cells (Dudley *et al.*, 1991; Dzau *et al.*, 1989); human fetal mesangial cells (Ray *et al.*, 1991); and adrenocortical cells (Gill *et al.*, 1977). It appears, however, that Ang II-induced proliferation only occurs in the presence of additional growth factors, i.e., platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and transforming growth factor (TGF) β, or under conditions where these factors can be synthesized by the cells (Dzau *et*

al., 1991; Stouffer and Owens, 1992). Thus under differential conditions, Ang II has been reported to induce hypertrophy rather than hyperplasia (Dzau *et al.,* 1989,1991) and to stimulate the expression of specific cytoskeletal proteins like α-actin (Turla *et al.,* 1991), suggesting that this peptide may also play a role in cell differentiation.

1.3.9 Other effect of Ang II:

Ang II stimulates the synthesis and release of prostaglandin (PG) and endothelial derived relaxation factors (EDRF) in blood vessels (Webbs, 1981; Toda, 1984). It also stimulates the release of atrial natriuretic factor (ANF) possibly by the increased blood pressure caused by Ang II (Katsube *et al.,* 1985). PG, EDRF, and ANF may induce vasorelaxation. Thus the vasoconstrictor, Ang II and the vasodilators have physiologically opposing actions in VSM. These effects are mediated by opposing intracellular signals.

1.4 REGULATION OF ANG GENE EXPRESSION

1.4.1 The structure of eukaryotic gene

Eukaryotic genes can be interrupted. The primary evidence of this interruption was a comparison between the structure of DNA and the corresponding messenger RNA (mRNA). The mRNA always includes a nucleotide sequence that corresponds exactly with the protein product according to the rules of the genetic code. But the gene may include additional sequences, introns, which lie within the coding region, interrupting the sequence that codes for the protein. The interrupted DNA coding sequences are named exons. This discrepancy between the structure of the DNA and mRNA is common in eukaryotes.

In addition to the coding region, genes also contain regulatory elements. Nucleotide sequences that influence the rate of transcription lie in region of DNA upstream to the transcription start site. They often include an element rich in adenine and thymine, known as the TATAA box, and other sequence motifs lying within about 100 bp of the start site. Collectively called the promoter region of a gene, these sequences comprise binding sites for

RNA polymerase and its numerous cofactors. The position of the promoter with regard to the transcription start site is relatively inflexible (McKnight, 1991; Rosenthal, 1994 a,b).

In contrast to promoters, other DNA regulatory elements, enhancers, occur in unpredictable locations, often at a considerable distance from the start site and augment transcription by the gene promoter. Enhancer-like promoters from binding site for regulatory proteins, but unlike promoters, the position and orientation of an enhancer are flexible with regard to the gene. Indeed, enhancers can dramatically increase gene transcription from positions within or on either side of the gene, even from thousands of bases away (Rosenthal, 1994b). Individual elements residing in the promoter or enhancer sequences that interact with specific transcription factors consist of small stretches of DNA (<30 bp) called cis elements (such as CRE, cAMP responsive element). The corresponding transcription factors are known as trans factors such as CREB (cAMP responsive element binding protein) that are important for the gene expression.

1.4.2 Initiation of gene transcription

Transcription is DNA directed RNA synthesis that is the first and usually most important step in the control of gene expression. There are three classes of transcription mediated by different RNA polymerases I, II, and III (Pol I, II, III). Pol I transcribes ribosomal pre-RNAs (rRNA); Pol II is the enzyme which facilitates synthesis of the mRNA and its encoding proteins; Pol III directs synthesis of transfer RNA (tRNA) and low-molecular-weight RNAs (Murray *et al.*, 1993).

The model for the assembly of the Pol II-directed transcription complex is based largely on kinetic assays, undenatured gel electrophoresis, and nuclease protein assay. TATAA box binding protein (TBP) forms an initial committed complex. TBP binds to the TATAA box of a promoter in the minor groove of DNA (while virtually all known DNA-binding proteins bind in the wild groove), with its larger outer surface (N-terminal) being available to extend contacts to other proteins. TBP is a small protein (about 30 kDa) that is

sufficient for recognition of a TATAA box and subsequent incorporation of other TBP-associated factors (TAFs). Whereas TBP is not enough to mediate transcriptional regulation by upstream regulator, which requires the entire transcription machinery, TBP is highly conserved during eukaryotic evolution (Buratowski, 1994). Transcription factor IIA (TFIIA) links the complex and may activate the TBP by reliving a repression caused by the TAFs. TFIIA contains three subunits and can associate with TBP or TFIID even in the absence of target DNA. TFIIA is not essential for basal transcription with other purified factors.

Transcription factor IIB (TFIIB) interacts directly with TBP and associates loosely with DNA downstream of the TATAA box, which (as a bridging protein) can recruit Pol II and transcription factor IIF (TFs) into the complex. This complex is stable in both kinetic assays and undenatured gel electrophoresis.

TFs contain two subunits. The larger subunit has an ATP-dependent DNA helicase activity that could be involved in melting the DNA at initiation. The smaller subunit, with some homology to the bacterial sigma factor that contains the core polymerase, binds tightly to Pol II. TFs may in fact bring Pol II to the assembling transcription complex and provide the means by which it binds. Interaction with TFIIB may be important when TFs-polymerase joins the complex. The yeast RNA polymerase II consists of 12 subunits, all of which have been cloned (Young, 1991). The mammalian counterparts of many of the subunit genes remain to be cloned.

Finally transcription factors TFIIE, and then TFIIH and TFIIJ join the complex. TFIIE is encoded by two genes and is probably a tetramer with two of TFIIH. TFIIH has a kinase activity that can phosphorylate the C-terminal domain of Pol II. It is possible that phosphorylation of the C-tail is needed to release Pol II from the transcription factors so that it can leave the promoter and start elongation. Once the complex is assembled, an ATP-dependent activation step is necessary for transcription to occur (Buratowski, 1994; Lewin, 1994). There are probably 20 proteins (a total mass of 500 kDa) involved in the basal transcriptional apparatus, excluding the Pol II that alone has 12 subunits with a mass of

another 500 kDa (Lewin, 1994). An upstream transcription factor (such as CREB) can interact directly with one of the basal transcription factors (such as TFIIB), or indirectly with the TAFs (such as CREB with TAF110). This kind of transcription factor may also indirectly with a coactivator (such as CREB with CBP, which then interacts with TFIIB), as will be discussed later.

1.4.3 The structure of ANG gene

In the 1980's ANG gene was cloned and characterized from different species. The rat (Ohkubo *et al,* 1983), mouse (Clouston *et al.,* 1988), and human (Kageyama *et al.,* 1984) ANG genes have been mapped to chromosome 19 (Mori *et al.,* 1989), 18 (Clouston *et al.,* 1989), and 1 (Gaillard-Sanchez *et al.,* 1990) respectively. All of three ANG genes are present as a single copy.

The rat ANG gene is roughly 12 kilobase pairs (kb) long and consists of five exons separated by four introns (Fig. 1-2). The first exon encodes only a portion of the 5' untranslated region of the ANG messenger RNA (mRNA). The 5'-terminal part of exon 2 encodes both the signal peptide and Ang I. The remainder of exon 2, exon 3, exon 4 and the initial part of exon 5 encode the sequence of des-Ang I. The 3'-terminal part of exon 5 encodes the C-terminal untranslated region of ANG mRNA. Like the rat ANG gene, the human ANG gene also consists of five exons and four introns, but it is longer, is roughly 13 kb. The first exon is very short (37 nucleotides) and corresponds to the 5' untranslated sequence of the mRNA. The second exon codes for 59% of the protein and contains the nucleotide sequences coding for the signal peptide and Ang I. Exon 3 and exon 4 codes for, respectively, 48 and 62 amino acid of the protein. The last exon codes for the C-terminal part of the protein and the 3' untranslated sequence of the mRNA (Jeunemaitre *et al.,* 1995).

Analysis of the nucleotide sequence 5' to the start site of transcription has revealed a number of DNA elements that may be involved in initiating ANG gene transcription. The

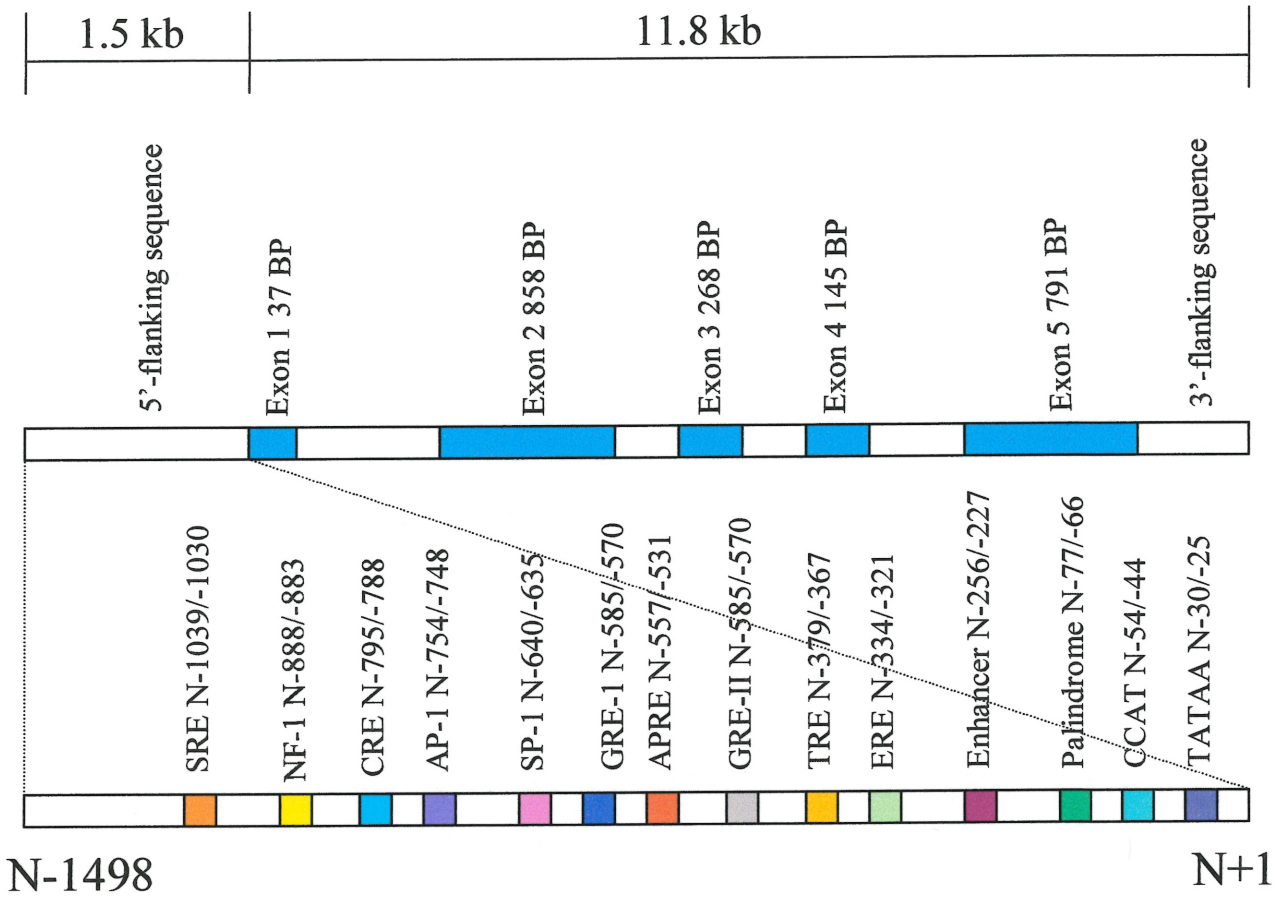


Fig. 1-2. Schematic structure of the rat angiotensinoge gene. The genomic structure spans 11.8 kb plus 1.5 kb of 5'-flanking region. The 5 exons are separated by 4 introns. Serveral putative consensus sequences of control elements have been identified and characterized in 5'-flanking region. SRE, serum responsive element; NF-1, nuclear factor-1 responsive element; CRE, cAMP responsive element; AP-1, c-FOS/c-JUN complex responsive element; SP-1, nuclear protein SP-1 responsive element; GRE, glucocorticoid responsive element ; APRE, acute phase responsive element;TRE, thyroid hormone responsive element; ERE, estrogen responsive element. (redrawn from: Chan et.al, 1990)

TATAA box and CAAT box are located at 30 bp and 50 bp upstream from the transcription start site (Tanaka at al., 1984).

Two glucocorticoid responsive elements, GRE I and GRE II hexameric "half- site" sequence, have been identified at -585 / -570 and - 477 /-472, respectively (Ohkubo *et al.*, 1983).

A cis-acting element centered on a 16-base-pairs palindrome (5'-GTTGGGTTTC CAAC-3' is similar to the NFκB transcription factor binding site (APRE) was found to function in the acute phase response (Brasier and Li, 1996).

An upstream silencer element (5'-CTCTGTACAGAG-3') has been identified at -108 /- 60, and inhibits the expression of ANG gene. However, when moved further away from the initiation site of transcription it functions as a transcriptional enhancer (Ron *et al.*, 1990 a,b).

Rat ANG mRNA is roughly 1.8 kb long, consisting of 61,1431, and 200-400 nucleotides of 5' untranslated, coding, and 3' untranslated sequence, respectively. The size of the ANG mRNA is heterogeneous. The reason is that a single ANG gene is transcripted into at least four different mRNA, which differ only in the lengths of their 3'-untranslated regions, as 4 different polyadenylation sites are used. The 4-polyadenylation sites are located at 1650, 1785, 1800 and 1840 nucleotides. Since one AUUAAA and two AAUAAA sequences have been found 10-30 nucleotides upstream from the 4-polyadenylation sites, these polyadenylation signals could generate 4 different mRNA species. Treatment with glucocorticoids increases liver ANG mRNA and elicits the use of two additional, upstream transcription site that are located at -328 nucleotides (Ben-Ari and Lynch, 1989). Although there is size heterogeneity of ANG mRNA, these extended ANG mRNAs apparently have the same coding potential as the predominant mRNA and there is no evidence for alternate splicing of the ANG pre-mRNA.

Although liver is the major organ that synthesizes ANG, ANG mRNA has also been found in various extrahepatic organs. By using Northern blotting with an ANG cDNA probe, ANG mRNA has been detected in the rat and mouse liver, heart, Kidney, and adrenal gland (Ohkubo *et al.,* 1986; Dzau*et al.,* 1987). There was no detectable ANG mRNA in rat testes and salivary gland. In contrast to the rat, ANG mRNA was present in mouse testes and submandibular gland (Ohkubo *et al.,* 1986; Dzau *et al.,* 1987).

1.4.4 Hormonal regulation of ANG gene transcription

1.4.4.1 Glucocorticoids

Glucocorticoids have been demonstrated to be able to stimulate the production of ANG. This augumentation has been known clinically for a long time, as an increase in serum renin substrate levels occurs during hypercortisolism (Cushing's syndrome) (Krakoff, 1973), and a decrease in these levels is observed during adrenal insufficiency (Addison's syndrome) (Stockigt *et al.,* 1979). Administration of DEX to intact animals will increase plasma ANG levels (Freeman and Rostorfer, 1972; Reid, 1977; Krakoff and Eisenfeld, 1977). Liver slices from DEX treated rat releases more ANG into the incubation medium than liver slices from control rats (Clauser *et al.,* 1983; Dzau and Herrmann, 1982). ANG secretion by hepatocytes and hepatoma cell lines can be stimulated by incubation with hydrocortisone (Stuzmannet *et al.,* 1986) or DEX. Administration of cortisol increases the rate of synthesis of ANG in perfused livers (Hasegawa *et al.,* 1973). Furthermore, adrenalectomy results in diminished liver ANG synthesis, and this deficit is reversed on glucocorticoid administration.

There is an increased liver ANG mRNA sequence accumulation in response to glucocorticoids applied to intact animals (Kalinyak and Perlman, 1987), dispersed hepatocytes (Ben-Ari and Garrison, 1988) and cultured cell lines (Chang and Perman, 1988). The increase in ANG mRNA accumulation occurs rapidly. In isolated hepatocytes exposed to DEX, the ANG mRNA started to increase within less than 1 hr, followed with a further time lag of about 2 hr, and by an increase in secretion rate of ANG (Klett *et al.,*

1992). The increased ANG mRNA accumulation is sensitive to protein synthesis inhibitors, and is blocked by the glucocorticoid antagonist RU486 (Ben-Ari and Garrision, 1988). These observations, along with the existence of glucocorticoid response elements upstream of the ANG gene suggest that the activated glucocorticoid receptor interact directly with this gene.

The increased ANG mRNA accumulation induced by glucocorticoids may be explained by:

(a). Glucocorticoid causing an increased transcription rate, since glucocorticoid response elements have been identified in the promoter region of ANG gene (Coezy *et al.*, 1984).

(b). Glucocorticoids inducing the accumulation of novel ANG gene transcripts. Ben-Ari et al. (Ben-Ari *et al.*, 1989) have reported that glucocorticoids stimulate the accumulation of a novel, 2.25-kb transcript of ANG gene, both in isolated hepatocytes and intact liver. The 2.25-kb ANG mRNA consists of two larger forms of ANG mRNA extended at their 5' ends. The novel transcripts are generated by the use of two new transcription initiation sites in the ANG gene, located at nucleotide positions -328 and -386 relative to the start site at position +1 used in the absence of hormone. A consensus TATAA box and a TATAA-like sequence are found at the expected position 25-30 nucleotide upstream from the glucocorticoid inducible transcription start site (at position -328 and -386) respectively. As they have the same coding potential as the predominant mRNA species, the larger ANG mRNAs do not appear to code for a novel form of ANG protein. It appears that glucocorticoids can directly induce the transcription from a second promoter in the ANG gene and that this promoter is absolutely dependent on this hormone.

c). Glucocorticoids can decrease the degradation of ANG mRNA, as studies have shown that DEX may stabilize the ANG mRNA (Brasier *et al.*, 1986).

1.4.4.2 Estrogen

Estrogen may increase plasma ANG, as the treatment with an oral contraceptive containing an estrogen component, or treatment with estrogen alone, will increase plasma ANG in both adult men and women (Newton *et al.,* 1968; Oelkers *et al.,* 1976). Also a significant positive correlation between estradiol and ANG has been noted in normal pregnant women whose endogenous estrogen level is increased comparing with non-pregnant women (Immonen *et al.,* 1983). On the average, the ANG level will be increased four to five-fold during pregnancy. In late pregnancy, about 15% of the total ANG will be high-molecular-weight ANG (HMrA). Estrogen administration will cause a proportionally greater increase in HMrA concentration than in low-molecular weight ANG (LMrA) concentration (Tewksbury and Dart, 1982). The reason is unclear at present.

The administration of estrogen causing a rise in both the plasma ANG concentration has ben demonstrated in animal experiments (Helmer and Griffith, 1952; Menard *et al.,* 1970; Saruta *et al.,* 1983; Dzau and Herrmann, 1982), in liver perfusion studies (Nasjletti and Masson, 1972), cultured liver slices (Clauser *et al.,* 1983; Dzau and Herrmann, 1982), and Hepa G2 cells (Coezy *et al.,* 1987). Treatment with estrodiol in the intact animal caused an increase in plasma ANG, which became first apparent after 9 hr, and resulted in plasma concentration 1.9-fold higher than controls in 24 hr. These changes were preceded by comparable increases in hepatic ANG mRNA (Klett *et al.,* 1992).

The putative estrogen responsive elements have been identified in the promoter regions of the human, rat and mouse ANG gene (Fukamizu *et al.,* 1986; Campbell and Habener, 1986; Clouston *et al.,* 1988). However, some studies have showed that relatively high concentration of 17β-estrodiol ($1X10^{-7}$ M) did not increase ANG mRNA in cultured rat hepatoma cells (Fatigatietal, 1987) It is possible that these cultured cells had some alteration in their receptors which made them refractory to estrogen treatment. In a hepatoma cell line stably transfected with the human estrogen receptor, estrodiol induced an

increase in ANG mRNA and in secretion of ANG with the same characteristics as in hepatocytes. (Klett *et al.,* 1992).

1.4.4.3 Androgen

Androgen stimulates the ANG level, as renal ANG mRNA concentration in male rat increases significantly during puberty (Ellison *et al.,* 1989). Whereas the renal ANG mRNA level in the adult female rat is the same as that in pre-puberty and is considerably lower than that in the adult male rat (Ellison *et al.,* 1989). Ellison et al. (Ellison *et al.,* 1989) have investigated the role of androgen in renal ANG mRNA expression, and their studies demonstrated that castration lowered ANG mRNA levels in the male kidney by > 60% compared with the control. Their results suggest that androgen may be involved in renal ANG gene regulation. Moreover, male rats castrated in weanlings and normal adult female rats implanted with testosterone displayed significant increases in renal ANG mRNA levels.

Klett et al. (Klett *et al.,* 1992) reported that dihydrotestosterone induced a rapid increase in total hepatocyte RNA and ANG mRNA with a peak at 2 hr. Surprisingly, this increase in ANG mRNA was not translated into ANG secretion. No convincing explanation can be given for these observations. One possible interpretation, which would integrate both effects, is that dihydrotestosterone induces transient sequestration of mRNA into a non-degradable, and at the same time, non-translatable form compartment.

1.4.4.4 Thyroid hormones

Thyroid hormones have been shown to be involved into the regulation of ANG gene expression. Thyoidectomized rats exhibited a significant decrease in plasma ANG level that could be corrected by the administration of triiodothyonine (T3) or thyroxine (T4) (Bouhnik *et al.,* 1981; Clauser *et al.,* 1983). Measurement of plasma ANG in thyroidectomized rats by direct radioimmunoassay and the indirect enzymatic assay gave equivalent results, indicating that the decrease is due to decreased production and not to increased consumption (Bouhnik *et al.,* 1982). Chronic hypothyroidism resulted in approximately a 50% decrease in plasma

ANG and ANG mRNA concentration in liver. In contrast, plasma ANG and liver ANG mRNA levels were elevated by about 75% during hyperthyoidism (Hong-Brown and Deschepper, 1992). *In vitro* studies with rat liver slices have also demonstrated that both the hepatic content and the release of ANG are decreased by thyroidectomy and increased by thyroid hormones (Clauser *et al.,* 1983; Ruiz *et al.,* 1987). In cultured rat hepatoma cell line, T3 also increased ANG mRNA concentration.

1.4.4.5 Angiotensin II (Ang II)

Angiotensin II increases plasma ANG levels. The current data support the concept that there is a positive-feedback loop in which, as renin consumes ANG, the production and release of ANG is stimulated by the final product, Ang II. Pharmacological doses of Ang II increased plasma ANG levels in rats (Nasjletti and Masson, 1973; Khayyall *et al.,* 1973) and dogs (Blair-West *et al.,* 1974). Kohara et al (Kohara *et al.,* 1992) demonstrated that plasma Ang II increased the levels of liver ANG mRNA. It has also been shown that the addition of Ang II to the perfusion medium would increase the rate of synthesis of ANG in the isolated rat liver preparation (Nasjletti and Masson, 1973). In most studies, the administration of ACE inhibitors resulted in a decrease of plasma ANG levels (Herrmann and Dzau, 1983; Radziwill *et al.,* 1986).

Ang II-mediated increase of liver ANG mRNA depends on an inhibition of adenylyl cyclase, not on the stimulation of phospholipase C. Therefore, the agents known to affect intracellular Ca^{++} concentrations (i.e. calcimycin, or methoxamine) failed to influence the snthesis of ANG (Klett *et al.,* 1993). Recently, using transcription assays in AT1 receptor-complemented human hepatocytes, studies (Brasier and Li, 1996) have shown that the ANG multihormonal response element, spanning nucleotide -615 to -470, is an Ang II-inducible enhancer. Mutations in this region not only abolished Ang II induction of the transfected ANG transgene, but also blocked NF-κB binding (Brasier and Li, 1996).

Since Ang II induced mRNA increased very rapidly (Klett *et al.,* 1993), it may suggest that the Ang II-induced ANG level does not depend on de novo protein synthesis but rather on an ANG post-translational modification, such as phosphorylation or dephosphorylation. Ang II activated a 12-kDa protein that may interact with the 3'-untranslated ANG mRNA and increase the half-life of ANG mRNA three to four-fold (Klett *et al.,* 1995). Although different mechanisms appear to be involved in the stabilization process for the various mRNAs (Nielsen and Shapiro, 1990), a general feature appears to be the binding of proteins to recognition sites at the 3'-untranslated region of the mRNA, either in the poly(A) tail region or somewhat upstream. In the later case, the base sequence AUUUA (in a single or more frequently, in a multiple repeat) seems to be an essential signal. The ANG mRNA does contain this sequence in its 3'-untranslated tail (Okhubo *et al.,* 1983). These results suggested that ANG level may be also regulated via stabilizing ANG mRNA. This could also be in accordance with the transient change in cAMP, since such a mechanism has been described as being responsible for the stabilization of mRNA coding for cytokines (Gillis and Malter, 1991; Malter and Hong, 1991).

1.4.4.6 Inflammation

Inflammation increases ANG levels, and is considered an acute-phase response protein. During injury, elevated plasma ANG levels were observed (Kageyama *et al.,* 1985; Bing, 1972), Hoj Nielsen (1987) measured ANG with proven acute inflammatory disease and a control group without infection that was matched with age and sex. The median value of the plasma concentration of ANG was increased 70% in the inflammation group compared with that of the control group. Kageyama et al. (1985) also demonstrated that ANG mRNA in liver and brain increased 5-fold during the first 5-hr of injection with E.coli lipopolysaccharide in rats.

A cis-acting DNA element, centered on a 16-base-pair palindrome (5'-GTTGGGATTTCCCAAC-3'; N-552 / -537 from the transcription site) in the 5'-flanking region of the ANG gene (Brasier and Li, 1996), was identified by DNase I footprinting, and

was similar to the NF-κB transcription factor binding site. It was found to function in the acute-phase response (i.e. transcriptional activation in response to cytokines) (Ron *et al.*, 1990a) and therefore was named as acute-phase responsive element (APRE). This element, when fused upstream of a cytokine-unresponsive gene, confers a cytokine-responsive phenotype. The APRE of ANG gene is flanked by two classic GREs (GREI and GREII) located on -582 and -475 respectively. Some experiments have shown that glucocorticoids are necessary for the increased ANG mRNA that is induced by cytokines (Ron *et al.*, 1990b; Ohtani *et al.*, 1992). Ron et al. (Ron *et al.*, 1990c) have demonstrated that this synergistic effect between cytokines and DEX is due to an interaction, in the cis region, between the glucocorticoid-inducible GREs and the cytokine-inducible APRE.

It has reported recently that the NF-κB is regulated not only by the cytokines but also by Ang II (Brasier and Li, 1996). NF-κB is a multiprotein complex encoded by different genes. These members include Rel A (P65), Rel B NF-κB, (NF-kB P50), and NF-kB2 (NF-kB P49) (Baeuerle, 1991). Rel A is a powerful transactivating NF-κB family member by virtue of its unique COOH-terminal transactivating domain (Schmitz, 1991; Ruben *et al.*, 1992). By contrast, NF-κB1 is an inert DNA binding protein (Blank *et al.*, 1992; Palombella *et al.*, 1994). Rel A / NF-κB1 heterodimers have slightly different DNA binding preference than either protein alone, and in the appropriate cell line they are inactivated by association with inhibitory cytoplasmic proteins. In resting hepatocytes, NF-κB is sequestered in the cytoplasm though reversible interaction with a family of inhibitory proteins termed I-κB (Baeuerle and Baltimore, 1989; Urban and Baeuerle, 1990; Beg and Baldwin, 1993). I-κBα binds to NF-κB1 / Rel A though repetitive, conserved domains homologous to erythrocyte ankyrin. In doing so, I-κB prevents DNA binding activity of Rel A and masks its nuclear localization signal (Hatada *et al.*, 1993). Cytokines, such as TNF-α activates NF-κB by disrupting its association with the I-κB inhibitor though a coupled phosphorylation-I-κB degradation step, allowing the NF-κB complex to enter the nucleus, bind to inducible promoters, and stimulate their transcription. TNF-α activates NF-κB though two

mechanisms: (a) a phosphatidylcholine-specific phospholipase C coupled activation of an acidic sphingomyelinase with formation of ceramide as a second messenger and (b) 1,2-diacylglycerol formation resulting from PKC activation (Meichle *et al.,* 1990; Bouscarel *et al.,* 1988; Wiegmann *et al.,* 1992, 1994). Either of these pathways appears to be sufficient to induce the proteolysis of I-κB though a coupled phosphorylation / degradation pathway.

In contrast, on binding to the hepatocyte type I receptor, Ang II stimulates the formation of 1,2-diacylglycerol with consequent PKC activation, mobilization of intracellular calcium, and inhibition of hormone-stimulated adenylate cyclase activity (Murphy *et al.,* 1991; Bouscarel *et al.,* 1988; Griendling *et al.,* 1986; Lang and Valloton, 1987). The demonstration that certain PKC isoforms are physically associated with the I-κB molecule (kinases that can phosphorylate and inactivate this inhibitor in vitro) makes PKC a likely candidate for coupling the activated AT1 receptor to modulating NF-κB activity. This finding provided a mechanism for the transcriptional component of ANG gene synthesis in the positive feedback loop. The induction of the ANG gene by diverse physiological stimuli is mediated through changes in the nuclear abundance of sequence-specific transcription factors. The intracellular convergence of cytokine- and Ang II-induced signaling pathways on the nuclear factor-κB transcription factor provides a point for "cross-talking" between Ang II- and cytokine-activated second messenger pathways (Brasier and Li, 1996).

1.5 cAMP RESPONSIVE ELEMENT (CRE) AND ITS BINDING PROTEINS (CREB)

1.5.1 cAMP responsive element (CRE)

Genes whose transcription is regulated by cAMP contain the palindromic sequence 5'-TGACGTCA-3', or close variations of it, in their promoter region (Deutsch *et al.,* 1988a; Roesler *et al.,* 1988). This sequence is known as the cAMP responsive element (CRE), and its integrity is required for transcriptional response to cAMP. Variant CREs include asymmetrical or atypical sequences that differ from the consensus motif by single or

multiple nucleotide deletions or substitutions. Examples of these include CRE-like sequences in the rat insulin (Philippe and Missoten, 1990), vasoactive intestinal polypeptide (VIP) (Deutsch *et al.,* 1988a,b), and rat ANG gene promoter. These CRE-like sequences are still capable of binding the regulatory factors and mediating transcriptional regulation by cAMP, albeit to a lesser extent than the consensus palindromic CRE. The CRE sequence may be repeated in a promoter, such as VIP gene promoter, where are two core-CRE octamers are presented. Studies suggeste that two or more CRE sequences in a promoter may come together to form a stable hairpin structure, to which the proteins bind much stronger than to the individual CRE (Spiro *et al.,* 1993).

The transcriptional activity of CRE is influenced by flanking sequences in different promoters (Deutsch *et al.,* 1988; Kanei-Ishii and Ishii, 1989; Lee *et al.,* 1989). Such as the CREs in somatostatin and VIP gene promoters behave differently in F9 embryonal carcinoma cells. Both promoters exhibit a differentiation-dependent cAMP response (Masson *et al.,* 1992), they can be activated in differentiated F9 cells but can't be activated in undifferentiated cells even in the presence of both CREB and the PKA catalytic subunit (cPKA). However, somatostatin promoter can be activated by exogenous CREB and cPKA, an increased levels are believed to overcome the negative effect of some inhibitors of CREB which is specifically expressed in undifferentiated cells. In contrast, exogenous CREB and cPKA can't activate VIP promoter.

Not only CRE variants, but also consensus palindromic CRE sequences may be different in basal activities and relative responsiveness to cAMP-induced transcriptional activation. One reason for this variation is that the sequence adjacent to the core palindromic octamer can influence the binding stability and / or transactivation functions of proteins bound to the core CRE (Deuthsch *et al.,* 1988). The flanking sequences can serve as binding sites for additional DNA-binding proteins that interact with proteins bound to the core CRE octamer (Muro *et al.,* 1992; Ikuyama *et al.,* 1992; Miller *et al.,* 1993). Additionally, flanking sequences can influence the stability of protein binding to the core

octamer (Ryseck and Bravo, 1991). Results from DNase I footprinting and methylation interference experiments show that proteins in nuclear extracts and purified CRE-binding protein (CREB) make base contacts that are well outside the core octamer (Andrisani *et al.*, 1988; Powers *et al.*, 1989; Knepel *et al.*, 1990; Vallejo *et al.*, 1992). Consequently, it is not surprising that sequences flanking the core CRE can influence the binding or function of transcription factors bound to the CRE.

CRE function may also be influenced by additional trans-acting factors. CREB is a member of the activating transcription factor multigene family, whose member has similar DNA-binding specificity and can interact with CREs (Hardy and Shenk, 1988; Hyman *et al.*, 1988; Lin and Green, 1988). CRE in SV40 promoter showed inducible activity in cell lines PC12, NIH3T3 and CV1, but not in cell lines: L (mouse connective tissue), Hela (human epitheloid carcinoma), KB (human epidermoid carcinoma), or A431 (human epidermoid carcinoma). The cell-type specificity of the inducible enhancer activity of CRE was not related to the extent of increase in the intracellular cAMP level by 8-Br-cAMP treatment. These results raised the possibility that the CRE-binding trans-activator, which is responsible for the inducible enhancer activity, exists only in cells such as PC12 cells, indicating that the cell-type specificity of constitutive enhancer is different from that of the inducible enhancer. Taking together, these findings suggeste that there are at least two different trans-activators bound to CRE that have different cell-type specificity: one for inducible enhancer activity and the other for constitutive enhancer activity. The selection of trans-activator bound to CRE depends on the promoter, because the trans-activator bound to CRE and the factor bound to the promoter must interact.

1.5.2 CREB Cell signal transduction

In almost all living organisms cells communicate by sending and receiving chemical signals in the form of neurotransmitters and hormones. These signals induce specific cellular responses, for example, changes in plasma membrane properties (ion channels or receptors), cellular growth and metabolism, or gene expression, depending on the nature of the signal

and the specific cell type involved. To elicit their actions, the signal neurotransmitter and hormone molecules must first bind to specific high-affinity cellular receptors that reside in the cytoplasm (e.g., steroid hormone receptors), in the nucleus (e.g., thyroid hormone and retinoid receptors), or on the cell surface (e.g., plasma hormone receptors) of target cells. Small lipophilic molecules such as steroid hormones, thyroid hormones, and retinoids can readily diffuse across plasma membranes. After gaining access to the interior of the cells, these hormones bind to and activate nuclear or cytoplasmic receptors (Fuller, 1991; Gronemeyer, 1992; Simons *et al.,* 1992; O'Malley, 1990; Beato, 1989). Often, the ligand-bound activated receptors are sequence-specific DNA-binding proteins that regulate the transcription of specific sets of target genes. Larger and more complex ligands such as peptide hormones cannot diffuse across plasma membranes. These molecules bind to and activate receptors located on the plasma membranes of target cells (Dohlman *et al.,* 1991). The activated cell surface receptors then transduce signals to the interior of the cell by coupling to GTP-binding proteins (G proteins) (Gilman, 1987; Simon *et al.,* 1991) or to autophosphorylate itself in response to conformational changes induced by the binding of ligand (Ullrich and Schlessinger, 1991). Thereby the receptor serves as the communicative link between the outside and inside of the cell. The active G proteins can be stimulatory (G_s) or inhibitory (G_i). The G_s protein activates adenylate cyclase leading to the production of the important second messenger cAMP which in turn activates protein kinase A (PKA) respectively (Hunter and Karin, 1992). Proteinases and the corresponding phosphatases (Cohen, 1989) are critically important in life processes because phosphorylation of proteins and lipids is a universal mechanism for regulation. One major role of protein kinases and phosphatases is to regulate the activities of DNA binding proteins (transcription factors) that interact with specific DNA control elements located in the promoter regions of gene and to thereby activate or repress transcription (Ptashne, 1988; Mitchell and Tjian, 1989).

In many cases these signal transduction pathways lead to the nucleus in order to regulate gene transcription. There are several well-characterized pathways by which signals can be transmitted from the cell surface to the nucleus. These pathways can be

conceptualized as having four components, or "messenger systems" (Meyer and Habener, 1993): first, the ligand / receptor / protein complexes; second, the activator substances (e.g. cAMP, diacylglycerol, calcium-calmodulin); third, the protein kinases; and fourth, the DNA binding proteins (Fig. 1-3) (Meyer and Habener, 1993). Each of these messengers individually consists of complex families of many related but distinct substances or proteins.

1.5.3 CREB and related proteins, CREM and ATF-1

Forty years have elapsed since the initial discovery of cAMP, the important second messenger and mediator of cellular signal transduction (Sutherland, 1972). Subsequently followed the discoveries of the cellular receptors that serve as sensor of hormones and other extracellular signaling molecules, the transducers consisting of stimulatory GTP-binding proteins (Gilman, 1989) involved in the signal transduction cascade ultimately leading to the regulation of gene expression. It has been only during the past several years that the final mediators in the cAMP-dependent signaling cascade have been identified. These are the cAMP-responsive transcription factors, DNA-binding proteins whose functions are to stimulate or repress the transcription of target genes. The first described CRE-binding factor is CREB (CRE-binding protein) with a molecular weight of 43 kDa and binds to the CRE of the rat somatostatin gene. CREB was first purified from both PC12 pheochromocytoma cells and rat brain and found to stimulate transcription of the cAMP-responsive gene when in dimeric form (Montminy and Bilezikjian, 1987; Yamamoto *et al.,* 1988). One year later Hoeffler et al isolated a cDNA from a human placental cDNA library, which encodes of 327 amino acids (CREB-327). (Hoeffler *et al.,* 1988, 1989). After that, cDNAs encoding CREB-341 (Gonzalez *et al.,* 1989) from rat brain, 505 amino acids from human brain (Maekawa *et al.,* 1989), 130 kDa from Hela cells (Kihara, F. and Ariga, H., 1991), CREB-BP (CRE-binding protein) 1,2,3 (Georgopoulos *et al.,* 1992), CREB2 (Willems *et al.,* 1992), CREBβ (Blendy *et al.,* 1996), etc. were reported independently. Sequence analysis CREB in human (Hoeffler *et al.,* 1990) and rat (Yamamoto *et al.,* 1990) tissues revealed that the CREB-327 and CREB-341 proteins are isoforms expressed from the same

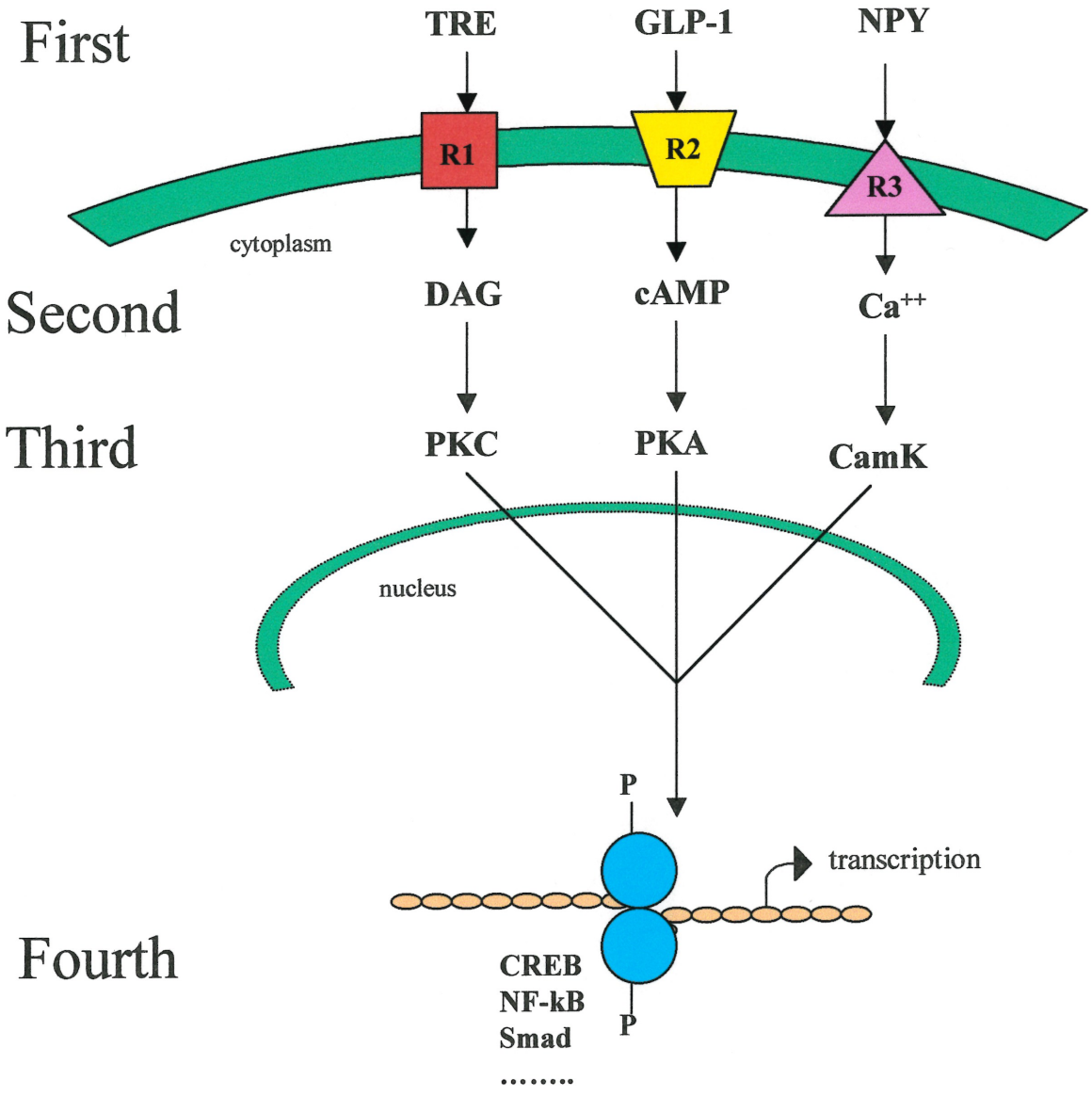


Fig. 1-3. Signal transduction pathways. The diagram depicts three representative pathways involved in the conveyance of the environmental extracellular signal (peptide hormone ligands bound to cell surface-located receptors R1, R2, R3) to the nucleus where gene transcription is controlled by phosphorylated DNA-binding proteins. The DNA-binding phosphoprotein CREB is activated by the cAMP-dependent signalling pathway. The steps in the signaling pathways are designated as first through fourth component messengers. Abbreviations: TRH, thyroid hormone-releasing hormone; GLP-1, glucagon-like peptide-1; NPY, neuropeptide Y; PKA protein kinase A; PKC, protein kinase C; CamK, calcium calmodulin kinase.(redrawn from: Meyer & Habener,1993)

homologous human and rat genes and differ by the presence or absence of an alternatively spliced exon encoding 14 amino acids. So far, at least three groups of cAMP-responsive DNA-binding proteins have been clearly identified to bind to CRE sequence: CREB (cAMP-responsive element binding protein), CREM (cAMP-responsive element modulator), and ATF-1 (activating transcription factor 1). Like CREB, CREM and ATF-1 also exist as multiple isoforms due to the alternative splicing of the primary mRNAs.

CREB, CREM, and ATF-1 are structurally similar (Fig. 1-4). They are all the members of a super-family known as bZIP proteins (table 1-1). bZIP proteins are so named because of the structural similarity of their DNA-binding domains that consists of a basic region (b) involved in recognition and binding to DNA, and a leucine zipper (ZIP), a coiled-coil structure with heptad repeats of leucines responsible for dimerization (Fig. 1-5). The members in this family include many other transcriptional factors such as jun, fos myc, c/CBP, CRE-BP1, and the yeast cellular transcriptional activator protein GCN4 (Gill and Ptashne, 1987; Hope *et al.,* 1988). The basic region is approximately 30 amino acids in length, is well conserved, and contains a relatively high proportion of the positively charged amino acids lysine and arginine. The leucine zipper lies immediately c-terminal to the basic region and consists of a region of amino acids with leucines occurring at every seventh position. The leucine zipper forms an amphipathic α-helix, with hydrophobic residues (including leucines) along one face of the helix and hydrophilic residues along the opposite face. Dimerization of bZIP proteins occurs by formation of a parallel coiled-coil structure with the hydrophobic surfaces of two leucine zipper α-helices facing each other (Landschultz *et al.,* 1988; Vinson *et al.,* 1989). Dimerization brings the positively charged basic regions into a configuration that facilitates recognition of target DNA sequences through contacts of the basic regions with nucleotides in the major groove of the DNA helix. Dimers of bZIP proteins are thought to assume a Y-shaped structure in which the stem of the Y is formed by the juxtaposed leucine zippers and the arms by the basic regions. Evidence suggested that lysine residues 289 and 291 of the basic region are important for the dimerization of CREB (Andrisani and Dixon, 1991), and the carboxyl-terminal 66 amino

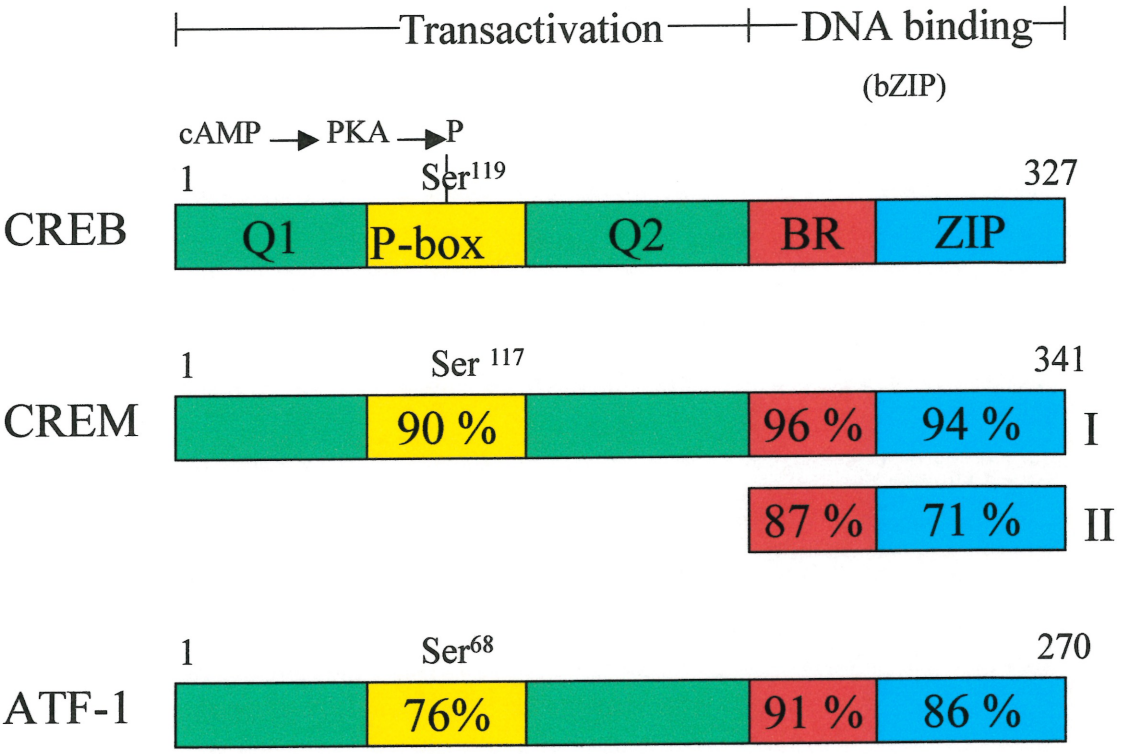


Fig. 1-4. cAMP-responsive bZIP proteins CREB, CREM, and ATF-1. These three transcription factors constitute distinct superfamily of bZIP proteins characterized by highly conserved basic region (BR), leucine zippers(ZIP), and phosphorylation regions (P box, also known as the kinase-inducible domain (KID), and percentage of amino acid similarity to those of corresponding regions of CREB are indicated. CREM I and CREM II are generated by alterative exon splicing, resulting in proteins with different DNA binding domains. Locations of the glutamine-rich regions (Q1 and Q2) and the protein kinase A (PKA) phosphorylation site, Ser 119, are also shown. P, phosphate.(redrawn from:

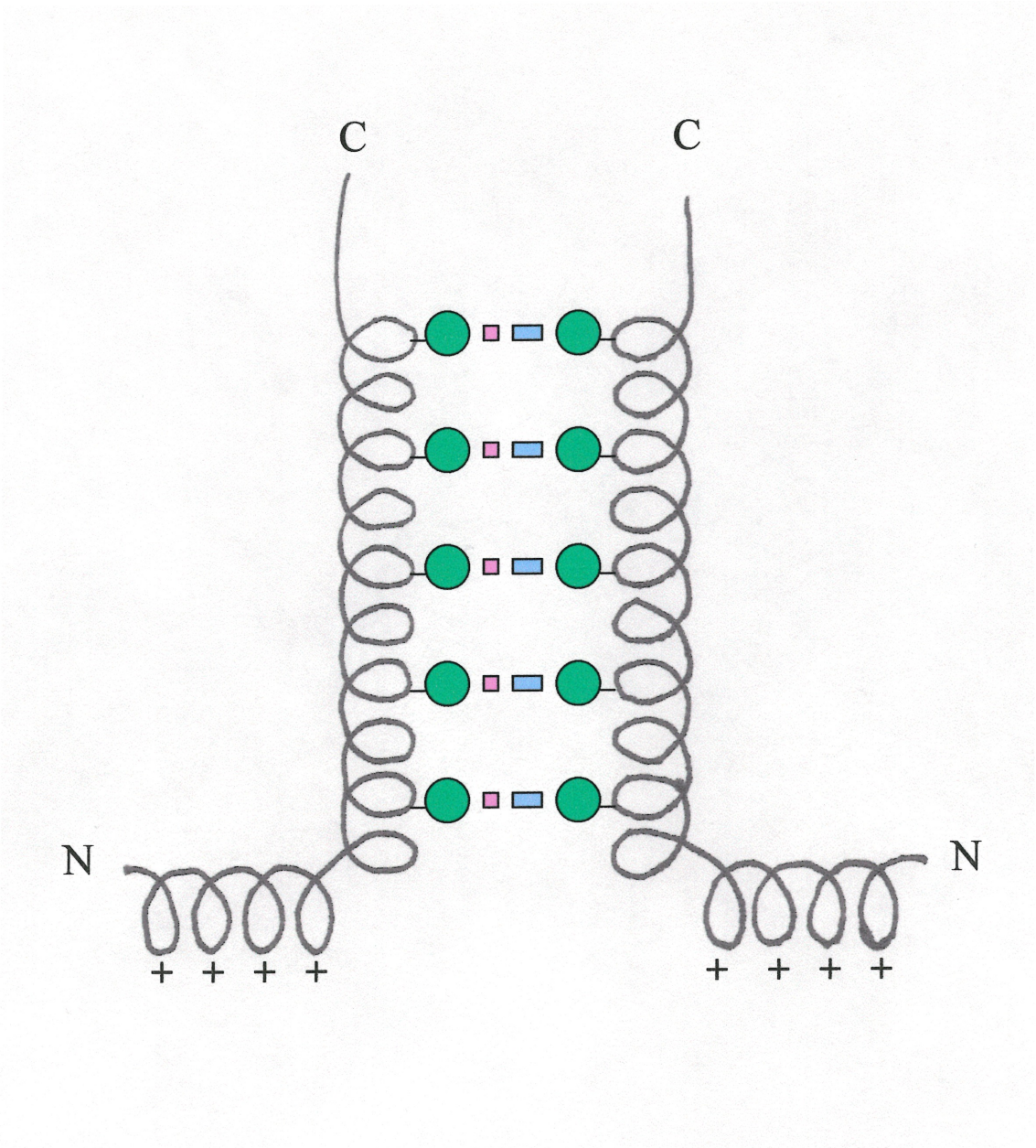


Fig. 1-5. Two leucine zippers in parallel orientations could form a dimeric structure. Note that if the α-helix has the usually 4 residues per turn, there will be a protruding leucine leucine almost every other turn. (redrawn from: Lewin, 1994)

acids are self-sufficient for DNA-binding and dimerization (Dwarki *et al.,* 1990; Yun *et al.,* 1990). The amino terminal region of the CREB protein consists of a randomly-coiled structure with a net negative charge, the counterpart of which has been showed to be important in the transactivation functions of the yeast transcriptional activators, GAL4 and GCN4 (Ma and Ptashne, 1987; Hope *et al.,* 1988). The transactivating domains of the yeast proteins that stimulate transcription by RNA polymerase II are acidic with no obvious sequence homology. These studies suggest that the extent of acidity *per se* may be related to the potency of the transcription (Sigler, 1988; Ptashne, 1988; Struhl, 1988) and may form amphipathic α-helical structures (Giniger and Ptashne, 1987). Studies of the GCN4 transcription factors revealed that negatively charged α-helical regions correlate with transcriptional activity (Struhl, 1988). Characterization of the structures of the relevant transcriptional activation domains of other DNA-binding proteins indicate that the acidic, glutamine rich (Sp1: Courey *et al.,* 1989;) and proline-rich (CTF/NF-1: Mermod *et al.,* 1989) domains are likely to be regions that function by contacting other proteins. The acidic activation domain of the yeast GAL4 and mammalian ATF have been shown to facilitate the formation of transcriptional initiation complexes by interacting with the general transcription factor, TFIID (the mammalian TATA box binding factor) (Horikoshi *et al.,* 1988).

The CREB genes from human (Hoeffler *et al.,* 1990) and mouse (Cole *et al.,* 1992) have been isolated. The human CREB gene is located on the long arm of chromosome 2 mapped to 2q32.3-q34 (Taylor *et al.,* 1990). The mouse CREB is located on chromosome 1 (Taylor *et al.,* 1990). The CREB genes consist of multiple, at least 12 exons, spanning an estimated 80-100 kb (Hoeffler *et al.,* 1990; Ruppert *et al.,* 1992). CREB-327 and CREB-341 differ by an alternatively spliced exon D encoding 14 amino acids (Hoeffler *et al.,* 1990).

1.5.4 Roles of CREB and CREM in the physiological regulation of gene transcription

Although the exact physiological role of CREB has not been completely established, increasing evidence indicates that CREB may regulate certain cellular processes in several

tissues of the intact organism, including the testes, the pituitary gland, and the brain.

1.5.4.1 Testis:

The CREB and CREM genes are highly expressed in the testes. The primary transcripts of these two genes undergo complex patterns of alternative splicing of exons during both the postnatal developmental maturation of spermatogenesis and the endogenous cycling of the seminiferous tubules in the adult animal (Waeber and Habener, 1992). In premeiotic germ cells the CREMα repressor isoform is expressed in low amounts. During the developmental transition of the spermatocytes through the pachytene stages, the CREMτ activator isoform is expressed and accumulates in high amounts. During the early stage of spermatogenesis, when the round spermatids elongate to spermatozoa, a new repressor isoform of CREM, CREMΔC-G, is expressed (Walker *et al.,* 1994). These circumstances suggest that during spermatogenesis, CREM gene expression undergo a biphasic transition of isoforms: from repressor, to activator, to repressor. These splicing-dependent changes in the transcriptional functions of CREM, along with the transitions in CREB splicing, are proposed to have an important role in programming the expression of cAMP-regulated target genes during spermatogenesis, although the detailed mechanisms involved are not yet understood (Walker *et al.,* 1995). Studies have demonstrated that spermatogenesis requires CREM (Bertherat, 1996), and severe impairment of spermatogenesis in mice lacking the CREM gene has been documented (Blendy *et al.,* 1996).

1.5.4.2 Anterior pituitary gland:

In the anterior pituitary CREB may be involved in the regulation of the cAMP-dependent proliferation of somatotrophs. This is supported by loss-of-function experiments in transgenic mice (Struthers *et al.,* 1991) and by gain-of-function experiments (Burton *et al.,* 1991). These experiments employed the transgenic mice carrying a chimeric gene encoding an intracellular form of cholera toxin under the control of the growth hormone gene promoter. In these animals cholera toxin specifically expressed in somatotrophs

irreversibly stimulates Gs protein-mediated activation of adenyl cyclase (AC), resulting in permanently elevated concentrations of cAMP. The phenotype of mice carrying this transgene is characterized by gigantism, hyperproliferation of somatotrophs, and pituitary hyperplasia (Burton *et al.,* 1991). A clinical correlate of these observations is found in patients with pituitary adenomas due to constitutively active mutant forms of G_s proteins (Landis *et al.,* 1989). The possible existence of mutated CREB in other somatotrophy adenoma has not been examined.

1.5.4.3 Brain:

In the brain, the distribution of mRNA encoding CREB and CREMτ is diffuse, whereas the distribution of mRNA encoding repressor isoform of CREM is restricted to specific areas (Mellstrom *et al.,* 1993). This pattern of expression suggests that in the central nervous system the presence of CREM antagonists may determine region-specific differences in CREB- (or CREMτ-) mediated responses to cAMP stimulation. Peripheral hyperosmotic stimulation results in CREB phosphorylation in the hypothalamic supraoptic and paraventricular nuclei (Borsook *et al.,* 1994). In addition, osmotic stimulation results in the induction of CREMα and CREMβ in neurons of the supraoptic nucleus (Mellstrom *et al.,* 1993). These findings suggest that CREB and CREM be involved in the control of hypo-thalamic homeostatic mechanisms that maintain plasma osmolality.

CREB and CREM are also thought to play an important role in the physiological mechanisms of the regulation of circadian rhythms (Ginty *et al.,* 1993; Stehle *et al.,* 1993) that control neuronal and hormonal response to light-dark cycles.

Emerging evidence indicates that CREB is also required for the normal process of higher brain functions such as memory consolidation (Frank and Greenberg, 1994; Stevens, 1994). Studies (Yin and Tully, 1996; Goda, 1995) suggest that CREB plays a central role in the formation of long-term memory in drosophila, aplysia and mice. Agents that disrupt the activity of CREB specifically block the formation of long-term memory, whereas agents that increase the amount or activity of the transcription factor accelerate the process. Analysis of

mice carrying the targeted mutation in the CREB gene revealed a deficiency in long-term memory although short-term memory is normal (Bourtchuladze *et al.,* 1994). It is hypothesized that CREB is pivotal in the switch from short-term to long-term memory (Yin and Tully, 1996). The role of CREB in memory consolidation is presumably related to the cAMP-dependent transcriptional regulation of genes whose expression increases synaptic strength in specific neuronal populations.

1.6 MECHANISMS OF CREB-INDUCED TRANSACTIVATION

Studies have shown that the transcriptional induction by cAMP is rapid, peaking at 30 min and declining gradually over 24 hr (Sasaki *et al.,* 1984; Lewis *et al.,* 1987). This burst in transcription is resistant to inhibitors of protein synthesis, suggesting that cAMP may stimulate gene expression by inducing the covalent modification rather than de novo synthesis of specific nuclear factors. Since all of the known cellular effects of the cAMP occur via the catalytic subunit of cAMP-dependent PKA, it appears likely that this enzyme mediates the phosphorylation of factors that are critical for the transcription response.

1.6.1 Phosphorylation of CREB, CREM and ATF-1 in the P-box

Studies on CRE-binding proteins have discovered that the PKA phosphorylation motif shared by CREB, CREMτ, and ATF-1, RRPSY, is located in a region of approximately about 60 amino acids known as the KID (kinase-induced domain) or P-box (Fig. 1-5). This region contains an abundance of serines and acidic residues arranged in sequence motifs such that serines, and possible threonines, are phosphorylated by cAMP-dependent PKA and possible also by several other protein kinases (Gonzale and Montminy, 1989; Roch, 1991; Gonzalez *et al.,* 1991). Two glutamine rich regions (Q1 and Q2) have been found on both sides of the P-Box. Experiments have demonstrated that phosphorylation of CREB at Ser133 CREB is induced 6-fold *in vivo*, following treatment of PC12 cells with forskolin (Gonzalez and Montminy, 1989). By contrast, no such induction was observed in the PKA-deficient PC12 line A126. Inactivating the PKA phosphorylation

site by *in vitro* mutagenesis of the cloned CREB cDNA at Ser133 completely abolished CREB transcriptional activity (Gonzalez and Montminy, 1989). It has been demonstrated that Ser133 in CREB-341 corresponds to the Ser119 in the CREB-327 isoform, Ser117 in CREMτ, and Ser68 in ATF-1, respectively. Phosphorylation by PKA on these sites is essential for protein function. PKA-deficient cell, are unable to stimulate somatostatin gene transcription in response to forskolin (Montminy *et al.,* 1986), and microinjection of the C-subunit of PKA into cells can directly activate CRE-dependent transcription without simultaneous addition of cAMP (Riabowol *et al.,* 1988). Increase in intracellular cAMP has been showed to induce the phosphorylation of CREB *in vivo* (Gonzalez and Montminy, 1989). Thus, the phosphorylation of CREB *in vivo* at Ser133 is critical to the activation of gene transcription by cAMP. Although the phosphorylation converts Ser133 or Ser119 from an uncharged into a negatively charged amino acid, it appears that the gain of a negative charge per se is not sufficient for CREB activation, because Ser133 can not be substituted for by other negatively charged residues (Gonzale and Montminy, 1989). Therefore, the mechanism by which phosphorylation by PKA activates CREB remains unclear. It has been proposed that phosphorylation induces a conformational change in CREB that induces its transcriptional activity (Brindle *et al.,* 1993; Gonzalez *et al.,* 1991; Lee, 1990).

Increasing evidence indicates that Ser133 provides a common phosphorylation site at which different signal transduction pathways converge. Thus, CREB mediates some of the transcriptional changes observed after stimulation by membrane depolarization and calcium influx into cells as a result of phosphorylation of Ser133 by Ca^{+2} / calmodulin-dependent kinases (e.g., CaMK) (Enalen *et al.,* 1994; Matthews *et al.,* 1994; Schwaninger *et al.,* 1993; Bading *et al.,* 1993). CaMK also phosphorylates CREM (DeGroot *et al.,* 1993b). ATF-1 has also been shown to mediate Ca^{+2}-induced transcriptional responses (Liu, 1993). Therefore, CREB, CREM, and ATF-1 are common targets that integrate signals converged by the activation of two different transduction pathways, one cAMP dependent and the other Ca^{+2} / calmodulin dependent. Both Ca^{+2} and cAMP-dependent pathways interact at different levels of the signal transduction cascade. For example, cAMP-induced

phosphorylation regulates the activity of specific voltage-sensitive Ca^{+2} channels (Sculptoreanu *et al.,* 1993), and in turn, Ca^{+2} affects the activity of certain phosphatases (Cohen, 1989) and adenylyl cyclase isoforms (Katsushika *et al.,* 1992; Yoshimura and Cooper, 1992).

1.6.2 Glutamine-rich regions: Q1 and Q2

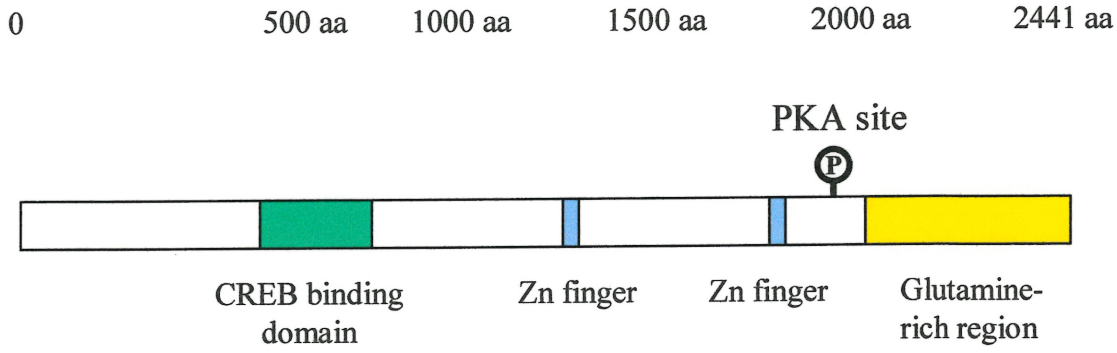
CREB affects gene transcription through its transactivation domain that includes the P-box/KID and its flanking glutamine-rich region, Q1 and Q2 region. Mutagenesis studies have revealed that the P-box functions synergistically with the adjacent Q regions (Brindle *et al.,* 1993; Quinn, 1993). The deletions of Q1 or Q2 result in a remarked reduction of CREB transcriptional activity (Brindle *et al.,* 1993 Gonzalez *et al.,* 1991; Quinn, 1993). In the absence of phosphorylation by PKA, the Q1 and Q2 domains are thought to be important to maintaining the basal activity of CREB. Hybrid proteins consisting of the N-terminal transactivation domain of CREB (amino acids 1-248) fused to DNA binding domain of B-cell activator protein (BSAP-1) that binds its regulatory element as a monomer, activate transcription constitutively independent of phosphorylation by PKA (Krajewski and Lee, 1994). By mutational deletion analyses, the transactivational activity of the CREB/BSAP-1 hybrid protein appears to be mediated by the glutamine-rich region, not by the P-box/KID domain. This activity may be due to the interactions of the Q1 and/or Q2 domains with other transcription factors bound to neighboring sites located in proximity to a CRE. It has been proposed that the Q1 and Q2 regions may provide interaction surfaces for the coupling of transcription factors with specific activator proteins associated with the RNA polymerases II complex and may result in the activation of transcription of target genes (Dynlacht *et al.,* 1991; Hoey *et al.,* 1993). *In vitro* experiments have demonstrated that wild-type CREB, but not Q2 mutant CREB, could bind to TAF 110, one of the proteins associated with the basic transcriptional machinery (Hoey *et al.,* 1993; Brindle *et al.,* 1993; Quinn, 1993; Ferreri *et al.,* 1994). Q1 and Q2 regions may be corresponding to constitutive activator domains that become exposed for interactions with target coactivator proteins upon phosphorylation

of the adjacent KID by PKA (Brindle *et al.,* 1993; Ferrieri *et al.,* 1994; Quinn, 1993; Krajewski and Lee, 1994).

1.6.3 CREB-binding protein (CBP)

CREB may activate gene transcription through interaction with another effector or coactivator proteins, CBP (CREB binding protein), a large nuclear protein of 2442 amino acid residues (265 kDa) (Chrivia *et al.,* 1993). Analysis of the amino acid sequence of CBP deduced from its cloned cDNA revealed the presence of at least three consensus phosphorylation sites for CaMK-II and one for PKA, as well as two putative zinc finger domains. In addition, the C-terminal region contains a glutamine-rich sequence that was thought to be the transactivation domain. The CREB binding domain, determined by deletion studies (Chrivia *et al.,* 1993), is located within the N-terminal region (Fig. 1-6). Recently, a novel transcriptional activation domain in the N-terminal was identified by deletion analysis (Bisotto *et al.,* 1996). This domain consists of the first 714 amino acids of CBP and is sufficient for high levels of transcriptional activity. CBP does not regulate the DNA binding, dimerization, or nuclear targeting properties of CREB, but binds selectively to the kinase-inducible 60 amino acid transactivation domain (KID) of CREB, critical for PKA inducible transcription (Montminy *et al.,* 1996). CBP is recruited to the CREB-DNA complex in the promoter of target genes upon phosphorylation of CREB. CREB mutated at the PKA phosphorylation site did not allow CBP to activate expression.

CBP functions as a coactivator. Known CBP functions can be roughly divided into six groups, ranging from the molecular to the organismal level: (1) it creates a physic bridge between various transcription factors and the basal transcription machinery; (2) It is a potent histone acetytransferases, which links transcription to chromatin remodeling; (3) it mediates both negative and positive cross-talk between different signaling pathway; (4) it participate in basic cellular functions, including DNA repair, cell growth, differentiation and apoptosis; (5) it plays pivitoal roles in embryonic development; and lastly, (6) it functions as tumor suppressor proteins and mutated in human cancers (Giles, et al, 1998, Goodman and



CREB binding protein (CBP)

Fig. 1-6. Schemic diagrams of the domain structures of CREB binding protein (CBP.

Smolik, 2000, Freedman,1999). CBP comprises two of seven known human histone acetyltransferases (HATs) and it is also the only HATs capable of acetylating all 4 core histones. Targeted (HAT) is thought to nutralize the positive charge of histone tails and relax the interaction between histone and negative charged DNA. Targeted gene promoters thus become accessible (Fig. 1-7) (Giles, et al, 1998). In this manner CBP mediates cAMP-independent CREB-induced transcriptional responses. Microinjection of anti-CBP antibodies into the nuclei of cells inhibits transcriptional responses elicited by cAMP stimulation, suggesting that CBP is essential for the activation of transcription of cAMP-responsive genes.

The discovery of CBP has revealed interesting implications for the alterations in gene transcription elicited by certain viral proteins mediated via CRE-like elements. CBP contains regions with amino acid sequences homologous to those of equivalent regions in the protein p300, another nuclear protein that interacts with the adenovirus E1A oncoprotein and may mediate E1A-induced proliferation of cells (Arany *et al.,* 1994). Both proteins share 85-95 % similarity over several segments, one of which is also homologous to ADA2 (Arany *et al.,* 1994; Chrivia *et al.,* 1993), a coactivator protein found in yeast (Berger *et al.,* 1992). Based on sequence similarities shared by p300 and CBP, it has been proposed (Arany *et al.,* 1994) that interactions between CREB and p300, as well as between viral E1A (or its putative cellular counterpart) and CBP may occur in cells. In addition, CBP interacts with phosphorylated c-Jun (Arias *et al.,* 1994), which activates gene transcription in response to mitogenic stimuli. Accordingly, these interactions among CREB, CBP and p300 may provide a molecular substrate for the observed effects elicited by cAMP on cell proliferation and differentiation.

1.7 THE OBJECTIVES OF THIS STUDY

Multiple factors are involved in the regulation of ANG gene expression, and some of them function through the changes of intracellular cAMP levels. The mechanism of cAMP has been revealed partly because of the discovery of CRE-binding proteins. Increased

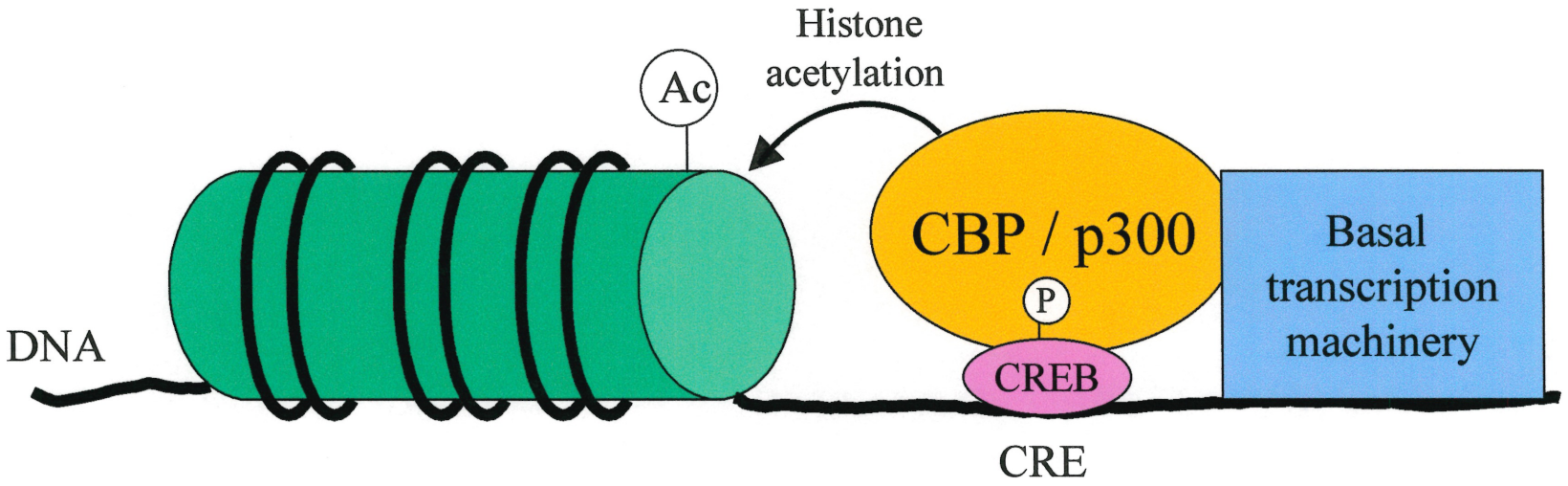


Fig. 3-7. CBP/p300 coordinate transcription with chromatin remodeling. CBP/p300 functions as a co-activator via binding to CREB. CBP / p300 acetylate all histone components of the nucleosome, thereby relaxing the chromatin structure the exposing the DNA to transcription elements. CRE, cAMP responsive element; P, phosphorylation group; Ac, acetyl group. (redrawn from: Giles R.H. et al: Trends Genet. 14; 178-183)

intracellular cAMP level results in the activation of PKA, which in turn phosphorylates the 43 kDa-CREB and /or related proteins. These phosphorylated proteins bind to the specific sequences (CRE) of promoter in target genes and affect the gene transcription. Previous studies in our laboratory on the DNA structure of the 5'-flanking sequence of the rat ANG gene showed that the DNA sequence of nucleotides N-795 / -788 (TGACGTAC) is almost identical to the CRE (TGACGTCA) of the somatostatin (SOM) gene except that the last two nucleotides are in reverse orders. With such a homology, we asked the question as to whether the sequence ANG N-795/-788 could be a putative CRE-motif. Indeed, our gene transfection experiments demonstrated that the addition of forskolin or 8-Br-cAMP stimulated the expression of the pTKCAT (ANG N-806/-779), but not the mutants of ANG N-806/-779 (Wang et al, 1999). These studies suggested that the DNA fragment ANG N-796/-788 could be the CRE of the rat ANG gene. However, the magnitude of the intracellular cAMP levels stimulated by either 8-Br-cAMP or forskolin as well as isoproterenol just shows of the maximum 2.5-3.0 fold over the control, even with the introduction of extraneous 43 kDa-CREB cDNA (Ming *et al.,* 1993; Ming *et al.,* 1995; Qian *et al.,* 1996). This stimulation is relatively low compared with other expression system. In human TSHβ gene expression system, forskolin induced CAT activity by about 12-fold (Hans, *et al.,* 1991; Lee *et al.,* 1990), and in human parathyroid hormone gene expression system, forskolin stimulated the promoter activity up to 29-fold (Rupp *et al.,* 1990). Increasing doses of the drugs or the 43 kDa-CREB could not enhance the stimulated level of ANG gene promoter (Qian *et al.,* 1996). It seems that the cAMP-PKA-CREB pathway was blocked. We speculate that there might be some inhibitory protein(s) that block the stimulatory effect of the 43 kDa-CREB by either competing with the 43 kDa-CREB to bind to ANG-CRE or interacting with the 43 kDa-CREB to form a complex and reduce the binding ability of the 43 kDa-CREB to ANG-CRE. The objective of the present study is to test the existence of this inhibitory protein(s), furthermore, to clone and characterize the protein. The project was divided into three parts:

Part A. To demonstrate the existence of the nuclear protein which binds to ANG-CRE and to answer the following questions:

1. Is there a nuclear protein binding to ANG-CRE?

2. Is this protein the same as the previous reported CRE-binding proteins?

3. What is the molecular weight of the ANG-CRE binding protein?

4. Does the protein show the ANG-CRE sequence specificity?

5. Does the protein exist in the different tissues?

Part 2. To clone the ANG-CRE binding protein. Based on the interactions between the ANG-CRE and the protein, the synthetic oligonucleotide that represents the ANG-CRE can be used to screen the cDNA expression library. Once the cDNA is cloned the following questions will be answered.

1. What is the sequence of the cDNA and deduced amino acid of the ANG-CRE binding protein?

2. Does the amino acid sequence of the protein reveal a similarity to the members of the bZIP superfamily?

Part 3. Use transient gene transfection to investigate the biological functions of the ANG-CRE binding protein

2. Chapter 2. Materials And Methods

2.1 MATERIALS

2.1.1. Enzymes

All restriction and modifying enzymes were purchased from Bethesda Research Laboratories (BRL), (Burlington, Ontario, Canada), or Boehringer-Mannheim, (Doval, Quebec, Canada), or Pharmacia Inc., (Baie d'Urfe, Quebec, Canada).

2.1.2. Vectors

pGEM-3 was purchased from Fisher-Promega (Montreal, Quebec, Canada); pcDNAI and PCRII were purchased from Invitrogen (La Jolla, CA, U.S.A.); pMalc was purchased from New England Biolabs,Ldt.(Mississauga, Ont, Canada).

2.1.3. Oligonucleotides

All oligonucleotides (see Fig. 2-1) were synthesized either in our lab. or purchased from Bio-synthesis Inc. (Lewisville, TX, U.S.A.). Polynucleotide oligo (dI/dC), (Cat.No.,27-7880), used for non-specific competition in gel shift assays, was purchased from Pharmacia Inc., (Baie d'Urfe, Que, Canada).

Synthesized oligonucleotides were double-stranded by mixing the equal molar of the sense and antisense in annealing buffer.

A.

B.

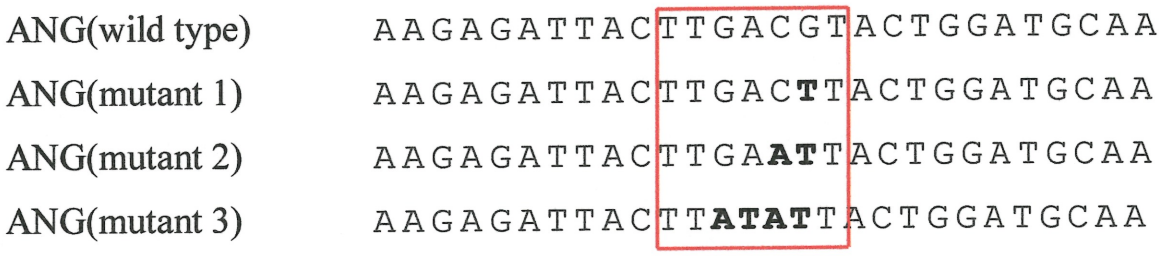


Fig. 2-1. **A**. Sequences of the cAMP responsive element (CRE from angiotensinogen gene (ANG-CRE); somatostatin gene (SOM-CRE)(Montminy, MR *et al.*, 1986); phosphoenopyruvate carboxylkinase gene (PEPCK-CRE)(Bokar, JA *et al.*, 1988) and tyrosine amino transferase gene (TAT-CRE)(Ganss, R *et al.*, 1994). **B**. Sequences of the wild type and mutants of angiotensinogen gene CRE used in the experiments.

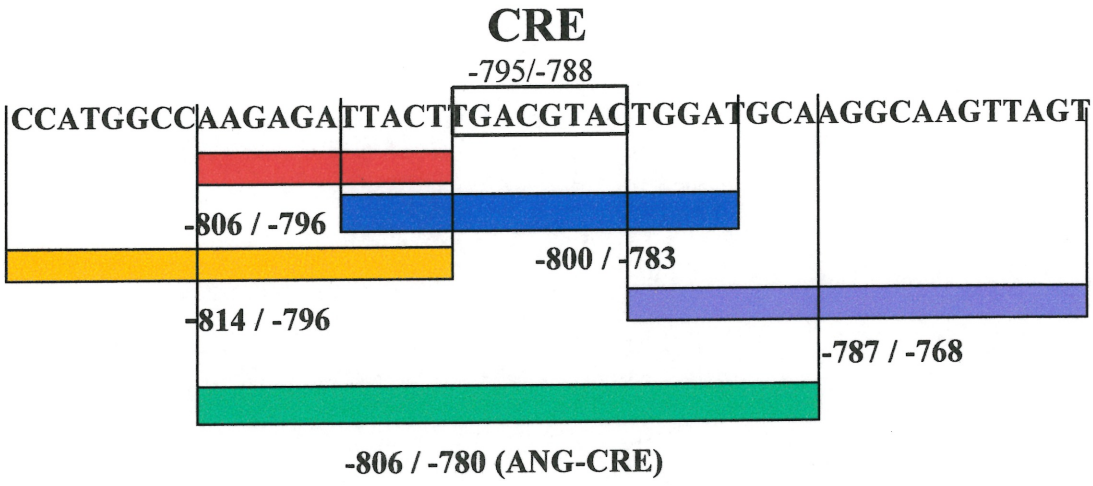


Fig. 2-2. Schematic diagrams of the oligonucleotides used in the gel mobility shift and southwestern blot assays. The corresponding locations on the rat angiotensinogen gene are marked.

2.1.4. Proteins

The basic leucine-zipper domain of the cAMP responsive element binding protein (CREB), (bZip, amino acid 254-327, Cat.No., sc-4002); Activating transcription factor-1(ATF-1 amino acids 39-271, Cat.No., sc-4006); and cAMP responsive element modulator (CREM, full length, Cat.No. sc-4005) were all purchased from Santa-Cruz Biotechnology, Inc., CA, U.S.A.

2.1.5. Antibodies

Rabbit polyclonal (Cat. No., sc-58A) and monoclonal (Cat. No., sc-240) antibodies against CREB were purchased from Santa-Cruz Biotechnology Inc., CA, U.S.A. Goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate, (Cat. No. 172-1013) was purchased from Bio-Rad, Laboratories (Canada) Ltd, (Mississauga, Ont, Canada).

2.1.6. Proteinase inhibitors

Aprotinin, (Cat. No., A-4529); leupeptins, (Cat. No., A-2884); antipain, (Cat. No., A-6271); PMSF, (Cat. No., A-7626); and DTT, (Cat. No., A-0632) were all purchased from Sigma, (St. Louis, MO, U.S.A.). AEBSF, (Cat.,No., 1429 868) was purchased from Boehring Mannheim, (Laval, Que, Canada).

2.1.7. Antibiotics

Ampicillin was purchased from Boehringer Mannheim (Laval, Que, Canada). Kanamycin was purchased from Sigma, (St. Louis, MO, U.S.A.).

2.1.8. 8-Br-cAMP and dexamethasone (DEX)

8-Br-adenosine-3', 5' monophosphate cyclic (8-Br-cAMP), (Cat.No. B7880), and Dexamethasone (DEX), (Cat.No. D9402), were purchased from Sigma, (St. Louis, MO, U.S.A.).

2.1.9. Kits

Polymerase reaction chain (PCR) Kit was purchased from Perkin Elmer Cetus, (CA., U.S.A.). Gene clean II kit was purchased from Bio101 Inc., (La Jolla, CA, U.S.A.). PCR II kit was purchased from Invitrogen (La Jolla, CA, U.S.A.). DNA sequencing kit (T7) and T7QuickPrimeTM kit were purchased from Pharmacia Inc., (Baie d'Urfe, Que, Canada). Phage DNA purification kit was purchased from Qiagen Inc. (Chatsworth, CA, U.S.A.).

2.1.10. Cells

Opossum Kidney (OK) cells and mouse hepatoma (Hepa1-6) cells were purchased from the American Type Tissue Culture Collection (ATCC) (Rockville, MD., U.S.A.); the bacterial strains HB101, JM109, TB-1 were purchased from New England Biolabs Ltd, (Mississauga, Ont, Canada). DH5α competent cell was purchased from GIBCO BRL, (Burlington, Ont. Canada).

2.1.11. Animals

Babl/c mouse and New Zealand white rabbits were purchased from Charles River Inc. (St. Constant, Que. Canada).

2.1.12. Radioactive isotopes

^{32}P-α-dCTP (800 ci / mmol), ^{32}P-γ-ATP (3000 ci / mmol), ^{32}P-α-CTP (3000 ci/mmol), and ^{35}S-dATP (>1000 ci/mmol) were all purchased from New England Nuclear, Dupont (Boston, MA. U.S.A.).

2.1.13. DNA library

Mouse liver cDNA library, (Cat.No., ML10356, Lot. No., 27415) was purchased from Bio/Can Scientific (CloneTech.) (La Jolla, CA. U.S.A.).

2.1.14. Others

Cell culture medium, DMEM was purchased from GIBCO BRL, (Grand Island, N.Y., U.S.A.). Protein dye and HRP color development reagent, 4CN (4-chloro-1-naphthol) were

purchased from Bio-Rad, Laboratories (Canada) Ltd, (Mississauga, Ont, Canada); Sephadex-50 was purchased from Pharmacia Inc., (Baie d'Urfe, Que, Canada). Nitrocellulose membrane, was purchased from S&S, distributed by Mandel Scientific Company Ltd. (Guelph, Ont, Canada), or from Dupont, Boston, (MA, U.S.A.); and Plastic petri dishes, was purchased from Polar Plastic Ltd., (St. Laurent, Que, Canada).

2.2 EQUIMENT

2.2.1. Centrifuge

Microcentrifuge, Model 235C, Fisher Scientific Ltd., (Montreal, Que. Canada); Beckman L8-70M Ultracentrifuge, Beckmen Instrument Inc., (Alto, CA, U.S.A.); and Sorvall RC-5B Refrigerated Superspeed Centrifuge, Dupont Canada Inc., (Mississauga, Ont., Canada).

2.2.2. Electrophoresis

Horizon 11.14 and 58, and Model S2, Bethesda Research Laboratory (BRL), (Burlington, Ontario, Canada). Protean II Xi Cell and Transblot Cell, Bio-Rad Laboratories (Canada) Ltd, (Mississauga, Ont, Canada).

2.2.3. Sonifier Cell Disrupter

Model W185D, Heat System-Ultrasonics, Inc. (Plainview, NY, U.S.A.).

2.2.4. DNA synthesizer

391, Applied Biosystems (Foster City, CA., U.S.A.)

2.2.5. Thermostatic circulator,

2219. multitemp II; LKB, Bromma.

2.2.6. Gel dryer

Model 583, and Vapor Tra, Bio-Rad Laboratories (Canada) Ltd (Mississau Ont, Canada).

2.2.7. CO_2 incubator,

Moquin Premier; Par/by Revco.(Montreal, Que. Canada)

2.2.8. Speed vac. concentrator

(Savant) (Montreal, Que. Canada)

2.2.9. DU-6 Spectrophotometer

Beckmen Instrument Inc., (Alto, CA, U.S.A.).

2.2.10. DNA Thermal cycler,

Perkin Elmer Cetus, (CA, U.S.A.).

2.2.11. IS-1000 digital imaging system,

Alpha Innotech Corporation (U.S.A.)

2.3 PREPARATION OF SOLUTIONS

2.3.1. General stock solutions

2.3.1.1 Glucose (1M):

Glucose was dissolved in water at the concentration of 1M. The solution was sterilized by filtration, and stored at RT.

2.3.1.2 Tris-HCl (1 M, pH 8.0):

Trise base was dissolved in water. The pH of the solution was adjusted with the concentrated HCl to 8.0. The solution was stored at RT.

2.3.1.3 EDTA-Na_2 (0.5 M):

EDTA-Na_2 was dissolved in water and the pH was adjusted to 8.0 with NaOH. The solution was stored at RT.

2.3.1.4 NaOH (10 N):

NaOH was dissolved in water at the concentration of 10 N. The solution was stored at RT.

2.3.1.5 SDS (20 %):

SDS was dissolved in water. The pH was adjusted with NaOH to 7.0. The solution was stored at RT.

2.3.1.6 NaAc (3 M, pH 5.2):

NaAc was dissolved in water. The pH was adjusted to 5.2 with HCl. The solution was stored at RT.

2.3.1.7 TBE (20 X, pH 8.3):

To prepare1000 ml: 216 g Tris base; 110 g boric acid; and 15 g EDTA-Na_2 were dissolved in 950 ml of dH_2O. The pH was adjusted to 8.3. The solution was stored at RT.

2.3.1.8 TAE (50 X):

To prepare 1000 ml: 242 g Tris base was dissolved in 843 ml of dH_2O. 57.1 ml of glacial acid, and 100 ml of 0.5 M EDTA-Na_2 (pH 8.0) were added. The solution was stored at RT.

2.3.1.9 KCl (3 M):

KCl was dissolved in dH_2O at the concentration of 3 M, the solution was autoclaved and stored at RT.

2.3.1.10 $MgCl_2$ (1 M):

$MgCl_2$ was dissolved in dH_2O at the concentration of 1 M, the solution was autoclaved and stored at RT.

2.3.1.11 NaCl (5 M):

NaCl was dissolved in dH_2O at the concentration of 5 M, the solution was autoclaved and stored at RT.

2.3.1.12 KAc (5 M):

KAc was dissolved in dH_2O at the concentration of 5 M and the solution was stored at RT.

2.3.1.13 NaH_2PO_4 (1 M):

NaH_2PO_4 was dissolved in dH_2O, autoclaved and stored at RT.

2.3.1.14 K_2HPO_4 (1 M):

K_2HPO_4 (1 M) was dissolved in dH_2O, autoclaved and stored at RT.

2.3.1.15 HEPES (1 M, pH 7.6):

HEPES was dissolved in water. The pH was adjusted to 7.6 with KOH. The solution was autoclaved and stored at 4 °C.

2.3.1.16 Spermin (0.5 M):

Spermin was dissolved in water, and stored at -20 °C in aliquots.

2.3.1.17 Spermidine (1 M):

Spermidine was dissolved in water and stored at -20 °C in aliquots.

2.3.1.18 IPTG (1 M):

IPTG (isopropyl-B-thiogalactopyranoside) was dissolved in water and stored at -20 °C in aliquots.

2.3.1.19 Dendhardt (50 X):

K_2HPO_4 Poplyvinylpyrrolidone (PVP), bovine serum albumin (BSA) and Ficoll were dissolved in water at the final concentration of 1% each, sterilized by filtration and stored at -20 °C.

2.3.1.20 Salmon (or herring) sperm DNA (10 mg / ml):

Desired amount of DNA was cut with a clean scissors and dissolved in water at the concentration of 10 mg/ml. The DNA was sheared by passing solution once forcefully through a 23-gauge needle, and then stored at -20 °C in aliquots.

2.3.1.21 Antibiotics solutions

Sodium salt of ampicillin (or tetracycline) was dissolved in water at the concentration of 25 mg/ml. The solution was sterilized by filtration and stored in aliquots (1 ml / tube) at -20 °C.

2.3.1.22 RNase A (or T1) stock solution:

RNase A (or T1) was dissolved in 10 mM Tris-HCl (pH 7.5) at the concentration of 10 mg /ml, aliquoted and stored at -20 °C.

2.3.1.23 Proteinase K stock solution:

Proteinase K was dissolved in water at the concentration of 10 mg /ml. The solution was heat at 100 °C for 15 min and cooled down to RT. Then the solution was aliquoted and stored at -20 °C.

2.3.1.24 Aprotinin (Trasylol):

Aprotinin was dissolved in 0.01 M HEPES (pH 8.0) at the concentration of 10 mg / ml.

2.3.1.25 Leupeptins:

Leupeptins was dissolved in autolaved water at the concentration of 10 mg /ml.

2.3.1.26 Pepstatin A:

Pepstatin A was dissolved in ethanol at the concentration of 1 mg / ml.

2.3.1.27 Antipain:

Antipain was dissolved in autoclaved water at the concentration of 1 mg/ml.

2.3.1.28 PMSF (phenylmethy-sulfony fluorid):

PMSF was dissolved in isopropanolol at the concentration, 1.74 mg / ml (10 mM).

2.3.1.29 AEBSF (Pefabloc SC):

AEBSF was dissolved in water at the concentration of 100 mg / ml.

2.3.1.30 DTT (dithiothreitol):

DTT was dissolved in autoclaved water at the concentration of 1 M.

2.3.1.31 LB bacteria culture medium

LB broth: 10 g of bacto-trypone, 5 g of yeast extract, and 10 g of NaCl were dissolved in 1 liter of water, autoclaved, and then stored at RT or 4 °C.

2.3.2. Solutions for plasmid DNA extraction

2.3.2.1 Solution I:

The following stock solutions were mixed at the final concentration: glucose, 0.1 M; Tris-HCl, 25 mM; and EDTA-Na_2, 50 mM.

2.3.2.2 Solution II:

The NaOH and SDS stock solutions were diluted and mixed at the final concentrations, 0.2 N and 1 %.

2.3.2.3 Solution III:

The KAc and glacial acetic acid stock was diluted and mixed at the final concentration, 3 M and 11.5%.

2.3.3. Solutions for transformation

2.3.3.1 FSB:

The following chemicals were dissolved in water at the final concentrations: KAc, 10 mM; KCl, 100 mM, $MnCl_2.4H_2O$, 45 mM, $CaCl_2.2H_2O$, 10 mM, and $HACoCl_3$ (hexammine cobalt (III) chlorid), 3 mM. Glycerol was added to 10%, autoclaved and stored at 4 °C.

2.3.3.2 SOB:

The following were dissolved in water at the final concentrations: bacto-tryptone, 2%; yeast extract, 0.5%; NaCl, 0.0584%; and KCl, 0.0186%, autoclaved and stored at 4 °C.

2.3.3.3 SOC:

The following stock solution were added into SOB at final concentrations: $MgCl_2$, 10 mM, $MgSO_4$, 10 mM, and glucose, 20 mM, autoclaved and stored at 4 °C.

2.3.3.4 Denaturation buffer:

NaCl and NaOH were dissolved in water at the concentrations of 1.5 M and 0.5 M respectively. The solution was stored at RT.

2.3.3.5 Neutralization buffer:

The Tris base and NaCl were dissolved in water at the concentrations of 1 M and 1.5 M respectively. The pH was adjusted to 8.0 with HCl. The solution was stored at RT.

2.3.4. Solutions for the DNA sequencing

2.3.4.1 NaOH (2 N):

10 N stock solution was diluted to 2 N with dH_2O.

2.3.4.2 TBE (1 X):

20 X stock solution was diluted to 1 X.

2.3.4.3 Polyacrylamide urea gel (PAG) (8%):

Polyacrylamide and N,N-methylene bisacrylamide were dissolved in water. Then 20 X TBE and urea were added. When the urea was dissolved completely (with help of heating)), the solution volume was made up with water to the final concentrations:

polyacrylamide, 7.6%; N, N-methylene bisacrylamide, 4%; TBE, 1 ×; and urea, 8 M. The solution was filtered with a 3 -MM Whatman filter, and then stored in a brown bottle at RT.

2.3.4.4 Ammonia persulfate ($(NH_4)_2S_2O_8$) (10%):

Ammonia persulfate was dissolved in water at the concentration 10%, and stored at 4 °C.

2.3.5. Solutions for nuclear extract from cultured cells

2.3.5.1 Buffer A:

The following stock solutions were diluted and mixed at the final concentrations: HEPES, 10 mM; KCl, 10 mM; $MgCl_2$, 1.5 mM; and DTT, 0.5 mM.

2.3.5.2 Buffer B:

The following solutions were diluted and mixed at the final concentrations: HEPES, 0.3 M; KCl, 1.4 M; and $MgCl_2$, 0.03 M.

2.3.5.3 Buffer C:

The following tock solutions were added into 25% glycerol solution at the concentrations: HEPES, 20 mM; NaCl, 420 mM; $MgCl_2$, 1.5 mM; EDTA-Na_2, 0.2 mM; and DTT, 0.5 mM.

2.3.5.4 Buffer D:

The following stock solutions were added into 20% glycerol solution at the concentrations: HEPES, 20 mM; KCl, 100 mM; EDTA-Na_2, 0.2 mM; and DTT, 0.5 mM.

All solutions were stored at 4 °C. Just before use, proteinase inhibitors were added to the buffers at the final concentrations: Aprotinin, 1-2 μg / ml; leupeptins, 1-2 μg / ml; Pepstatin A, 1 μg / ml; Antipain, 1-2 μg /ml; PMSF, 100 μg / ml (or AEBSF, 96-958 μg / ml).

2.3.6. Solutions for nuclear extract from fresh tissues

2.3.6.1 Sucrose cushion:

Sucrose was dissolved in 10% glycerol solution at the final concentration 2 M (heating might be needed). Then the following stock solutions were added to the final concentrations: HEPES, pH 7.9, 10 mM; KCl, 15 mM; spermine, 0.15 mM; spermidine, 0.5 mM; and EDTA-Na_2, 1 mM.

2.3.6.2 Homogenization buffer (H buffer):

Sucrose was dissolved in 5% glycerol solution at the concentration of 2.2 M. Then the following stock solutions were added to the final concentrations: HEPES, pH 7.9, 10 mM; KCl, 15 mM; spermine, 0.15 mM; spermidine, 0.5 mM; and EDTA-Na_2, 1 mM.

2.3.6.3 Lysis buffer (L buffer):

The following stock solutions were added to a 10% glycerol solution at a final concentration: HEPES, pH 7.9; KCl, 100 mM; $MgCl_2$, 3 mM; and EDTA-Na_2, 0.1 mM.

All solutions were stored at 4 °C. Just before use, the proteinase inhibitors were added at the concentrations: Aprotinin, 1-2 μg / ml; leupeptins, 1-2 μg / ml; Pepstatin A, 1 μg / ml; Antipain, 1-2 μg /ml; PMSF, 100 μg / ml (or AEBSF, 96-958 μg / ml).

2.3.6.4 Dialysis buffer:

The following stock solutions were diluted and mixed at the final concentration: glycerol, 20%; HEPES, 20 mM; KCl, 0.1 mM; EDTA-Na_2, 0.2 mM; and DTT, 2 mM.

2.3.7. Solutions for gel mobility shift assay

2.3.7.1 TBE (0.25 X):

20 X TBE stock was diluted with water to 1 X.

2.3.7.2 Poly (dI / dC):

Double strand polynucleotide was dissolved with water at the concentration of 1 μg/μl. For high molecular weight molecule (> 800 bp), the sonication was needed. The solution was incubated at 45 °C for 1 hr, aliquoted, and stored at -20 °C.

2.3.7.3 Bovine serum albumin (BSA)(1μg / μl) solution:

BSA was dissolved with autoclaved water at the concentration, and then stored at -20 °C in aliquots.

2.3.7.4 Binding buffer (5 X):

The following stock solutions were mixed with 50 % glycerol solution at the final concentrations: HEPES, 20 mM; KCl, 50 mM; spermidine, 2 mM; EDTA-Na_2, 1 mM; DTT, 1 mM; and PMSF, 0.5 mM. The buffer was aliquoted, and stored at -20 °C.

2.3.7.5 Polyacrylamide gel (6 %):

50% polyacrylamide gel (A / B = 49 / 1) was diluted with 0.25 X TBE buffer to the final concentration.

2.3.7.6 Loading dye:

Bromophenol blue was dissolved in 10% glycerol at the concentration of 0.02%.

2.3.8. Solutions for Western and Southwestern blots

2.3.8.1 Polyacrylamide gel (30%) stock:

Polyacrylamide and N, N-methylene bis-acrylamide were dissolved in water at the concentrations, 29% and 1% respectively. The solution was stored at RT.

2.3.8.2 Gel buffer (4 X):

Tris base was dissolved in 0.4% of SDS solution at the final concentration of 1.5 M. The pH was adjusted to 8.8 with HCl, and then the buffer was stored at RT.

2.3.8.3 Stacking gel buffer (2 X):

Tris base was dissolved in 0.2% SDS solution at the final concentration of 0.25 M. The pH was adjusted to 6.8 with HCl, and the buffer was stored at RT.

2.3.8.4 Electrode buffer (10 X):

Tris base and glycine were dissolved in 1% SDS at the concentrations of 0.25 M and 14.4% respectively. The pH should be 8.3.

2.3.8.5 Sample buffer (2 X):

Tris base and bromophenol blue were dissolved in 4% of SDS at the concentrations of 0.125 M and 0.02% respectively. Then, the glycerol and B-mercaptoenthanol were added at the concentrations of 10% and 4%. Finally, the pH was adjusted to 6.8 with HCl.

2.3.8.6 Protein staining dye:

Coomassie brilliant blue was dissolved in 10% acetic acid 50% methanol at the concentration of 0.2%.

2.3.8.7 Destaining buffer:

Methanol was added into 10% acetic acid solution at the concentration of 30%.

2.3.8.8 Transfer buffer:

Tris base and glycine were dissolved in 20% methanol at the concentration of 0.24% and 1.15% respectively. The pH was adjusted to 8.3 with HCl.

2.3.8.9 Protein / DNA binding buffer:

The following stock solutions were added into 0.25% non-fat milk with the final concentrations: HEPES, 10 mM; $MgCl_2$, 10 mM; NaCl, 50 mM; EDTA-Na_2, 5 mM; and glycerol, 2.5%.

2.3.8.10 Blotting buffer:

Non-fat milk powder was dissolved in binding buffer at the concentration of 5%.

2.3.9. Solutions for cloning

2.3.9.1 Maltose (20%):

Maltose was dissolved in water at the concentration of 20%, sterilized by filtration and stored at 4 °C.

2.3.9.2 $MgSO_4$(1 M):

$MgSO_4$ was dissolved in water at the concentration of 1 M, autoclaved and stored at RT.

2.3.9.3 Lamda dilution buffer (LDB):

NaCl and $MgSO_4$ were dissolved in 35 mM of Tris-HCl (pH 7.5) at the final concentration of 10 mM each.

2.3.9.4 Denature buffer (for cDNA probe):

See: 2.3.29.4.

2.3.9.5 Neutralization buffer (for cDNA probe):

See: 2.3.29.5.

2.3.9.6 Hybridization buffer (for cDNA probe):

The following stock solutions were diluted and mixed at the final concentrations: deionized formamide, 50%; SSC, 5 X; denhardt, 5X; and SDS, 1%.

2.3.9.7 Hybridization buffer (for double strand oligonucleotide probe):

The DNA / protein binding buffer: (see 2.2.11.9).

2.3.9.8 Wash buffer (SSC / SDS):

SSC and SDS stock solutions were diluted and mixed at the final concentrations: 2 X and 0.05%.

2.3.10. Solutions for fusion protein

2.3.10.1 Sonication buffer:

The NaH_2PO_4 and NaCl stock solutions were diluted and mixed at the concentrations of 50 mM and 300 mM respectively. The pH was adjusted to 7.8 with NaOH.

2.4 EXPERIMENTAL PROCRDURES

2.4.1. Preparation of plasmid DNA

2.4.1.1 Mini-Prep

A single bacterial colony was picked and inoculated in 10 ml of LB medium which contained AMP (50 μg/ml). The tube was shacked at 2,500 rpm, in a 37 °C incubator overnight. Next day, 1.5 ml of the overnight culture was transferred into a microcentrifuge tube and centrifuged for 1 min. The bacterial pellet was resuspended in 100 μl of solution I. The suspension was left on ice for 5 min. Then 200 μl of solution II was added and mixed well. The tube stood on ice for 5 min, 150 μl of solution III was then added, mixed and stood on ice for an additional 5 min. The tube was centrifuged at 4 °C for 10 min and the supernatant was transferred into a fresh microcentrifuge tube. Equal volume of phenol / chloroform was added. The tube was vortexed for 30 sec, and centrifuged at RT for 5 min. The aqueous phase was transferred into a fresh microcentrifuge tube, and 2 volume of 95 % alcohol was added. The tube stood at -80 °C for 15 min. The tube was centrifuged at 4 °C for 5 min., the DNA pellet was washed once with 1 ml of 75 % alcohol, and vacuum-dried. Subsequently the pellet was resuspended in 100 μl of dH_2O. Then, 1 μl of 5M NaCl, 5 μl of RNase A, and 5 μl of RNase T1 were added. The tube was incubated at 37 °C water bath for 1 hr. Then 1 μl of 20 % SDS and 5 μl of proteinase K were added. The tube was further incubated in at 37 °C for an additional 1 hr, and 100 μl of phenol/ chloroform was added. The tube was vortexed and centrifuged for 5 min. The aqueous phase was transferred into a new microcentrifuge tube, and 0.5 volume of 7.5 M NH_4Ac and 2.5 volume of 95 % alcohol were added. The tube was kept at -80 °C for 30 min. Then the tube was centrifuged for 5

min to pellet the DNA. The DNA pellet was washed once with 0.5 ml of 75 % alcohol, and vacuum dried, then resuspended in 10 µl of dH_2O for analysis or stored at -20 °C until use.

2.4.1.2 Maxi-prep

200 µl of bacterial from mini-culture or storage was inoculated in 250-500 ml of LB medium containing 50 µg/ml of AMP. The bottle was shaked at 2,500 rpm, in a 37 °C incubator overnight. Next day, the bacterial culture was centrifuged at 2,500 g (Sorvall, GSA rotor, 4000 rpm) to pellet the bacteria. The pellet was resuspended in 15 ml of solution I, and left on ice for 10 min, and then 30 ml of solution II was added, mixed and the tube was kept on ice. After 10 min, 22.5 ml of solution III was added, mixed well and the tube was left on ice for an additional 10 min. The bacterial mixture was centrifuged at 8,000 g (Sorvall GSA rotor, 7000 rpm) for 30 min. The supernatant was transferred to a new tube by passing two layers of cheesecloth. 2 volume of 95 % alcohol was added, mixed and the tube was stood at RT for 10 min, and centrifuged at 10,000 g (Sorvall, GSA rotor, 8,000 rpm) for 30 min. The DNA pellet was resuspended in 4.5 ml of TE buffer, and aliquoted into 5 microcentrifuge tubes (1 ml / tube). 0.5 ml of 7.5M NH_4Ac was added into each microcentrifuge tube, and kept the tubes on ice. After 30 min. the tube was centrifuged at 4 °C for 5 min. The supernatant was pooled into a 15-ml Corex tube, and 15 ml of 95 % cold alcohol was added. The tube was kept at -80 °C for 2 hr or more, and then centrifuged at 17,000 g (Sorvall, SS-34 rotor, 12000 rpm) for 30 min to pellet the DNA. The DNA was vacuum-dried, and resuspended in 1 ml of 1X TE, and aliquoted into 5 microcentrifuge tubes (200 µl / tube). 2 µl of 5 M NaCl, 10 µl of RNase A, and 10 µl of RNaseT1 were added. The tubes were incubated in 37 °C water bath for 1 hr. Then 2 µl of 20 % SDS, 10 µl of proteinase K was added. The tubes were further incubated in 37 °C water bath for 1 hr. Then, the DNA solution was extracted once with an equal volume of phenol/chloroform. The aqueous phase was transferred into fresh microcentrifuge tubes, and 1/10 volume of 3 M NaAc and 2.5 volume of 95 % alcohol were added, mixed. The tubes were kept at -80 °C for 2 hr or more, and then centrifuged at 4 °C for 5 min. The DNA pellet was washed with

0.5 ml of 75 % alcohol, vacuum-dried, and resuspended in 50 (or more) µl of dH_2O. The DNA was ready to be used.

2.4.2. DNA sequencing

2.4.2.1 Preparation of sequencing gel

The glass plates were cleaned with Windex and 75% alcohol, and siliconized with Sigmacote. Two plates were separated by a pair of spacer and immobilized with plastic tapes. 80-100 ml of 8 % polyacrylamide urea solution was mixed with 250 µl of TEMED and 175 µl of 10 % ammonia persulfate in a squeeze bottle, and then carefully poured into the space of two glass plates. The plates with the polyacrylamide urea solution was left polymerize at RT for at least 2 hr or more.

2.4.2.2 Sequencing reaction with T7-DNA Sequencing Kit

2-3 µg of plasmid DNA (from maxi-prep or mini-prep) was pipetted into a microcentrifuge tube and made up the volume to 8 µl with dH_2O. Then 2 µl of 2 N NaOH was added, mixed, and incubated at RT for 10 min. Then 3 µl of 3 M NaAc, 7 µl of dH_2O, and 60 µl of 95% alcohol were added in order. After mixing, the tube was kept at -80 °C for 30 min or more, and then centrifuged at 4 °C for 10 min. The supernatant was aspirated carefully. The DNA pellet (might not be seen) was washed once with 100 µl 75% alcohol, and vacuum-dried. The DNA pellet was resuspended in 10 µl of dH_2O, mixed with 2 µl of annealing buffer, 2 µl of primer (10 µg/ml) and incubated the in 37 °C water bath for 15 min, and then cooled down slowly at RT. After 15-20 min, 3 µl of labeling mixA, 1 µl of ^{35}S-dATP, and 2 µl of diluted DNA polymerase (1.5-2.0 units) were added, mixed and incubated at RT for 5 min. This primered mixture was aliquoted into 4 microcentrifuge tubes (4.5 µl / tube) containing 2.5 µl of short (or long) G.A.C.T. respectively. The tubes were incubated in 37 °C water bath for 5 min and then 5 µl of stop buffer was added to each tube. The reaction mixture was ready to be heated and applied to sequencing gel (or stored at -20 °C).

2.4.2.3 Running the sequencing gel

The reaction tubes were heated at 95 °C for 5 min, and chilled on ice immediately. After cooling down, the reaction mixtures were applied onto the gel. The gel was run at constant voltage 1,700-2,000V, with 1 X TBE as running buffer. Finally, the gel was vacuum-dried on the gel dryer and autoradiographed with an X-ray film.

2.4.3. DNA construction

2.4.3.1 Insertion of a DNA fragment into a plasmid

The plasmid was digested or cleaved with one or two different restriction enzymes. If linearized with one enzyme, the linearized plasmid was treated with calf intestinal alkaline phosphatase (CIP) to prevent the plasmid religated back. The enzymatic treated plasmid DNA was purified by running a mini-agarose gel and recovered by Gene Clean II Kit. This purified linear plasmid DNA was incubated with DNA fragment (or synthetic oligonucleotide) which had the restriction sites at both ends corresponding to the plasmid DNA, in the presence of 1 unit of T4 ligase, at 15 -16 °C water bath for 16 hr or more.

2.4.3.2 Preparation of competent cells

A single bacterial colony was picked and inoculated in 10 ml of LB medium. The tube was shacked in 37 °C incubator for overnight. Next day, the bacterial culture was diluted at 1:100 with SOB (or SOC), and incubated at 37 °C with shaking until $O.D._{650}$ = 0.3 to 0.4. The bacterial culture was poured into two pre-chilled 50 ml plastic tubes, and centrifuged at 4 °C, 780 X g (ICE Centra-8R centrifuge, 2,500 rpm) for 12 minutes to pellet the bacteria. The bacteria was resuspended in 1/3 volume of FSB and incubated on ice for 10-15 min. Then the tubes were centrifuged at 4 °C, 780 X g for 10 minutes. The pellet was resuspended in 1/12.5 volume of FSB, and 140 µl of DMSO was added into each tube. The tubes were stood on ice. After 5 min, an additional DMSO 140 µl was added. The tubes were left on ice for another 10 min. The bacterial was aliquoted in microcentrifuge tubes with 200 µl / tube. The tubes were frozen in dry ice/ alcohol, then stored at -80 °C for use.

2.4.3.3 Transformation

The competent cells were thawed by hand and left it on ice for 10 min. The plasmid DNA was added into the competent cells and mixed well. The tube was left on ice for 30 min. Then the tube was transferred to 42 °C water bath to heat-shock for 90 sec. Then, 0.8 ml of SOC was added immediately. The tube was incubated at 37 °C with shaking for 1 hr. Finally, the transformed cells were spread on a LB-agar plate which contained antibiotics (e.g. 50 μg / ml of AMP). The plate was incubated in 37 °C incubator for overnight.

2.4.3.4 Screening of bacterial colonies by DNA radioactive probe

The bacterial colonies were transferred onto a nitrocellulose membrane by covering the bacterial plate with a membrane for 30 seconds and then removed immediately. Then the membrane was carefully loaded on the surface of 10 ml denaturation buffer (bacterial upside), for 15 min. Then the membrane was transferred onto the surface of 10 ml of neutralization buffer for 15 min. The membrane was air dried, and baked at 80 °C vacuum oven for 2 hr. Then, the membrane was placed in a plastic bag, and pre-hybridized with 10 ml of hybridization buffer which contained herring sperm DNA, 100 μg /ml, in 42 °C incubator with rotation for 2 hr. Then the radioactive DNA probe was added at 10^6 cpm /ml, and further incubated at 42 °C with rotation for overnight. Next day, the membrane was washed at RT with 2 X SSC / 0.5% SDS. The radioactivity on the membrane was checked with Geiger counter until the cpm was about 1000 cpm. Finally, the membrane was air dried and exposed with a X-ray film.

2.4.4. Preparation of radioactive probes

2.4.4.1 Preparation of ^{32}P-cDNA probe with T7 Quickprimer™ kit

DNA (about 50 ng in 10 μl of dH_2O) in microcentrifuge tube was heated at 95 °C water bath for 10 min, and immediately chilled on ice. Then 24 μl of dH_2O, 10 μl of reagent mixture, 5 μl of ^{32}P-α-dCTP, and 1 μl of DNA polymerase were added. The tube was spined briefly, and then incubated in 37 °C water bath for 1 hr. Meantime, a Sephadex G-50

column was packed with a Pasteur pipette and equilibrated with dH_2O. The reaction mixture was applied to the column, and eluted with dH_2O. The first 500 µl of elute was collected in the one tube, then every 100 µl was collected in the following tubes. 1 µl from each tube was taken to count. The three peak tubes were pooled and kept at -20 °C for use.

2.4.4.2 Preparation of ^{32}P-oligonucleotide probe with polynucleotide T4 kinase

In a microcentrifuge tube, 13 µl of dH_2O, 2 µl of kinase buffer, 1 µl of oligonucleotide (20 pmol / µl), 1 µl of T4 kinase (1 unit / µl), and 3 µl of ^{32}P-γ-ATP were added. The reaction mixture was centrifuged briefly and incubated in 37 °C water bath for 1 hr. The labeled oligonucleotide was separated from the free ^{32}P by passed a Sephadex G-50 column as described above (2.4.4.1.).

2.4.5. Transient gene transfection assays

2.4.5.1 Cell culture

The mouse hepatoma (Hepa 1-6) cells or opossum kidney (OK) cells were grow in 100 X 20 mm plastic Petri dishes using Dulbecco's Modified Eagle's Medium (DMEM), pH 7.2, supplemented with 10 % fetal bovine serum (FBS), 50 units/ml of penicillin and 50 µg/ml of streptomycin. The cells were incubated in a humidified atmosphere of 95 % O_2, and 5% CO_2 at 37 °C. For subculture, cell were trypsinized (0.5% trypsin and EDTA) and plated at 3.5 X 10^4 cells/cm^2.

2.4.5.2 DNA transfection

Calcium phosphate was used for the DNA transfection in the present study. The detail of the transfection in Hepa 1-6 or OK cells has been described previously (Ming *et al.*, 1993). Briefly, the cells cultured in DMEM containing 10 % FBS were seeded in 6-well-plate at 2 x 10^5 / well 24 hr before the transfection. A total of 10 to 20 µg of supercoiled DNA was gentally mixed with $CaCl_2$ in HBSP to form the DNA-Ca++ particles. The DNA-Ca++ particles was then gentally loaded on the surface of the cells. 24 hr after the

transfection, the media were replaced with fresh media without FBS. The drugs were added 24 hr after apply of serum-free medium. The cells were harvested 48 hr later and assayed for CAT activity.

2.4.5.3 Chloramphenicol acetyl transferase (CAT) assay

The cell extracts (20-50 μg protein) x μl, 4 mM acetyl coenzyme A (0.53 mM) 20 μl, [^{14}C] chloramphenicol solution (70 μl of 1 M Tris-HCl pH7.6 + 0.25 μl of [^{14}C] chloramphenicol for each sample) 71 μl (0.1 μci), and 0.25 M Tris-HCl buffer (pH 7.8) was added to bring the final volume to 146 μl.

OK cell extracts were incubated for 30-60 min at 37 °C. Whereas Hepa 1-6 cell extracts were incubated initially for I hr, then another 10 μl of 4 mM acetyl coenzyme A was added and the system was incubated for another 15 hr at 37 °C.

At the end of incubation, the reaction mixtures were extracted once with 1 ml of 100% ethyl acetate. The organic phase was taken and dried by speed-vacuum. The final samples were resuspended in 10 μl of ethyl acetate and loaded onto the thin layer chromatography (TLC) plates. The TLC plates were developed in a saturated mixture of 190 ml of chloroform and 10 ml methanol (19:1) for 1 hr in the TLC tank. Then the plates were dried in the air, and subjected to autoradiography for 1-2 days at room temperature. The bands of acetylated and unacetylated forms of [^{14}C] chloramphenicol were separated from the TLC plates, and mixed with 10 ml of scintillation liquid (950 ml toluene + 50 ml liquifluor), respectively.

CAT activity was quantified by counting the radioactivity of the bands containing either the acetylated or unacetylated form of [^{14}C] chloramphenicol in the liquid scintillation counter. The results were expressed as the percentage of [^{14}C] chloramphenicol converted to the acetylated forms.

2.4.6. Extraction of cellular nuclear proteins

2.4.6.1 Preparation of nuclear protein from cultured cells

This method is based on the method of J.D. Dignam et al. (Nucleic acid Research 1983) with some modifications.

The cultured cells (20 plates, 150 x 20 mm) were collected by centrifugation at 4 °C, 1000 X g (Sorvall HG4L rotor, 2000 rpm). After washing with 5 volume of cold PBS (1 M, pH 7.2), the pellet was resuspended in 5 volume of buffer A, incubated on ice for 10 min, and then centrifuged at 4 °C, 1000 g for 10 min. The pellet was resuspended in 2 volume of buffer A, and homogenized to break the cells with Kontes glass Dounce homogenizer, B type pestle. This step was monitored by examination under the microscope to check for cell lysis. The homogenate was centrifuged at 4 °C, 1000 X g for 10 min. The nuclear pellet was resuspended in 2 volume of buffer B, and centrifuged at 25,000 X g (Sorvall ss-34 rotor, 14,500 rpm) for 20 min. The pellet was resuspended with buffer C, at 10^9 cells / 3 ml, and homogenized 10 strokes with the Dounce homogenizer with B type pestle. Then, the homogenate was mixed with a magnetic stir at 4 °C for 30 min, and centrifuged at 25,000 X g for 30 min. The supernatant was dialyzed against 50 volume of buffer D. Finally, the nuclear protein dialysate was aliquoted and stored at -80 °C or in liquid nitrogen.

2.4.6.2 Preparation of nuclear protein from fresh tissue

This method based on the method of M.Hattori et al. (DNA and cell biology, 1990) with some modifications.

For each nuclear extract preparation, 15 male Babl/c mouse were anesthetized and killed by decapitation. The mouse tissues were rinsed with 0.9 % of NaCl, and cut with scissors into approximately 3-mm cubes in 10 ml homogenization buffer. Additional homogenization buffer was added into the minced tissue until the volume was 25 ml. The tissue was homogenized in a 55-ml Potter homogenizer with a motor-driven Teflon pestle. The homogenate was laid carefully on the surface of 3 ml of cushion buffer in 15-ml ultracentrifuge tubes. The tubes were centrifuged at 4 °C, 80,000 X g for 1 hr (Beckman

SW 41 rotor, 25,000 rpm). The supernatant was removed and the tube was inverted for 10 min on ice to drain the remaining buffer from the nuclear pellet. The pellet was resuspended 5 ml of lysis buffer by several strokes of a pestle of a 40-ml Dounce homogenizer. Then, 20 µl of homogenate was taken and mixed with 980 µl of 0.5 % SDS to measured the O.D. at 260 nm. According to the calculated DNA concentration, additional lysis buffer was added to adjust the DNA concentration until 0.5 mg / ml. Subsequently, the 3 M KCl was added to the final concentration of 0.55 M (i.e. 0.176 ml of KCl per ml of homogenate, taking into consideration that lysis buffer already contains 0.1 M KCl). The homogenizer was kept on ice for 30 min with occasional mixing, and then the homogenate was transferred into Type Ti45 (Beckman) tubes, and centrifuged at 95,000 X g (34,500 rpm) for 30 min. The supernatant was transferred into a fresh Ti45 tube, and finely powdered $(NH_4)_2SO_4$ was added, (i.e. 0.3 mg/ml). The tube was shaked gently to dissolve the salt and then kept on ice for 1 hr. The tube was centrifuged at 95,000 X g for 20 min. The pellet was resuspended with dialysis buffer, and the protein concentration was adjusted to 10 mg / ml, and then transferred to a sterile, washed dialysis tubing. The nuclear protein extract was dialyzed against 250 ml of dialysis buffer at 4 °C with stirring. After 4 hr or more of dialysis, the nuclear extract was clarified by centrifugation at 4 °C for 10 min to remove the visible particles. The supernatant was aliquoted and stored at -80 °C for use.

2.4.7. Gel mobility shift assay

In a microcentrifuge tube, the followings were added: 4 µl of 5 X binding buffer, 2 µl of poly (dI/dC) (1 µg / µl), 5 -10 of µg nuclear extract (or fusion protein) and dH_2O to a final volume of 18 µl. After mixing by spinning the tube, the mixture was incubated at RT for 30 min. Then the ^{32}P-oligonucleotide (20-40,000 cpm, 0.1 pmol) was added, and the tube was kept at RT for additional 30 min. After the incubation, the reaction mixture was mixed with1 µl of loading dye and applied to 6-8 % polyacrylamide gel. The gel was run at constant voltage, 200V, with 0.25 X TBE as running buffer. After 2 - 2.5 hr, the gel was

placed on a piece of 0.3-MM Whatman filter paper, and vacuum-dried. Finally, the dried gel was exposed on X-ray film. 10 μg of BSA was used as negative control.

2.4.8. Southwestern blot assay

Nuclear proteins (50 - 200 μg) were resolved according to the size by applying to a 4-20% SDS gradient polyacrylamide gel, running at constant voltage, 100-120 V. Then the proteins were electro-transfered onto a nitrocellulose membrane with constant current, 125 mA for overnight. The membrane was soaked in blotting buffer with shaking at 4 °C for 2 hr more, and then rinsed with binding buffer. The membrane was incubated with ^{32}P-oligonucleotide at 10^5 - 10^6 cpm / ml in binding buffer in the presence of salmon sperm DNA, 300 μg / ml at 4 °C for overnight. The membrane was washed 3-4 times with binding buffer at 4 °C for 20 min. Finally the membrane was air-dried and exposed on an X-ray film.

2.4.9. Southern blot

DNA was separated on a 0.8% agarose gel and then transferred on to a nitrocellulose (or nylon) membrane. The DNA was denatured by carefully loading the membrane on the surface of denaturation buffer for 15 min, then re-natured by transferring the membrane on to the surface of the neutralization buffer for 15 min. After air-dried, the membrane was baked at 80 °C for 2 hr, and hybridized with the radioactive probe.

2.4.10. Western blot

Proteins were separated on 10% SDS polyacrylamide gel, and then electro-transfered onto a nitrocellulose membrane, at the constant current 125 mA for overnight. The membrane was blocked with 5% non-fat milk in 0.1 M PBS for 2 hr more, and then incubated with the first antibody (1:1000 dilution) in 0.1 M PBS at 4 °C for overnight. Next day, the membrane was rinsed with 5% milk in 0.1 M PBS and incubated with the second antibody (1: 2000) that is conjugated with HPR in 0.1 M PBS at 4 °C for 4 hr. Then the membrane was rinsed with 0.1 M PBS, and stained in 50 ml of development reagent solution for 10 min. The specific band(s) will be seen.

2.4.11. Cloning

The procedures are based on the instruction of CloneTech laboratories, Inc.

2.4.11.1 Tittering of phage library

A single host bacterial (Y1090r -) colony was inoculated in 20 ml of LB medium containing ampicillin, 50 μg / ml, 0.2% maltose and 10 mM $MgSO_4$. This bacteria tube was incubated in 37 °C incubator with shaking until the $O.D._{600}$ of the culture reached to 2.0. The library was diluted from $1:10^2$ to $1:10^{10}$ with Lamda dilution buffer, each dilution with a final volume of 100 μl, and was incubated with 200 UL of the bacterial culture in 37 °C water bath for 15 min. Then, this bacteria-phage library mixture was mixed well with 3 ml of 0.75% agarose (45-55 °C), and poured onto the pre-warmed LB-agar plate (37-42 °C). The plate was left at RT for 20 min to allow the inoculum to soak into the agar, and then transferred to a 37 °C incubator for overnight. Next day, the phage plaques were counted and calculated the titer (pfu / ml):

pfu / ml = # of plaques/μl used X dilution factor X 10^3 μl / ml

2.4.11.2 Preparation of the bacteria / phage plates

This method is based on that of Singh et al. (1990) with some modification.

A single bacteria (Y1090r-) was inoculated into 10 ml of LB broth, containing ampicillin, 50 μg / ml, 0.2% maltose and 10 mM $MgSO_4$. This bacteria tube was incubated in 37 °C incubator with shaking until the $O.D._{600}$ of the culture reached to 2.0 (6 - 8 hr). Then 20 microcentrifuge tubes were prepared. For each tube, 200 μl of the Y1090r $^-$ culture was mixed with 100 μl of 1 X λ dilution buffer containing 5 X 10^4 pfu of λgt11 phage. The mixture was incubated in 37 °C water bath for 15 min for the adsorption of phage to the cells. Then each bacteria / phage tube was mixed with 7 ml of 0.75 % agarose (45-55 °C), and poured onto 20 LB - agar plates (37-42 °C) (150 mm) respectively. After air-dried, the plates were transferred into 37 °C incubator for overnight.

2.4.11.3 Preparation of nitrocellulose filter

40 pieces of nitrocellulose filters were saturated in 20 ml of 10 mM of IPTG for 10 min, and then air-dried on Whatman paper. The nitrocellulose filters were marked for 20 plates with duplicate (filter A and B). The bacteria / phage plates were cooled on ice for 1 hr. Then the nitrocellulose filter A was carefully placed on the surface of 20 plates one by one, avoiding trapping air bubbles. Mark the filter in 3 asymmetric locations by stabbing through the filter and into the agarose with an 18-gauge needle. The plates with filters were stood on ice for 10 min, then the filter were removed. The filter B was overlaid onto the plates, and the plates / filters were incubated in 37 °C incubator for 2 hr. Then the plates were removed and stood on ice for 1 hr, and then the filter B was removed. The filters removed from the plates were air dried for 20 min, and soaked in 10% non-fat milk in binding buffer for at least 2 hr.

2.4.11.4 Probe the filters with double strand oligonucleotide

The filters, blotted with 10 % non-fat milk, were rinsed with binding buffer, then incubated with ^{32}P-double strand oligonucleotide (ANG-CRE, ANG N-806/-779), 10^6cpm / ml, in binding buffer in the presence of herring sperm DNA, 100 μg / ml, at 4 °C for overnight with gentle shaking. Next day, the filters were washed with binding buffer at 4 °C, for 4 X 20 min. And then the filter was air-dried, and exposed on X-ray films for overnight at -20 °C. The positive colonies were picked from the bacteria / phage plates with small pipette tips according to the X-ray films from both filter A and B. The agar blocks containing positive clones were kept separately in different microcentrifuge tubes containing 0.5 ml of 1 X Lamda dilution buffer and 50 μl of chloroform. The tubes were kept at 4 °C for the next cycle of screening in which much lower titer of phage was used per plate (300-500 pfu / plate) to obtain the single, isolated positive clones.

2.4.11.5 Screening of the library with the cDNA probe

The bacteria / phage plates and nitrocellulose filters (A and B) were prepared as

described above. The differences are: the filter removed from the plates containing the phage DNA was denatured by loading the filter on the surface of denaturation buffer for 15 min, then re-natured by transferred the filters onto the surface of neutralization buffer for 15 min. The filters were air-dried and then baked at 80 °C oven with vacuum for 2 hr. Then, the filters were prehybridized with hybridization buffer at 42 °C for 2 hr, and subsequently incubated with radioactive cDNA (95 °C boiled for 10 min, chilled on ice immediately) or single oligonucleotide probe in the 42 °C incubator for overnight. Next day, the filters were washed and exposed on to an X-ray film as described above.

2.4.12. Phage DNA extraction

2.4.12.1 Bacteria / phage lysate preparation

250 ml of host cell Y1090r - was grown in a 500-ml flask in the presence of 10 mM of $MgSO_4$ and 0.2 % maltose until the $O.D._{600}$ reached to 0.6. Then, 1 X 10^{10} pfu of phage was added into the bacterial culture. The flask was continued to grow in 37 °C incubator with shaking for 6 - 8 hr until lysis was apparent (when lysis occurred, the culture looked clear with some bacterial debris. If lysis do not occur, the culture will appear turbid, in this case small amount of lysozyme powder was added into the culture, and stood the flask at RT for 20 min.).

2.4.12.2 Preparation of Lamda phage

The bacteria / phage lysate was centrifuged at 2500 g (Sorvall GAS, 8000 rpm) for 20 min. The supernatant was collected with a 500-ml bottle. 250 µl of DNase I (10 mg/ml), and 250 µl of RNase A (20 mg/ml) were added, and the bottle was incubated at 37 °C water bath for 1 hr. Then, 1/10 volume of chloroform was added into the bottle. The bottle was shacked with hands. Then lysate with chloroform was transferred into 2 centrifuge tubes and centrifuged at 2500 g (GSA rotor, 10,000 rpm) for 30 min. The aqueous phase was carefully collected into a 500 ml bottle and added 1 /3 volume of pre-cold 30% PEG / 3 M NaCl, mixed and stood on ice for at least for 1 hr or overnight. The lysate was transferred

into 2 centrifuge tubes and centrifuged at 2500 g (GSA rotor, 10,000 rpm) for 30 min. The pellet was resuspended with 10 ml of 1 X Lamda dilution buffer and extracted once with equal volume of chloroform. The aqueous phase was transferred to a Beckman SW41 tube, and added CsCl powder, 0.5 g / ml. After dissolving of CsCl, the tube was centrifuged at 4 °C (Beckman SW41 rotor, 27,000 rpm) for 2 hr. The clear, sticky phage pellet was resuspended in 1 ml of 1 X Lamda dilution buffer.

2.4.12.3 Preparation of phage DNA

1 ml of Lamda phage solution was separated into 2 microcentrifuge tubes, and to each tube, 20 µl of 0.5 M EDTA-Na_2, 6.3 µl of 20 % SDS, and 2.5 µl of proteinase K (10 mg/ml) were added. The tubes were incubated at 65 °C for 1 hr. Then extracted with equal volume of phenol/chloroform once and equal volume of chloroform once. Then, 1/10 volume of 3 M NaAc, and 2.5 volume of 95% cold alcohol were added into the tubes, and the tubes were stood at -80 °C for at least for 1 hr. The tubes centrifuged in microcentrifuge at 4 °C for 10 min. The phage DNA was washed once with 75% alcohol, vacuum-dried, and resuspended in 30 µl of TE buffer. The phage DNA was checked with mini-gel, if there was RNA contamination, the phage DNA was redigested with RNases.

2.4.12.4 Excision of the insert from phage DNA and subclone into pGEM-3 vector

10 -15 µg of purified phage DNA was incubated with EcoRI at 37 °C water bath for 2-3 hr. The digest was analyzed on a 0.8 % mini-agarose gel with DNA maker to estimate the size of the insert. Then cut out the gel block(s) containing the DNA insert and the insert was recovered with Gene Clean II kit. The insert DNA was subcloned into PGEM-3 vector and sequenced from both end with Sp6, T7 and synthetic primers with the procedures as described previously.

2.4.13. Polymerase chain reaction (PCR) with PCR kit

In a 0.5-ml PCR tube, the followings were added in order: 68 µl of H_2O; 10 µl of 10

X PCR buffer, 16 μl of dNTP mixture (1.25 mM each), 1 μl of DNA template (about 2-3 nM), 2 μl of primer 1 (50 pmol /μl); 2 μl of primer 2 (50 pmol / μl); and 1 μl of Taq DNA polymerase. The total volume was 100 μl. Then 2 drops of mineral oil were carefully loaded on the surface of the mixture, and the tube was covered. The PCR was started with the delay program: 95 °C for 5 min to denature the non-specific enzyme(s) (in case there was contamination). Then the PCR cycles was stated: 94 °C for 1 min and 30 sec, 50 °C for 1 min 30 sec, and 72 °C for 2 min with 2 sec extension every cycle. The number of total cycle was 35. Finally, the tube was kept at 4 °C.

After the PCR, the mineral oil was removed carefully from the surface of the reaction mixture. The latter was analyzed with a 0.8 % mini-agarose gel with a DNA marker to check the size of the PCR product. The gel block(s) containing the PCR product was cut out, and the DNA was recovered by Gene Clean II kit. The PCR product was ready to be used.

2.4.14. Fusion protein expression

2.4.14.1 Construction of fusion expression vector

The open reading frame of the cDNA coding for ANG-CREB was inserted into bacterial expression vector pMALc downstream from the malE gene, at EcoRI site. The reversed orientation of the cDNA was also constructed as a control. These vectors containing the cDNA were transformed into the host cells, TB -1 with the procedures described above.

2.4.14.2 Small-scale expression culture

Single colonies of transformants was picked and inoculated in 10 ml of LB broth containing ampicillin. The tubes were grown in a 37 °C incubator for overnight with shaking. Next day, 1 ml of the bacterial culture was transferred into 9 ml of LB broth, and continued to grow until the $O.D._{600}$ reached to 0.5 - 0.6. Then, 200 μl of 1 M IPTG was added until the final concentration was 2 mM, and continued to grow for 3 hr. The tube was

centrifuged at 12,500 X g, and bacteria pellet was dissolved with 2 ml of sonication buffer, and frozen at -20 °C for overnight. Next day, the frozen bacteria was thawed in cold water (alternatively, lysozyme was added to 1 mg/ml and incubate on ice for 30 min), and then sonicated on ice, 4 X 1 min, 50 watt. The cell breakage was monitored by the release of nucleic acid measured at 260nm wavelength, until it reached a maximum. The cell lysate was centrifuged at >10,000 X g for 10 min, and the supernatant was collected and stored at -20 °C in aliquots.

2.4.15. Preparation of antiserum against ANG-CREB

The peptide corresponding to the residues 227-236 from the N-terminus of ANG-CREB, NH_2-SGDLETRYWG was synthesized, and the purified peptide was then conjugated to KLH (keyhole limpet hemocyanin). About 100 μg of this conjugated peptide was emulsified in Freund's complete adjuvant and injected into New Zealand rabbits. Four weeks later, the rabbits received another injection of 100 μg of the peptide emulsified with Freund's incomplete adjuvant. Blood was withdrawn from the rabbits after 10 days. Blood was allowed to clot at 4 °C overnight and the antiserum was obtained by centrifugation at 3000 g at 4 °C.

3. Chapter 3. Results

3.1 REGULATION OF ANG GENE EXPRESSION BY cAMP

It has been reported previously that the addition of isoproterenol or cAMP or forskolin stimulates the expression of the ANG gene in OK cells (Ming *et al.*, 1995) and Hepa 1-6 cells (Ming *et al.*, 1993). Studies have indicated that there is a putative cAMP

responsive element (CRE) in the rat ANG gene (ANG-CRE), TGACGTAC, located at -795/-788 (Chan *et al.,* 1990). In comparison with the previous reported parlindromic CRE octamer TGACGTCA (Montminy *et al.,* 1986), the last two nucleotides are in reverse order in ANG-CRE. To investigate whether this DNA sequence would mediate the effect of cAMP on the expression of ANG-CAT fusion genes, series fusion genes were constructed with various length of 5-flanking region with or without putative ANG-CRE fused to TK promoter. These constructs were transfected into Hepa 1-6 cells by Ms. Ming, another Ph.D. student in our laboratory. The results showed that the addition of 8-Br-cAMP (10^{-3} mol/l) stimulated the expression of pTKCAT (ANG-814/-689), pTKCAT(ANG N-814/-761) by 1.4- and 2.2 fold as compared to the control (in the absence of 8-Br-cAMP). Similarly, the addition of forskolin (10^{-5} mol/l) stimulated the expression of these two fusion genes by 1.5- and 3.0 fold (Ming, PhD thesis). To further investigate whether the TGACGTCA (ANG N-806/-779) octamer is responsible for the effect of cAMP on the expression of ANG-CAT fusion gene, a 28-mer oligonucleotide that contains this sequence (Fig. 2-1) was chemically synthesized. The oligonucleotide was cloned into the expression vector (pTKCAT) at the 5'-end of the thymidine kinase (TK) promoter fused to the chloramphenicol acetyl transferase (CAT) coding region in the expression vector. This construct then were transfected into OK cells and assayed for CAT expression either in the absence or presence of 8-Br-cAMP (10^{-3} M) or forskolin (10^{-5} M). The results as shown in Fig.3-1, demonstrated that the addition of both 8-Br-cAMP and forskolin stimulated the the expression of pTKCAT(ANG N-806/-779) by 2.1- and 1.9 fold respectively as compared to the control. Furthermore, a dose-dependent relationship between the various concentrations of 8-Br-cAMP (10^{-9}-10^{-3} mol/l) and the stimulation of expression of the pTKCAT (ANG N-806/-779) was observed in Hepa 1-6 cells. And the maximal stimulation (2.2-fold, $p \leq 0.01$) was found when using 10^{-3} mol/L of 8-Br-cAMP (Ming, PhD thesis). These results demonstrated that the putative ANG-CRE octamer conferred an increased expression of fusion genes in the presence of 8-Br-cAMP or forskolin.

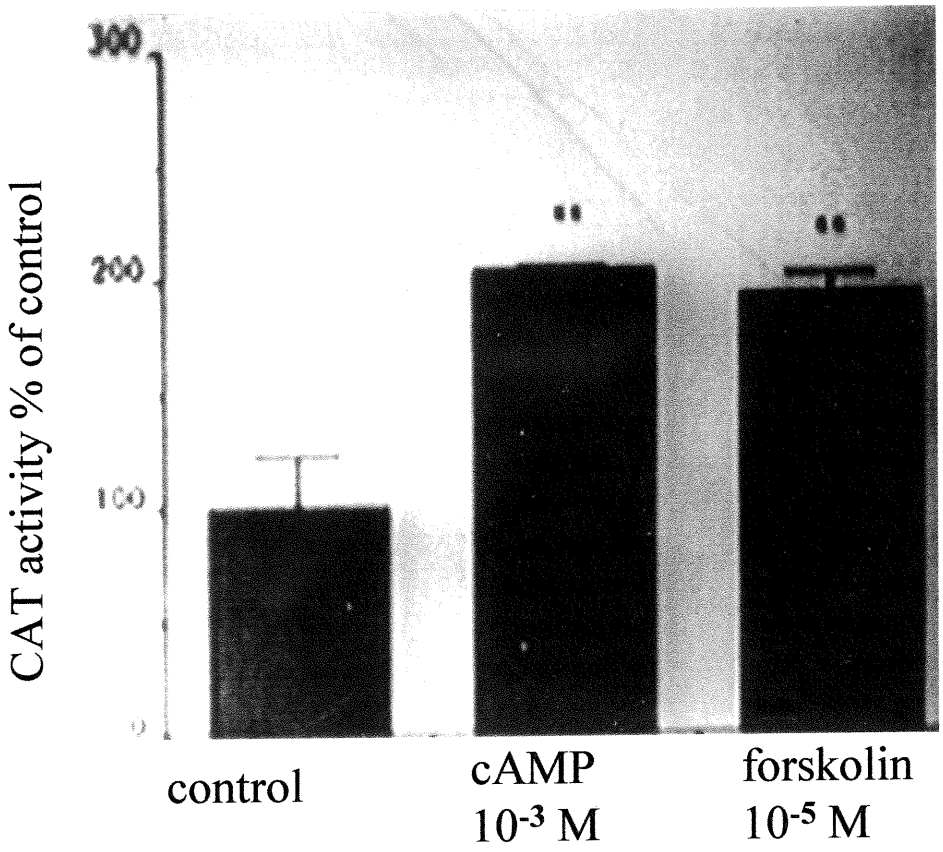


Fig. 3-1. Effect of 8-Br-cAMP (10^{-3} mol/l) and forskolin (10^{-5} mol/l) on the expression of fusion gene containing the putative cAMP responsive element fused with thymidine kinase (TK) promoter, pTKCAT(ANG N-806/-779), in OK cells. Results are expressed for the mean SD as a percentage of control (without the addition of 8-Br-cAMP or forskolin, 100%) (**P<0.001)

3.2 ANG-CRE BINDING PROTEIN

Studies have revealed that CRE functions through the binding of the specific regulatory factor known as cAMP responsive element binding protein (CREB) (Montminy *et al.,* 1988, 1989; Meyer & Habener, 1993). To address the question whether the nuclear protein(s) bind to the putative ANG-CRE of ANG gene, DNase I footprinting assay was performed by Miss Qin Jiang, a former MSc student in our laboratory. In the experiment, ^{32}P labeled DNA fragment, ANG N-821/-689, was used as the probe. The nuclear extracts were prepared from mouse liver. The result showed that a region at nucleotide -799/-788, in which the putative ANG-CRE site was located, was protected by the nuclear protein(s), (Wu *et al.,* 1998). This result indicates that nuclear protein(s) bind to ANG-CRE sequence. To further study the ANG-CRE binding protein (ANG-CREB), we used the ANG-CRE (N-806/-779) flanked on either side by seven bases of their natural environment (Fig. 2-1). The flanking sequences were restricted to this length to avoid complication of protein binding patterns due to the adjacent sequences. The element was labeled with ^{32}P and used for gel mobility shift assay. Briefly, the mouse liver nuclear extracts was incubated with the ^{32}P-labeled ANG-CRE in the presence of 2 μg of poly (dI/dC), and then separated by a 4% polyacrylamide gel. The result is shown in Fig. 3-2. Lanes 1 and 2 show the ^{32}P-ANG-CRE probe alone and probe with BSA respectively; both serve as non-specific binding controls. Lane 3 shows the probe with 10 μg of nuclear extracts. As expected, a strong DNA/protein complex band is observed. Lane 4 shows that the DNA/protein complex band is abolished by the presence of 200 molar excess (20 pmol) of unlabeled ANG-CRE. Lanes 5 and 6 show that the DNA / protein binding is not affected by the presence of 200 molar excess (20 pmol) of CRE (lane 5) and TRE (lane 6) from c-fos gene. The DNA/protein binding can be displaced by unlabeled ANG-CRE, but not by unlabeled CRE and TRE from c-fos gene indicating this protein has sequence specificity. In addition, these data suggest that the mouse liver nuclear protein(s) specifically interact with ANG-CRE and the ANG-CRE flanking sequences are recognized.

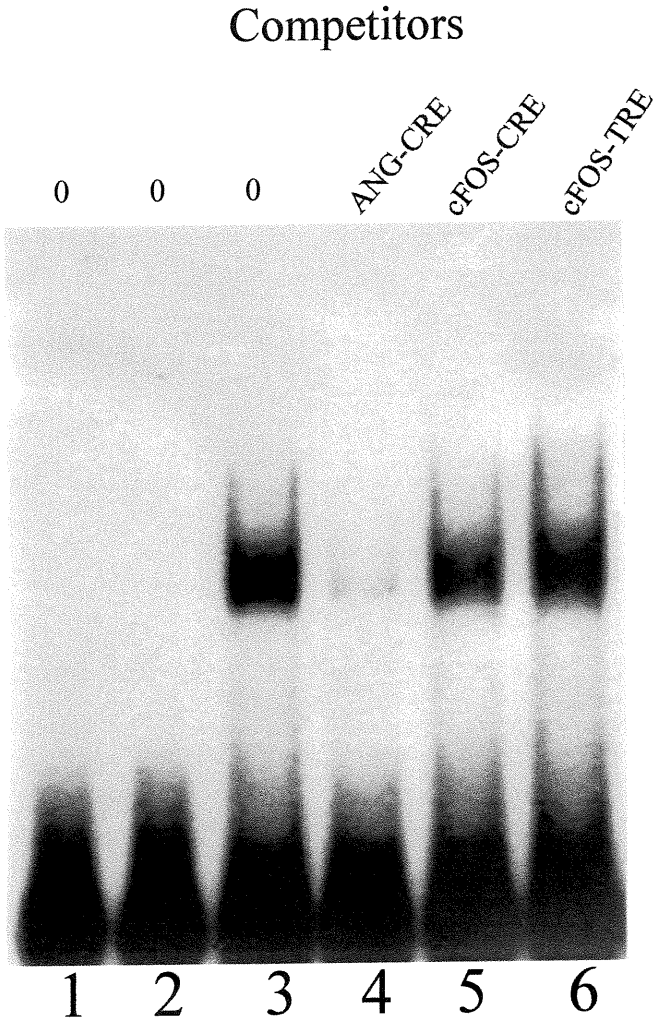


Fig.3-2. Gel mobility shift competition assay.ANG-CRE was labeled with ^{32}P as the probe, and 0.1 pmol was used for each lane. Nuclear extracts (NE) were prepared from mouse liver. Lane 1, probe alone without any protein added; Lane 2, 10 μg of BSA as nonspecific control; Lanes 3-6, 10 μg of nuclear extract were used for each lane. Lanes 4-6 , 20 pmol of competitors was used as indicated on the top of the figure. The concentration of polyacrylamide gel is 4%.

3.3 IMMUNOLOGICAL DIFFERENCE BETWEEN ANG-CREB AND the 43 kDa-CREB

As discussed in chapter 1, the CRE sequence may interact with a group of proteins that belong to ATF/CREB superfamily. The family members were originally defined by their specific binding to a palindromic sequence (TGACGTCA), known as the CRE, which has been found in many cAMP-inducible cellular genes and in several viral promoters. All of those proteins share a common basic DNA binding domain followed by a leucine zipper dimerization domain. Among the increasing number of ATF/CREB family members, the 43 kDa-CREB is the best-characterized one. The 43 kDa-CREB has been shown to be involved in the regulation of expression of many genes. Therefore, we have reason to believe that it might also bind to ANG-CRE, and regulate the ANG gene expression. Assuming the DNA/protein complex showed on the Fig. 3-2 is due to the 43 kDa-CREB binding to ANG-CRE, the removing of the 43 kDa-CREB from mouse liver nuclear extracts by immunoprecipitation of the nuclear extracts with the antiserum against the 43 kDa-CREB could result in the disappearance of the DNA/protein complex band. To test this possibility, immunoprecipitation / Southwestern blot was performed. Briefly, the mouse liver nuclear extracts were incubated with the pre-immune serum, the antisera against the 43 kDa-CREB, and the activating transcription factor 2 (ATF-2) for 16 hr respectively. Then the Protein A was added into each tube. After another incubation of 4 hr the nuclear extracts were subjected to the centrifugation. The supernatant was separated by a 4-20% gradient SDS-polyacylamide gel. And then the proteins were electro-transfered onto a nitrocellulose membrane. The nitrocellulose membrane was probed with the ^{32}P-labeled ANG-CRE. The result is shown in Fig. 3-3. Lane 1 shows the nuclear extracts treated with pre-immune serum, the band that represents the DNA /protein complex is observed. Lanes 2 and 3 show the nuclear extracts were treated with antisera against the 43 kDa-CREB (lane 2) and ATF-2 (lane 3) respectively, the DNA/protein complex band has not been abolished but significantly attenuated. This result suggests that multiple nuclear proteins bind to ANG-

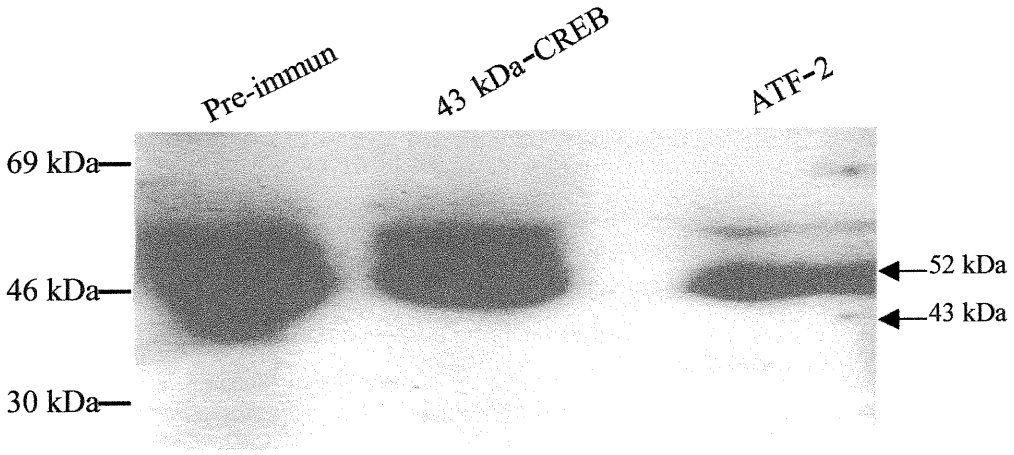


Fig. 3-3. Southwestern blot. ANG-CRE was labeled with ^{32}P as the probe. Before southwestern blot the 100 μg of mouse liver nuclear extracts were incubated with pre-immune serum, the 43 kDa-CREB, and ATF-2 antiserum respectively for 16 hr, and then immunoprecipitated with Protein A.

CRE, including 43kDa-CREB, ATF family members and some unknown factors. The immuno-precipitation with antiserum against the 43 kDa-CREB or ATF-2 removed CREB or ATF-2-related proteins, but not all. Therefore, the DNA/protein complex band was attenuated but not abolished. To further confirm the ANG-CRE does interact with different proteins, we performed the gel mobility supershift assay with 43-kDa-CREB antiserum. The gel mobility supershift is different from the regular gel mobility shift by the pre-incubation of the nuclear extracts with the 43-kDa-CREB antiserum before the co-incubation of DNA and the nuclear extracts. If the protein interacts with the antiserum, the mobility of the DNA/protein complex will be affected because of the possible changes of the electric charges and the molecular weight of the DNA/protein complex. Usually, the gel shift band will be up-shifted. Alternatively DNA/protein complex may disappear because the banding of the antibodies to nuclear protein may cause the later lossing the banding activity to DNA. The result of the supershift assay is shown in the Fig. 3-4. To increase the resolution the higher concentration of polyacrylamide (6%) was used. Lane 1 shows the BSA, which serves as the non-specific binding control. Lanes 2-4 show the nuclear extracts without treatment of any serum, the protein binds to ^{32}P-ANG-CRE and formed DNA/protein complexes. Lanes 5-6 show the nuclear extracts treated with pre-immune serum, no supershift of DNA/protein complex was observed. Lanes 7-12 shows the nuclear extracts treated with the 43-kDa-CREB antiserum produced in our laboratory, the DNA/protein complexes are partially up-shifted compared with lanes 5-6. Lanes 13-15 show the nuclear extracts treated with the 43-kDa-CREB antiserum purchased from the company, similar results to lanes 7-12 has been shown. These results, taking together with the results from the immunoprecipitation / Southwestern blot, demonstrated that a protein that is immunologically different from the 43 kDa-CREB and ATF-2 can band to ANG-CRE. This protein might be a novel transcription factor. We designated this ANG-CRE binding protein as the ANG-CREB.

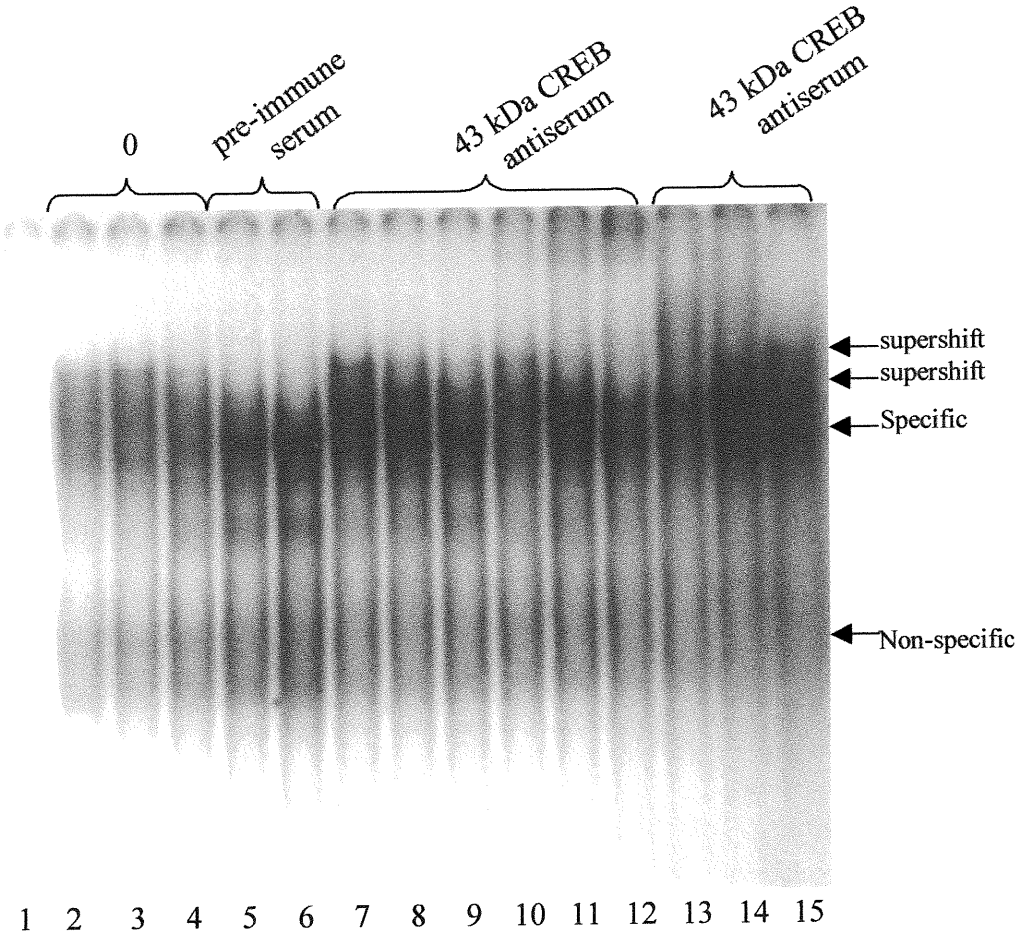


Fig.3-4. Gel mobility supershift assay. ANG-CRE was labeled with ^{32}P as the probe. The nuclear extracts were prepared from Mouse liver Lane 1, BSA was used instead of nuclear extracts. Lanes 2-4, the nuclear extracts were not incubated with serum. Lanes 5-6, nuclear extracts were incubated with pre-immune serum. Lanes7-12, nuclear extracts were incubated with 43 kDa-CREB antiserum prepared in the laboratory. Lanes13-15, antiserum from commercial. The polyacrylamide gel is 6%.

3.4 MOLECULAR WEIGHT OF ANG-CREB

Previous experiments have demonstrated that ANG-CRE mediates the cAMP response of ANG gene (Fig. 3-1), and this DNA element interacts with nuclear protein(s) (Fig. 3-2). Initially we thought that this ANG-CRE binding protein was the well characterized 43 kDa-CREB. The 43-kDa-CREB phosphorylated by cAMP-dependent PKA binds to ANG-CRE to regulate the gene expression. However, the results from immunoprecipitation (Fig. 3-3) and gel mobility supershift assay (Fig. 3-4) indicate that in addition to the 43kDa-CREB, there is another nuclear protein interacting with ANG-CRE. This ANG-CRE binding protein is immunologically different from the 43 kDa-CREB, suggesting that this ANG-CRE binding protein is distinct from the bZIP family. Although the CRE-binding proteins identified so far are all belong to the bZIP family, recent studies showed that CRE-binding protein might be structurally distinct from CREB / ATF family members (Jansen *et al.,* 1997). If the ANG-CREB is novel CRE binding protein, the first question is what is the molecular weight of this protein? To address this question, we performed the Southwestern blot assay. Briefly, the mouse liver nuclear extracts were separated by a 4-20% gradient SDS polyacrylamide gel and then electrotransfered onto a nitrocellulose filter. The nitrocellulose filter was then probed with the ^{32}P-labeled ANG-CRE. The result is shown in Fig. 3-5. The molecular weight of the protein marker was noted on the left of the figure. Different amounts of the nuclear extracts were used as shown in Fig. 3-5. Two DNA-protein complexes that represent two nuclear proteins were observed. Compared with the protein marker, the two proteins have an apparent molecular weight of 52 kDa and 43 kDa respectively. The 52 kDa one shows a much stronger radioactive intensity than the 43 kDa one, suggesting either a higher amount of the protein in the nuclear extracts or/and a stronger binding affinity of the protein to the ^{32}P-ANG-CRE. The 52-kDa-protein showed a good stability and was reproducible in the different preparation of the nuclear extracts (data not showed). Whereas the 43-kDa-band is less dense and only appeared when a large amount of nuclear protein was used in the assay as showed in the Fig. 3-5. According to the size of the protein, the 43-kDa-protein might be the 43 kDa-

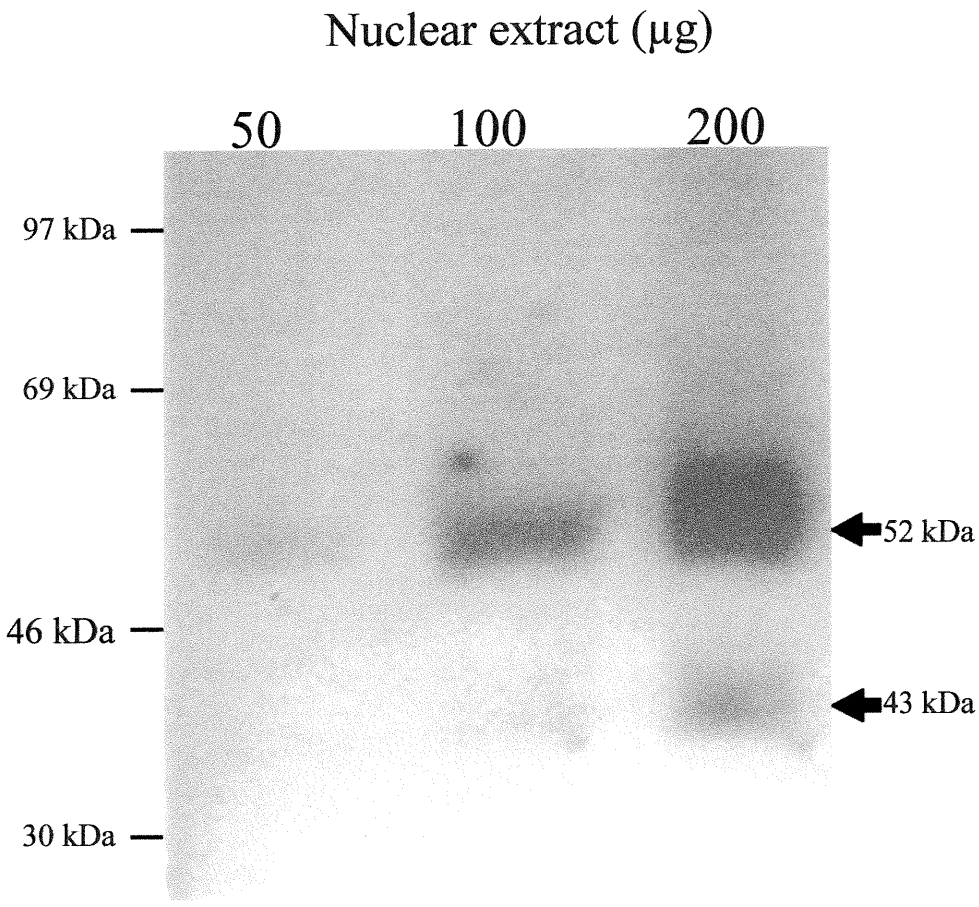


Fig. 3-5. Southwestern blot. ANG-CRE was labeled with ^{32}P as the probe. Various amount of mouse liver nuclear extract were used as indicated on the top of the fig. The protein markers were noted on the left of the figure. The molecular weight of the ANG-CRE binding proteins are noted on the right of the figure.

CREB that was first reported by Montminy et al in 1989. However, the uncertainty of this protein in different nuclear extract preparations supports the possibility that it may be derived from the degradation of the 52-kDa-protein.

As discussed above, the mouse liver nuclear protein that binds to the ANG-CRE has a molecular weight at around 52 kDa.

3.5 THERMOSTABILITY OF ANG-CRE BINDING PROTEIN

As discussed above, the appearance of the 52-kDa-protein was very reproducible for different preparation of the nuclear extracts. The protein does not appear to be sensitive to the temperature during the experiments. To test the thermostability of this protein, we heated the nuclear extracts at 95 °C for 10, 20, and 30 min respectively before they were used for the Southwestern blot analysis. The result is shown in Fig. 3-6. In lanes 1-2, the nuclear extracts were not heated, and the protein can bind to the ^{32}P-ANG-CRE probe. Lanes 3-8, the nuclear extracts were heated at 95 °C heating block for 10 (lanes 3-4), 20 (lanes 5-6), and 30 (lanes 7-8) min respectively. Compared with the control (lanes 1-2), the heating of nuclear extracts at 95 °C for 30 min (lanes 7-8) did not significantly affect the protein binding to the ANG-CRE. At present we do not know whether the heating will change its biological functions.

3.6 TISSUE DISTRIBUTION OF ANG-CRE BINDING PROTEIN

The results presented above revealed that a 52-kDa nuclear protein from mouse liver can bind to the ANG-CRE, which may play some roles in the expression of the ANG gene. As discussed in the chapter 1, the ANG gene is not only expressed in the liver, but is also expressed in many other tissues. The 52-kDa-protein may be also widely expresed in different tissues. To test the tissue distribution of the 52-protein, the Southwestern Blot was performed with the nuclear extracts from mouse liver, kidney, testis, lung, brain, heart, and

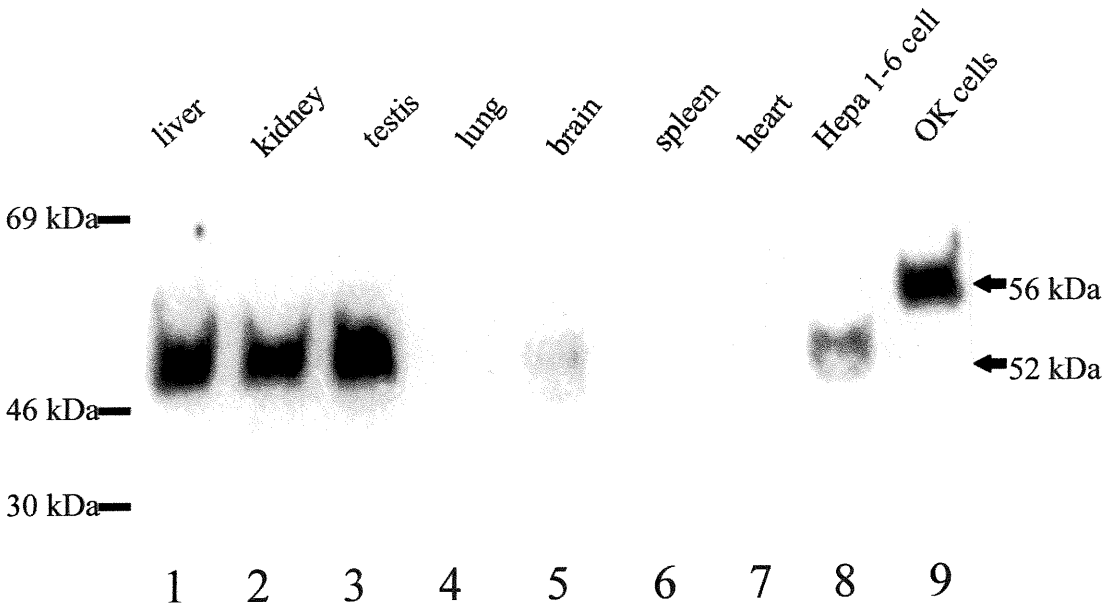


Fig. 3-7. Southwestern blot. ANG-CRE was labeled with ^{32}P. Nuclear extract from mouse liver, kidney, testis, lung brain, spleen heart and two cell lines, Hepa 1-6 and OK were used in the blot. The protein marker was noted on the left, and the molecular weight of the ANG-CRE binding protein are noted on the right of the figure.

spleen as well as two cell lines: Hepa 1-6 and OK. The result is shown in Fig. 3-7. The molecular weight of the protein marker was noted at the left of the figure. Lanes 1-9 show the nuclear extracts from the different tissues or cells as indicated on the top of the figure. The same amount of nuclear extracts from different tissues were loaded in the experiment except brain, lung and heart because of the rich of lipids and connective tissue which result in the difficulty of the preparation of nuclear extracts from these two tissues. As shown in the Fig.3-7, the 52-kDa protein was presente in the liver, kidney, testis, brain, and two cells lines, Hepa1-6 and OK, but was not presente in the lung, heart and spleen. Although the lung and heart were negative, these might not be true because of the modest amount of protein loaded. The OK cells a higher molecular weight (56 kDa) protein, was detected, as compared with that from other tissues and Hepa 1-6 cells. This difference of the molecular weight might be explained by the different isoform of the protein that may contribute to the difference of the ANG gene expression in Hepa 1-6 and OK cells as observed in our laboratory (Ming *et al.,* 1993).

3.7 AFFINITY OF ANG-CREB FOR ANG-CRE

The results from the above experiments above suggest that 52-kDa-protein is a novel CRE binding protein. We then asked whether the 52-kDa protein binds to the CRE sequences from different genes and is involved in the regulation of gene expression. We synthesized the CREs from the rat somotostatin gene (SOM-CRE), phosphoenopyruvate carboxyl kinase gene (PEPCK-CRE), and tyrosine amino transferase (TAT-CRE). Like ANG-CRE, these elements are flanked on both ends by four bases of their natural environment. We labeled these CREs with ^{32}P and performed the Southwestern blots. Briefly, the mouse liver nuclear extracts were separated by a 4-20% SDS-polyacylamide gradient gel and electrotransfered onto a nitrocellulose filter. The filter was cut into 4 pieces by scissors, and these filter pieces were probed with ^{32}P-ANG-CRE, ^{32}P-SOM-CRE, ^{32}P-PEPCK-CRE, and ^{32}P-TAT-CRE respectively. As shown in Fig. 3-8, lanes 1-2 show the

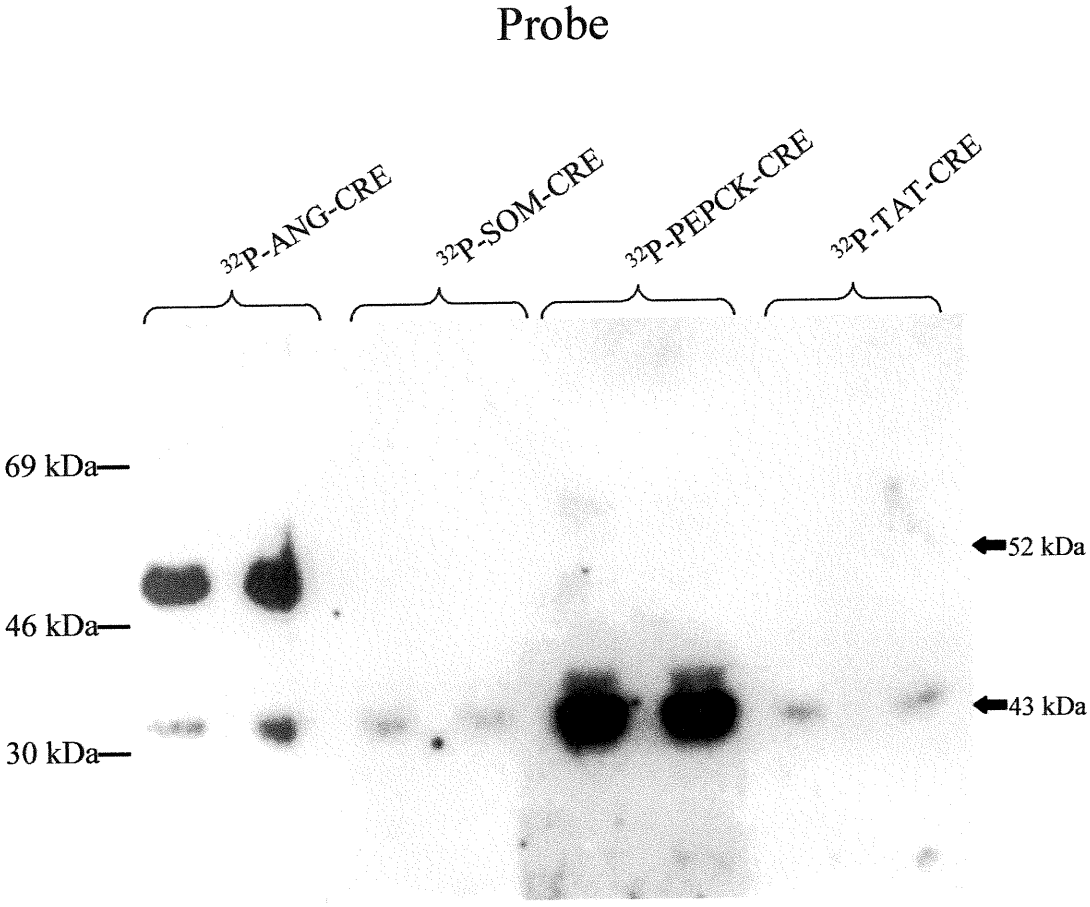


Fig. 3-8. Southwestern blot.ANG-CRE, SOM-CRE, PEPCK-CRE, and TAT-CRE were labeled with ^{32}P as the probes. The same amount of mouse nuclear extracts (100 ug) were used for each probe. The protein marker was noted on the left of the figure.

ANG-CRE can bind to two proteins with molecular weights 52 kDa and 43 kDa respectively. While the CREs from SOM (lanes 3-4), PEPCK (lanes 5-6) and TAT (lanes 7-8) CREs bind only to the protein with a molecular weight of 43 kDa. These results demonstrate that the 52-kDa-protein could bind to the ANG-CRE but not to the SOM-CRE, PEPCK-CRE, and TAT-CRE. Also, these results support the premise that the 43-kD-protein might be the 43 kDa-CREB, because the SOM-CRE, PEPCK-CRE and TAT-CRE are known to bind to this CREB protein. To further determine the DNA sequence specificity of the 52-kDa-protein, we performed Southwestern competition assay. Briefly, the mouse nuclear extracts were separated by a 4-20% of SDS-polyacylamide gradient gel and then electro-transfered onto a nitrocellulose filter. The filter was cut into different pieces, and then these filter pieces were pre-incubated with the 200-fold of unlabeled ANG-CRE, SOM-CRE, PEPCK-CRE, and TAT-CRE respectively. After the incubation the filter pieces were probed with the ^{32}P-ANG-CRE in the presence of different competitors respectively. The result is shown in the Fig. 3-9. The different unlabeled competitors were indicated on the top of the figure. For each competitor, the two concentrations of nuclear extracts (50 and 100 μg / lane) were used. Lane 1 shows the normal DNA / protein binding without any competitor. Lanes 2 and 3 show the ^{32}P-ANG-CRE / protein binding was blocked by the presence of 200-fold of unlabeled ANG-CRE. Lanes 4-9 shows the ^{32}P-ANG-CRE / protein binding was not affected by the presence of either 200-fold excess of SOM-CRE (lanes 4 and 5), PEPCK-CRE (lanes 6 and 7), or TAT-CRE (lanes 8 and 9). These results, together with the results shown in Fig. 3-8, strongly demonstrate that 52-kDa-protein can bind specifically to the ANG-CRE but does not interacted with SOM-CRE, PEPCK-CRE, and TAT-CRE. Thus the 52-kDa-protein might be a novel CRE-binding protein.

The results from the Southwestern blots analysis (Fig. 3-8 and Fig. 3-9) demonstrate that the 52-kDa-protein can't bind to the CREs from the SOM, PEPCK, and TAT genes. However, because of the sensitivity of Southwestern blot is much lower compared with the gel mobility shift assay, there might be a possibility that ANG-CREB can bind to the CREs from SOM, PEPCK, and TAT genes, but the binding affinity to these CREs is much lower

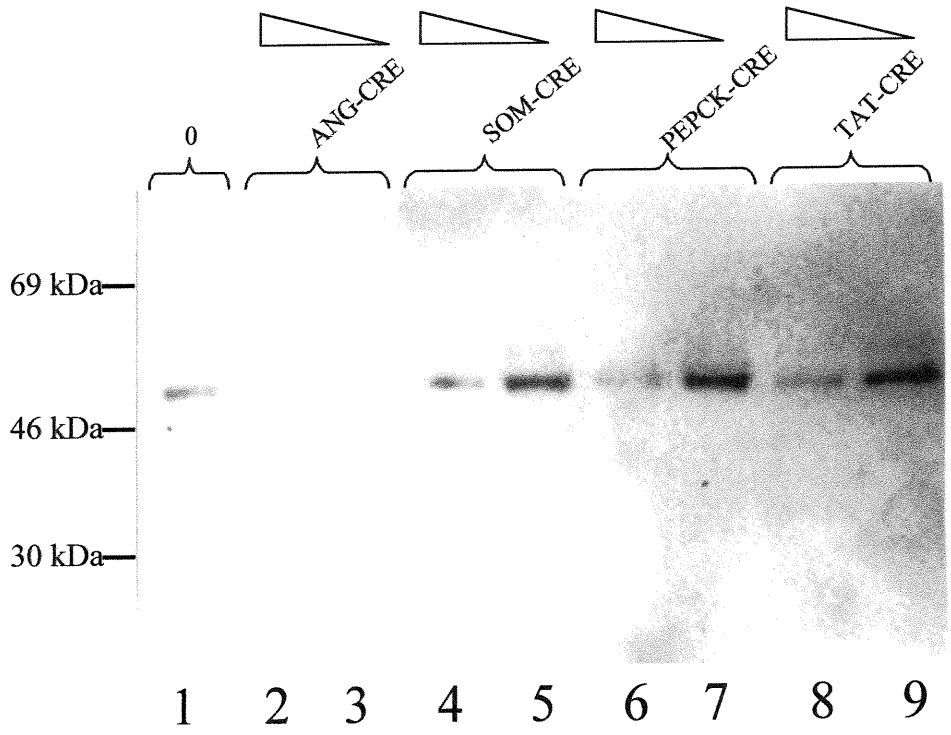


Fig. 3-9. Southwestern competition assay. ANG-CRE was labeled with ^{32}P as the probe. Nuclear extracts were prepared from mouse liver. For each competitor, 50 (lanes, 1, 2,4,6, and 8) and 100 (lanes 3,4,5,7, and 9) μg of nuclear extracts were used. Lane 1, the nuclear protein bind to ^{32}P-ANG-CRE without any competitors; Lanes 2-3, the binding of nuclear protein to ^{32}P-ANG-CRE was displaced by the presence of 200 fold of ``cold`` ANG-CRE; Lanes 4-9, nuclear protein bind to ^{32}P ANG-CRE in the presence of 200 molar excess of competitors as indicated on the top of the figure.

that that to ANG-CRE. To test this possibility we performed the gel mobility shift competition assay. Briefly, the mouse nuclear extracts were pre-incubated with 100 or 200 fold of unlabeled ANG-CRE, SOM-CRE, PEPCK-CRE, or TAT-CRE before the addition of the probe, ^{32}P-ANG-CRE. The nuclear extracts were then separated by a 4% of polyacylamide gel. The result is shown in the Fig. 3-10. Different unlabeled competitor sequences are indicated in the figure. Lanes 1-2, the BSA as nonspecific binding controls. Lanes 3-4 shows the ^{32}P-ANG-CRE / protein binding without any competitor. Lanes 5-6, unlabeled ANG-CRE competes with the ^{32}P-ANG-CRE to bind with the protein, and result in disappearance of the radioactivity. Lances 7-8 shows the presence of 100- or 200-fold of SOM-CRE did not displaced the ^{32}P-ANG-CRE binding, indicating the affinity between the SOM-CRE and the protein is very low. Lanes 9-12 show the presence of 100- or 200-fold of the PEPCK-CRE (lanes 9-10) and TAT-CRE (lanes 11-12) could partially abolished the ^{32}P-ANG-CRE / protein binding, suggesting the protein may bind to the PEPCK-CRE or TAT-CRE, but the affinity is much lower than ANG-CRE. Fig. 3-10 suggests that affinity order of the 52-kDa-protein to these CREs is: ANG-CRE, PEPCK-CRE, TAT-CRE, and SOM-CRE.

3.8 THE BINDING SITE OF ANG-CRE BINDING PROTEIN

A mutation within the palindromic CRE octamer will reduce or eliminate the binding of the 43 kDa-CREB to this sequence. Our studies have demonstrated that the 52-kDa-protein is immunologically different from the 43 kDa-CREB, indicating that the protein might be structurally distinct from the CREB/ATF family members. To test the importance of the ANG-CRE sequence for protein binding, the 28-mer-oligonucleotide (ANG N-806 /-779) was cleaved into three smaller fragments: -806 /-796, -800 / -783, and -787 /-768. These fragments represent sequences of upstream, ANG-CRE, and downstream respectively. These fragments are 4 bases overlapped each other (Fig. 2-2). With these elements, we performed a Southwestern blot. The results reveal shown on Fig. 3-11. The

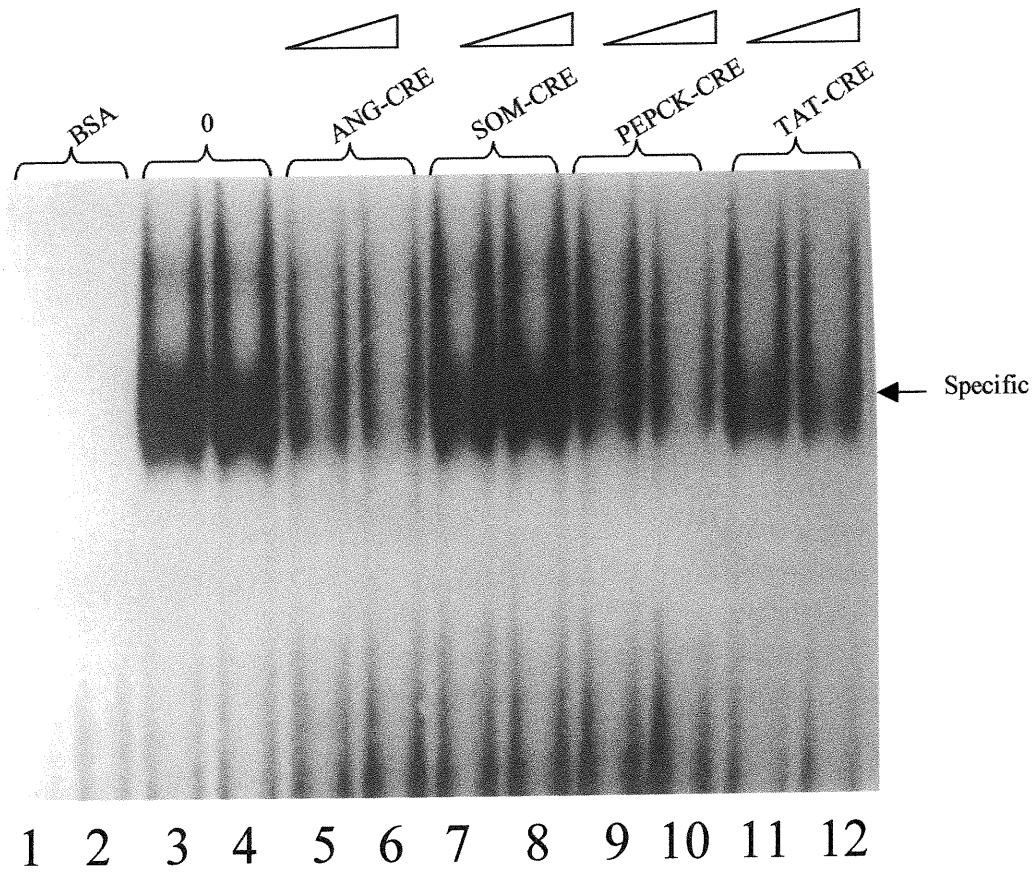


Fig. 3-10. Gel mobility shift competition assay. ANG was labeled with ^{32}P as the probe. Nuclear extracts were prepared from mouse liver. Lanes 1and 2, BSA control; Lanes 3 and 4, the nuclear protein bind to ^{32}PANG-CRE without competitors; Lanes 5-12, the nuclear protein bind to ^{32}P-ANG-CRE in the presence of 100 (lanes 5,7,9,11) and 200 (lane 6,8,10,12) molar excess of competitors as indicated on the top of the figure.

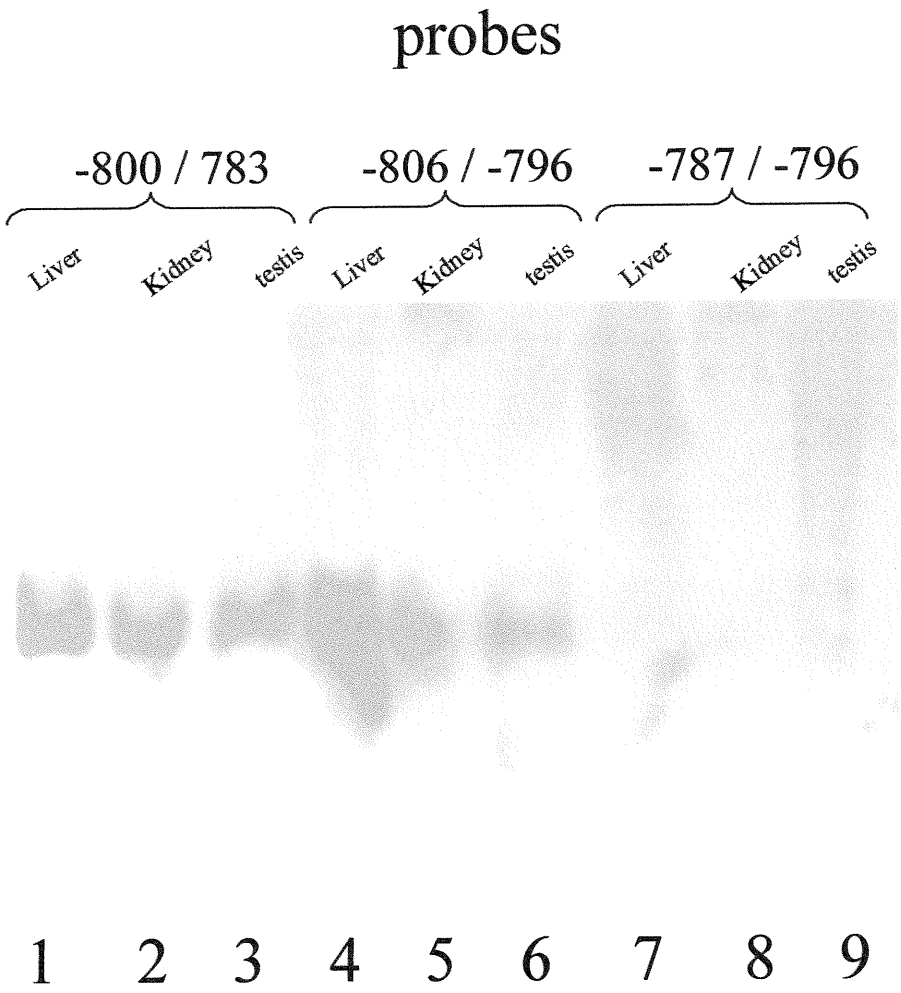


Fig. 3-11. Southwestern blot. The fragments -806 / -796 (upstream of ANG-CRE) (lanes 4-6), -800 / -783 (ANG-CRE), and -787 / -796) (down stream of ANG-CRE) were labeled a with ^{32}P as probes. Nuclear extracts were prepared from mouse liver, kidney, and testis as indicated on the top of the figure.

nuclear extracts were prepared from mouse liver, kidney, and testis. The result shows that the nuclear protein binds to the probes ^{32}P-800/-783 (lanes 1-3) and ^{32}P-814/-796 (lanes 4-6) but not the probe ^{32}P-787/-769 (lanes 7-9), suggesting that the ANG-CRE and its upstream sequence is the protein-binding site. The similar result was obtained from the gel mobility shift competition assays. As shown in Fig. 3-12, fragment -814/-796 (upstream of the ANG-CRE) was labeled as the probe, and nuclear extracts were prepared from OK cells. Lane 1 shows the BSA control. Lanes 2-3 shows the DNA/protein binding without any competitor. Three bands were observed. In the presence of the 200-600 fold of unlabeled fragment -814/-796 (upstream of ANG-CRE) (lanes 4-6) or -800/-783 (ANG-CRE) (lanes 7-9), or -806/-779 (containing ANG-CRE) (lanes 13-15) the band 3 is completely abolished. However, the presence of 200-600 fold of unlabeled fragment -787/-768 (downstream of ANG-CRE) (lanes 10-12) does not affect band 3. These results indicate that this protein binds to the ANG-CRE and its upstream sequence (the probe). To compare the importance of the ANG-CRE octamer and its upstream sequence, we labeled ANG-CRE core sequence -800/-783 with ^{32}P to performed gel mobility competition assays. The results are shown in Fig. 3-13. The nuclear extracts were prepared from mouse liver. Lane 1 shows the BSA control. Lanes 2-3, show the DNA/protein binding without any competitors. This DNA /protein binding was not affected by the presence of 200-600 fold of -814/-796 (sequence upstream of the ANG-CRE) (lanes 4-6) and -787/-768 (sequence downstream of the ANG-CRE) (lanes 10-12). The probe /protein binding was completely abolished by the presence of 200-600 fold of -800/-783 (lanes 7, 8, and 9) and -806/-779 which contains the ANG-CRE. The exact same results were obtained from the gel mobility shift competition assays with the nuclear extracts from Hepa 1-6 cells (Fig. 3-14). These results demonstrate that the ANG-CRE core sequence and its upstream 4 bases are necessary for the 52-kDa-protein binding. Taken togther this is the 52-kDa protein-binding site. Because the fragment upstream the ANG-CRE (-814/-796) has only 4 bases which overlap the ANG-CRE core sequence (-800/-783), it is uderstandable that the ANG-CRE fragment can compete with upstream fragment (Fig.3-12, lanes 7-9 and lanes 13-15) to abolish the protein binding. The

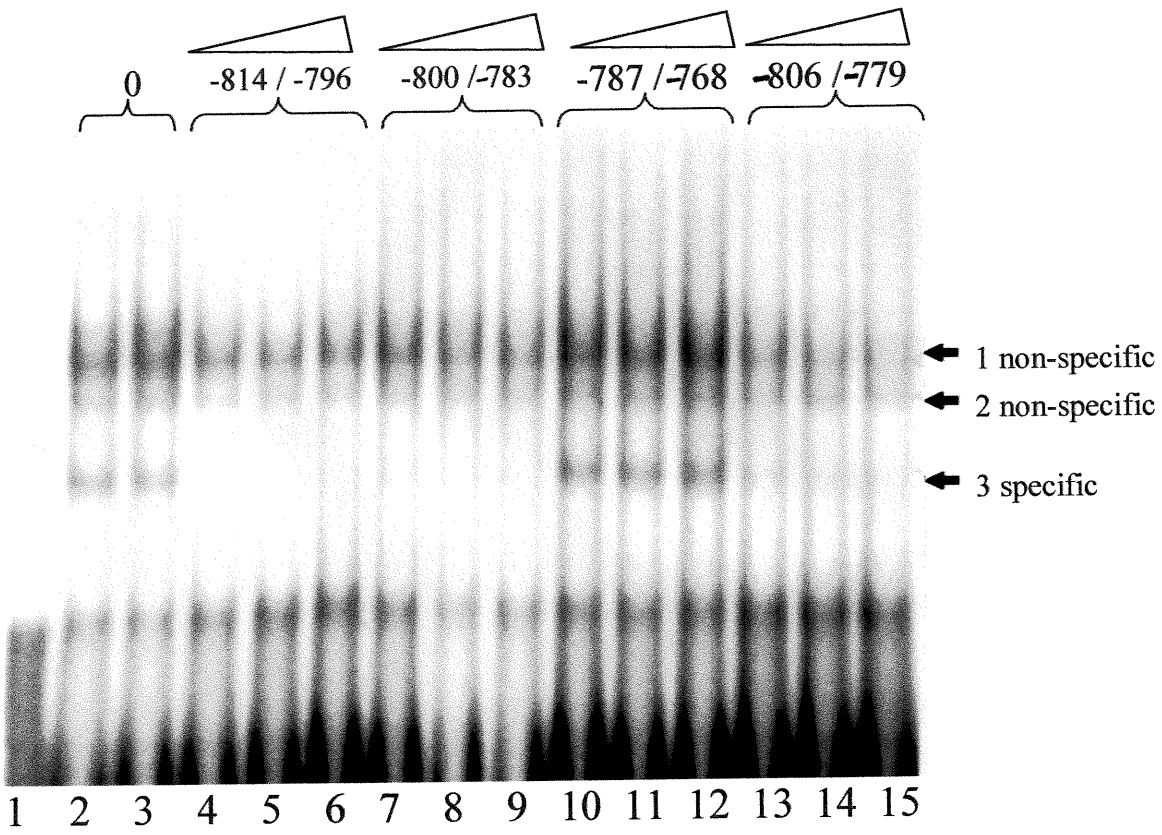


Fig. 3-12. Gel mobility shift competition assay. The fragment -814 / -796 (upstream of ANG-CRE) was labeled with ^{32}P as the probe. Nuclear extracts were prepared from OK cells. Lane 1, BSA control; Lanes 2 and 3, the nuclear protein bind to ^{32}P-814 /-796 without any competitors; Lanes 4-15, the nuclear protein bind to ^{32}P-814 / -796 in the presence of 200 (lanes 4,7,10,and 13), 400 (lanes 5,8,11, and 14), and 600 (lanes 6, 9, 12, and 15) molar excess of competitors as indicated on the top of the figure.

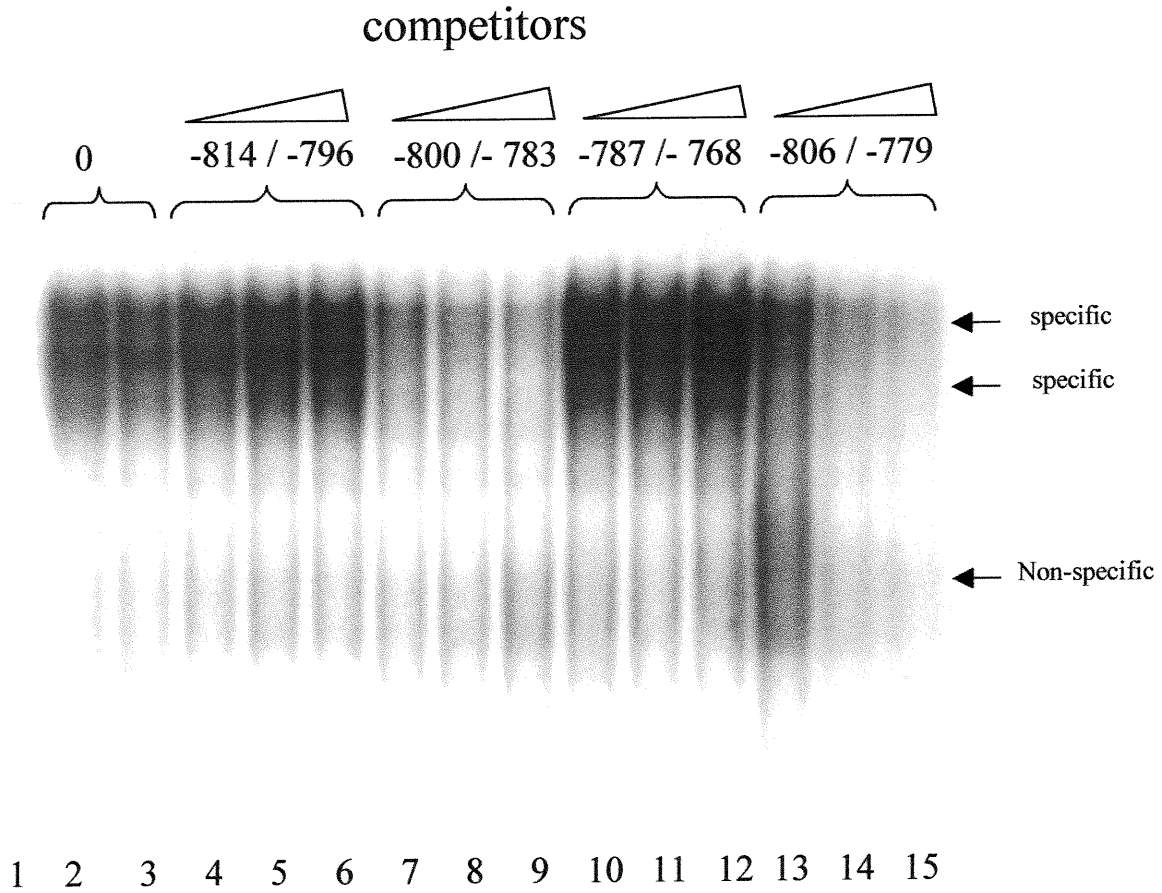


Fig. 3-13. Gel mobility shift competition assay. The fragment -800 / -780 (ANG-CRE) was labeled with ^{32}P as the probe. sNuclear extracts were prepared from mouse liver. Lane 1, BSA control; Lanes 2 and 3, the nuclear protein binds to ^{32}P-800 / -783 without any competitors; lanes 4-15, the nuclear protein binds to ^{32}P-800 / -783 in the presence of 200 (lanes 4,7,10,and 13), 400 (lanes 5,8,11, and 144), and 600 (lanes 6,9,12,and 15) molar excess of competitors as indicated on the top of the figure.

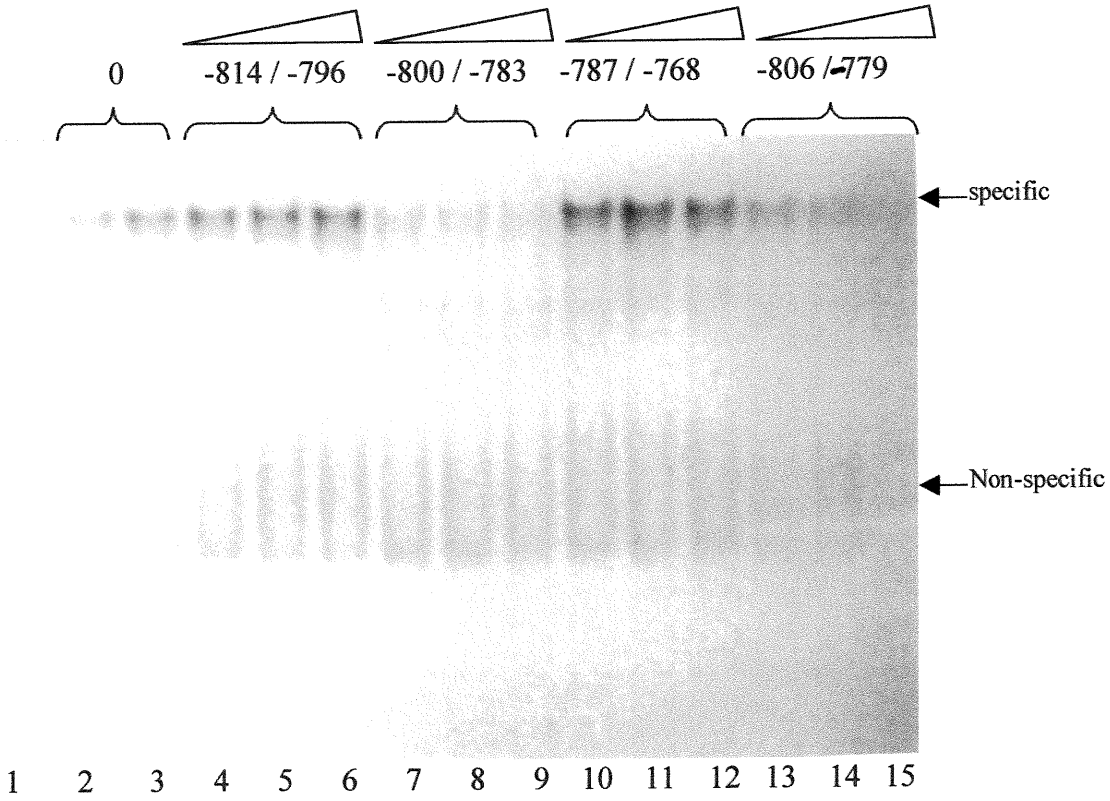


Fig. 3-14. Gel mobility shift competition assay. The fragment -800 / -780 (ANG-CRE) was labeled with ^{32}P as the probe. Nuclear extracts were prepared from Hepa 1-6 cells. Lane 1, BSA control; Lanes 2 and 3, the nuclear protein bind to ^{32}P-800 / -783 without any competitors; Lanes 4-15, the nuclear protein bind to ^{32}P-800 / -783 in the presence of 200 (lanes 4,7,10, and 13), 400 (lanes 5,8,11, and 14), and 600 (lanes 6,9,12,and 15) molar excess of competitors as indicated on the top of the figure.

upstream fragment (-814/-796) is not able to compet with ANG-CRE core sequence (-800/-783), and thus is unable to abolish protein binding (Fig. 3-13 and Fig. 3-14, lanes 4-6). To study wether the intact ANG-CRE is necessary for the 52-kDa-protein binding, we generated 3 mutants of ANG-CRE fragments (Fig.2-1 B). With these mutants we performed gel mobility competition assay and the results are shown in Fig. 3-15. The ANG-CRE (-806/-779) was labeled as the probe, and the nuclear extracts were prepared from Hepa 1-6 cells. Lane 1, shows the BSA control. Lanes 2-3, show the probe/protein binding without any competitor. Lanes 4-6 show the probe/binding was completely abolished by the presence of 100-300 fold of the unlabeled -806/-779. The probe/protein binding can not abolished by the presence of 100-300 of ANG-CRE mutant 1(M-1, lanes 7-9), mutant 2 (M-2, lanes 10-12), and mutant 3 (M-3, lanes 13-15). These results demonstrated that intact ANG-CRE octamer is necessary for the protein binding.

Thus, these results have demonstrated that the ANG-CRE sequence and it's 4 upstream bases represent the binding site of the 52-kDa protein, and mutations within the ANG-CRE results in the loss of the binding between of the DNA and the protein.

3.9 CLONING OF ANG-CRE BINDING PROTEIN

The results from the above experiments suggest that the 52-kDa-protein might be a novel CRE-binding protein that is structurally distinct from the CREB/ATF family members. In addition, this protein shows the ANG-CRE sequence specificity, which may play an important role in the regulation of ANG gene expression. Based on the strong binding of this protein to ANG-CRE under the conditions we used in the Southwestern blot, we decided to clone the cDNA of this protein by direct cloning according the procedures developed by Singh et al. (1988). Briefly, a 28-mer double-strand oligonucleotide (-806/-779) corresponding to the ANG-CRE was labeled with ^{32}P and used to screen a λgt11 cDNA expression library prepared from mouse liver (purchased from CloneTech). About 1×10^6 recombinants were inoculated evenly onto 20 plates (5000 pfu / plates). 2 mM of

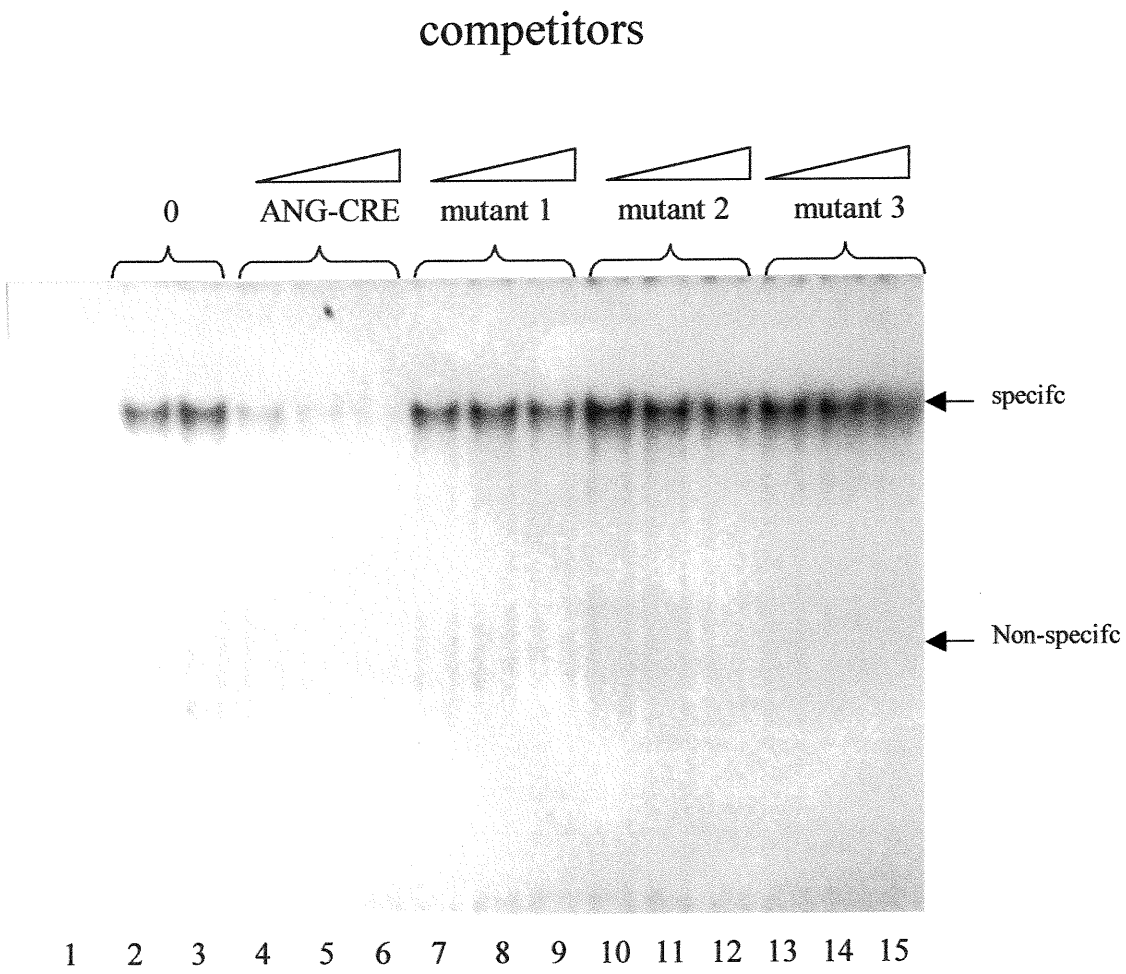


Fig. 3-15. Gel mobility shift competition assay. ANG-CRE was labeled with ^{32}P as the probe. Nuclear extracts were prepared from Hepa 1-6 cells. Lane 1, BSA control; Lanes 2 and 3, the nuclear protein bind to ^{32}P-800 / -783 without any competitors; lanes 4-15, the nuclear protein bind to ^{32}P-ANG-CRE in the presence of 100 (lanes 4,7,10,and 13), 200 (lanes 5,8,11, and 14), and 300 (lanes 6,9,12,and 15) molar excess of ANG-CRE mutants competitors as indicated on the top of the figure.

IPTG was used for the induction of the protein expression. Four phage clones were found to bind with the DNA probe in all four rounds of screening. The inserts from these 4 positive clones were excised by the restriction enzymes EcoRI, and analyzed on a mini-agarose gel. To compare the cDNA size, the cDNA coding the 43 kDa-CREB was loaded beside. The DNA was then transferred on to a nitrocellulose membrane. The membrane was then probed by the ^{32}P-sequence of clone 1. As showed in Fig. 3-16 A, the insert from all four clones show the same molecular weight, about 1.4 Kb, while the cDNA of the 43 kDa-CREB has a molecular weight at around 1.1 Kb. Fig.3-16 B shows the result from the Southern blot analysis. All inserts gave strong signal but the cDNA of the 43 kDa-CREB was not detected. These results suggest that the inserts 1, 2, 3, and 4 have similar DNA sequence with a little homology to the cDNA of the 43 kDa-CREB.

The sequence analysis of the isolated cDNA revealed a >98% identity to the sequence reported by A.H. Li, *et al.* (1996). The amino acid sequence, deduced from the cDNA as predicted, shows no apparent bZIP structure in the molecule (Fig. 3-17).

3.10. EXPRESSION OF ANG-CREB IN A BACTERIAL SYSTEM

To further characterize this protein, we expressed the protein in a bacterial system. The isolated cDNA was cloned into the prokaryotic expression vector pMalc in frame at EcoRI site that is downstream of the maltose binding protein (MBP). The reversed orientation (or antisense) was constructed as a control. The constructs of both orientations were transformed into bacterial TB-1, and the bacteria were grown in 2.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) for induction. After the incubation, 10 ml of the bacterial culture was subjected to the repeated frozen-thaw and ultrasonication, and the bacterial extracts were used for the gel mobility assays. The results are shown in Fig. 3-18. Lane 1, the bacteria transformed with the empty expression vector alone (pMalc) as nonspecific binding control. Lanes 2-3, the bacteria transformed with pMalc containing the reversed orientation (or antisense) of 52-kDa-protein cDNA. Lane 4, the bacteria transformed with

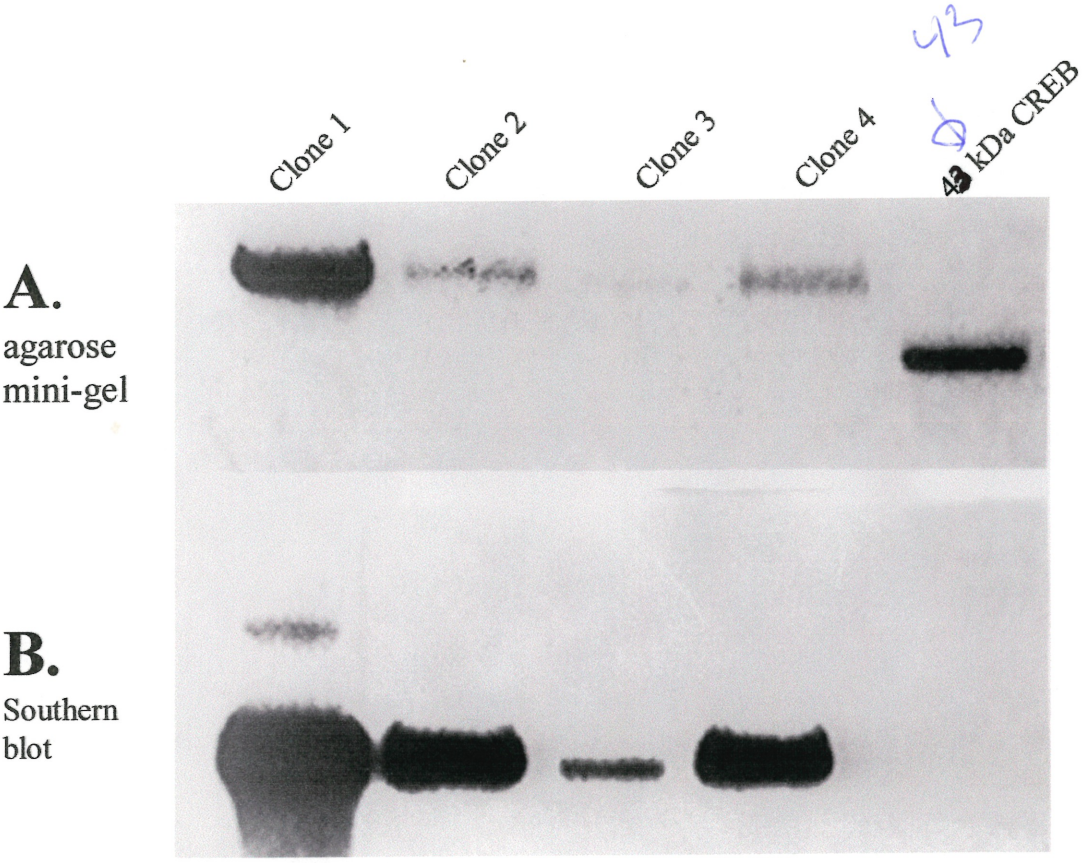


Fig. 3-16. **A.** Inserts from isolated 4 clones were analysized by 0.8 % agarose gel. Lanes 1-4 show the inserts from 4 isolated clones are the same size. Lane 5 show the cDNA of the 43 kDa CREB. **B.** Southern blot. The DNA were transferred on to a nitrocellulose filter from the mini-gel (A) and probed with ^{32}P labeled insert from clone 1.

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DNA: AGAATGACAAGCCAAATAACTAGTAATTCGCCCGTAAAATGGAAACGTAGA      51
AA :    M  T  S  Q  I  T  S  N  S  P  V  K  W  K  R  R      16

DNA: ACTCGTCATGACACTTGTGGTAAAATAACATTATTCGCCTTGCAAAAAACG     102
AA : T  R  H  D  T  C  G  K  I  T  L  F  A  L  Q  K  T      33

DNA: ATGTACTTTGACAATTTAAGCACAAAAAAACACAACCTCGACAATTTAGAG     153
AA : M  Y  F  D  N  L  S  T  K  K  H  N  L  D  N  L  E      50

DNA: TTAGAGAGGTCGTGTTTTGAGATTAGTGAGACTATGGATAGTTCATTAAGT     204
AA : L  E  R  S  C  F  E  I  S  E  T  M  D  S  S  L  S      67

DNA: ATTATAACATACTCAATGAGTGTACATAGTCTCCAGAAAAATGGAGAACCC     255
AA : I  I  T  Y  S  M  S  V  H  S  L  Q  K  N  G  E  P      84

DNA: ATGAAACAAGAAAAACAGCAGTTTATAAAAACGGATTGGACGATTGACATG     306
AA : M  K  Q  E  K  Q  Q  F  I  K  T  D  W  T  I  D  M     101

DNA: ATAAATGCCGTCGGCAATTTGAGAAATATGCCATTGATATTTCTCACAGCT     357
AA : I  N  A  V  G  N  L  R  N  M  P  L  I  F  L  T  A     118

DNA: ACTAAGGATATTCATCGCGGTGGATGGGTAGAACGAATTGATAATAAGGCT     408
AA : T  K  D  I  H  R  G  G  W  V  E  R  I  D  N  K  A     135

DNA: TGGCAGTATGTCAGAGTTTATGAAAATGGCGACATTGAAGTTTTAATTACA     458
AA : W  Q  Y  V  R  V  Y  E  N  G  D  I  E  V  L  I  T     153

DNA: CTTCAGATAATAAGATTCCACAAATATTTTGATACACCAGTTTGGTTACAG     510
AA : L  Q  I  I  R  F  H  K  Y  F  D  T  P  V  W  L  Q     169

DNA: TTTAACCCGAATCATCTTTTACCCGCAGATTATGTGCAACTTGATCATGTG     561
AA : F  N  P  N  H  L  L  P  A  D  Y  V  Q  L  D  H  V     186

DNA: ATGAAATATGTTGATCACTCACATTTAACTCGCGCCGATTTAGCAAATGAT     612
AA : M  K  Y  V  D  H  S  H  L  T  R  A  D  L  A  N  D     203

DNA: ATTTATAACATCAACTTGCAACGTTACGATTTTGGATTGTTTGGAGTAACT     663
AA : I  Y  N  I  N  L  Q  R  Y  D  F  G  L  F  G  V  T     220

DNA: AGAGATATCTATCGTAGTTTATCTGGTGATTTAGAAACACGGTATTGGGGA     714
AA : R  D  I  Y  R  S  L  S  G  D  L  E  T  R  Y  W  G     237

DNA: CGCCGAAAAAGTGAGCGCCAAATTCGTCTTTATGACAAAATGCGCGAGATG     765
AA : R  R  K  S  E  R  Q  I  R  L  Y  D  K  M  R  E  M     254

DNA: AAAAAACATGGCAAAGCAGATGATATTCCAGACGGTATTACTGATTGGTGG     816
AA : K  K  H  G  K  A  D  D  I  P  D  G  I  T  D  W  W     271

DNA: CGTTTAGAATTTCAATTTAGAGGTGGCAAGGTTGAGTCATGGCAAGAAGAA     867
AA : R  L  E  F  Q  F  R  G  G  K  V  E  S  W  Q  E  E     288

DNA: GTAATGGACAAAATGCAGTCGTTCCATGTTCTTGCTGTTGATGATAATGAT     918
AA : V  M  D  K  M  Q  S  F  H  V  L  A  V  D  D  N  D     305

DNA: GATTTAAGTGAAATAGATAAAGCCATTTTAGCACGTGTTAATGCCGATAAA     969
AA : D  L  S  E  I  D  K  A  I  L  A  R  V  N  A  D  K     322
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DNA: TTTGATTTTAAAAGAGTCGGTAAACGTTATGCTGCAAAAATTCGGAAAATG      1020
AA : F  D  F  K  R  V  G  K  R  Y  A  A  K  I  R  K  M       339

DNA: GTACGTGAAAACGTTGGTTTTGATACGACCGTTGCAGAATTATCTCTCAAA      1071
AA : V  R  E  N  V  G  F  D  T  T  V  A  E  L  S  L  K       356

DNA: ACATTCAACGAACAAAAAGATGAGTTGCAGAGACAACTAGATAGCATGCTT      1122
AA : T  F  N  E  Q  K  D  E  L  Q  R  Q  L  D  S  M  L       373

DNA: GCAAAATATAATATCGGTGCACAAACAGAAGAAATGACAGCATATTTTGAG      1173
AA : A  K  Y  N  I  G  A  Q  T  E  E  M  T  A  Y  F  E       390

DNA: GAAGAGCTTAAACAAACAGGTAATCTTGATTTTTCTGTTGTTGAGAGTGAA      1224
AA : E  E  L  K  Q  T  G  N  L  D  F  S  V  V  E  S  E       407

DNA: TCTGCGTTAGAGCGTAATGTGATACGAAATAGTGCTAAAACTTGGCGTGAA      1275
AA : S  A  L  E  R  N  V  I  R  N  S  A  K  T  W  R  E       424

DNA: GAAAATAGTTTAACGCACAGAACTAATACTAATAGTTGATGCGTATTTTTT      1326
AA : E  N  S  L  T  H  R  T  N  T  N  S  *                   436

DNA: ATTTTTCTAATAT                                            1339
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Fig.3-17. The nucleotide sequence and deduced amino acid sequences of ANG-CREB. The nucleotides underlined are different from c-50 Nucleotides bolded are absent in c-50. The deduced amino acid residues are depicted below the open reading frame. The nucleotide and amino acid sequence are numbered.

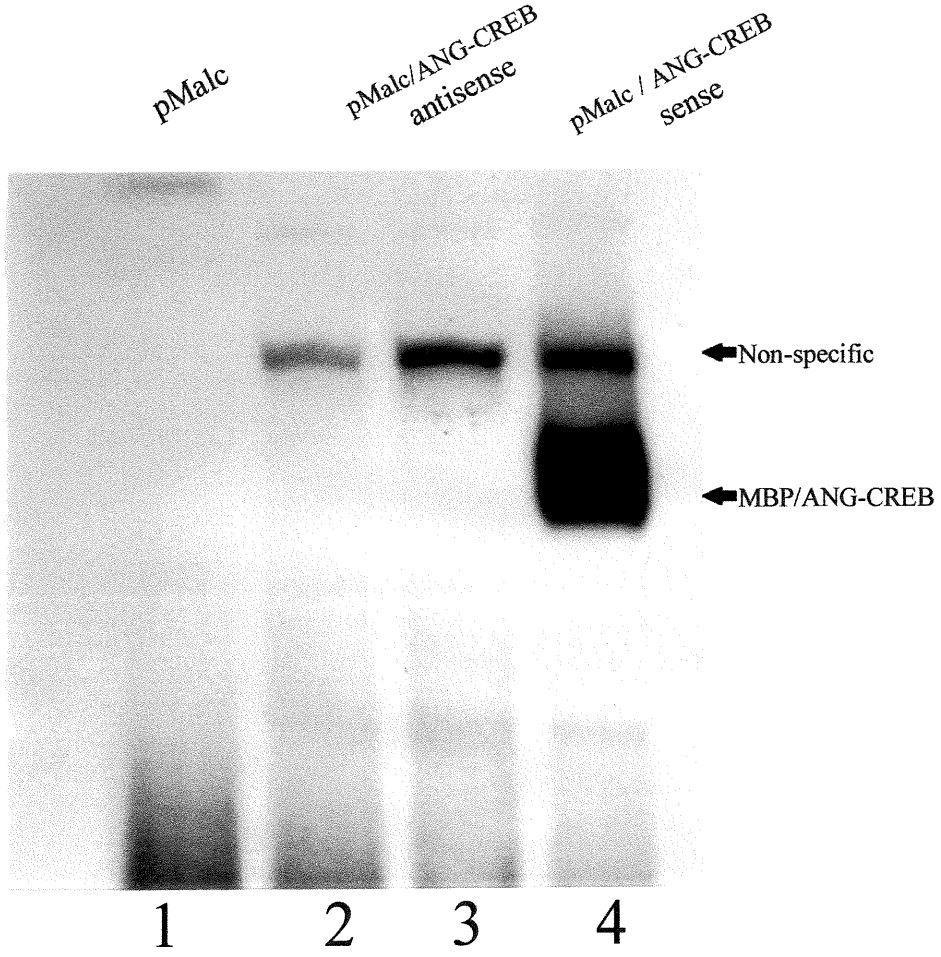

Fig. 3-18. Gel mobility shift assay. ANG-CRE was labeled with ^{32}P as the probe. The bacterial extracts were prepared from the bacteria transfected with empty expression vector (pMalc) (lane 1), pMalc fused with ANG-CREB antisense (lanes 2 and 3) or sense (lane 4). The specific band is markered with arrow

pMalc containing the sense of 52-kDa-protein cDNA in fram. Only the bacterial extracts from the bacterial transformed with pMalc containing the sense of 52-kDa-protein cDNA binds with ^{32}P-ANG-CRE (lane 4).

Gel mobility shift competition assays performed with the bacterial extract reveals the binding specificity of ANG-CRE of the binding. As shown in Fig. 3-19, lane 1 is the BSA control. Lanes 2 and 3, the bacterial extracts from the bacteria transformed with the empty expression vector alone (pMalc) and treated with IPTG, no specific probe/protein interaction was observed. Lanes 4 and 5, the bacterial extracts from the bacterial transformed with pMalc containing the sense of 52-kDa-protein cDNA, and treated with IPTG, the probe/protein complex was observed. Lanes 6-9 demonstrates the probe/protein binding in presence of the 100-300 fold of unlabeled ANG-CRE. In the presence of 300 fold of unlabeled ANG-CRE, the probe/protein was abolished (lanes 8 and 9). Lanes 10-13 shows the presence of 100-300 fold of unlabeled SOM-CRE could not compete with the ^{32}P-ANG-CRE probe nor abolish the probe/protein binding. Also, the presence of 300 fold of unlabeled ANG-CRE mutant 3 (Fig. 2-1) could not abolish the probe/protein binding (lanes 14 and 15). These results demonstrate that the intact ANG-CRE is necessary for the protein binding and is in good agreement with the results from the nuclear extracts from mouse liver.

3.11 TRANSCRIPTIONAL ACTIVITY OF ANG-CREB

The results of experiments (Fig. 3-9 and Fig. 3-10) indicate that ANG-CREB can binding to ANG-CRE. The sequence deduced amino acid reveals no apparent leucine zipper structure (Fig. 3-17), which distinct from the superfamily members of CRE binding proteins reported so far. We next asked whether this protein is functional in vivo. To answer this question, The cDNA of the 52-Kda protein was constructed into the pRSV mammalian expression vector at EcoRI site in both orientation, and the constructs were then co-transfected into Hepa 1-6 cells respectively with pTKCAT expression vector with ANG-

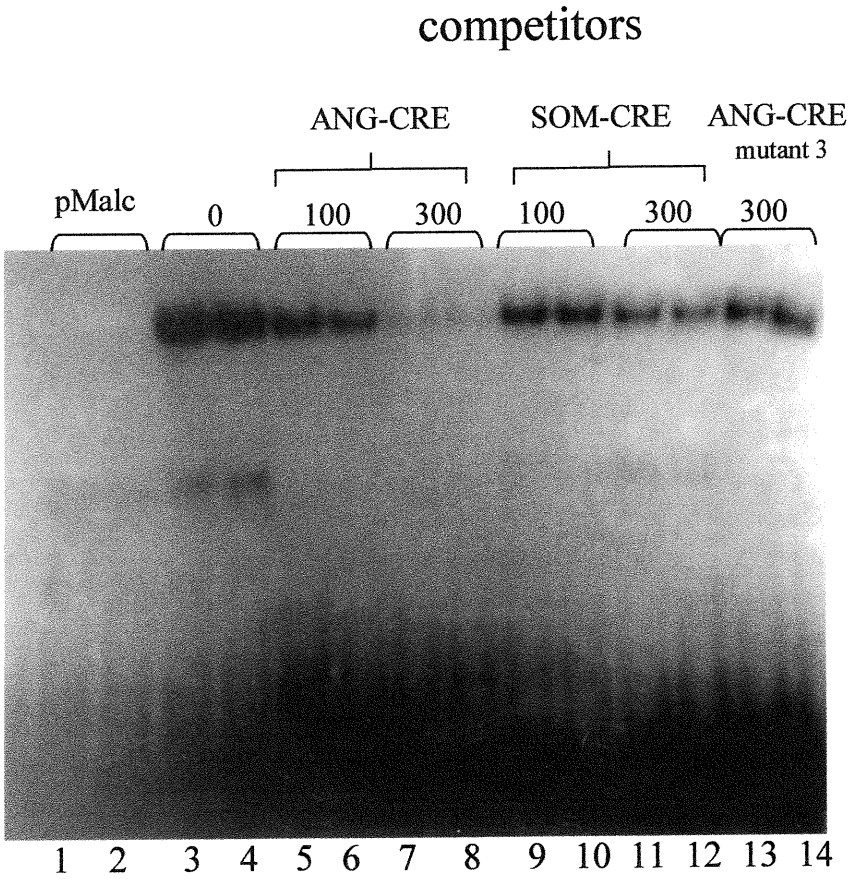

Fig.3-19. Gel mobility competition assay. ANG-CRE was labeled with ^{32}P as the probe. The bacterial extracts were prepared from the bacterial transfected with empty expression vector (pMalc) (lanes 1 and 2), pMalc fused with sense (lanes 3 -14). Lanes 4 and 5, the ANG-CREB / MBP fusion protein bind to ^{32}P-ANG-CRE in the absence of competitors. Lanes 6-14, the fusion protein / ^{32}P-ANG-CRE in the presence of 100-300 molar excess of competitors as indicated.

CRE sequence upstream of TK promoter. The CAT activity was assayed in either absence or presence of isoproterenol. The results are shown in Fig. 3-20. As expected, the isoproterenol stimulates the CAT activity (black bar) compared with control (empty bars) (control group). The same result was obtained when the pTKCAT(ANG-CRE) expression vector was co-tranfected with pRSV with the antisense of 52-kDa protein cDNA, suggesting the antisense of the cDNA has no effect on both basal (empty bars) or stimulated levels (black bars) (groupantisense). However, when pTKCAT(ANG-CRE) expression vector was co-transfected with pRSV containing the sense of 52-kDa protein cDNA, the stimulatory effect of isoproterenol on the CAT activity is suppressed. This result indicates that the stimulation of isoproterenol on the ANG-CRE/TK promoter was blocked by the expression of the 52-kDa-protein. Compare the basal levels (untreated by isoproterenol, empty bars) of both orientation of the 52-kDa-protein cDNA fusion gene and control plasmid (pGEM-3), expression of the 52-kDa-protein shows little effect on the CAT activity. In other words, 52-kDa-protein down-regulates the transactivity of ANG-CRE/TK promoter only on the stimulated level but not on the basal level. This result highlights the functionally difference between the 52-kDa protein and the 43 kDa-CREB. The 43 kDa-CREB was demonstrated to stimulate the transactivity of ANG gene promoter (ANG N-1498/+18) (Qian *et al.,* 1997). The addition of isoproterenol further enhanced the stimulatory effect of pRSV/CREB (Qian *et al.,* 1997).

3.12THE EXPRESSION OF THE ANG-CREB IS REGULATED BY cAMP AND DEX

As discussed in chapter 1, the expression of ANG gene is regulated by glucocorticoids. Previous studies in our laboratory have demonstrated the addition of DEX could stimulate ANG gene expression in both Hepa 1-6 and OK cells (Ming *et al.*, 1993; 1995). The adrenalectomy results in the decrease of the ANG gene expression because of the lack of the stimulation by glucocorticoids. The administration of DEX could reverse the decreased ANG level to some extent (unpublished data in our laboratory). During the

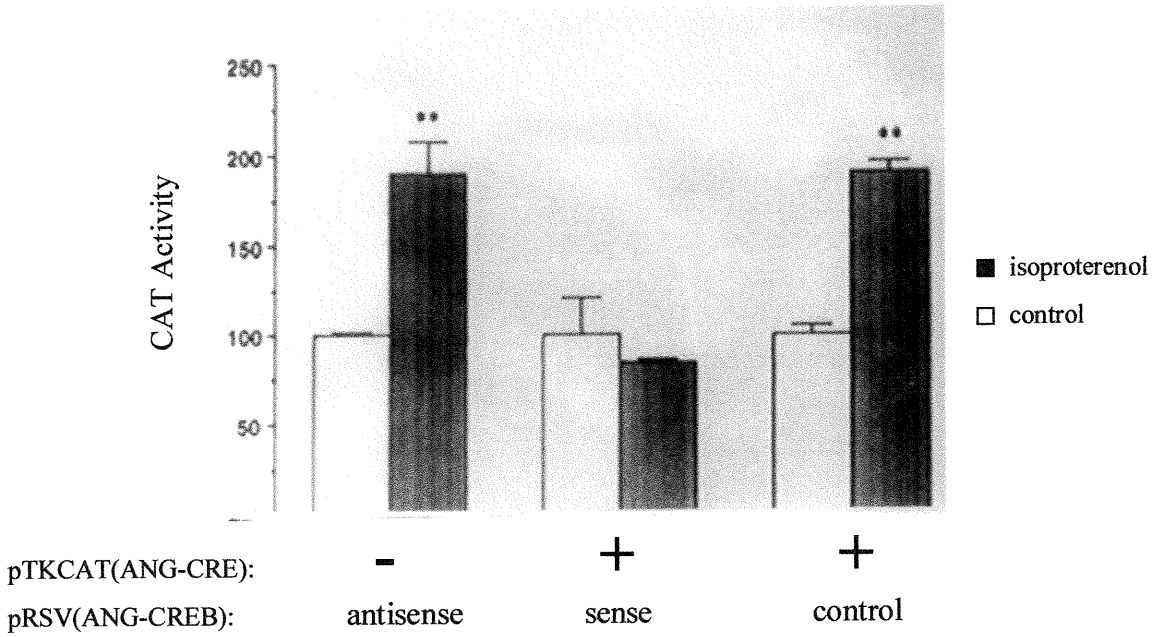

Fig.3-20. Transient gene transfection assay. Effect of isoproterenol (10^{-5} mol / l) on the expression of pTKCAT(ANG N-806/-779) in Hepa 1-6 cells cotransfected with pRSV(ANG-CREB) or control plasmid PGEM-3. Results are expressed for the mean+ SD as a percentage of control (without the addition of isoproterenol 100%) (**p±0.01).

studies on the effect of the DEX and cAMP on the adrenalectomized mouse, we used the mouse liver nuclear extracts for the gel mobility shift and Southwestern blot assays. Interestingly, we found that the adrenalectomy decreased the ANG-CREB level. As showed in Fig. 3-21, in normal mouse liver, two ANG-CRE binding protein are observed, and the 52-kDa protein is predominant (lane 1). Adrenalectomy resulted in the decrease in the 52-kDa-protein and an increase of the 43 kDa-CREB (lane 2). The injection of DEX (lane 3) or cAMP (lane 4) alone could not reverse the adrenalectomy-reduced level of 52-kDa-protein significantly. However, the injection of the combination of the DEX and cAMP, restored the level of the 52-kDa protein close to the normal level (lane 5). This result indicates that the 52-kDa-protein may be regulated by DEX and cAMP.

3.13 ROLES OF THE 43 kDa-CREB ON THE ANG GENE EXPRESSION

As discussed in chapter 1, we know that CRE interacts with a group of proteins with similar bZIP structure. Our present studies reveal a non-b-ZIP protein that can also bind to ANG-CRE and regulate the gene expression. Studies demonstrate that the 52-kDa-protein is a novel ANG-CRE binding protein that is different from CREB both structurally and functionally. However, whether the 43 kDa-CREB interacts with ANG-CRE and subsequently regulates ANG gene expression is unknown. Southwestern blot (Fig. 3-5, 3-8) assays with mouse nuclear extracts demonstrated that the 43-kDa-protein binds to ANG-CRE *in vitro*. Immunoprecipitation/Southwestern blot (Fig. 3-3) and gel mobility supershift (Fig. 3-4) assays with 43 kDa-CREB antiserum demonstrate that the 43 kDa-CREB does bind to ANG-CRE. To investigate whether the 43 kDa-CREB functions *in vivo*, the cDNA coding for 43 kDa-CREB was cloned and constructed into the mammalian expression vector. The construct (pRSV/CREB) was transfected into OK 27 cells (a stable OK cell line with a fusion gene containing the 5'-flanking regulatory sequence of the rat ANG gene fused with a human growth hormone (hGH) gene as a reporter, pOGH(ANG N-1498/+18)). The level of expression of the pOGH (ANG N-1498/+18) in OK 27 cells was

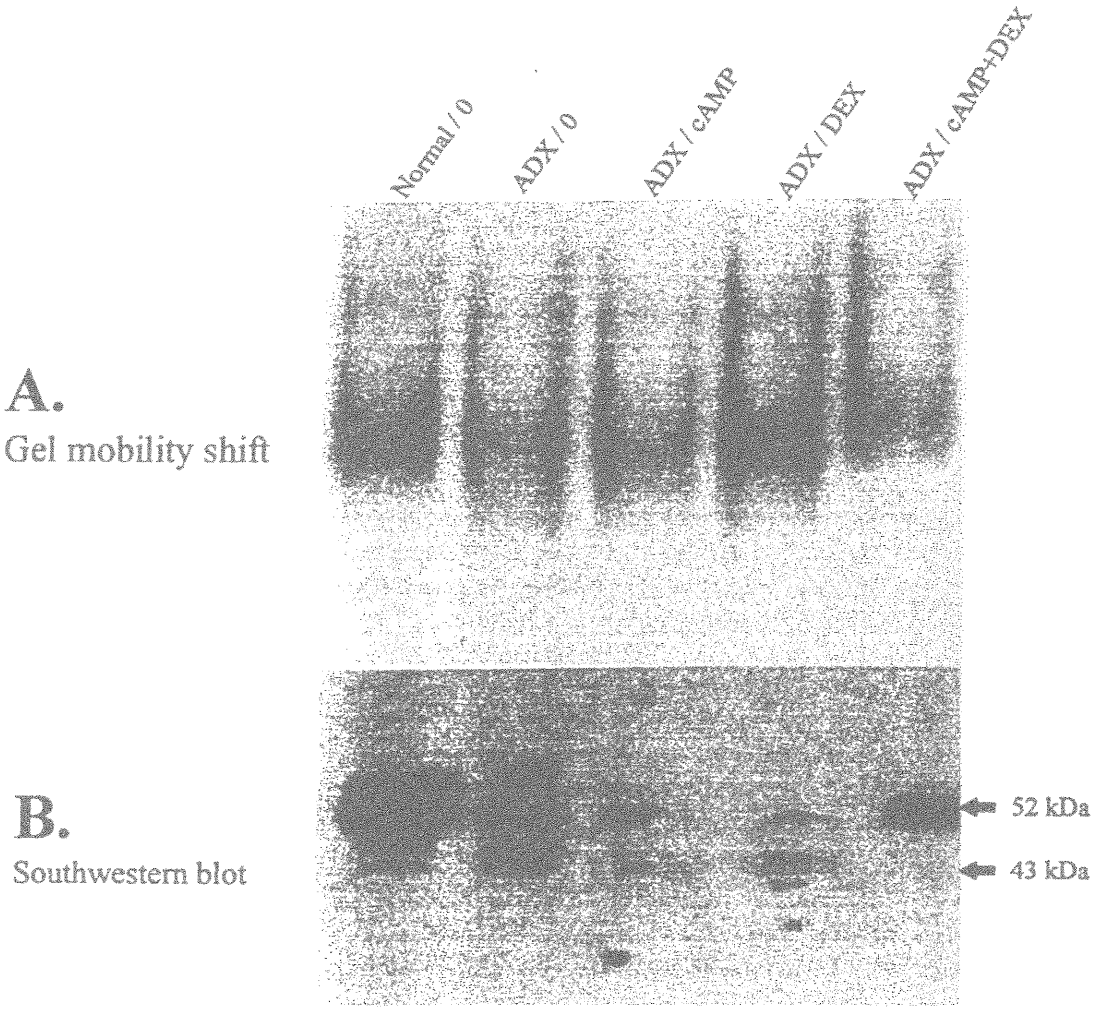

Fig.3-21. The effect of cAMP and dexamethasone (DEX) on the expression of the 52 kDa protein in adrenectomized (ADX) mouse. The nuclear extracts were prepared from mouse liver. The probe was ^{32}P-ANG-CRE. Lane1, normal mouse without injection of either 8-Br-cAMP or DEX; Lane 2, adrenectomized mouse without injection of either 8-Br-cAMP or DEX; Lane 3, adrenectomized mouse with injection of 8-Br-cAMP. Lane 4, adrenectomized mouse with injection of DEX; Lane 5, adrenectomized mouse with injection of both 8-Br-cAMP and DEX.

estimated by the amount of immunoreactive hGH secreted into the culture medium. The results demonstrate that the transfection of pRSV/CREB alone stimulated the expression of pOGH(ANG N-1498/+18), and the addition of isoproterenol or forskolin further enhanced the stimulatory effect of pRSV/CREB on the expression of pOGH(ANG N-1498/+18) (Qian *et al.,* 1996). The enhancing effect of isoproterenol was inhibited by the presence of propranolol (an inhibitor of β-adrenoceptors) and R-p-adenosine-3'5'-cyclic monophospho-orthioate ((Rp)-cAMP, an inhibitor of cAMP-dependent protein kinase AI and II) (Qian *et al.,* 1996). Transfection of pRSV/CREB had no effect on the expression of the fusion gene containing the thymidine kinase promoter fused with an hGH gene as a reporter (pTKGH) (Qian *et al.,* 1996). Because isoproterenol is known to stimulate the synthesis of intracellualr cAMP via β-adrenergic receptor, and also forskolin is a wildly used cAMP activator, these studies suggest isoproterenol stimulates the expression of ANG gene via the cAMP-dependent protein kinase A (PKA) and probably via the interaction of the 43 kDa-CREB with ANG-CRE, the nuclear 43-kDa-CREB may play a modulatory role on the expression of the ANG gene in OK cells.

4. Chapter 4 Discussion

4.1 ANG GENE EXPRESSION IS REGULATED BY cAMP

The systemic renin-angiotensin system is designed to maintain cardiovascular homeostasis in response to hypotension. Under normal conditions, angiotensinogen (ANG) concentrations circulating in the plasma are rate limiting for the maximum formation of angiotensin I. In the liver, the major site of circulating ANG synthesis, ANG expression is under tight hormonal control. Hormonal regulation of expression of ANG gene can occur on different levels dependent on different hormones which function via different pathways, such as, transcription rates, mRNA stability and processing, and translational initiation rates. Some hormones may function via different mechnismslevels. Ang II increases ANG gene expression by both transcription (Chan 1992) and stabilization of the transcripts (Klett *et al.*, 1993).

Although the regulation of ANG gene expression may occur on different levels, changes in transcriptional initiation rate have been observed to be a common and important

mechanism in controlling genes expressed by the mammalian hepatocyte. Studies have showed the treatment of rats with a combination of dexamethasone (DEX), estradiol and L-T3 increases ANG mRNA synthesis in liver and other tissues (Campbell and Habener, 1986; Kalinyak and Perlman, 1987). It has been also demonstrated that a combination treatment with DEX and L-T3 resulted in a synergistic increase in ANG mRNA levels in the rat hepatoma cells (Reuber H35) (Chang and Perlman, 1988). DEX may increase ANG mRNA levels in rat pancreatic islet cells (1056A) in a time- and dose-dependent manner (Brasier *et al.,* 1986). In our laboratory, studies employing gene transfection assays with the different fragments of rat ANG gene 5`-flanking regulatory region fused to a reporter gene (CAT) demonstrated that 8-Br-cAMP (a cAMP analogue) and forskolin (can increase intracellular cAMP) stimulate ANG gene expression in OK cells and Hepa 1-6 cells.

The cAMP effect on ANG gene expression in OK cells: cAMP or forskolin directly stimulated the expression of pOCAT(ANG N-1498/+18) in a dose dependent manner in cultured OK cells. It has been found that pOCAT (ANG N-1136/+18), pOCAT(ANG N-960/+18), pOCAT(ANG N-814/+18), and pOCAT (ANG N-688/+18) are also responsive to the addition of cAMP but not pOCAT (ANG N-280/+18), pOCAT(ANG N-196/+18), pOCAT (ANG N-110/+18), pOCATANG (N-53/+18), and pOCAT(ANG N-35/+18) (Ming *et al.,* 1995).

The gene transfection experiments revealed that the addition of 8-Br-cAMP or forskolin stimulated the expression of pTKCAT(ANG N-814/-761) by 2- to 3-fold, compared with the controls (in the absence of either cAMP or forskolin), whereas it stimulated the expression of pTKCAT(ANG N-814/-689) by 1.3- 1.2-fold. Comparing with of pTKCAT(ANG N-814/-761), we do not know the reason for a lower stimulation by cAMP on pTKCAT(ANG N-814/-689). One possible explanation may be that the downstream DNA sequence N-760/-689 may interact with a CREB-associated protein(s), which may modulate or inhibit the effect of cAMP. Indeed, this possibility is supported by the report (Miller *et al.,* 1993) that CREB-associated proteins bind to the DNA sequence adjacent to the CRE motif of glucagon gene and inhibit the response of the glucagon gene to

cAMP. Deletion of the fragment ANG N-814/-761 to ANG N-806/-779, the pTKCAT(ANG N-806/-779) is also responsible to either 8-Br-cAMP (2.1-fold) or forskolin (1.9-fold). These studies further provide evidence that the CRE motif is probably located in ANG N-795/-788 (5'-TGACGTAC-3') in rat ANG gene (Ming *et al*, 1997).

The addition of Rp-cAMP (10^{-4} mol/L), an inhibitor of cAMP-dependent protein kinase AI and II, blocks the effect of 8-Br-cAMP on the expression of pTKCAT (ANG N-806/-779) in OK cells (Ming et al, 1997). These studies demonstrated that the stimulatory effect of 8-Br-cAMP on the ANG gene expression was mediated by the PKA pathway. The addition of DEX further enhanced the stimulatory effect of cAMP or forskolin on the expression of pOCAT(ANG N-1498/+18) in OK cells (Ming *et al*, 1995), which showed that glucocorticoid and 8-Br-cAMP may significantly stimulate the transcription of the ANG gene in kidney cells. Moreover, the studies showed that the stimulatory effect of 8-Br-cAMP plus DEX on the expression of pOCAT(ANG N-1498/+18) was no more than 2- to 3-fold, compared with control (in the absence of both DEX and cAMP). Such observations are in agreement with the studies of Ohtani *et al.* (1992), which showed that the maximal effect of DEX and dibutyryl $(DB)_2$cAMP on ANG secretion by primary cultured hepatocytes is about 2- to 3-fold over the control.

Unlike in OK cells, 8-Br-cAMP alone did not stimulate the expression of the full length ANG-CAT fusion gene, pOCAT(ANG N-1498/+18) in Hepa 1-6 cells. However, it can stimulate the expression of the fusion genes of ANG / thymidine kinase promoter /CAT reporter: pTKCAT(ANG N-814/-761) and pTKCAT(ANG N-806/-779) (Ming et al, 1995, 1997). The action of 8-Br-cAMP on pOCAT(ANG N-1498/+18) required the addition of DEX. DEX alone can stimulate the pOCAT(ANG N-1498/+18) in Hepa 1-6 cells. The combination of 8-Br-cAMP and DEX stimulated the expression of pOCAT(ANG N-1498/+18) at least 1.5-fold higher than the DEX alone. This synergistic effect of 8-Br-cAMP and DEX was also shown in studies by Ohtani *et al.* (1992) who reported that in the primary culture of rat hepatocytes, the basal secretion of ANG decreased during culture. The addition of DEX and $(Bu)_2$-cAMP completely prevented this decrease. ANG secretion

by freshly plated hypatocytes was slightly increased in response to DEX, but after 24 hrs in culture, hepatocytes no longer responded to DEX alone. When hypatocytes were treated with $(Bu)_2$-cAMP, or glucagon, or forskolin, ANG secretion increased in response to DEX in a concentration-dependent manner. This synergistic effect of 8-Br-cAMP and DEX was also observed in OK cells as well as hepa1-6 cells (Ming *et al*, 1995)

The molecular mechanism of the difference of the same gene pOCAT(ANG N-1498/+18) in Hepa 1-6 and OK cells is unclear at present. We hypothesize that there is an inhibitory factor in Hepa 1-6 cells, which may inhibit the stimulatory effect of 8-Br-cAMP on the expression of the ANG gene, by binding to a neighboring sequence. The addition of DEX may decrease the effect of inhibitory factors. Thus the effect of cAMP on ANG gene expression appeared only in the presence of DEX (Ming *et al.*, 1997). Indeed, 8-Br-cAMP alone may induce the fusion genes pTKCAT(ANG N-814/-761) and pTKCAT(ANG N-806/-779) to which the element(s) that supposed to be the binding site of the inhibitory factors has been deleted.

4.2 cAMP RESPONSIVE ELEMENT (CRE) OF RAT ANGIOTENSINOGEN GENE

Eukaryotic gene transcription is regulated by DNA sequence-specific transcription factors that bind to cis-acting enhancer and promoter element. The cAMP effect is often mediated by a short parlindromic core motif, 5'-TGACGTCA-3' (designated as a cAMP-responsive element, CRE) in the 5'-flanking region of a specific gene (Montminy *et al.*, 1986; Silver *et al.*, 1987; Philippe, 1991). The CRE is highly conserved among the cAMP-inducible promoters. The cAMP-responsive element displays properties of a classical enhancer sequence, stimulating transcription at a distance and functionally independent of orientation. The CRE core motif was also observed in promoters of certain viruses including HTLV-I and -II, cytomegalovirus and adenovirus. It has been reported that a putative CRE, 5'-TGCGTCA-3' is localized in the 5'-flanking region (ANG N-839/-833) of the human ANG gene (Fukamizu *et al.*, 1990). Previous studies in our laboratory (Chan *et al*, 1990) on

the sequence of the 5`-flanking region of the rat ANG gene demonstrated that the DNA sequence of nucleotides N-795/-788 (5'-TGACGTCA-3') was almost identical to the consensus CRE of the somatostatin gene (Montminy *et al.,* 1986), except the last two nucleotides are in reverse order. Thus this observation raises the possibility that cAMP may stimulate the expression of the ANG gene via this putative ANG-CRE in the 5`-flanking region of the rat ANG gene. Indeed, the gene transfection experiments demonstrated that the addition of 8-Br-cAMP or forskolin stimulated the expression of pTKCAT(ANG N-814/-689), and pTKCAT(ANG N-814/-761) as well as pTKCAT(ANG N-806/-779), but not the mutants of ANG N-806/-779 (Ming *et al.,* PhD thesis).

The expression of the ANG gene in mouse hepatoma cells (Hepa 1-6) has shown that isoproterenol or 8-Br-cAMP enhances the stimulatory effect of dexamethasone on the expression of the ANG gene in Hepa 1-6 cells (Ming *et al.,* 1993; Ming *et al.,* 1995). The enhancing effect of isoproterenol is blocked by the presence of propranolol (β-adrenergic receptor blocker), ICI 118,551 (β2-adrenergic receptor blocker) and Rp-cAMP (an inhibitor of cAMP-dependent protein kinase A I and II), but only minimally by atenolol (β1-adrenergic receptor blocker). These findings demonstrated that the enhancing effect of isoproterenol is mediated predominantly via the β2-adrenergic receptor and the cAMP dependent protein kiase A (PKA) signal transduction pathway.

The molecular mechanism(s) responsible for the enhancing effect of isoproterenol or 8-Br-cAMP with DEX on the expression of the ANG gene in Hepa 1-6 cells has not been defined. One possibility may be that the addition of isoproterenol stimulates the synthesis of intracellular cAMP, since β-adrenergic receptors are linked through guanine nucleotide regulatory protein (G-protein) to adenylyl cyclase on the inner part of the plasma membrane of target cells (Rizza *et al.,* 1980). The intracellular cAMP then binds to the regulatory subunit of cAMP-dependent PKA and releases the catalytic subunit of PKA. The free catalytic subunit of PKA then translocates into the nucleus and subsequently phosphorylates the nuclear 43 kDa-CREB and / or other CRE-binding proteins. These nuclear proteins interact with the putative ANG-CRE in the 5'-flanking region of the rat ANG gene (Chan *et*

al., 1990), and interact with the activated-glucocorticoid receptor complex (GRC) which is bound to the glucocorticoid-responsive element (GREs) in the 5'-flanking region of the rat ANG gene when activated by DEX. Subsequently, the bound CREB / GRC unit will act synergistically with the pre-initiation complex to enhancing the expression of the ANG gene.

4.3 THE 43 kDa-CREB IS INVOLVED IN THE ANG GENE REGULATION

Within the last 10 years at least 20 different CRE-binding proteins (CREBs) have been cloned, including CREBs, CREM and ATF family (Meyer and Habener, 1993). CREB 341 (341 amino acid residues) and CREB 327 (327 amino acid residues) (they are tentatively designated as 43 kDa-CREB) are the best-characterized CREBs. They are encoded from the same gene by two alternatively spliced messenger RNAs. The 43 kDa-CREB homodimers bind strongly to the symmetrical palindrome but less well to the asymmetrical sequence, such as the CRE (TAGCGTAA) of the phosphoenopyruvate carboxykinase (PEPCK) gene and CRE (TGACTTCA) of the α-subunit of human glycoprotein. Thus, the symmetry of the CRE-sequence and its flanking sequence determine the relative binding affinity and specificity of the 43 kDa-CREB. The binding of CREB to CRE in the regulatory region of cAMP induciable gene is thought to be an important step in the regulation of the cAMP regulated gene expression. Elevated intracellular cAMP activates the protein kinase A (PKA) that dissociates into regulatory subunit and catalytic subunits. The catalytic subunits of PKA then translocated into nucleus, and results in the phosphorylation of the 43 kDa-CREB and / or other functional nuclear proteins. The phosphorylated nuclear protein then binds to specific region of the DNA (CRE) and interacts with other transcriptional activators to influence the gene expression (Fig.1-17). Therefore, the cAMP functions via the 43 kDa-CREB or / and other nuclear proteins.

Our studies demonstrate that the 43 kDa-CREB does interact with ANG-CRE. From mouse liver nuclear extracts we found a 43-kDa protein binds to ANG-CRE (Fig. 3-5). This 43 kDa protein also bind to somatostain gene CRE (SOM-CRE), phosphoenol pyruvate

carboxyl kinase gene CRE (PEPCK-CRE) and tyrosine amino transferase gene CRE (TAT-CRE) (Fig. 3-8). Immunoprecipitation (Fig. 3-3) and gel mobility supershift (Fig. 3-4) assays with the 43 kDa-CREB antiserum suggest this 43-kDa protein is the 43 kDa-CREB. We have also demonstrated that either the full length of the 43 kDa-CREB or the bZIP domain can bind to ANG-CRE *in vitro* (unpublished data in the laboratory). The experiments showed that the mutations within ANG-CRE sequence resulted in the loss of the binding activity of the ANG-CRE with the 43 kDa-CREB (unpublished data in the laboratory). These results indicate that the intact CRE-motif of ANG-CRE (TGACGTAC) is essential for the binding with the 43 kDa-CREB.

The 43 kDa-CREB can interact with ANG-CRE *in vitro*. We speculate that the 43 kDa-CREB might mediate the effect of forskolin or 8-Br-cAMP on the expression of ANG gene in via the binding to the putative CRE. We have previously reported that isoproterenol enhances the stimulatory effect of DEX on the expression of ANG gene in mouse hepatoma cells via the β2-adrenergic receptor and cAMP-dependent protein kinase A (PKA) pathway (Ming *et al.,* 1993; 1995). To study the effect of the 43 kDa-CREB on the expression of the ANG gene, we constructed the fusion gene pRSV/CREB, by fusing the full length of the 43 kDa-CREB cDNA with SV promoter. The fusion gene was introduced into a opossum kidney (OK) cell line, OK27 cells which is a stable transfectant with a fusion gene (pOGH(ANG N-1498/+18)) containing the 5'-flanking regulatory sequence of the rat ANG gene fused with a human growth hormone gene as a reporter. The level of expression of the pOGH(ANG N-1498/+18) in OK27 cells was estimated by the amount of immunoreactive human growth hormone secreted into the culture medium. Transfection of pRSV/CREB alone stimulated the expression of pOGH(ANG N-1498/+18). The addition of isoproterenol (an activator of both β1- and β2-adrenergic receptor) or forskolin which are known to increase intracellular cAMP levels further enhanced the stimulatory effect of pRSV/CREB on the expression of pOGH(ANG N-1498/+18) (Qian *et al.*, 1996). The stimulatory effect of isoproterenol was blocked in the presence of propranolol (an inhibitor of β-adrenoceptors) and Rp-cAMP (an inhibitor of cAMP-dependent protein kinase AI and II)

(Qian *et al.*, 1996). Transfection of pRSV/CREB had no effect on the expression of human growth hormone that was fused to thymidine kinase promoter (TK) in another stable transfectant OK13 (Qian *et al.*, 1996). These studies demonstrate that isoproterenol stimulates the expression of ANG gene via the cAMP-dependent protein kinase A, and probably via the interaction of the 43 kDa-CREB with the putative CRE in 5'-flanking region of the ANG gene. Or the nuclear 43 kDa-CREB may have a modulatory role on the expression of the ANG gene.

4.4 ANG-CRE BINDING PROTEIN (52-kDa protein)

The cAMP effects on ANG gene transcription observed in our studies revealed a stimulation factor of approximately 2.5 fold (Ming *et al* 1993, 1995). Compared to other cAMP-responsive system, however, the induction of the ANG promoter achieved in our assay system is relatively low. For instance, the appropriate regions (CRE) of the human gonadotropin alpha subunit gene and somatostatin gene promoters conferred over 10-fold stimulation by 8-Br-cAMP (Hoeffler, 1992) or forskolin (Brindle *et al*, 1995). This may be due to the deviation in the end of the ANG-CRE from the consensus sequence (TGACGTAC instead of TGACGTCA). However, a putative CRE with the similar deviation as in the ANG promoter is found in the promoter of the PEPCK or TAT genes. There might be a possibility that some element in the vicinity of ANG-CRE octamer exerts additional effects partly obscuring the cAMP induction mechanism in ANG gene. Alternatively, the stimulatory effect of cAMP on the expression of ANG gene is decreased by an inhibitory protein. This inhibitory protein functions via either blocking the binding of 43 kDa-CREB to ANG-CRE or attenuating the transcriptional activity of the 43 kDa-CREB. The latter possibility is supported by the results from the present studies.

In the present study, a double stranded oligonucleotide of the putative CRE element on the rat ANG gene was used to isolate a cDNA from mouse liver library. This cDNA encodes a nuclear protein, designated 52-kDa-protein, which binds to ANG gene CRE-like

sequence in vitro. The apparent structure of the 52-kDa-protein is not similar to either CREBs or ATF-1 or CREM-related proteins (Meyer and Habener, 1993) that are the proteins demonstrated to be able to bind to CRE sequence. These CRE-binding proteins belong to the bZIP superfamily, in which the members all contain the basic DNA-binding domain and the leucine zipper dimerization domain (bZIP). However, Southern blot (Fig.3-16) demonstrated that the 52-kDa-protein has little sequence homology to the 43 kDa-CREB. Not surprisingly, the sequence analysis of the cDNA and deduced amino acid of the 52-kDa protein show no the basic zipper structure. It is understood that the addition of the antibodies can decrease but not eliminate the binding of the labeled ANG-CRE with the nuclear proteins as showed in Fig.3-3. Also addition of rabbit polyclonal antibodies against the 43 kDa-CREB can not yield a complete supershift band in the gel mobility shift assays by employing the labeled ANG-CRE as probe (Fig. 3-4). These studies support the notion that the 52-kDa-protein is immunologically different from the 43 kDa-CREB. These results demonstrate that antibodies against the CREB or ATF-1 protein did not interfere with the binding of the labeled ANG-CRE with the 52-kDa-protein. The 52-kDa-protein is a non-bZIP CRE binding protein. Indeed, the previous studies have suggested that the CRE binding protein could be structurally distinct from CREB / ATF family members. Jansen *et al.* (1997) reported a protein that binds to the CRE of the prohormone convertase 1 promoter (PC1). Moreover, this protein did not cross-react with the antibodies against the bZIP family members: ATF-1, 2, 3, CREB-1, CREM, C/EBP, AP-1 (c-Jun), and c-Fos in the supershift assays, indicating that this protein is not a known member of the bZIP superfamily. UV cross-linking experiments demonstrated this potential novel protein was ~100 kDa in size. This protein has not yet been cloned or characterized further. This work is the first of identification of a non-bZIP CRE-binding protein

4.5 CLONING AND EXPRESSION OF ANG-CREB IN BACTERIAL SYSTEM

The cDNA of ANG-CREB is isolated by the strategy that was developed by Signs et al (1988). Briefly, the double strands DNA (or oligonucleotide) that represents the protein site was labeled with radioisotope, ^{32}P. The λgt11 library culture was treated with IPTG to induce the expression of proteins. The clone that expresses the DNA-binding protein will give the positive signal after incubation with the radioactive labeled DNA. So, this strategy is based on the DNA-protein interactions. Compared with the conventional cloning which is based on DNA-DNA interactions, this strategy was designed to clone genes encoding sequence-specific DNA-binding proteins, and undertaken with the particular aim of isolating genes specifying mammalian transcriptional regulatory proteins. This method is first established by Singh et al. (1988) in the isolation of the MHC class 1 H-2Kb gene with a recognition site probe, H2TF1. The vector used for the construction of the library must be an expression vector, or it must have the translation machinery, and express protein, in high concentration. The IPTG may be used as a inducer for the Lac promoter. IPTG functions by binding to the repressor protein and interrupted the binding of repressor to the gene operator, and therefore stimulates gene expression. Lastly, the protein can bind to the labeled DNA probe with relatively high binding constants since only these are likely to form complex with half-lives long enough to withstand the wash protocol. It may be possible to isolate recombinants encoding proteins with binding constant of 10^9 M^{-1} or lower (Singh *et al.,* 1988). Using DNA probes containing multiple binding sites that are spaced so that the probe can simultaneously bind two or more immobilized protein molecules might significantly enhance the sensitivity of this strategy for low affinity proteins. Alternative, it may be possible to covalently stabilize protein-DNA complexes by procedures such as UV-irradiation. Since the binding constants of regulatory proteins are dependent on ionic strength, temperature, and pH, these factors might also be manipulated to enhance detection. From Southwestern blot assay, we know that a 52-kda protein has a high affinity to ANG-CRE. Under the same condition, we screened a λgt11 cDNA library with an oligonucleotide containing a single ANG-CRE sequence. We isolated a cDNA clone that is 1345 base long. During this study, we found that a very similar cDNA sequence was

published by Li *et al* (1995). Li *et al.* used a polyclonal antibody against the prostaglandin D synthetase to screen the a human brain cDNA expression library, isolated a full-length cDNA of 2295 bp coding for a human cerebrospinal fluid protein designated cerebrin-50. Comparison of cDNA sequences of our clone and cerebrin-50, we found that our clone corresponded to the coding region of cerebrin-50, the identity is over 95% (Fig.3-17). Because such a high homology exists, there is a possibility that our clone and cerebrin-50 are counterparts in different species. By hot-nested PCR, the cerebrin-50 mRNA was found in human spleen, brain, liver, testis, and T lymphoblastoid cells (Li *et al.,* 1995). The present study, by Southwestern blot, we demonstrated that the ANG-CREB exists in liver, kidney, testis, brain, probably heart, but not in the spleen (Fig. 3-7). The difference in spleen might be result from the different species: human and mouse, or from the techniques used. In comparison with the Southwestern blot, PCR is much more sensitive.

For the further study we tried to express the protein in bacterial expression system. We constructed the cDNA in both orientations, sense and antisense, into bacterial expression vector pMalc, and expressed in TB1 cells. The pMalc vector alone (without insertion of cloned cDNA) was used as the control. After incubation, the bacterial extracts were prepared, and used for gel mobility shift and competition assays. The results were shown in Fig. 3-18 and Fig. 3-19. Only the bacterial extract from the pMalc/cDNA sense reveals specific binding. The cell extract from the bacteria transformed with pMalc alone or with reversed orientation of the cDNA showed no the specific binding.

4.6 THE FUNCTION OF ANG-CREB

ANG-CREB is suggested to be novel CRE binding protein. To study its physiological functions, we inserted the ANG-CREB cDNA into the mammalian expression vector, pRSV, in both orientations, sense and antisense, and co-transfected them with the pTKCAT/ANG-CRE into the Hepa 1-6 cells respectively. The results showed that without any treatment of the cells, compared with the control, the transfection of the 52-kDa-protein

cDNA has no effect on the ANG gene promoter activity (Fig. 3-20). However, when the cells were treated with isoproterenol, as reported previously, the ANG promoter activity was stimulated (Ming *et al*, 1995). This stimulation of isoproterenol was inhibited by the transfection of the sense ANG-CREB whereas the antisense ANG-CREB showed no effect on the stimulated levels (Fig.3-20). This primary data obtained from transient gene transfection assays indicated that the ANG-CREB functions as a repressor on the ANG gene promoter, which is contrary to that of 43 kDa-CREB. We may hypothesize that ANG-CREB works together with the 43 kDa-CREB or other transactive factors to assure that ANG gene expresses at a proper level. Usually ANG-CREB exists at a very low level or inactive, once the ANG gene was stimulated at a high level, ANG-CREB level may be increased and become bioactive. Indeed, our primary *in vivo* studies showed the increased 52-kDa-protein when the mouse was injected with the combination of cAMP and DEX (Fig. 3-21). This combination synergistically stimulated the ANG-gene expression as previously reported (Ming *et al.,* 1993). ANG-CREB and the 43 kDa-CREB were demonstrated to regulate the ANG gene promoter activity in the opposite ways that assure the ANG level is at a proper level. The interruption of this balance might result in physiological problems. In fact, this biological balance may be regulated at different levels, such as the dissociation /association of protein kinase A regulatory subunit (R) and catalytic subunit (C). Newly synthesized cAMP molecules bind to regulatory R subunits in A kinase holoenzymes, causing them to release their catalytic (C) subunits. These free C subunits then phosphorylate the substrates until the cAMP level falls, the R subunits regain their affinity for free C subunits, and thus form inactive holoenzymes again. However if cAMP levels remain persistently elevated, many cells change their A kinase system. Some cells alter the rate of degradation of subunits, and some cells change the level or stability of the messengers encoding subunits. Cellular behavior often changes if cAMP levels remain elevated: many cells differentiated, some cells proliferate, and some cells die, depending on the stage of the cell cycle. The two forms of A kinase holoenzyme (type I and type II) contain identical C subunits, but contain either a RI dimer or an RII dimer. In some tissues,

type II holoenzyme is compartmentalized to subcellular organelles via specific anchoring proteins, whereas type I holoenzyme is generally cytosolic. Free RI subunits turn over more rapidly than free RII subunits in most cells, but all free subunits are degraded more rapidly than when they are associated together in holoenzymes. Free C subunits can phosphorylate a broad spectrum of proteins in both the cytoplasm and nucleus, depending on the type of cell, its state of differentiation, and the hormone. If free C subunit is microinjected into the cytoplasm of some intact cells, it migrates to the nucleus, whereas if free R subunit is microinjected, it remains in the cytoplasm. If both subunits are co-injected, R subunit blocks the nuclear migration of the C subunit. A major nuclear target for free C subunits is the CREB family of nuclear proteins, which bind to cAMP response element (CRE) in the promoter regions of cAMP-responsive genes. Phosphorylation of CREB proteins alters their ability to form dimers and to interact with CREs. Many CREB proteins can be phosphorylated by other kinase as well, indicating that this represents a mechanism by which cells coordinate cAMP- and non-cAMP-mediated gene response. However, interactions between CREB and a number of other nuclear proteins with which they can dimerize, especially proteins whose levels are rapidly altered in response to hormones, provide an even higher degree of complexity of gene regulation than is possible from various kinases phosphorylating the different sites in CREB proteins.

4.7 THE MECHANISMS OF THE ANG-CREB FUNCTION

The physiologically role(s) of ANG-CREB is not clear at present. We have initially demonstrated that the ANG-CREB can bind to the ANG-CRE (Fig.3-2), and repress isoproterenol-stimulated level of ANG gene promoter in the transient gene transfection assays (Fig. 3-21). The mechanism about how the ANG-CREB works is not clear. Our studies have shown that not only the 52-kDa-protein, but also the 43 kDa-CREB could bind to ANG-CRE. The 43 kDa-CREB showed a stimulatory effect on the ANG promoter in

expression of pOCAT (ANG N-1498/+18) in a dose-dependent manner. The maximal and one-half maximal stimulation of the expression of the pOCAT (ANG N-1498/+18) were found with 10^{-3} and 10^{-5} mol/L of 8-Br-cAMP, respectively. However, when the same fusion gene was transfected into mouse hepatoma cells (Hepa 1-6), 8-Br-cAMP could not stimulate the gene expression significantly. The 8-Br-cAMP function required the addition of dexamethasone (DEX) in Hepa 1-6 cells. DEX alone stimulated the fusion gene expression when transfected into both OK and Hepa 1-6 cells. In Hepa 1-6 cells, DEX stimulated fusion gene expression with a maximal stimulation at 10^{-4} mol/L. cAMP alone did not stimulate gene expression significantly. The combination of DEX (10^{-6} mol/L) and 8-Br-cAMP (10^{-3} mol/L) was synergistic. Considering the difference in the molecular weight of this ANG-CRE binding protein in Hepa 1-6 cells and OK cells, the cell-specificity of the fusion gene expression could be in part explained the result from the modification of 52-kDa protein in OK cell. The higher molecular weight of the protein in the OK cell may suggest a structural difference. Although this difference does not show the effect on the protein binding to DNA in vitro (Fig.3-7), it might affect the transcriptional activity and results in the different expression pattern of ANG gene. In Hepa 1-6 cells, the ANG-CRE binding protein exerts inhibitory function, the addition of the DEX can release the inhibition. We hypothesize that the glucocorticoid receptor interacts with the 52-kDa protein or 43 kDa-CREB or both, to change their DNA binding affinity and results in the changes of transcriptional activity. At present, we miss the experiments to prove that glucocorticoid receptor can interact with the 52-kDa-protein, but it was reported to interact with 43 kDa-CREB. Nevertheless more experiments are required to reveal the molecular mechanism of the ANG-CREB functions.

5. Conclusion

1. A cAMP-responsive element (CRE) was identified on the 5' regulatory region of rat angiotensinogen (ANG) gene. This ANG-CRE motif was located at -795 / -788. Compared to the previously reported parlindromic CRE in the somatostatin gene, the last two nucleotides are in reverse order in ANG-CRE.

2. A full length of cDNA was isolated from mouse liver cDNA library. This cDNA encodes a nuclear protein that binds to ANG-CRE in vitro. Because of its molecular weight, we designated it as 52-kDa-protein.

3. The 52-kDa-protein exists in the mouse liver, kidney, testis and brain but not the spleen.

4. The 52-kDa protein is thermostable.

5. DNA sequence demonstrates that the 52-kDa protein is different from the members of leucine zipper family, although all CRE-binding proteins identified so far belong to this family.

6. The 52-kDa-protein can decrease the stimulated levels of ANG gene promoter activity in cultured cells. It may play an important physiological role in keeping the angiotensinogen at a proper level.

6. Future Work

As discussed above, the 52-kDa-protein is a novel CRE-binding protein that is structurally different from the members of CREB/ATF family identified so far. As a novel regulatory protein, the information we have obtained is limited. We believe that the following experiments will be of interest in the future:

1. The most important aspect is to elucidate the physiological role of the 52-kDa protein. Although the results from transient gene transfection assay suggested that ANG-CREB might function as an inhibitor on the ANG gene expression, this data is preliminary.

2. Our studies have demonstrated that both the 52-kDa-protein and the 43 kDa-CREB interact with ANG-CRE and play a role in the expression of the ANG gene. It is possible that these two proteins may interact directly or indirectly, and regulate the gene expression cooperatively. Experiments are needed to reveal the relationship between the 52-kDa protein and the 43 kDa-CREB.

3. Phosphorylation is known to be important to the functional 43 kDa-CREB, and it could also be important to the functional 52-kDa-protein. Experiments are needed to study whether the phosphorylation may affect either the DNA-binding ability or transactivity of the 52-kDa protein, or both.

4. To better understand how the 52-kDa protein works, experiments are needed to further characterize the structure of the 52-kDa-protein. Such as the DNA binding domain, transactive domain, and probably a protein-protein interaction domain by which the ANG-CREB interacts with the 43 kDa-CREB. For this purpose different DNA constructs should be made.

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transient gene transfection assays (J.F. Qian *et al.,* 1997), which is contrary to that of 52-kDa-protein which exhibited repressor activity. These two proteins are able to bind to the same DNA element, and in this regard we hypothesize that there might be a competition between the 43 kDa-CREB and ANG-CREB for the ANG-CRE sequence. Our previous studies have shown that either 8-Br-cAMP or forskolin or isoproterenol could just stimulate ANG gene promoter activity by a maximum 2.5-3.0 fold, even if the extraneous 43 kDa-CREB cDNA is introduced. This stimulation is relatively low compared with other expression systems, such as human TSHβ gene expression system, as forskolin induced CAT activity by about 12-fold (Hans, *et al.,* 1991). Increasing doses of the drugs or CREB could not elevate the stimulation level of ANG gene promoter. It seems that the cAMP-PKA-CREB pathway was blocked. The 2.5-fold induction might be thought to be due to the nonspecific effect of the drugs (Hans, *et al.,* 1991). Alternatively, these results might be explained by the hypothesized molecular mechanism: the 52-kDa protein functions as a repressor protein that competes with the 43 kDa-CREB to bind to ANG-CRE. Because of the much higher affinity of the 52-kDa protein to ANG-CRE than 43 kDa-CREB, which has been demonstrated by gel mobility shift and Southwestern blot assays in vitro, the presence of the 52-kDa protein effectively blocks the binding of 43 kDa-CREB to ANG-CRE. In addition to the competition hypothesis, we can also assume that the 52-kDa protein and the 43 kDa-CREB may interact and form a heterodimer complex. Compared with the 43 kDa-CREB homodimer, this complex may have lower DNA binding affinity and transcriptional activity to ANG gene, and can't effectively stimulate gene expression. This model may explain that introduce of 43 kDa-CREB cDNA in the transient gene transfection system resulted in higher level of the promoter activity compared with control. Because the over expressed CREB can saturate the endogenous 52-kDa-protein and the rest can form the homodimer which bind to ANG-CRE effectively, and stimulate the gene expression.

As we mentioned in the beginning of the result section, transient gene transfection in Hepa 1-6 cells and OK cells revealed the different results. When the transfection assays were carried in opossum kidney (OK) cells, 8-Br-cAMP (10^{-7}-10^{-3} mol/L) stimulated the

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

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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 interacted 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.

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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-^{32}P]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 μg/ml aprotinin, 2 μg/ml leupeptin and 1 μg/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 μg/ml aprotinin, 2 μ/ml leupeptin, 1 μg/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 $MgCl_2$/0.1 mM EDTA/1.0 mM DTT/0.1 mM PMSF/2 μg/ml leupeptin/2 μg/ml aprotinin/1 μg/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 $(NH_4)_2SO_4$ 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 *Hin*dIII (polylinker site of pGEM-3 plasmid) and end-labelled with T_4 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 μg) or mouse liver nuclear extract (0–20 μg) in a total volume of 50 μl of buffer containing 1 μg 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 $MgCl_2$, 1 mM $CaCl_2$ 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 μl of 100 mM Tris/HCl, pH 7.6, containing 100 mM NaCl, 15 mM EDTA, 0.375% SDS, 150 μg/ml proteinase K and 100 μg/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-^{32}P]ATP by using T_4 polynucleotide kinase. Mouse liver nuclear proteins (10 μg) or BSA (10 μg) 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 μg) 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 °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 μg/ml non-denatured herring sperm DNA at 4 °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.

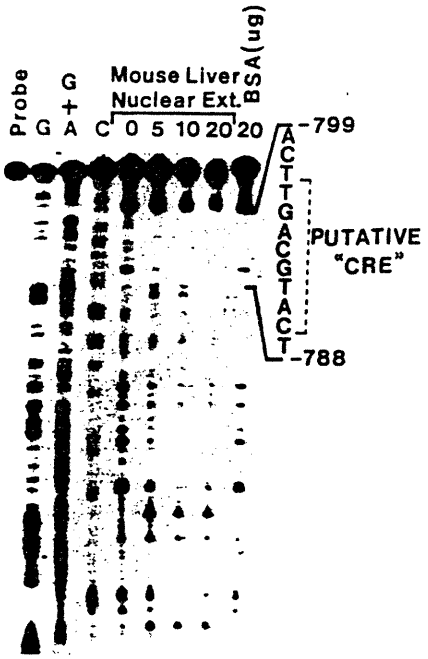

Figure 1 DNase 1 footprinting analysis of the DNA fragment, ANG nt −814/−689, of the ANG gene

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: ug, μg.

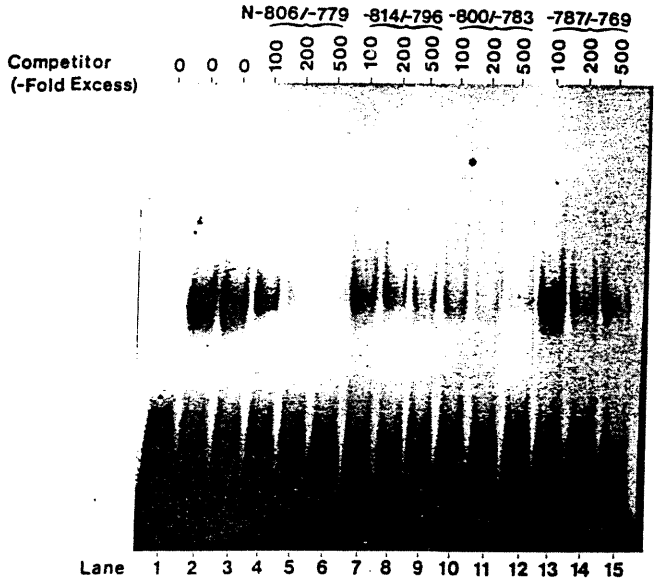

Figure 2 Gel mobility-shift assay of the radioactively labelled DNA fragment ANG nt −806/−779 with the mouse liver nuclear proteins

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.

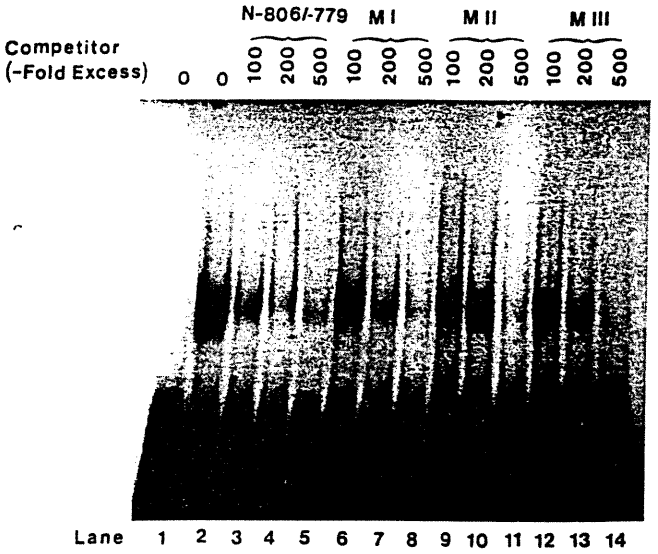

Figure 3 Autoradiography of the gel mobility-shift assay of the radioactively labelled DNA fragment ANG nt −806/−779 with the mouse liver nuclear proteins

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

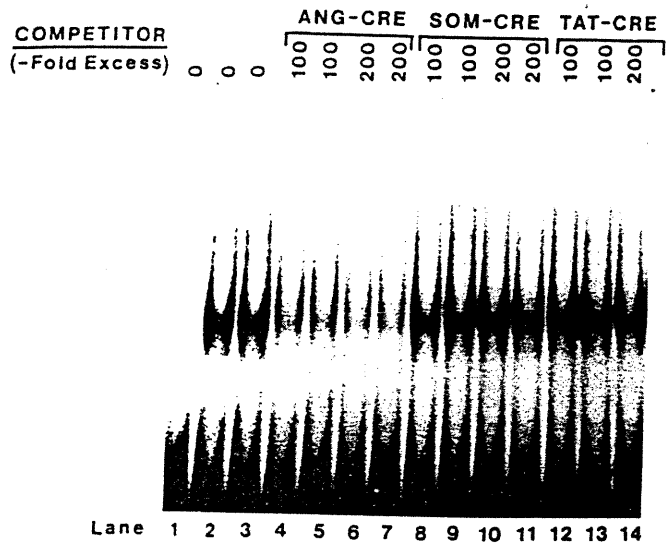

Figure 4 Gel mobility-shift assay of the radioactively labelled DNA fragment ANG nt −806/−779 with the mouse liver nuclear proteins

The labelled DNA probe (0.1 pmol) was incubated with BSA (10 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, 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.

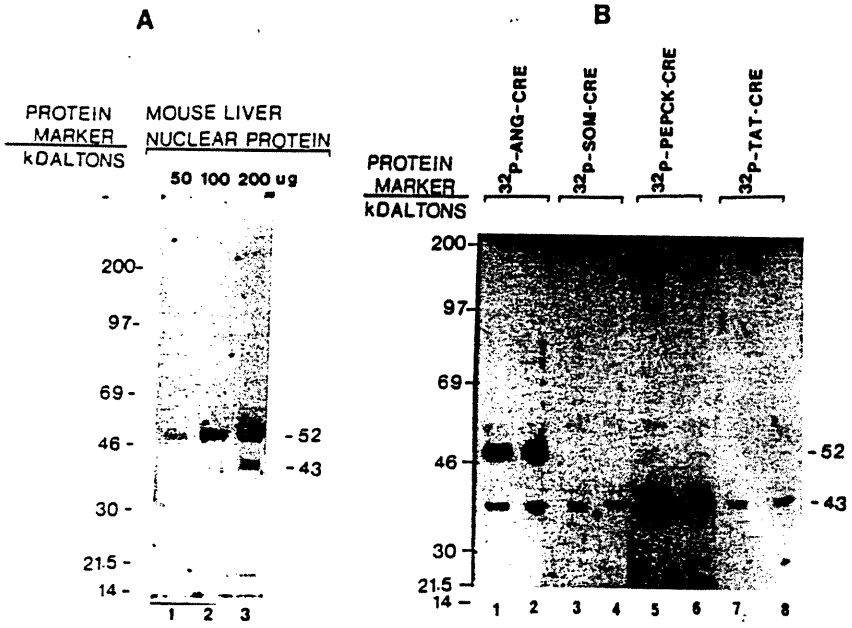

Figure 5 Southwestern analysis with the labelled DNA probes

(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 μg of nuclear proteins was loaded per well).

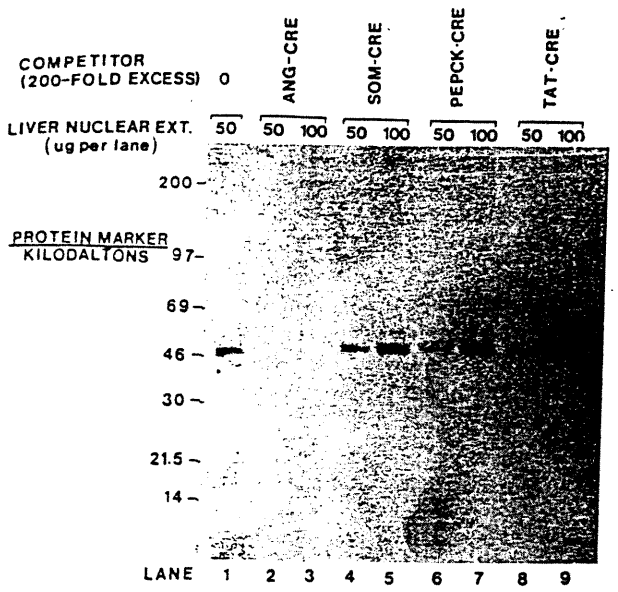

Figure 6 Effect of addition of the competitor DNA in the Southwestern analysis with labelled ANG nt −806/−779

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 °C. Then the membrane was hybridized with radioactively labelled ANG nt −806/−779, washed and subjected to autoradiography.

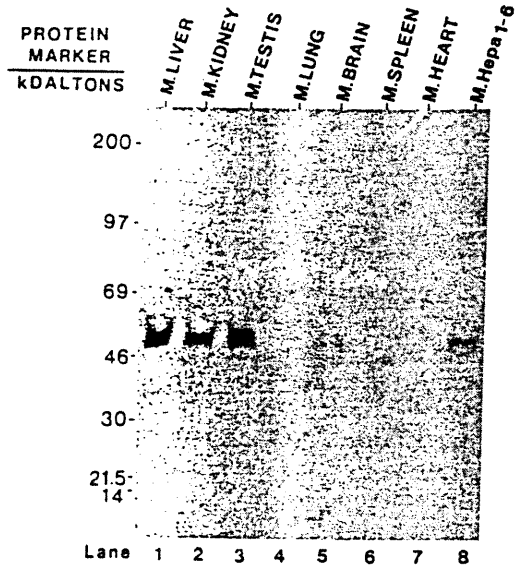

Figure 7 Distribution of the 52 kDa protein in various mouse tissues as analysed by Southwestern blot analysis

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.

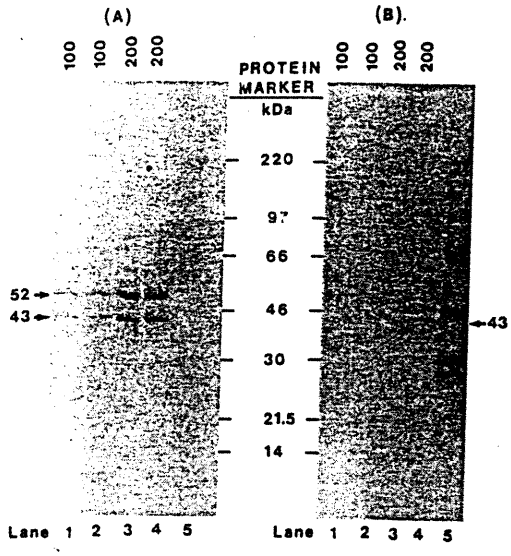

Figure 8 Southwestern and Western blot analysis of immunoreactive 43 kDa CREB from the mouse liver nuclear extract

(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 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

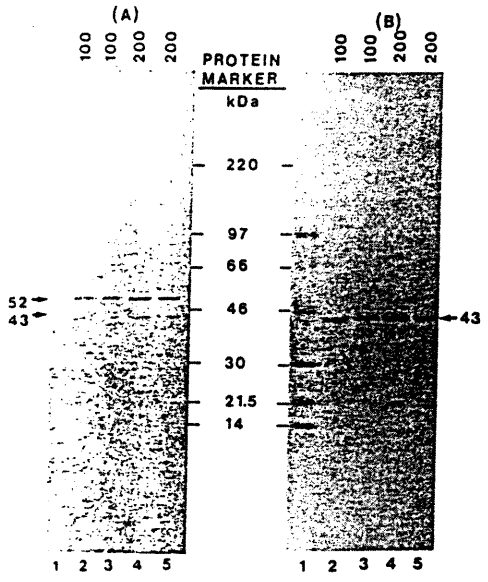

Figure 9 Southwestern and Western blot analysis of 43 kDa CREB from the mouse liver nuclear extract

(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 I footprinting protection and gel mobility-shift assays. Our DNase I 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 I 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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Angiotensinogen Gene Expression Is Stimulated by the cAMP-Responsive Element Binding Protein in Opossum Kidney Cells

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Abstract. It has been reported previously that the addition of isoproterenol or forskolin stimulates the expression of the angiotensinogen (ANG) gene in opossum kidney (OK) 27 cells, an OK cell line with a fusion gene containing the 5′-flanking regulatory sequence of the rat ANG gene fused with a human growth hormone (hGH) gene as a reporter, pOGH (ANG N-1498/+18), permanently integrated into their genomes. To investigate whether the effect of isoproterenol or forskolin on the expression of the ANG gene is mediated via the nuclear 43-kD cAMP-responsive element binding protein (CREB), OK 27 cells were transiently transfected with an expression plasmid containing the cDNA for the 43-kD CREB (pRSV/CREB). The level of expression of the pOGH (ANG N-1498/+18) in OK 27 cells was estimated by the amount of immunoreactive hGH secreted into the culture medium. Transfection of pRSV/CREB alone stimulated the expression of pOGH (ANG N-1498/+18). The addition of isoproterenol or forskolin further enhanced the stimulatory effect of pRSV/CREB on the expression of pOGH (ANG N-1498/+18). The enhancing effect of isoproterenol was inhibited by the presence of propranolol (an inhibitor of β-adrenoceptors) and (*R*)-*p*-adenosine 3′5′-cyclic monophospho-orthioate (Rp)-cAMP (an inhibitor of cAMP-dependent protein kinase A I and II). Transfection of pRSV/CREB had no effect on the expression of thymidine kinase growth hormone in OK 13 cells, an OK cell line with a fusion gene containing the promoter/enhancer DNA sequence of the viral thymidine-kinase gene fused with an hGH gene as a reporter, thymidine kinase growth hormone, permanently integrated into their genomes. These studies demonstrate that isoproterenol stimulates the expression of ANG gene via the cAMP-dependent protein kinase A and probably via the interaction of the 43-kD CREB with the 5′-flanking region of the ANG gene. Our data indicate that the nuclear 43-kD CREB may have a modulatory role on the expression of the ANG gene in OK cells. (J Am Soc Nephrol 8: 1072–1079, 1997)

Angiotensinogen (ANG) mRNA has been localized in rat renal proximal tubules by the techniques of *in situ* hybridization (1) and PCR (2). Furthermore, studies by Wolf and Neilson (3) and Tang *et al.* (4) showed that ANG mRNA is expressed in mouse and rat immortalized proximal tubular cell lines, respectively. We (5), as well as Ingelfinger *et al.* (6), have also demonstrated that the ANG mRNA is expressed in opossum kidney (OK) proximal tubular cells. Thus, these studies demonstrate that the intrarenal angiotensin (Ang) I and II are probably derived from the ANG synthesized in renal proximal tubules.

We have reported previously that thyroid hormone ($L\text{-}T_3$), dexamethasone, 8-bromo-cAMP (8-Br-cAMP), and forskolin stimulate the expression of the ANG gene in OK cells *in vitro* in a dose-dependent manner (5,7,8). We have also demonstrated that isoproterenol stimulates the expression of the ANG gene in OK cells (9). The effect of isoproterenol is mediated via the β_1-adrenoceptor and is blocked by the presence of propranolol (β-adrenoceptor blocker), atenolol (β_1-adrenoceptor blocker), and (*R*)-*p*-adenosine 3′5′-cyclic monophospho-orthioate (Rp)-cAMP (an inhibitor of cAMP-dependent protein kinase A I and II) (9), but not by ICI 118,551 (β_2-adrenoceptor blocker). The specificity of the β_1-adrenoceptor is supported further by our more recent studies that isoproterenol stimulates the expression of the ANG gene when cotransfected with an expression vector containing the cDNA for the β_1-adrenoceptor, but not the cDNA for the β_2-adrenoceptor (10). Our studies confirm the report of Nakamura and Johns (11) that the administration of atenolol blocks the effect of renal nerve stimulation after the increase of the ANG mRNA level in the rat kidney *in vivo*. Furthermore, our studies (9,10) and those of Nakamura and Johns (11) together suggest the presence of a functional relationship between the renal sympathetic nervous system and the activation of local renal renin-angiotensin system. Thus, the local formation of renal Ang II may play an important role in the modulation of the physiology of the renal proximal tubular cells (*i.e.*, sodium and fluid reabsorption) (12–17).

The exact molecular mechanism(s) for the effect of isoproterenol on the expression of the ANG gene in OK cells is unknown. One possibility may be that the addition of isopro-

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terenol stimulates the synthesis of intracellular cAMP, which we have demonstrated previously (9). The intracellular cAMP then binds to the regulatory subunit of cAMP-dependent protein kinase A (PKA) and releases the catalytic subunit of PKA. The free catalytic subunit of PKA then translocates into the nucleus and subsequently phosphorylates the nuclear cAMP-responsive element binding protein (CREB) (18) or CREB-like proteins. The phosphorylated CREB or CREB-like protein(s) then interacts with the putative cAMP-responsive element (CRE) (*i.e.*, ANG N-795 to N-788, TGACGTAC) in the 5′-flanking region of the rat ANG gene (19) and enhances the expression of the ANG gene.

Thus, in the present studies, we investigated the possibility of whether the 43 kD-CREB will directly enhance expression of the ANG gene in OK cells. Our studies demonstrated that the transient gene transfection of the plasmid containing the cDNA for the 43-kD CREB (pRSV/CREB) stimulates the expression of the fusion gene pOGH (ANG N-1498/+18) in OK 27 cells (9), an OK cell line into which has been stably integrated the fusion gene pOGH (ANG N-1498/+18) containing the 5′-flanking regulatory sequence of the rat ANG gene fused with a human growth hormone (hGH) gene as a reporter. The addition of isoproterenol or forskolin further enhanced the stimulatory effect of pRSV/CREB on the expression of pOGH (ANG N-1498/+18). Finally, the addition of propranolol and Rp-cAMP blocked the enhancing effect of isoproterenol on the expression of the pOGH (ANG N-1498/+18) in OK 27 cells when transfected with pRSV/CREB.

Materials and Methods

Materials

The fusion gene pOGH (ANG N-1498/+18) containing the 5′-flanking sequence (1498 bp) upstream of the transcriptional site plus 18 bp of Exon I of the rat ANG gene fused with an hGH gene, has been described previously (19). The plasmid pRSV-Neo containing the coding sequence for Neomycin (Neo) with the Rous sarcoma virus (RSV) enhancer/promoter sequence fused in the 5′-end of the Neomycin gene was a gift from Dr. Teresa Wang (Department of Pathology, Stanford University, Stanford, CA). The plasmid pTKGH containing the thymidine kinase (TK) enhancer/promoter sequence fused to the 5′ end of the hGH gene was purchased from the Nichols Institute of Diagnostics (La Jolla, CA). The plasmid pGEM-3 as well as the pTKCAT and pRSVCAT vectors containing the coding sequence for chloramphenicol acetyltransferase (CAT) with TK or RSV enhancer/promoter sequence in the 5′ end of the CAT gene, respectively, were a gift from Dr. Joel F. Habener (Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Boston, MA).

The expression plasmid containing the cDNA for the 43-kD CREB, pRSV/CREB, was constructed as follows. Briefly, total cellular RNA was prepared from adult, male rat liver (Wistar, Kyoto, Japan) by guanidium isothiocyanate/cesium chloride gradient as described previously (5). Twenty micrograms of total RNA was used to synthesize the double-strand cDNA for CREB by the method of reverse transcription and amplification by PCR (Thermal Cycler) according to the manual supplied by the manufacturer (Perkin Elmer Cetus, Inc., Foster City, CA). The nucleotide sequences for two oligonucleotide primers corresponding to the first 18 nucleotides (5′-ATG ACC ATG GAC TCT GGA-3′) and the last 18 nucleotides (5′-TTA ATC TGA CTT GTG GCA-3′) in the opening reading frame of the rat CREB (18), respectively, were used in PCR. The oligonucleotides were synthesized by Biosynthesis, Inc. (Lewisville, TX). One-kilobase CREB cDNA fragment was amplified and then subcloned in the plasmid pBluescript (SK^-) (Stratagene, Inc., La Jolla, CA). The sequence for the CREB cDNA was confirmed by dideoxy sequencing with T_3 and T_7 primers (Stratagene, Inc.). Finally, the CREB cDNA containing the entire coding sequence was isolated and inserted into a mammalian cell expression vector pRC/RSV (purchased from Invitrogen, Inc., San Diego, CA). The plasmid pRC/RSV contains the RSV/long terminal repeat promoter and enhancer plus a bovine growth hormone polyadenylation signal for polyadenylation of transcribed mRNA.

The RIA kit for hGH (RIA-hGH) was a gift from National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. The RIA procedure has been described previously (19). National Institute of Arthritis, Metabolism, and Digestive Diseases-hGH-I-1 (AFP-4793 B) was used for both iodination and as a hormone standard. The limit of sensitivity of the assay was 0.1 ng/ml. The inter- and intra-assay coefficients of variation were 10% (n = 10) and 12% (n = 10), respectively.

R(−)-isoproterenol (+)-bitartrate salt, forskolin, *S*(−)-propranolol hydrochloride, and Rp-cAMP (an inhibitor of the cAMP-dependent protein kinase A I and II) (20) were all purchased from Research Biochemicals, Inc. (Natick, MA).

Na-^{125}I was purchased from Dupont, New England Nuclear (Boston, MA). Calcium chloride was purchased from Mallinckrodt, Inc. (Montreal, Quebec, Canada), and Geneticin (G 418) was purchased from Bethesda Research Laboratories (Gibco-BRL, Burlington, Ontario, Canada). Other molecular biology grade reagents were obtained either from Sigma (St. Louis, MO), Gibco-BRL, Boehringer-Mannheim (Dorval, Quebec, Canada), Pharmacia, Inc. (Baie d'Urfe, Quebec, Canada) or Promega-Fisher, Inc. (Montreal, Quebec, Canada).

Cell Culture

The OK proximal tubular cell line was obtained from the American Tissue Culture Collection (Rockville, MD). This cell line is derived from the kidney of a female American opossum, retains several properties of proximal tubular epithelial cells in culture (21,22), and expresses a low level of ANG mRNA (5–6). The culture conditions of OK cells have been described previously (5,7–10).

OK Cell Stable Transformants

Clones OK 27 and OK 13 are stable transformants with pOGH (ANG N-1498/+18) and pTKGH integrated into OK cellular genomes, respectively. The characteristics of these clones have been reported previously (9). Briefly, these clones were grown in the medium containing 500 μg/ml G 418 for more than 3 mo and expressed a high amount of immunoreactive (IR)-hGH into the medium. The expression of pOGH (ANG N-1498/+18) and pTKGH in OK 27 and OK 13 cells, respectively, was time-dependent. The levels of IR-hGH in cellular extracts were consistently less than 5% of that found in the media, indicating that IR-hGH is not stored in the cell (9).

DNA Transfection

The method of transient gene transfection into OK 27 and OK 13 cells is similar to that described previously in OK cells (7–10). Briefly, cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) immediately after transfection. Twenty-four hours after the DNA transfection, the media were replaced with fresh media without FBS. The media and cells

were harvested 24 h later and assayed for IR-hGH and CAT activity, respectively.

To study the effect of isoproterenol or forskolin on the expression of the fusion gene (pOGH (ANG N-1498/+18)) in OK 27 cells transfected with pRSV/CREB, cells were incubated in DMEM containing 1% depleted FBS, and various concentrations of hormones were added on day 1 after DNA transfection. After a 24-h incubation period, the media and cells were harvested for hGH assays and CAT enzymatic assays, respectively. The control plasmid pRC/RSV was used as negative control.

To normalize the efficiency of transfection of the plasmid, 2 μg of pTKCAT (a vector with the TK enhancer/promoter fused to the coding sequence of CAT) was cotransfected with pRSV/CREB, pRC/RSV, or pGEM-3 as internal control for normalization. The levels of CAT activity in the control groups (*i.e.*, Figure 1, cells transfected with 2 μg of pTKCAT but without the cotransfection with either pRSV/CREB or pRC/RSV) were used as 100% transfection efficiency. The levels of CAT activity in other groups (*i.e.*, cells cotransfected with 2 μg of pTKCAT and pRC/RSV (at various concentrations) or pRSV/CREB (at various concentrations) were compared with the control group as percentage of transfection efficiency. Subsequently, the IR-hGH levels in groups cotransfected with either pRSV/CREB or pRC/RSV were normalized with the percentage of transfection efficiency as compared with controls (group 1). In each experiment, an additional group of cells was transfected with 2 μg of pRSVCAT (a plasmid with the RSV enhancer/promoter fused to the coding sequence of CAT) as the positive control for the comparison of transfection efficiency. The transfection efficiency of pTKCAT ranged from 25 to 55% compared with pRSVCAT.

The depleted FBS was prepared by incubation with 1% activated charcoal and 1% AG 1 × 8 ion-exchange resin (Bio-Rad Laboratories, Richmond, CA) for 16 h or more at room temperature as described by Samuels *et al.* (23). This procedure removed endogenous steroid and thyroid hormones from the FBS as demonstrated by Samuels *et al.* (23).

Effect of pRSV/CREB on the Expression of pOGH (ANG N-1498/+18) in OK 27 Cells

OK 27 cells were plated at a density of 1 to 2 × 10^5 cells/well in 6-well plates and incubated overnight in DMEM containing 10% FBS. Then, 2 μg of pTKCAT with or without 1 to 10 μg of pRSV/CREB or pRC/RSV per well were transiently cotransfected into OK 27 cells. After an overnight incubation, the media were replaced with fresh media without FBS and incubated for an additional 24 h. After incubation, cultured media and cells were harvested and kept at −20°C until assayed for IR-hGH and for CAT enzymatic activity, respectively.

Effect of Isoproterenol or Forskolin on the Expression of pOGH (ANG N-1498/+18) in OK 27 Cells Transfected with pRSV/CREB

OK 27 cells were plated at a density of 1 to 2 × 10^5 cells/well in 6-well plates and incubated overnight in DMEM containing 10% FBS. Then, 2 μg of pTKCAT and 5 μg of pRSV/CREB or pRC/RSV were transiently cotransfected into OK 27 cells. After an overnight incubation, the media were replaced with media containing 1% depleted FBS and various concentrations (10^{-13} to 10^{-5} M) of isoproterenol or forskolin and incubated for 24 h. At the end of the incubation period, media and cells were collected and kept at −20°C until assayed.

Effect of Propranolol or Rp-cAMP on the Expression of pOGH (ANG N-1498/+18) in OK 27 Cells Transfected with pRSV/CREB in the Presence of Isoproterenol

OK 27 cells were plated at a density of 1 to 2 × 10^5 cells/well in 6-well plates and incubated overnight in DMEM containing 10% FBS. Then, 2 μg of pTKCAT and 5 μg of pRSV/CREB or pRC/RSV were transiently cotransfected into OK 27 cells. After an overnight incubation, the media were replaced with media containing 1% depleted FBS, 10^{-9} M isoproterenol, and 10^{-6} M propranolol or 10^{-4} M Rp-cAMP. After 24 h of incubation, media and cells were collected and kept at −20°C until assay.

Effect of pRSV/CREB on the Expression of pTKGH in OK 13 Cells

OK 13 cells were plated at a density of 1 to 2 × 10^5 cells/well in 6-well plates and incubated overnight in DMEM containing 10% FBS. Then, 2 μg of pTKCAT with or without 1 to 10 μg of pRSV/CREB per well were transiently cotransfected into OK 13 cells. After an overnight incubation, the media were replaced with fresh media containing 1% depleted FBS and incubated for an additional 24 h. After incubation, cultured media and cells were collected and kept at −20°C until assayed.

Statistical Analyses

The experiments were performed at least three to four times in triplicate. The data were analyzed with *t* test or ANOVA. A probability level of $P \leq 0.05$ was regarded as significant.

Results

Effect of pRSV/CREB on the Expression of pOGH (ANG N-1498/+18) in OK 27 Cells

Figure 1 shows that the transfection of pRSV/CREB (1 to 10 μg DNA) per well stimulates the expression of pOGH (ANG N-1498/+18) in OK 27 cells. A dose-dependent relationship between pRSV/CREB and the stimulation of expression of the pOGH (ANG N-1498/+18) was observed for pRSV/CREB at 1 to 5 μg of DNA. It appears that the maximal stimulation was found with 5 μg of pRSV/CREB. At doses greater than 5 μg of pRSV/CREB, the expression of pOGH (ANG N-1498/+18) diminished. No significant stimulation of expression of the pOGH (ANG N-1498/+18) was observed with the control plasmid pRC/RSV (1 to 10 μg). These studies indicate that the expression of pOGH (ANG N-1498/+18) could be stimulated directly by CREB alone.

Dose–Response Curve of Isoproterenol or Forskolin on the Expression of pOGH (ANG N-1498/+18) Transfected with pRSV/CREB or pRC/RSV

Figure 2 shows that the addition of isoproterenol (10^{-11} to 10^{-5} M) stimulated the expression of the pOGH (ANG N-1498/+18) when transiently transfected with 5 μg of pRSV/CREB in a dose-dependent manner. It appears that the maximal effect of isoproterenol was found with 10^{-11} to 10^{-9} M isoproterenol. At concentrations greater than 10^{-9} M, the enhancing effect of isoproterenol was diminished.

Similarly, Figure 3 shows that addition of forskolin (10^{-11}

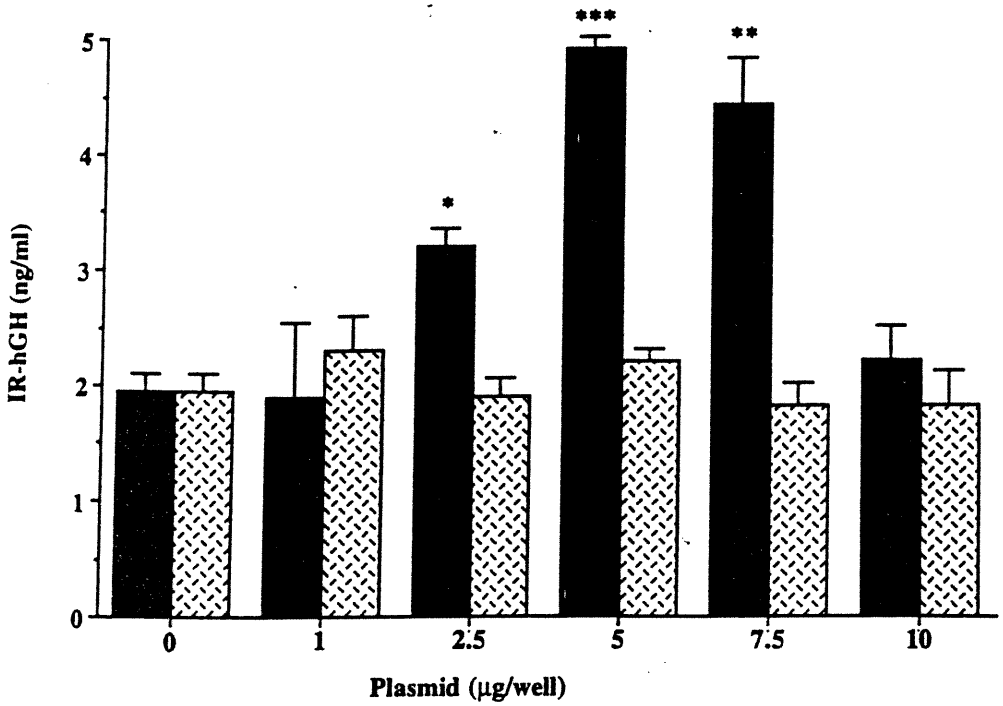


Figure 1. Basal expression of the pOGH (ANG N-1498/+18) in OK 27 cells when transiently transfected with different concentrations of plasmid containing the cDNA for the 43-kD CREB (pRSV/CREB) or with the control plasmid pRC/RSV. The levels of transcriptional activity of pOGH (ANG N-1498/+18) were quantified by the amount of immunoreactive human growth hormone (IR-hGH) in the medium assayed by a specific RIA for hGH (RIA-hGH). The concentration of IR-hGH in the medium of cells (2×10^5 cells per well) without transfection with pRSV/CREB or pRC/RSV is considered as the control level. Results are expressed as the mean ± SD of three determinations (triplicates). Solid bars, cells transfected with pRSV/CREB; stippled bars, cells transfected with pRC/RSV. Similar results were obtained from four independent xperiments. The data were normalized with the DNA transfection efficiency by cotransfection with 2 μg of pTKCAT as internal controls. The ransfection efficiency of pTKCAT in this experiment was approximately 40% compared with the positive control (*i.e.*, cells transfected with 2 μg of pRSVCAT. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.005$.

to 10^{-7} M) significantly stimulated the expression of the pOGH (ANG N-1498/+18) in OK 27 cells transfected with pRSV/CREB. The maximal and half-maximal effect of forskolin was found with 10^{-7} and 10^{-9} M forskolin, respectively. At concentrations greater than 10^{-7} M (*i.e.*, 10^{-5} M), the expression of the pOGH (ANG N-1498/+18) was inhibited.

Figure 4 shows that the addition of isoproterenol (10^{-9}) alone significantly stimulated the expression of pOGH (ANG N-1498/+18) in OK 27 cells compared with the control (without the addition of isoproterenol). The effect of isoproterenol was further enhanced ($P \leq 0.001$) when OK 27 cells were transiently transfected with pRSV/CREB (5 μg per well), but not when transfected with pGEM-3 (5 μg).

Effect of Propranolol or Rp-cAMP on the Expression of pOGH (ANG N-1498/+18) in OK 27 Cells Transfected with pRSV/CREB in the Presence of Isoproterenol

Figure 5 shows that addition of propranolol (10^{-5} M) or p-cAMP (10^{-4} M) blocked the stimulatory effect of isoproterenol (10^{-7} M) on the expression of pOGH (ANG N-1498/+18) in OK 27 cells when transiently transfected with pRSV/CREB.

Effect of pRSV/CREB on the Expression of pTKGH in OK 13 Cells

In OK 13 cells, transfection of pRSV/CREB (1 to 10 μg of DNA per well) had no effect on the expression of pTKGH compared with the controls (in the absence of pRSV/CREB) (Figure 6).

Discussion

Eukaryotic gene transcription is regulated by DNA sequence-specific transcription factors that bind to *cis*-acting enhancer and promoter elements. The CRE, one of the best studies of such an element, consists of a palindromic octanucleotide, TGACGTCA (24). Within the past 7 to 8 yr, at least 20 different CREB have been cloned, including CREB, activating transcription factor family, and cAMP response element modulator family (for review, see reference 25). CREB 341 (341 amino acid residues) and CREB 327 (327 amino acid residues) (they are tentatively designated as 43-kD CREB) are the best characterized CREB. They are coded from the same gene by two alternatively spliced mRNA. The CREB homodimers bind strongly to the symmetrical palindrome but less well to the asymmetrical sequence such as the CRE (TGACGTAA) of the phospho*enol*pyruvate carboxyl kinase gene (26) and CRE (TGACTTCA) of the α-subunit of human glycopro-

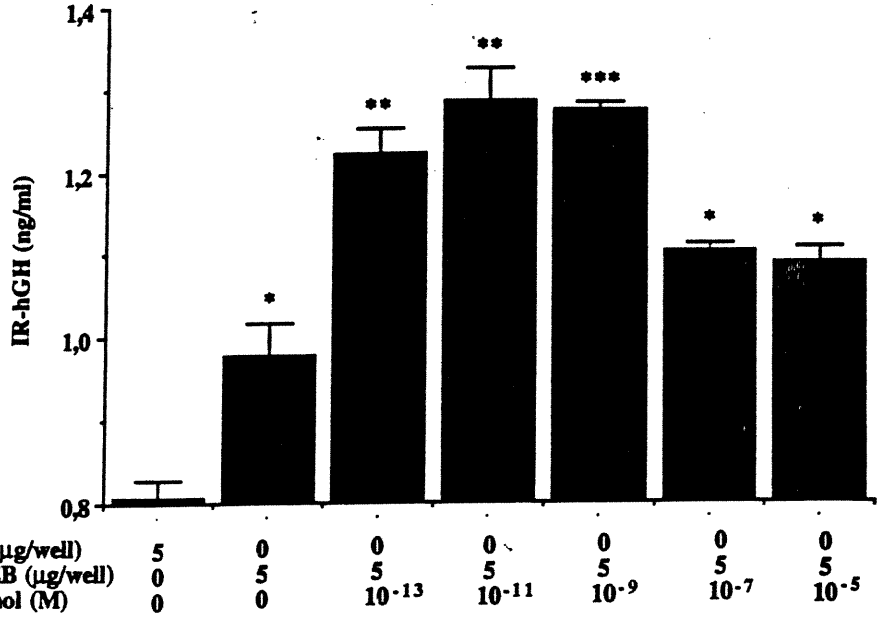


Figure 2. Dose–response relationship for the addition of isoproterenol on the expression of pOGH (ANG N-1498/+18) in OK 27 cells cotransfected with pRSV/CREB. Five micrograms of pRSV/CREB or pRC/RSV per well (1×10^5 cell) were used in the experiment. The effect of isoproterenol is compared with the control cells (transfected with 5 µg of pRSV/CREB but without the addition of isoproterenol). Results are expressed as the mean ± SD of a minimum of three determinations. Similar results were obtained in four independent experiments. The DNA transfection efficiency for this experiment was approximately 25% compared with pRSVCAT. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.005$.

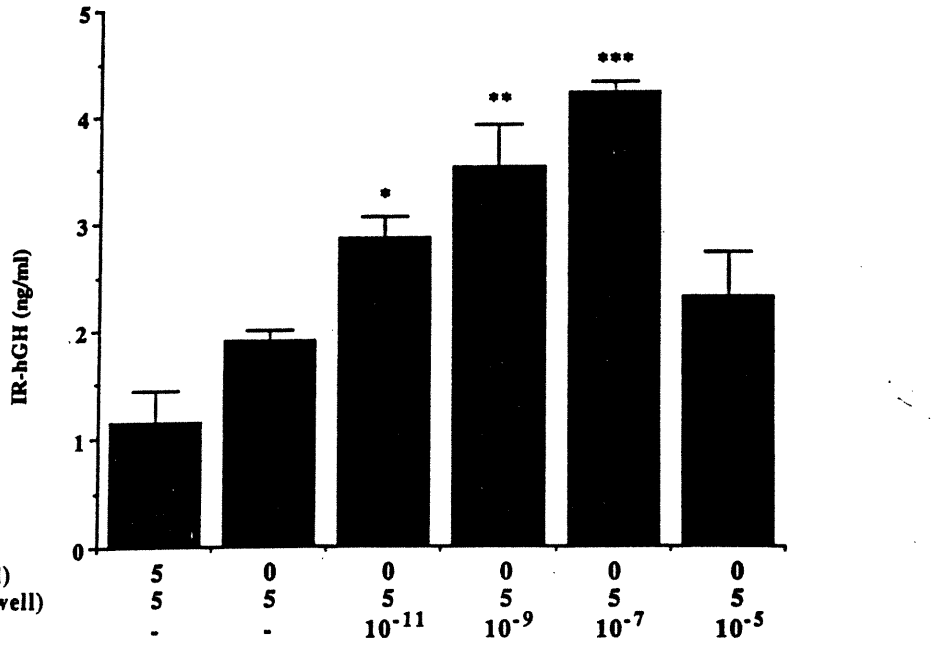


Figure 3. Dose–response relationship for the addition of forskolin on the expression of pOGH (ANG N-1498/+18) in OK 27 cells cotransfected with pRSV/CREB. Five micrograms of pRSV/CREB or pRC/RSV per well (2×10^5 cells) were used in the experiment. The effect of forskolin is compared with the control cells (transfected with 5 µg of pRSV/CREB but without the addition of forskolin). Results are expressed as the mean ± SD of a minimum of three determinations. Similar results were obtained in three independent experiments. The DNA transfection efficiency for this experiment was approximately 35% compared with pRSV/CAT. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.005$.

tein (27). Thus, the symmetry of the CRE sequence and its 5′- and 3′-flanking sequences determine the relative binding affinity and specificity of the CREB.

Our previous studies (19) on the DNA structure of the 5′-flanking sequence of the rat ANG gene showed that the DNA sequence of nucleotides N-795 to N-788 (TGACGTAC) is almost identical to the CRE (TGACGTCA) of the somatostatin gene (24), except that the last two nucleotides are in reverse order. With such homology, we raised the question of whether the sequence ANG N-795 to N-788 could be a putative

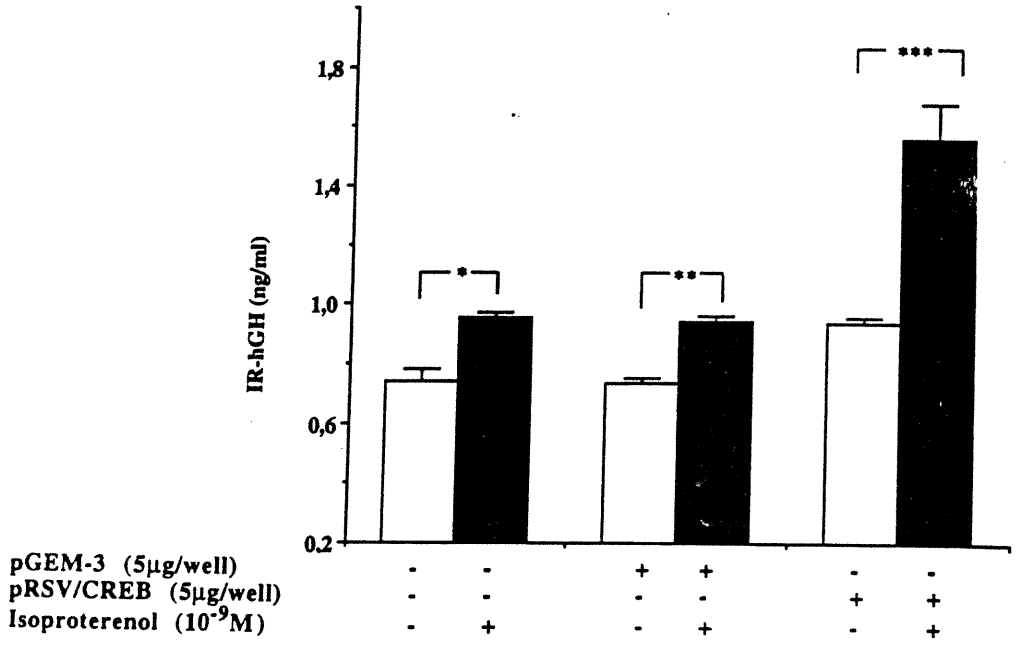
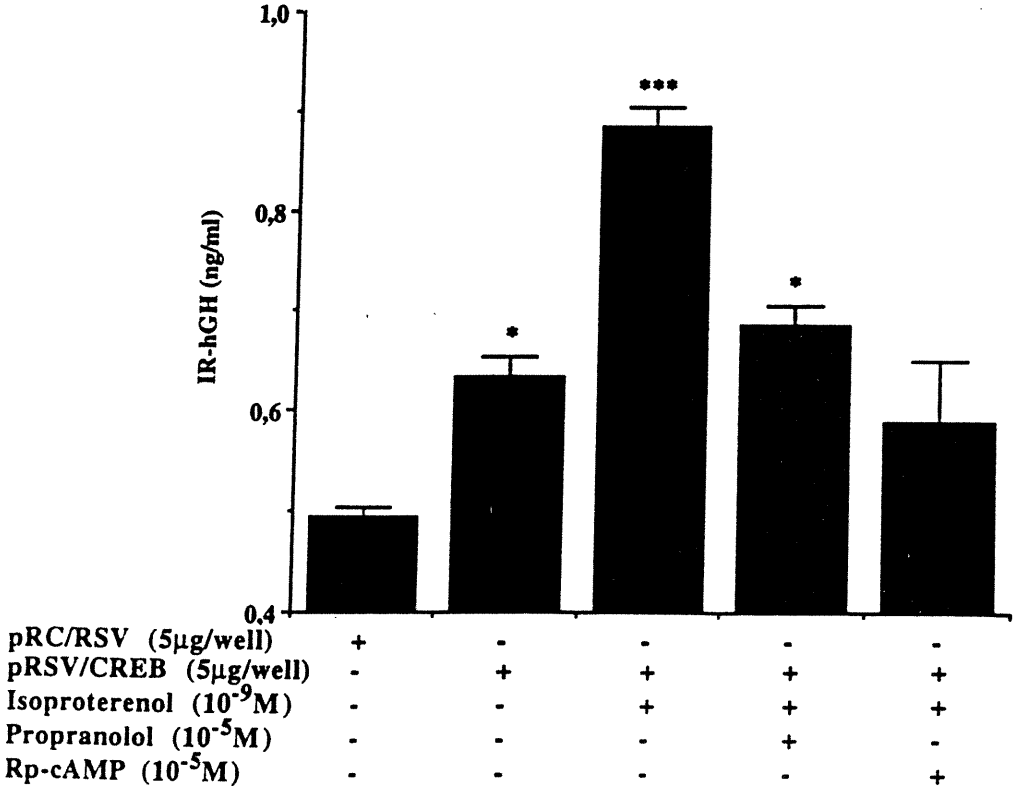


Figure 4. Effect of isoproterenol (10^{-9} M) on the expression of pOGH (ANG N-1498/+18) in OK 27 cells with or without the transfection of pRSV/CREB or pGEM-3. The concentration of IR-hGH in the medium of cells (1×10^5 cells) transfected with 5 μg of pGEM-3 or pRSV/CREB, but without the addition of isoproterenol (10^{-9} M), is considered as the control level. Results are expressed as the mean ± SD of a minimum of three determinations. Similar results were obtained from four additional experiments. The DNA transfection efficiency in this experiment was approximately 30% compared with pRSVCAT. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.005$.



igure 5. Inhibitory effect of propranolol or (*R*)-*p*-adenosine 3′5′-cyclic monophospho-orthioate (Rp-cAMP) on the expression of pOGH (ANG -1498/+18) in OK 27 cells when transfected with pRSV/CREB and stimulated by isoproterenol (10^{-9} M). Cells (1×10^5 cells per well) were ed for up to 24 h in the presence of isoproterenol (10^{-7} M) with or without propranolol (10^{-5} M) or Rp-cAMP (10^{-5} M). The centration of IR-hGH in the medium of cells (1×10^5 cells) transfected with 5 μg of pRSV/CREB, but without the addition of isoproterenol propranolol or Rp-cAMP, is considered as the control levels. The inhibitory effect of propranolol or Rp-cAMP was compared with cells that ere incubated with 10^{-9} M isoproterenol. Results are expressed as the mean ± SD of a minimum of three determinations. Propranolol (10^{-5}) and Rp-cAMP (10^{-5} M) consistently inhibited the stimulatory effect of isoproterenol in three experiments. The DNA transfection efficiency this experiment was approximately 55% compared with pRSVCAT. $*P \leq 0.05$; $***P \leq 0.005$.

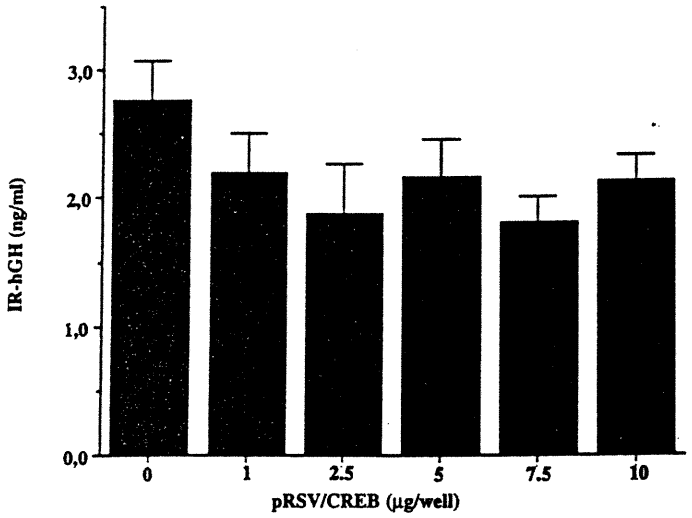


Figure 6. Expression of the pTKGH in OK 13 cells when transiently transfected with different concentrations of the plasmid pRSV/CREB. The levels of transcriptional activity of pTKGH were quantified by the amount of IR-hGH in the medium assayed by RIA-hGH. The concentration of IR-hGH in the medium of cells (1×10^5 cells) without transfection with pRSV/CREB is considered as the control level. Results are expressed as the mean ± SD of a minimum of three determinations. Similar results were obtained from three additional experiments. The DNA transfection efficiency in this experiment was approximately 35% compared with pRSVCAT.

CRE. Indeed, we have demonstrated that forskolin or 8-Br-cAMP directly stimulated the expression of pTKCAT (ANG N-814 to N-761) (a fusion gene containing the 5′-flanking region [nucleotides N-814 to N-761] of the rat ANG gene upstream of the TK promoter fused to a CAT gene in OK cells) (8). Thus, we speculate that CREB might mediate the effect of forskolin or 8-Br-cAMP on the expression of the ANG gene in OK cells via the binding to the putative CRE.

Our present gene transfection experiments showed that pRSV-CREB directly stimulated the expression of pOGH (ANG N-1498/+18) in OK 27 cells (Figure 1). The maximal stimulation was at 5 μg of DNA. At higher concentrations of DNA (*i.e.*, >5 μg), the effect of pRSV/CREB was diminished. At present, we do not know the reasons for this observation. One possible explanation may be that large amounts of DNA transfected into OK cells might exhaust the limited amount of cellular transcriptional factors. Nevertheless, more studies are warranted to clarify this observation.

Isoproterenol and forskolin are known to increase intracellular cAMP levels. Indeed, our previous studies have shown that the addition of isoproterenol increased the intracellular levels of cAMP in OK 27 cells (9). The present studies (Figures 2, 3, and 4) showed that addition of isoproterenol or forskolin further enhanced the stimulatory effect of pRSV/CREB compared with those without the transfection with pRSV/CREB. These studies support the hypothesis that isoproterenol stimulates the synthesis of intracellular cAMP. The elevated intracellular cAMP then activates the cAMP-dependent PKA and phosphorylates the nuclear CREB. Subsequently, the CREB binds to the CRE of the rat ANG gene and enhances the gene expression.

At present, we do not understand why forskolin at 10^{-5} M has an inhibitory effect on the expression of the pOGH (ANG N-1498/+18) in OK 27 cells when transfected with pRSV/CREB. One possible explanation may be that the high concentration of forskolin may exert other physiological effects (*i.e.*, Ca^{2+} transport) and subsequently alter the effect of CREB on the expression of the ANG gene in OK cells. Indeed, more experiments are required to clarify this observation.

Our results also showed that the addition of Rp-cAMP or propranolol blocked the enhancing effect of isoproterenol on the stimulatory effect of pRSV/CREB on pOGH (ANG N-1498/+18) gene expression in OK 27 cells (Figure 5). These studies suggest strongly that the effects of cAMP-dependent PKA and β-adrenergic receptor are involved in the expression of the ANG gene and probably are mediated via the CREB. Indeed, experiments are under way in our laboratory to investigate the direct involvement of cAMP-dependent PKA with the 43 kD CREB (*i.e.*, phosphorylation of 43-kD CREB).

Finally, it appears that pRSV/CREB had no effect on the expression of pTKGH in OK 13 cells (Figure 6). Because the expression of pTKGH in OK 13 cells is driven by the promoter/enhancer DNA sequence of the TK gene, these studies demonstrated that the promoter/enhancer DNA sequence of TK gene is not responsive to the addition of 43-kD CREB. The effect of CREB in OK 27 cells may be mediated via the interaction of CREB with the putative CRE (ANG N-795 to N-788) in the 5′-flanking region of the rat ANG gene of the fusion gene. Indeed, preliminary studies in our laboratory (28) have shown that the CREB is able to bind to the CRE of the ANG gene. Nevertheless, more experiments are under way in our laboratory to confirm this observation.

In summary, our present studies demonstrate that CREB directly stimulates the expression of pOGH (ANG N-1498/+18) in OK 27 cells. The stimulatory effect of CREB could be further enhanced by the presence of isoproterenol or forskolin. The addition of Rp-cAMP or propranolol could block the enhancing effect of isoproterenol. Our studies raise the possibility that the molecular mechanism(s) of the effect of renal nerve (that is, via the activation of β-adrenoceptors) on the expression of the renal ANG gene is probably mediated via the PKA and nuclear 43-kD CREB. The local formation of renal Ang II might then modulate the sodium and fluid reabsorption by the renal proximal tubular cells. Hence, the local intrarenal renin-angiotensin system might play a significant role in the modulation of sodium reabsorption.

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β-Adrenergic Receptors and Angiotensinogen Gene Expression in Mouse Hepatoma Cells In Vitro

Ming Ming, Jie Wu, Silvana Lachance, Aline Delalandre, Serge Carrière, John S.D. Chan

Abstract We have previously reported that addition of 8-bromo-cyclic AMP enhances the stimulatory effect of dexamethasone on the expression of the angiotensinogen gene in mouse hepatoma cells in vitro. Isoproterenol is known to stimulate the synthesis of hepatic intracellular cyclic AMP via β-adrenergic receptors. To study the possible effect of β-adrenergic receptors on the expression of the angiotensinogen gene in mouse hepatoma cells, we transiently transfected them with a fusion gene with the 5′-flanking region of the angiotensinogen gene linked to a bacterial chloramphenicol acetyltransferase coding sequence as a reporter, pOCAT (ANG N-1498/+18). The addition of isoproterenol (10^{-9} to 10^{-5} mol/L) alone had no stimulatory effect on the expression of pOCAT (ANG N-1498/+18). In the presence of dexamethasone (10^{-6} mol/L), however, isoproterenol enhanced the stimulatory effect of the dexamethasone on the expression of pOCAT (ANG N-1498/+18). The enhancing effect of isoproterenol was inhibited by the presence of propranolol (β_1- and β_2-adrenergic receptor antagonist) and ICI 118,551 (β_2-adrenergic receptor antagonist) but not by the presence of atenolol (β_1-adrenergic receptor antagonist). Furthermore, the addition of Rp-cAMP (an inhibitor of protein kinase A I and II) blocked the enhancing effect of isoproterenol. These studies demonstrated that isoproterenol enhances the stimulatory effect of dexamethasone on the expression of the angiotensinogen gene in mouse hepatoma cells via β_2-adrenergic receptor and cyclic AMP–dependent protein kinase pathways. Our data may be important in understanding the molecular mechanism(s) of the stimulatory effect of catecholamines glucocorticoid-induced expression of the angiotensinogen gene in the liver. (*Hypertension*. 1995;25:105-109.)

Key Words • β-adrenergic receptors • angiotensinogen gene • hepatoma cells

Circulating angiotensinogen (ANG) is synthesized mainly in the liver and cleaved successively by renin and angiotensin-converting enzyme to yield the bioactive peptide angiotensin II (Ang II). Recent studies by Kimura et al[1] and Fukamizu et al[2] demonstrated that transgenic mice that carry an exogenous ANG gene and/or renin gene and express high levels of plasma ANG and Ang II will develop high blood pressure. These studies demonstrated unequivocally that ANG is an important component for the development of hypertension.

We have previously reported the expression of the ANG gene in mouse hepatoma (Hepa 1-6) cells and shown that dexamethasone stimulates the expression of the fusion genes containing the 5′-flanking region of the rat ANG gene fused with a bacterial chloramphenicol acetyltransferase (CAT) coding sequence as reporter in a dose-dependent manner.[3] Furthermore, we have shown that addition of 8-bromo-cyclic AMP (8-Br-cAMP) enhanced the stimulatory effect of dexamethasone on the expression of angiotensinogen–chloramphenicol acetyl transferase (ANG-CAT) fusion genes. The addition of 8-Br-cAMP alone, however, had no stimulatory effect on the expression of the ANG-CAT fusion genes. These studies suggest that dexamethasone and cAMP might act synergistically or cooperatively to stimulate the expression of the ANG gene in the liver.

A classic example of the activation of the membrane adenylate cyclase system to increase intracellular cAMP in the liver is seen with catecholamines. Catecholamines (norepinephrine and epinephrine) are known to interact with both α- and β-adrenergic receptors.[4-6] 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.[7,8] The biological responses to interaction of isoproterenol (a β_1- and β_2-adrenergic receptor agonist) are generally mediated by an increase of intracellular cAMP, which subsequently initiates the biochemical cascade, including glycogenolysis in the liver.[9-12] It is not clear, however, whether isoproterenol has an effect on the expression of the ANG gene in the liver.

β-Adrenergic receptors are present in mouse liver.[13] The objective of our present study was to investigate whether addition of isoproterenol enhances the stimulatory effect of dexamethasone on the expression of ANG-CAT fusion genes, pOCAT (ANG N-1498/+18) in Hepa 1-6 in vitro. Our studies provide evidence that isoproterenol enhances the effect of dexamethasone and that the enhanced effect of isoproterenol is mediated via the β_2-adrenergic receptor and the cAMP-dependent protein kinase A pathway.

Methods

Materials

Both restriction and modifying enzymes were purchased either from Bethesda Research Laboratories, Boehringer-Mannheim, or Pharmacia Inc.

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The expression vectors (pOCAT and pRSVCAT containing the coding sequence for CAT without or with Rous sarcoma virus enhancer/promoter sequence fused to the 5′ end of the CAT coding sequence, respectively) were a gift from Dr Joel F. Habener (Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Boston).

α-[^{35}S]dATP (>1000 Ci/mmol), α-[^{32}P]CTP (800 Ci/mmol), γ-[^{32}P]ATP (3000 Ci/mmol), and D-*threo*-[1,2^{14}C]-chloramphenicol were purchased from New England Nuclear, Dupont.

R(−)-Isoproterenol(+)-bitartrate salt, *S*(−)-propranolol hydrochloride, *S*(−)-atenolol, ICI-118,551 HCl, and Rp-cAMP (an inhibitor of the cAMP-dependent protein kinase A I and II[14]) were all purchased from Research Biochemicals Inc.

Thin-layer chromatography plates were purchased from Fisher Scientific Ltd. Other reagents were molecular-biology grade and were obtained from Sigma Chemical Co, Bethesda Research Laboratories, Boehringer-Mannheim, or Pharmacia Inc.

Construction of Fusion Genes

The method of construction of ANG-CAT fusion genes has been described previously.[15] The sequences and orientation for all fusion genes were confirmed by dideoxy sequencing[16] with SP6 primers (Promega-Fisher, Inc) and restriction enzyme digestion mapping.

Cell Culture

The mouse hepatoma (Hepa 1-6) cell line was obtained from the American Type Culture Collection. The Hepa 1-6 cells were grown in 100×20-mm plastic Petri dishes (Gibco) using Dulbecco's modified Eagle medium (DMEM), pH 7.20, supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 μg/mL streptomycin. The cells were incubated in a humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C. For subculturing, cells were trypsinized (0.05% trypsin and EDTA) and plated at 3.5×10^5 cells per dish.

DNA Transfection

Plasmid or ANG-CAT fusion gene was transfected into Hepa 1-6 cells using calcium phosphate endocytosis as described previously.[3] We have shown previously that the optimal dose of DNA for gene transfection is 20 μg per 0.5 to 1×10^6 cells. Thus, in the present studies, a total of 20 μg of supercoiled DNA was routinely used in the cell transfection.

To study the effect of dexamethasone with isoproterenol and β-adrenergic receptor antagonists or Rp-cAMP on the expression of pOCAT (ANG N-1498/+18), cells were incubated in DMEM without FBS, and various concentrations of hormones or drugs were added on day 1 after DNA transfection. The cells were harvested on day 3 for CAT assays.[3] The plasmids pOCAT and pRSVCAT were used as negative and positive controls, respectively.

We have previously demonstrated that there is a dose-dependent relationship between dexamethasone concentration and the stimulation of expression of pOCAT (ANG N-1498/+18).[3] Dexamethasone at 10^{-6} mol/L consistently produced a 1.5- to twofold stimulation of expression of pOCAT (ANG N-1498/+18). Thus, we routinely used dexamethasone (10^{-6} mol/L) for the present studies.

To normalize the efficiency of transfection of various plasmids, 2 mg of pTKGH (a vector with the thymidine kinase enhancer/promoter fused to the 5′ human growth hormone gene) was cotransfected with pOCAT (ANG N-1498/+18). The presence of human growth hormone (National Institute of Arthritis, Metabolism, and Digestive Diseases–human growth hormone-I-1, AFP-4793B) or insulin growth factor-I (IGF-I, Sigma Chemical Co) at levels up to 4.5×10^{-9} mol/L and 12.8×10^{-9} mol/L, respectively, had no stimulatory effect on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells (M. Ming, unpublished observations, 1994). Similarly, the presence of isoproterenol (10^{-5} mol/L), propranolol (10^{-5} mol/L), atenolol (10^{-5} mol/L), ICI 118,551 (10^{-5} mol/L), or dexamethasone (10^{-6} mol/L), or a combination of both dexamethasone (10^{-6} mol/L) and isoproterenol (10^{-5} mol/L), had no stimulatory effect on pTKGH expression (M. Ming, unpublished results, 1994). However, the results presented here were normalized to the efficiency of transfection of pTKGH in the absence of various hormones or drugs added. The radioimmunoassay for human growth hormone was performed according to the method described previously.[17,18] The level of transfection efficiency for pOCAT (ANG N-1498/+18) ranged from 25% to 35% compared with pRSVCAT.

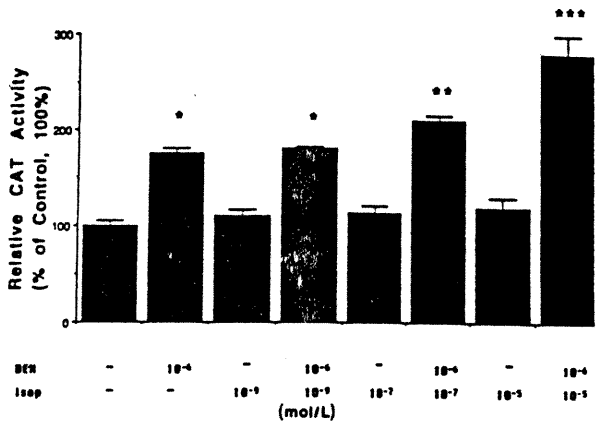

FIG 1. Bar graph shows the effect of isoproterenol (Isop; 10^{-9} to 10^{-5} mol/L) alone or combined with dexamethasone (DEX; 10^{-6} mol/L) on the expression of pOCAT (ANG N-1498/+18) in mouse hepatome (Hepa 1-6) cells. The chloramphenicol acetyltransferase (CAT) activity of the pOCAT (ANG N-1498/+18) in the absence of both dexamethasone and isoproterenol is expressed as 100% (control). Each point represents the mean±SD of a minimum of three determinations, and the probability values are derived from Student's *t* test (*$P\leq.05$, **$P\leq.01$, and ***$P\leq.005$).

Chloramphenicol Acetyltransferase Assay

The method for the CAT assay has been described previously.[3] The results of all CAT assays are given as a mean±SD of triplicates.

Statistical Analysis

The experiments were run three times in triplicate. Values are given as mean±SD (n=3), and statistical analysis was done with Student's *t* test. A level of $P\leq.05$ was regarded as significant.

Results

Effect of Isoproterenol and Dexamethasone on the Expression of the pOCAT (ANG N-1498/+18) Fusion Gene in Hepa 1-6 Cells

Fig 1 shows that addition of isoproterenol (10^{-9} to 10^{-5} mol/L) enhanced the stimulatory effect of dexamethasone (10^{-6} mol/L) on the expression of pOCAT (ANG N-1498/+18) in a dose-dependent manner. The maximal (280%) and half-maximal (220%) enhancing effect appeared to be 10^{-5} mol/L and 10^{-7} mol/L, respectively. On the other hand, isoproterenol alone had no effect on the expression of pOCAT (ANG N-1498/+18), whereas the addition of dexamethasone (10^{-6} mol/L) alone stimulated the expression of pOCAT (ANG N-1498/+18) by 1.8-fold ($P\leq.05$). These data suggest that isoproterenol and dexamethasone acted synergistically to stimulate the expression of pOCAT (ANG N-1498/+18).

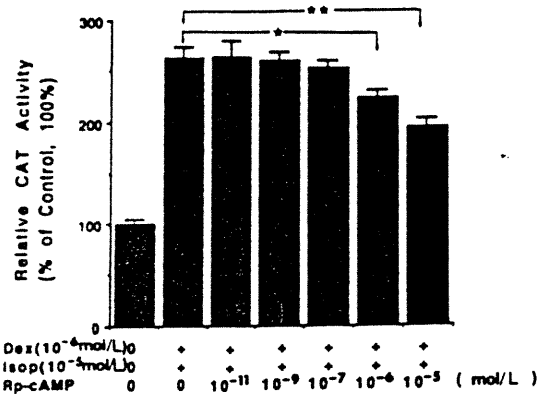

FIG 2. Bar graph shows the inhibitory effect of Rp-cAMP on the expression of pOCAT (ANG N-1498/+18) in mouse hepatoma (Hepa 1-6) cells stimulated by isoproterenol (Isop) and dexamethasone (DEX). Cells were incubated for up to 24 hours in the presence of dexamethasone (10^{-6} mol/L), isoproterenol (10^{-5} mol/L), and various concentrations of Rp-cAMP (10^{-11} to 10^{-5} mol/L). The chloramphenicol acetyltransferase (CAT) activity of the fusion gene in the absence of dexamethasone, isoproterenol, and Rp-cAMP is expressed as 100% (control). Each point represents the mean±SD of a minimum of three determinations (*$P \leq .05$, **$P \leq .01$, and ***$P \leq .005$).

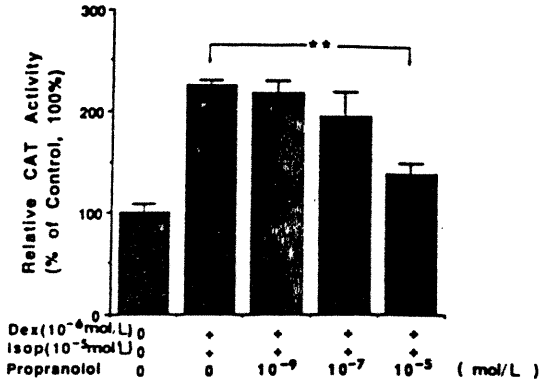

FIG 3. Bar graph shows the inhibitory effect of propranolol on the expression of pOCAT (ANG N-1498/+18) in the presence of dexamethasone (DEX) and isoproterenol (Isop) in mouse hepatoma (Hepa 1-6) cells. Cells were incubated for up to 24 hours in the presence of dexamethasone (10^{-6} mol/L), isoproterenol (10^{-5} mol/L), and various concentrations of propranolol (10^{-9} to 10^{-5} mol/L). The chloramphenicol acetyltransferase (CAT) activity of the fusion gene in the absence of dexamethasone, isoproterenol, and propranolol is expressed as 100% (control). Each point represents the mean±SD of a minimum of three determinations (*$P \leq .05$, **$P \leq .01$, and ***$P \leq .005$).

Effect of Rp-cAMP on the Expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 Cells

Fig 2 shows that addition of Rp-cAMP (an inhibitor of cAMP-dependent protein kinase A I and II) inhibits the enhancing effect of isoproterenol (10^{-5} mol/L) on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells in the presence of dexamethasone (10^{-6} mol/L). Maximal inhibition was found with 10^{-5} mol/L Rp-cAMP ($P \leq .01$). Rp-cAMP (10^{-6} mol/L) also produced a significant inhibitory effect ($P \leq .05$). These results indicate that the effect of isoproterenol was mediated via the cAMP-dependent protein kinase pathways.

Effect of β-Adrenergic Receptor Antagonists on the Expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 Cells

Fig 3 shows that addition of propranolol (a β_1- and β_2-adrenergic receptor blocker; 10^{-5} mol/L) blocked the enhancing effect of isoproterenol on the expression of pOCAT (ANG N-1498/+18) in the presence of dexamethasone (10^{-6} mol/L) ($P \leq .01$). It appears that lower concentrations of propranolol (less than 10^{-5} mol/L) did not exhibit an inhibitory effect.

Similarly, Fig 4 illustrates that the addition of β_2-adrenergic receptor antagonist (ICI 118,551) blocked the enhancing effect of isoproterenol in a dose-dependent manner. The maximal and half-maximal effects were 10^{-5} mol/L and 10^{-7} mol/L, respectively. On the other hand, atenolol (a β_1-adrenergic receptor antagonist) had no inhibitory effect on the expression of pOCAT (ANG N-1498/+18) at a dose as high as 10^{-5} mol/L (Fig 5). These results show that the enhancing effect of isoproterenol was mediated predominantly by the β_2-adrenergic receptors.

Discussion

β-Adrenergic receptors are among the best-studied receptors.[19] β-Adrenergic receptors were initially subdivided into two categories, β-1 and β-2, on the basis of the rank order of potency of agonists. Recent studies using recombinant DNA technology, however, have cloned three different β-adrenergic receptors.[20-23] Thus, it is now generally accepted that there are three types of β-adrenergic receptors.

Isoproterenol is known to stimulate the synthesis of hepatic intracellular cAMP via β-adrenergic receptors.[9-12,24,25] Our present studies (Fig 1) showed that addition of isoproterenol enhances the stimulatory effect of dexamethasone on the expression of the pOCAT (ANG N-1498/+18) in Hepa 1-6 cells. Thus, these studies are in agreement with our previous studies,[3] which showed that 8-Br-cAMP enhances the stimulatory effect of dexamethasone on the expression of the ANG gene. The present studies indicate that

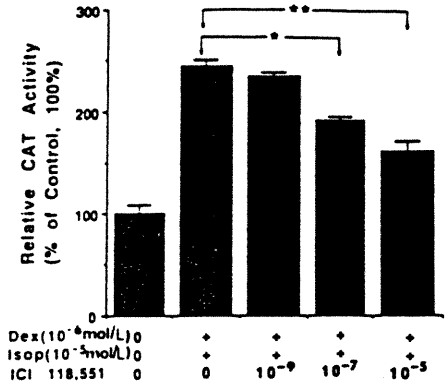

FIG 4. Bar graph shows the inhibitory effect of ICI 118,551 on the expression of pOCAT (ANG N-1498/+18) in the presence of dexamethasone (DEX) and isoproterenol (Isop) in mouse hepatoma (Hepa 1-6) cells. Cells were incubated for up to 24 hours in the presence of dexamethasone (10^{-6} mol/L), isoproterenol (10^{-5} mol/L), and various concentrations of ICI 118,551 (10^{-9} to 10^{-5} mol/L). The chloramphenicol acetyltransferase (CAT) activity of the fusion gene in the absence of dexamethasone, isoproterenol, and ICI 118,551 is expressed as 100% (control). Each point represents the mean±SD of a minimum of three determinations (*$P \leq .05$, **$P \leq .01$, and ***$P \leq .005$).

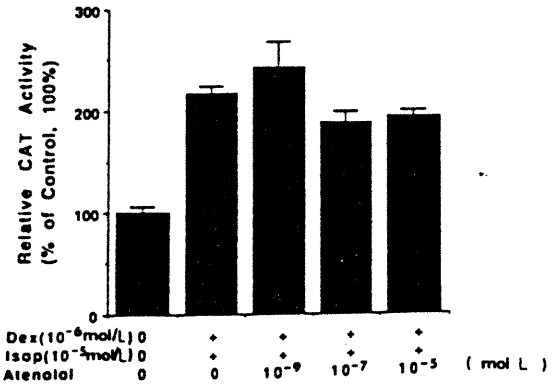

FIG 5. Bar graph shows the effect of atenolol (10^{-9} to 10^{-5} mol/L) on the expression of pOCAT (ANG N-1498/+18) in the presence of dexamethasone (DEX; 10^{-6} mol/L) and isoproterenol (Isop; 10^{-5} mol/L) in mouse hepatoma (Hepa 1-6) cells. The chloramphenicol acetyltransferase (CAT) activity of pOCAT (ANG N-1498/+18) in the absence of dexamethasone, isoproterenol, and atenolol is expressed as 100% (control). Each point represents the mean±SD of a minimum of three determinations (*$P \leq .05$, **$P \leq .01$, and ***$P \leq .005$).

the effect of isoproterenol may be via the cAMP-dependent protein kinase pathway. Indeed, our studies showed that addition of Rp-cAMP (an inhibitor of cAMP-dependent protein kinase A I and II[14]) inhibits the enhancing effect of isoproterenol (Fig 2). The present studies demonstrated that the effect of isoproterenol is mediated via the cAMP-dependent protein kinase pathway. Moreover, Fig 1 shows that the maximum enhancing effect of isoproterenol (10^{-5} mol/L) on the stimulatory effect of dexamethasone (10^{-6} mol/L) is no more than 1.5-fold compared with that of controls (in the presence of dexamethasone, $P \leq .05$). These observations are also in agreement with studies of Ohtani et al[26] and Ben-Ari et al,[27] who showed that cAMP enhances the effect of dexamethasone on ANG secretion by primary hepatocyte cultures and on the accumulation of the ANG mRNA transcripts in rat hepatoma cells by about 1.5- to twofold over that of controls, respectively.

It has been shown that β_1-, β_2-, and β_3-subtype adrenergic receptors are present in the liver and hepatoma cells.[13,24,28,29] Our present study showed that addition of propranolol or ICI 118,551 inhibits the enhancing effect of isoproterenol on the expression of pOCAT (ANG N-1498/+18) by dexamethasone, but the addition of atenolol has no inhibitory effect (Figs 3 through 5). These studies demonstrated that the enhancing effect of isoproterenol is mediated by the β_2-adrenergic receptors and not the β_1-adrenergic receptors. This is in agreement with the studies of Schmelck and Hanoune[24] and Graziano et al[28] showing that β_2-adrenergic receptors are predominant in the liver. Atenolol (10^{-5} mol/L) did not inhibit the enhancing effect of isoproterenol (Fig 5). A higher dose of atenolol (10^{-4} mol/L), however, was effective in inhibiting the enhancing effect of isoproterenol (M. Ming, unpublished results, 1993). Hence, it is possible that β_1-adrenergic receptors are also present in Hepa 1-6 cells but in a lesser amount than β_2-adrenergic receptors. Indeed, more experiments are definitely required to demonstrate the presence or absence of β_1-adrenergic receptors in mouse Hepa 1-6 cells.

Finally, an increased activation of the sympathetic nervous system[30,31] and of the renin-angiotensin axis[32,33] is believed to be involved in the pathogenesis of hypertension. Numerous studies have shown that Ang II enhances those responses (release of norepinephrine) that are elicited by postganglionic sympathetic nerve stimulation in vivo and in vitro.[34-36] The recent studies of Matsukawa et al[37] showed that administration of captopril (an angiotensin-converting enzyme inhibitor) significantly decreases the muscle sympathetic nerve activity in accelerated hypertensive compared with normotensive patients, suggesting that levels of plasma norepinephrine, which may reflect sympathetic nerve activity in the hypertensive patients, could depend on the concentration of Ang II (ie, activation of the renin-angiotensin system). On the other hand, there are only a few studies demonstrating that catecholamines have an effect on the release of Ang II.[38-42] Nakamaru et al[38] demonstrated that isoproterenol (10^{-9} to 10^{-6} mol/L) causes an increase in the release of Ang II from isolated perfused mesenteric arteries. The increase in Ang II release during isoproterenol infusion was blocked by propranolol. Captopril (2×10^{-6} mol/L) also inhibited the increase in Ang II induced by isoproterenol. Studies by Richards et al[39] also showed that a high dose of isoproterenol (100 mmol/L) significantly stimulates the release of Ang II from neuronal cultures. Recent studies by Tang et al[40] demonstrated that isoproterenol (10^{-7} to 10^{-5} mol/L) increases secretion of angiotensins from cultured bovine aortic endothelial cells in a dose-dependent manner. The addition of ICI 118,551 (10^{-6} mol/L) blocked the effect of isoproterenol but not atenolol (10^{-6} mol/L). More recent studies by Taddei et al[41,42] demonstrated that infusion of isoproterenol into the brachial artery of hypertensive subjects stimulates a local outflow of Ang II.

All of these studies demonstrate that release of locally generated Ang II by isoproterenol is mediated by β-adrenergic receptors. Unfortunately, there are no reports demonstrating that isoproterenol has an effect on the expression of hepatic and/or extrahepatic ANG gene. Our present study showed that high concentrations of isoproterenol enhance the stimulatory effect of dexamethasone on the expression of the ANG gene in Hepa 1-6 cells (Fig 1). This suggests that the sympathetic nervous system may have a regulatory role in the activation of the renin-angiotensin axis (ie, synthesis and release of Ang II). Hence, we speculate that the activation of the sympathetic nervous system during stress may enhance the effect of cortisol on the expression of the ANG gene in patients with Cushing's syndrome, since hypertension is present in 75% to 80% of those patients with hypercortisolism.[43,44] More experiments are definitely required to confirm this hypothesis.

In summary, we have demonstrated that the addition of isoproterenol enhances the effect of dexamethasone on the expression of the ANG gene in Hepa 1-6 cells. The enhancing effect of isoproterenol is mediated via the β_2-adrenergic receptors and cAMP-dependent protein kinase pathway. Our data may be useful for a better understanding of the molecular mechanism(s) of glucocorticoid/catecholamine(s)-induced hypertension.

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