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REGULATION OF THE EXPRESSION OF RAT ANGIOTENSINOGEN GENE IN MOUSE HEPATOMA AND OPOSSUM KIDNEY CELL LINES

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RÉSUMÉ

Notre connaissance de la régulation de l'expression du gène de l'angiotensinogène (ANG) est toujours réduite. L'importance du présent travail est soulignée par des données cliniques indiquant qu'il y a une coopération étroite entre les glucocorticoïdes et le système nerveux sympathique pour la régulation de la pression sanguine et le développement de l'hypertension. Puisque l'ANG est un précurseur de l'angiotensine II (AII), un élément clé du système rénine-angiotensine (RAS), dans la présente étude, nous avons examiné l'effet d'hormones stéroïdiennes et des catécholamines sur la régulation de l'expression du gène de l'angiotensinogène de rat dans une lignée cellulaire hépatocytaire (Hepa 1-6) et la lignée cellulaire rénale d'opossum (OK). Des gènes de fusion contenant des tailles variables de la région 5'-flanquante du gène ANG lié au gène rapporteur de la chloramphénicol acétyle transférase (CAT) bactérienne ont été introduits dans les cellules Hepa 1-6 ou OK. Les niveaux d'expression de ces gènes de fusion ont été enregistrés en utilisant l'activité enzymatique CAT exprimée par ces cellules transfectées suite à des traitements hormonaux ou avec des drogues.

La dexaméthasone (DEX) a stimulé l'expression de pOCAT (ANG N-1498/+18) de manière dose-dépendante dans les deux lignées cellulaires Hepa 1-6 et OK. La testostérone, les estrogènes et la progestérone n'ont pas eu d'effet significatif dans les deux lignées cellulaires. L'hormone thyroïdienne, la L-T3, stimula l'expression de pOCAT (ANG -1498/+18) dans les cellules OK mais non dans les cellules Hepa 1-6.

Dans les cellules Hepa 1-6, ni le 8-Bromo-cAMP ni la forskoline ne stimula significativement l'expression de pOCAT (ANG N-1498/+18), mais le 8-Bromo-cAMP ou la forskoline a potentialisé l'effet du DEX sur l'expression de ce gène de fusion. Dans les cellules OK, le 8-Bromo-cAMP ou la forskoline individuellement stimula l'expression de pOCAT (ANG N-1498/+18) de manière dose-dépendante. DEX et le 8-Bromo-cAMP agirent additivement sur l'expression de ce gène de fusion.

Dans les cellules Hepa 1-6 et OK, le 8-Bromo-cAMP ou la forskoline individuellement ont stimulé l'expression de pTKCAT (ANG N-814/-761) et de pTKCAT (ANG N-806/-779). Ces deux constructions contiennent l'élément putatif de réponse à l'AMP cyclique (CRE: 5'-TGACGTAC-3') du gène ANG de rat (N-795 à N-788). Ces résultats ont fourni l'évidence que l'effet de l'AMP cyclique est médié par ce fragment.

L'isoprotérénole seule ne stimula pas l'expression de pOCAT (ANG N-1498/+18) dans les cellules Hepa 1-6 ou OK. Dans les cellules Hepa 1-6, l'isoprotérénole a potentialisé l'effet du DEX pour stimuler l'expression de ce gène de fusion. L'effet potentialisateur de l'isoprotérénole fut inhibé en présence de l'inhibiteur du récepteur β2-adrénergique, le ICI 118,551, mais non en présence de l'inhibiteur du récepteur β1-adrénergique, l'aténolol. Ces résultats démontrèrent que l'effet de l'isoprotérénole était médié par le récepteur β2-adrénergique dans les cellules Hepa 1-6. Dans les cellules OK cotransfectées avec le cDNA (pBC-β1AR) du récepteur β1-adrénergique, l'isoprotérénole stimula l'expression de pOCAT (ANG N-1498/+18), et DEX et l'isoprotéténole agirent synergistiquement pour stimuler l'expression de ce gène de fusion. Cependant, lorsque les cellules OK furent transfectées

avec le cDNA (pBC-β2AR) du récepteur β2-adrénergique, l'isoprotérénole ne stimula pas l'expression de pOCAT (ANG N-1498/+18), et DEX et l'isoprotérénole n'agirent pas de manière synergistique sur l'expression de ce gène de fusion. Ainsi, l'effet de l'isoprotérénole sur l'expression du gène ANG fut médié par le récepteur β1-adrénergique dans les cellules OK. L'effet de l'isoprotérénole fut bloqué par le Rp-cAMP dans les deux lignées cellulaires Hepa 1-6 et OK, impliquant la voie protéine kinase A dans l'effet de l'isoprotérénole sur l'expression du gène ANG.

Pour étudier le(s) mécanisme(s) moléculaire(s) de l'effet synergistique de DEX plus isoprotérénole sur l'expression du gène ANG, des gènes de fusion variées ANG-TK-CAT avec ou sans l'élément de réponse à l'AMP cyclique (CRE), l'élément(s) de réponse aux glucocorticoïde GRE(s) ou les deux ont été transfectés dans les cellules OK. Le 8-Bromo-cAMP stimula uniquement les gènes de fusion contenant le CRE. L'effet du 8-Bromo-cAMP fut bloqué par le Rp-cAMP. DEX stimula uniquement l'expression des gènes de fusion contenant le GRE(s). L'effet du DEX fut bloqué par le RU 486, impliquant le récepteur aux glucocorticoïdes dans l'effet de DEX sur l'expression du gène ANG. L'effet additif du 8-Bromo-cAMP et du DEX ne fut observé que dans les gènes de fusion contenant les deux éléments de réponse CRE et GRE(s).

La présent étude a démontré que l'effet synergistique de l'isoprotérénole et de DEX sur l'expression du gène ANG fut médié par une communication croisée «cross-talk» entre la voie de signalisation du récepteur aux glucocorticoïdes stimulé par DEX et la voie PKA stimulée par l'isoprotérénole, qui fut activée via une interaction protéine-protéine entre le

récepteur aux glucocorticoïdes lié au GRE, et le CREB qui lié au CRE. L'effet synergistique ne fut pas médié par la phosphorylation du récepteur aux glucocorticoïdes via la voie PKA activée par l'isoprotérénole.

ABSTRACT

Our knowledge on the regulation of the expression of the angiotensinogen (ANG) gene is still meager. The importance of the current work is underscored by clinical findings that indicate that there is a cooperation between glucocorticoids and the sympathetic nervous system in the regulation of blood pressure and the development of hypertension. As ANG is a precursor of angiotensin II (AII), a key element in the renin-angiotensin system (RAS), in the present studies, we investigated the effect of steroid hormones and catecholamine on the regulation of the expression of the rat ANG gene in the mouse hepatoma cell line (Hepa 1-6) and the opossum kidney cell line (OK). Fusion genes containing various lengths of the 5'-flanking region of the rat ANG gene linked to the bacterial chloramphenicol acetyl transferase (CAT) reporter gene were introduced into Hepa 1-6 or OK cells. The expression levels of these fusion genes were monitored using CAT enzymatic activity, following treatment of the cells with hormones or drugs.

Dexamethasone (DEX) stimulated the expression of pOCAT (ANG N-1498/+18) in a concentration-dependent manner in both Hepa 1-6 and OK cells. Testosterone, estrogen, and progesterone did not have significant stimulatory effect in either cell line. Thyroid hormone, L-T_3, stimulated the expression of pOCAT (ANG N-1498/+18) in OK cells but not in Hepa 1-6 cells.

In Hepa 1-6 cells, neither 8-Bromo-cAMP nor forskolin significantly stimulated the expression of pOCAT (ANG N-1498/+18), but 8-Bromo-cAMP or forskolin potentiated the effect of DEX on expression of this fusion gene. In OK cells, 8-Bromo-cAMP or forskolin alone stimulated the expression of pOCAT (ANG N-1498/+18) in a concentration dependent manner. DEX plus 8-Bromo-cAMP acted additively on expression of this fusion gene.

In both Hepa 1-6 and OK cells, 8-Bromo-cAMP or forskolin alone stimulated the expression of pTKCAT (ANG N-814/-761) and pTKCAT (ANG N-806/-779), constructs which contain the putative CRE (5'-TGACGTAC-3') of the rat ANG gene (N-795 to N-788). These data provided evidence that the cAMP effect was mediated by this fragment.

Isoproterenol alone did not stimulate the expression of pOCAT (ANG N-1498/+18) in either Hepa 1-6 or OK cells. In Hepa 1-6 cells, isoproterenol potentiated the effect of DEX to stimulate the expression of this fusion gene. The potentiating effect of isoproterenol was inhibited by the presence of the β-adrenoceptor inhibitor, ICI 118,551, but not by the presence of the β-adrenoceptor inhibitor, atenolol. These results demonstrated that the effect of isoproterenol on ANG gene expression was mediated by the β_2-adrenoceptor in Hepa 1-6 cells. In OK cells cotransfected with β_1-adrenoceptor cDNA (pBC-β_1AR), isoproterenol stimulated the expression of pOCAT (ANG N-1498/+18), and DEX and isoproterenol acted synergistically to stimulate the expression of this fusion gene. However, when cotransfected with the β_2-adrenoceptor cDNA (pBC-β_2AR), isoproterenol did not stimulate the expression of pOCAT (ANG N-1498/+18), and DEX and isoproterenol did not act synergistically in stimulating expression of this fusion gene. Thus, the isoproterenol effect on ANG gene expression was mediated by the β_1-adrenoceptor in OK cells. The isoproterenol effect was blocked by Rp-cAMP in both cell lines, implicating the protein kinase A (PKA) pathway in the effect of isoproterenol on ANG gene expression.

To investigate the molecular mechanism(s) of the synergistic effect of DEX and isoproterenol on ANG gene expression, various ANG-TK-CAT fusion genes with or without the cAMP responsive element (CRE), the glucocorticoid responsive element(s) (GRE(s)) or both were transfected into OK cells. 8-Bromo-cAMP only stimulated the expression of the fusion genes which contained the CRE. The effect of 8-Bromo-cAMP was blocked by Rp-cAMP. DEX only stimulated the expression of the fusion genes which contained the GRE(s). The effect of DEX was blocked by RU 486, implicating the glucocorticoid receptor in the effect of DEX on

ANG gene expression. The additive effect of 8-Bromo-cAMP and DEX was observed only in the fusion genes which contained both CRE and GRE(s).

The present study demonstrated that the synergistic effect of isoproterenol and DEX on ANG gene expression was mediated by cross-talk between the DEX-activated glucocorticoid receptor pathway and the isoproterenol-activated PKA pathway, which occured via a protein-protein interaction between the glucocorticoid receptor bound to GRE and the CREB bound to the CRE. The synergistic effect was not mediated by phosphorylation of the glucocorticoid receptor via the isoproterenol-activated PKA pathway.

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LIST OF ABBREVIATIONS

8-Br-cAMP:	8-bromo adenosine 3', 5'-cyclic monophosphte
β-Gal:	β-galactosidase
A (1-7)	angiotensin 1-7
AI:	angiotensin I
AII:	angiotensin II
AIII:	angiotensin III
ACE:	angiotensin converting enzyme
ACTH:	adrenocorticotrophic hormone
ANF:	atrial natriuretic factor
ANG:	angiotensinogen
AP-1:	activator protein 1
APRE:	acute-phase response element
AT_1:	angiotensin receptor type I
AT_2:	angiotensin receptor type II
ATF:	activating transcription factor
ATP:	adenosine triphosphate
AVP	arginine vasopressin
bp:	base pair(s)
BSA:	bovine serum albumin
cAMP:	adenosine 3', 5'-cyclic monophosphte
CAT:	chloramphenicol acetyl transferase
CBP:	CCAAT box/enhancer binding protein
cGMP:	cyclic guanosine monophosphate
CIP:	calf intestinal alkaline phosphatase
CNS:	central nervous system
CRE:	cAMP response element
CREB:	cAMP response element binding protein
CREM:	cAMP response element modulator
CTP:	cytidine triphosphate
DAG:	1, 2-diacylglycerol
dbcAMP:	dibutal adenosine 3', 5'-cyclic monophosphtedes
(AI)-ANG:	the remainder of angiotensinogen after removal of AI
DBD:	DNA binding domain
DDW:	double distilled water
DEX:	dexamethasone
dFBS:	depleted fetal bovine serum
DMEM:	Dulbecco's modified eagle's medium
DMSO:	
DNA:	deoxyribonucleic acid
EDRE:	endothelium-derived relaxation factor
EGF:	epidermal growth factor
ELP:	embryonal long terminal repeat binding protein
ERE:	estrogen response element
ERK :	extracellular signal regulated kinase
FBS:	fetal bovine serum
FSH :	fellicle stimulation hormone
GFR:	glomerular filtration rate

G protein:	GTP-binding protein
GH:	growth hormone
GR-DBD:	glucocorticoid receptor-DNA binding domain
GRE:	glucocorticoid response element
GRU:	glucocorticoid responsive unit
GTP:	guanosine triphosphate
H-4-II-E:	rat hepatoma cells
HCG:	human chorionic gonadotrophin
HEPES:	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hGH:	human growth hormone
HMrA:	high molecular weight angiotensinogen
HRE:	hormone response element
hsp:	heat shock protein
ICSB:	interferon consensus sequence binding protein site
icv:	intracerebroventricular
IGF-I:	insulin-like growth factor I
IGF-II:	insulin-like growth factor II
IL-1:	interleukin 1
IL-6:	interleukin 6
IP_3:	1,4,5-triphosphate
IRS:	insulin response sequence
iv:	intravenous
kbp:	kelobase pair
kDa:	kiloDalton
KID:	kinase inducible domain
LH:	luteinizing hormone
LMrA:	low molecular weight angiotensinogen
MAP:	mitogen-activated protein
MMTV-LTR:	mouse mammary tumor virus long terminal repeat
mRNA:	messenger ribonucleic acid
MW:	molecular weight
NE:	norepinephrine
NGF:	neuron growth factor
NO:	nitric oxide
NRE:	negative regulatory element
OK:	opossum kidney
PABP:	poly(A) tail binding protein
pBC- β1AR:	the mammalian expression vectors, pBC-BC12 containing the cDNA for human β1-adrenoceptor
pBC- β2AR:	the mammalian expression vectors, pBC-BC12 containing the cDNA for human β2-adrenoceptor
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PEPCK:	phosphoenolpyruvate carboxykinase
pGC:	particulate guanylate cyclase
PIP2:	phosphatidylinositol-4, 5-bisphosphate
PKA:	protein kinase A
PKC:	protein kinase C
PLA_2:	phospholipase A_2
PLC:	phospholipase C
plfG:	proliferin gene enhancer element

Pol I:	DNA-dependent RNA polymerase I
Pol II:	DNA-dependent RNA polymerase II
Pol III:	DNA-dependent RNA polymerase III
POMC:	pre-opiomelanocortin
PRA:	plasma renin activity
PT:	proximal tubule
PTP:	phosphotyrosine phosphatase
PTX:	pertusis toxin
RAR:	retinoic acid receptor
RARE:	retinoic acid response element
RAS:	renin-angiotensin system
RIA:	radioimmunoassay
RIA-hGH:	radioimmunoassay for human growth hormone
RNA:	ribonucleic acid
RPF:	renal plasma flow
SDS:	sodium dodecyl sulfate
SMG:	submandibular gland
sORF:	short open reading frame
SSPE:	NaCl-NaH2PO4-EDTA (buffer)
SV40:	simian virus 40
T_3:	3,3'5-triiodo-L-thyronine
TAF1:	transcription activation factor-1
TAFs:	TATA binding protein (TBP) associated factors
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:	trascription factor IIJ
TK:	thymidine kinase
TNF:	tumor necrosis factor
TRE:	thyroid hormone response element
VIP:	vasoactive intestinal peptide
VSMC:	vascular smooth muscle cell

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DEDICATION

For my parents far away on the soil of my upbring and my aunt and uncle here in the land where I am studing, whose encouragements and support helped to make it all possible.

CHAPTER I INTRODUCTION AND LITERATURE REVIEW

I.1. BIOCHEMISTRY AND MOLECULAR BIOLOGY OF THE RENIN-ANGIOTENSIN SYSTEM

I.1.1. Angiotensinogen

It was first recognized in 1939 that renin was a proteolytic enzyme which released a hypertensive factor from a plasma protein (Munoz et al., 1939). Although many attempts were made to purify this plasma protein, definite information about its structure had not been obtained until 1957 (Peart, 1956). A tetradecapeptide was successfully purified from a tryptic digest of an ammonium sulfate fraction of horse plasma. Amino acid sequencing showed that this peptide contained angiotensin I (AI) at the amino terminal end plus four additional amino acid residues. Thus, the amino acid sequence around the renin cleavage site could be deduced (Peart, 1956).

From the 1960s to the 1980s, there was much effort on the purification of ANG from various species and the determination of their structures and properties. During this period ANG was purified from the plasma of hog (Skeggs et al., 1963), rabbit (Ryan and Mckenzie, 1978), human (Printz et al., 1977; Tewksbury et al., 1979), nephrectomized rats (Hilgenfeldt et al., 1980), sheep (Fernley et al., 1986), and dog (Moffett, 1987). In 1983-1984, the entire amino acid sequence of the rat (Ohkubo et al., 1983) and the human ANG (Kageyama et al., 1984) were determined from the nucleotide sequences of their respective cDNAs. These studies provided information on ANG synthesis, site of synthesis, and the regulation of synthesis.

I.1.1.1. Angiotensinogen Gene

The rat (Ohkubo et al., 1983), mouse (Clouston et al., 1988), and human (Kageyama et al., 1984) ANG genes have been cloned and characterized. The rat ANG gene has been mapped to chromosome 19 (Mori et al., 1989), the mouse ANG gene to chromosome 8 (Clouston et al.,

1989), and the human ANG gene to chromosome 1 (Gaillard-Sanchez et al., 1990). The gene in these three species is present as a single copy.

The rat ANG gene is approximately 12 kilobase pairs (kbp) long and consists of five exons separated by four introns. 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 AI. The remainder of exon 2, exon 3, exon 4, and the initial part of exon 5 encode the primary sequence of des-AI-angiotensinogen. The 3'-terminal part of exon 5 encodes the C-terminal untranslated region of the ANG mRNA (Figure 1-1).

The human ANG gene is approximately 13 kbp long and also consists of five exons separated by four introns. 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 AI. Exons 3 and 4 code for 48 and 62 amino acids of the protein, respectively, and 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 from the 5'-end to the start site of transcription has revealed a number of DNA elements that may be involved in initiating ANG gene transcription. A TATAA box and a CAAT box are located 30 bp and 50 bp upstream from the transcription start site, respectively (Tanaka et al., 1984). Two glucocorticoid response elements, GRE I (5'-AGAACATTTTGTTTC-3') and GRE II (5'-AGAACA-3'), a 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-pair palindrome [(5'-GTTGGGATTTCCCAAC-3', 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. In its natural position, it inhibits the expression of the ANG gene, but when moved further away from the initiation site of transcription, it functions as a transcriptional enhancer (Ron et al., 1990 a; Ron et al., 1990 b).

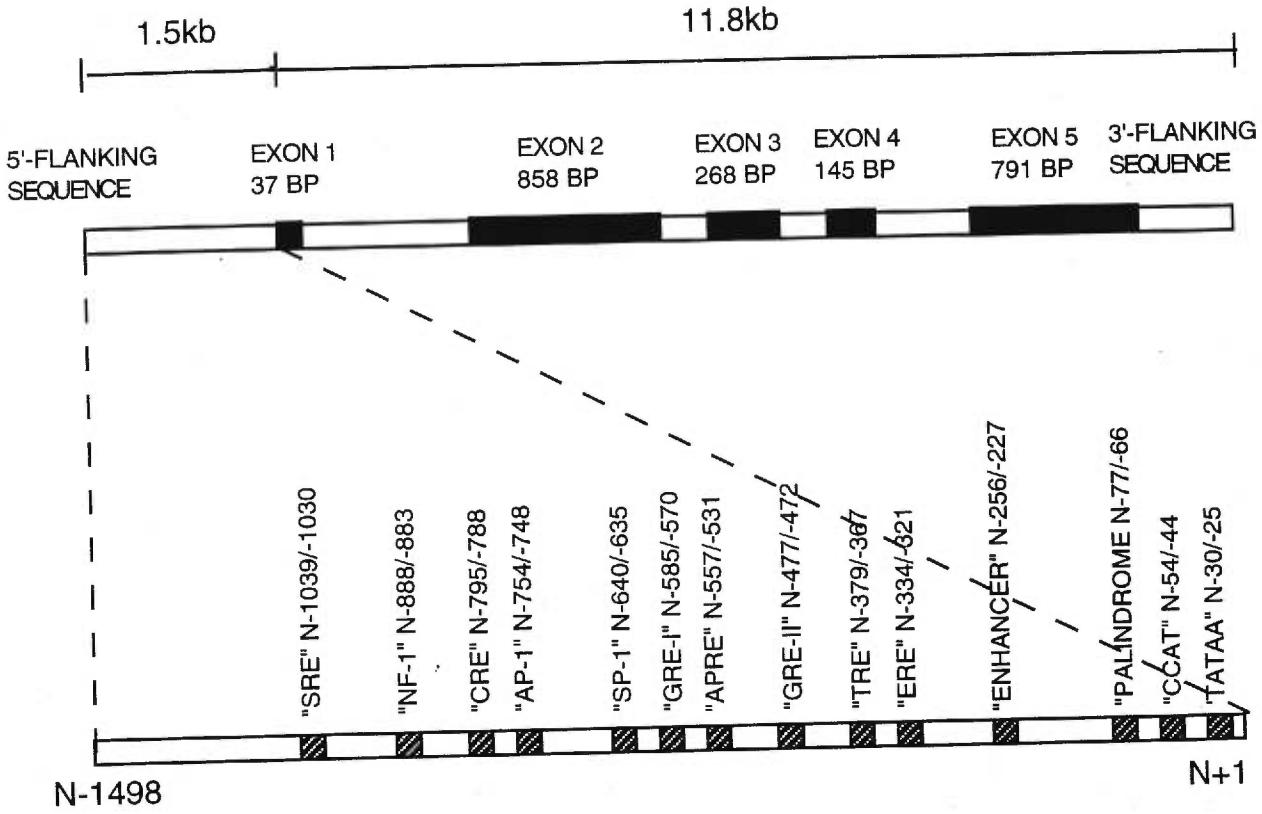


Figure 1-1. Schematic Structure of the Rat ANG Gene

The genomic structure spans 11.8 kbp plus 1.5 kbp of the 5'-flanking region. The 5 exons (black boxes) are separated by 4 introns. Several putative consensus sequences of regulatory elements have been identified and characterized in the rat ANG gene 5'-flanking regulatory region. SRE, serum response element; NF-1, nuclear factor 1 response element; CRE, cAMP response element; AP-1, c-FOS/c-JUN complex response element; SP-1, nuclear protein SP-1 response element; GRE, glucocorticoid response element; APRE, acute phase response element; TRE, thyroid hormone response element; ERE, estrogen response element.

The structure and function of the mouse ANG gene are not significantly different from the rat and human ANG genes (Clouston et al., 1988).

I.1.1.2. ANG Messenger RNA (mRNA)

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

Rat ANG mRNA is approximately 1,800 nucleotides long, consisting of 61, 1431, and 200-400 nucleotides of 5'-untranslated, coding, and 3'-untranslated sequence respectively (Lynch and Peach, 1991). The size of the ANG mRNA is heterogeneous. The reason is that a single ANG gene is transcribed into at least four different mRNAs, which differ only in the lengths of their 3'-untranslated regions, as four different polyadenylation sites are used. The four 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 four polyadenylation sites, the four different mRNA species could be generated by using these different polyadenylation signals. Treatment with glucocorticoids increases liver ANG mRNA and elicits the use of two additional, upstream transcription start sites that are located at -386 and -328 nucleotides (Ben-Ari et al., 1989). Although there is a size heterogeneity of ANG mRNAs, these extended ANG mRNAs apparently have the same coding potential as the predominant mRNA species and there is no evidence for alternative splicing of the ANG pre-mRNA.

I.1.1.3. Angiotensinogen

ANG is synthesized by a variety of cells, most prominently in hepatocytes, adipocytes, and astrocytes. The ANG protein is synthesized in the rough endoplasmic reticulum, then processed

and glycosylated in the Golgi apparatus. There is a signal peptide in pre-ANG. It contains 25 or 34 amino acid residues in the N-terminal (Lynch and Peach, 1991).

ANG is a ubiquitous, moderately abundant macroglycoprotein with plasma and cerebrospinal fluid concentrations of approximately 55-65 μg/ml and 11-13 μg/ml, respectively (Genain et al., 1984). As a member of the serpin gene superfamily, ANG is distantly related to α_1-antitrypsin, antithrombin, and ovalbumin (Lynch and Peach, 1991).

There is 87% amino acid sequence conservation between rat and mouse ANG, 64% between mouse and human, and 60% between rat and human (Lynch and Peach, 1991). 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; Clouston et al., 1988) and the mature ANG protein is 453 amino acids in rodents, and 452 amino acids in human. The invariant AI decapeptide is located at the amino terminus. The amino acid sequence immediately following this decapeptide differs in various species (Table 1-1). In humans, renin cleaves a Leu-Val bond to release AI, but in all other species, renin cleaves the Leu-Leu bond. Human ANG has histidine in position 13, whereas rat, dog, and horse have tyrosine at that position. It was suggested that these substitutions may contribute to the known species specificity exhibited by renin (Tewksbury, 1983).

ANG is a glycoprotein with a molecular weight (MW) of 55-65 kDa. There are three potential sites for N-linked glycosylation (Asn-X-Ser/Thr) in rat ANG. They are located at positions 47-49, 295-297, 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 molecular weight. The different glycosylation patterns are apparently responsible for the different isoelectric points and sizes of circulating ANG (Lynch and Peach, 1991).

There are species of ANG present in human plasma and amniotic fluid that exhibit much higher apparent molecular weights (Tewksbury, 1986). These have been referred to as high-

Table 1-1. Amino-Terminal Primary Structures of ANGs

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Human	Asp	Arg	Val	Try	Ile	His	Pro	Phe	His	Leu	Val	Ile	His	Asn	Glu
Ovine	..	..	..	..	..	..	..	..	..	..	Leu	Val	His	..	Lys
Rat	..	..	..	..	..	..	..	..	..	..	Leu	Tyr	Tyr	Ser	Ser
Mouse	..	..	..	..	..	..	..	..	..	..	Leu	..	His	..	..
Dog	..	..	..	..	..	..	..	..	..	..	Leu	Val	..	..	..
Horse	..	..	..	..	..	..	..	..	..	..	Leu	Val	..	..	..

Adapted from Tewksbury DA: (1990) Angiotensinogen biochemistry and molecular biology in hypertension. in Hypertension: Pathophysiology, Diagnosis, and Management; edited by Laragh JH and Brenner BM. Revan Press, LTD., NEW YORK. 1203.

molecular-weight-angiotensinogen (HMrA) (MW 450-500 kDa), whereas the other usually predominant form has been called low-molecular-weight ANG (LMrA) (MW 55-65 kDa). Two different subtypes of HMrA have been found, HMrA-1 and HMrA-2. HMrA-1 is composed of different subunits of which at least one is very similar to LMrA. HMrA-2 is composed of identical subunits which are very similar to LMrA. The subunits are bound together by disulfide bonds and hydrophobic interactions (Tewksbury, 1986). In plasma from men and menstruating women, only 3-7% of the total ANG is HMrA. But in plasma from pregnant women or women taking oral contraceptives containing estrogen, the HMrA is increased to 16-61%. As the HMrA subtypes are capable of being converted into AI, they may play an important role in the hypertension of pregnant women (Tewksbury, 1986).

I.1.1.4. Angiotensin I (AI)

AI is a decapeptide which is released from the N-terminus of ANG by renin. The amino acid sequence of AI in various species is identical. AI appears to function only as the precursor of AII and is not found to have any other established physiological action (Peach, 1977).

I.1.1.5. Angiotensin II (AII)

AII is an octapeptide which is formed from AI. ACE, a dipeptidyl-carboxypeptidase, splits off histidyl-leucine from the physiologically inactive AI, producing AII (Ng and Vane, 1967). The conversion of AI to AII is slow in isolated plasma but fast *in vivo*, indicating that most of the hydrolysis occurs by the action of ACE present in the endothelial cells, predominantly in the pulmonary capillaries (Campbell, 1987). AII is proteolysed rapidly; its half-life in human plasma is 1-2 minutes. The enzymes that cleave AII are grouped together under the term angiotensinase (Peach, 1977).

I.1.1.6. Angiotensin III (AIII)

AIII is a heptapeptide which is derived from AII. Angiotensinase or aminopeptidase removes

the Asp residue from the N-terminus of AII, to form AIII. In addition, aminopeptidase can act on AI to produce (des-Asp) AI, and this compound can be converted directly to AIII by the action of ACE (Peach, 1977). Angiotensin III, unlike the other peptide fragments, has about 40% of the pressor activity of AII but 100% of the aldosterone-stimulating activity. It has been suggested that AIII is the natural aldosterone-stimulating peptide, whereas AII is the blood-pressure-regulating peptide (Peach, 1977).

I.1.1.7. Angiotensin (1-7) [A (1-7)]

Angiotensin (1-7) [A (1-7)] is a bioactive component of the renin-angiotensin system (RAS). It is formed endogenously from either AI or AII. The first actions described for A (1-7) indicated that this peptide mimicked some of the effects of AII, including the release of prostanoids and vasopressin. However, accumulating evidence suggests that A (1-7) may antagonize the action of AII on vasoconstriction either directly or by stimulation of prostaglandins and nitric oxide. The counterregulatory actions of A (1-7) imply that it may function as an antihypertensive peptide within the cascade of the RAS (Ferrario, et al., 1997).

I.1.1.8. Des(AI)-Angiotensinogen

The remainder of ANG, after removal of AI, is des(AI)-ANG. Its physiological function is not clear. The suggestion that des(AI)-ANG is a renin inhibitor (Hackenthal et al., 1977) has not been confirmed experimentally (Poulsen and Jacobsen, 1986).

I.1.2. Renin

Renin is a proteolytic enzyme, a member of the aspartyl proteases. It cleaves the first 10 amino acid residues from the N-terminus of ANG, releasing AI and des-(AI)-ANG. Renin has species specificity. Human ANG can be cleaved only by primate renin. Human renin can cleave other mammalian ANGs, but other mammalian renins cannot cleave human ANG (Burton and Quinn, 1988).

I.1.2.1. The Renin Gene

There is only one renin gene in humans (Hobart et al., 1984), rats (Burnham et al., 1987) and certain strains of mice with low levels of submandibular gland (SMG) renin, such as C57/BL10 (Mullins et al., 1982), whereas mouse strains with high levels of SMG renin, such as DBA/2J, carry two renin genes (Ren 1d and Ren 2d) (Mullins et al., 1982). The single renin gene is approximately 11-12 kbp in length and consists of nine exons and eight introns. The amino acid sequence predicted 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 acid residues, which include mature renin and a 66-residue amino-terminal prepropeptide (Morris, 1986). The homology of the renin gene between rat and mouse is 85%, whereas between rat and human it is 68% (Krieger and Dzau, 1991).

There are two TATAA box sequences in the promoter region of the renin gene. In humans they are 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 mouse kidney a minor proportion of transcripts initiates at promoter 2 (Mullins et al., 1982; Hobart et al., 1984; Burnham et al., 1987). Extrarenal tissues of the two renin gene mice, use promoter 1 (Fukamizu et al., 1986) while the SMG uses promoter 2 as well. Associated with the TATAA boxes are two CAAT boxes at positions -51 and -92 (Fukamizu et al., 1986) of the human renin gene. Several regulatory sequences in the 5'-flanking region of the human renin gene have been found. The hexanucleotide sequences TGTCCT, TGTTCT, and AGTCCT proposed as the motifs of the glucocorticoid response element (GRE) are located at -190 to -185, -465 to -461, and +233 to +238 (first intron) (Fukamizu et al., 1986). There are two DNA sequences at -999 to -981 (5'-ATCTCCTTGGGGGTTAGTT-3') and -318 to -300 (5'-GTCCCAGTTTTGA-3') with different degrees of similarity to the consensus sequence of the progesterone response element (PRE) (Fukamizu et al., 1986). The DNA sequence (5'-GGAGCTGGAAA-3') has been proposed as an estrogen response element (ERE) (Fukamizu et al., 1986). The sequence at -145 to -117 (5'-

CCCTTCACCCACCTAGCTCTGTCCCGCAG-3') has been proposed as a cAMP response element (CRE) (Fukamizu et al., 1986).

I.1.2.2. Renin mRNA

In situ hybridization studies have localized renin mRNA to renal juxtaglomerular cells (Samani et al., 1988), testicular Leydig cells (Samani et al., 1988), adrenal zona glomerulosa cells (Samani et al., 1988), pituitary gonadotrophs (Kettani et al., 1991), the ovarian mature follicle (Shaw et al., 1989), and the corpus luteum (Duncan et al., 1990), as well as in granular duct cells of mouse SMG. The PCR technique has detected renin mRNA in the following tissues (approximate relative concentrations): kidney, 100%; adrenal, 10%; testis, 1%; ovaries and liver, 0.1%; brain, spleen, lung and thymus, 0.01% (Paul et al., 1993).

I.1.2.3 Renin Protein

The plasma renin is secreted by the kidney, especially by the juxtaglomerular cells. It is an acidic protease and a glycoprotein hormone with a molecular weight of 43 kDa in humans. There are two potential sites for N-linked glycosylation (Asn-X-Ser/Thr) in renin. They are located at amino acid positions 5-7, and 75-77, respectively. The carbohydrate accounts for about 5% of the total glycoprotein (Galen et al., 1979). The renin protein is composed of two domains, between which the activation sites of the enzyme are located in a deep cleft. Two aspartic acid residues, one at position 32 and one at position 215, are juxtaposed at the mouth of the cleft and are essential for activity (Sielecki et al., 1989).

Like insulin, renin is synthesized as a large preprorenin molecule. It contains 406 amino acid residues in humans. After removal of a leader sequence of 23 amino acid residues from the N-terminus, prorenin is released. It contains 383 amino acid residues and has relatively little biological activity. After removal of the pro-sequence from the N-terminus of prorenin, the active renin which contains 340 amino acid residues is released (Morris, 1986). The renin in mouse

salivary glands undergoes an additional cleavage near the C-terminal end to produce 2 separate peptide chains connected by a disulfide bond. The half-life of human renin in the circulation is 30-80 minutes (Konrads et al., 1981). Very little prorenin is converted to renin in the kidney, with most of it being directly secreted into the circulation. So prorenin appears to be the major circulating form of renin, accounting for as much as 90% of the enzyme in human plasma (Sealey et al., 1986; Glorioso et al., 1986). One of the important roles of prorenin could be to provide a transport form of renin for delivery to the tissues, where it is converted to renin by tissue kallikrein, an enzyme that generates kinin by hydrolysing kininogen. There is very little conversion to active renin in the circulation (Morris, 1986).

I.1.3. Angiotensin Converting Enzyme (ACE)

ACE was discovered by Skeggs and coworkers in 1954 (Skeggs et al., 1954a, 1954b). It is a ubiquitous mammalian dipeptidyl carboxypeptidase and plays a role in blood pressure regulation by converting the inactive AI to active AII. ACE also inactivates the vasodepressor bradykinin (Figure 1-2) (Ng and Vane, 1967; Ng and Vane, 1968).

I.1.3.1. ACE Gene

Only a single ACE gene exists within the mammalian genome. It is 21 kbp in length and consists of 26 exons separated by 25 introns (Hubert et al., 1991). Two isozymes of angiotensin converting enzyme (ACE) are present in mammals. One of them, having a molecular mass of 170 kDa, is produced by a variety of tissue types including the vascular endothelium, renal tubular epithelium, ciliated gut epithelium, and macrophages, and is known as somatic ACE. The other which is known as testicular ACE has a molecular mass of 90 kDa and is produced only in the testis by developing germ cells. Both of them are coded by a single ACE gene with different promoters. The somatic ACE promoter contains typical sequences (TATAA box and several SP1 binding sites) identified in the 5' flanking region (Hubert et al., 1991).

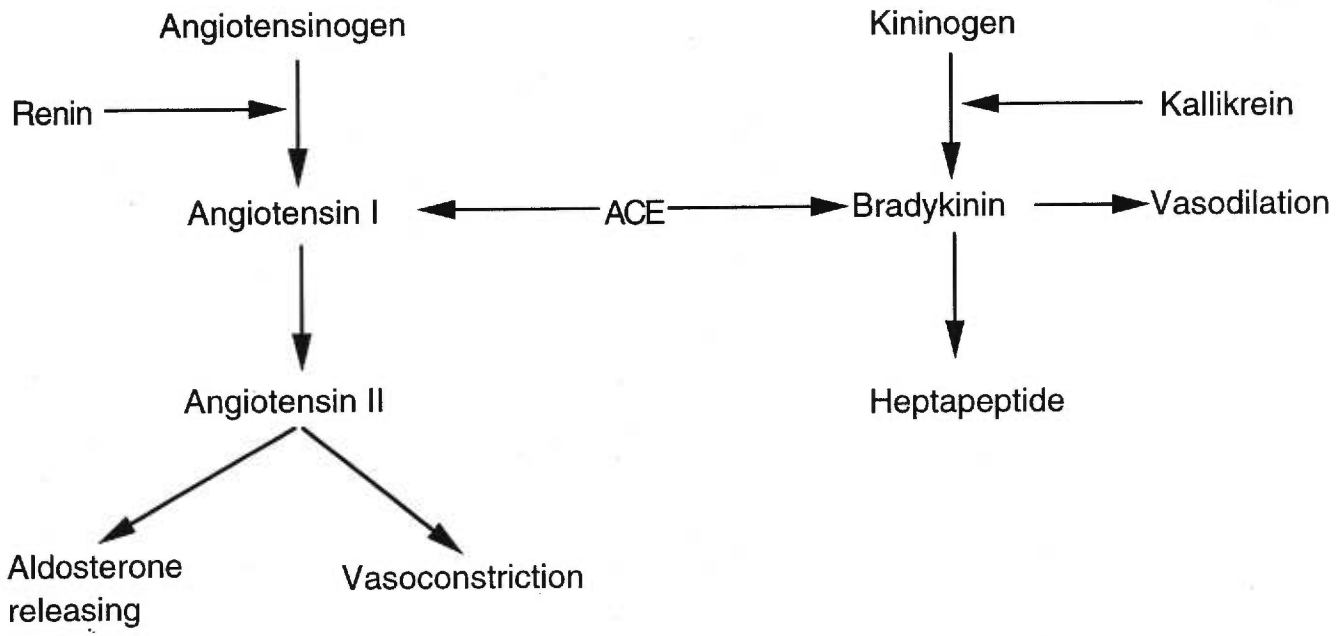


Figure 1-2. Enzymatic Effect of Angiotensin I Converting Enzyme

Adapted fom Ehlers MRW and Riordan JF: (1990) Angiotensin-converting enzyme. In: Hypertension: Pathophysiology, Diagnosis, and Management; edited by Laragh JH and Brenner BM. Revan Press, Ltd., New York. 1218.

I.1.3.2. The ACE mRNA

The somatic ACE mRNA (4.3 kb) is transcribed from exons 1 to 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 exons 14 to 26 (Hubert et al., 1991).

I.1.3.3. The ACE Protein

Early studies have elucidated several of the most important properties of ACE, including its chloride dependence, metalloprotein nature, and specificity for the removal of intact dipeptide units from the C-terminus of peptide substrates.

Current evidence suggests that ACE is fundamentally similar in most mammalian species. The molecular weight of somatic ACE appears to be between 140 and 160 kDa. It is an acidic glycoprotein containing a high percentage of acidic residues (21-22%). There is a molar equivalent of zinc in the single, large polypeptide chain of the ACE molecule. Removal of zinc abolishes all activity, and activity can be restored by addition of various divalent metal cations (Cushman and Cheung, 1971a; 1971b; Bunning and Riordan, 1981).

The complete amino acid sequence deduced from the somatic ACE cDNA consists of 1306 residues, beginning with a signal peptide of 29 amino acids. There is considerable interspecies homology at the N-terminus. A highly hydrophobic sequence located near the C-terminus of the molecule most likely constitutes the plasma membrane anchor. A hypothesis has been proposed that ACE is anchored through its C-terminus, either by a glycolipid moiety containing covalently attached phosphatidylinositol or by the hydrophobic C-terminal amino acid sequence directly (Hooper et al., 1987).

The sequence of ACE reveals a high degree of internal homology between two large domains, suggesting that the molecule resulted from a gene duplication. Each of these two domains contains short amino acid sequences identical to those located around critical residues of the active site of

other metallopeptidases (thermolysin, collagenase), and therefore bears a putative active site (Hubert et al., 1991).

I.1.4. Angiotensin II Receptors

I.1.4.1 Types of Angiotensin II Receptors

Angiotensin II elicits cellular responses in all target tissues by binding to specific high-affinity cell surface receptors. Gunther (1984) measured the binding of [^{125}I] AII to liver membranes and found two types of binding sites, one with high affinity (dissociation constant Kd~ 0.35 nM) and another one with a lower affinity (Kd~3.1 nM). Only the high affinity site was inactivated by treatment with the reducing agent, dithiothreitol. Furthermore, the availability of highly specific and selective AII receptor ligands has clearly established that there are at least two types of angiotensin receptors. These are now referred to as the AT_1 type (blocked by Losartan [Dup753] and dithiothreitol) and the AT_2 type (blocked by PD 123177). The AT_1 receptor shares only 34% amino acid sequence identity with the AT_2 receptor (Heerding et al., 1998). Among the AT_1 receptors, two subtypes, AT_{1A} and AT_{1B}, have been identified in rats and mice, but not in humans (Iwai and Inagami, 1992, Kakar et al., 1992; Sandberg et al., 1992). There is 96% amino acid homology between the two subtypes, but only the AT_{1B} receptor is suppressed by estrogen (Kakar et al., 1992). Mutation of either the AT_{1A} (Ito et al., 1995) or AT_{1B} receptor (Guo et al., 1994) affects blood pressure (Zhu et al., 1998).

The AT_1 and AT_2 receptors are not uniformly distributed in all somatic tissues (Chang and Lotti, 1991). Some tissues have a nearly homogeneous population of either the AT_1 or AT_2 receptor, while others are characterized by a mixture of both types. Tissues such as the liver, lung, kidney, placenta, urinary bladder, gastrointestinal tract, and aortic smooth muscle cells express only AT_1 receptors (Bottari et al., 1993). In the brain and heart tissues, the expression of the AT_1 receptor is regulated in a cell-specific manner. AT_1 receptor expression is very low in neurons and myocytes, whereas the glial cells and cardiac fibroblasts abundantly express the AT_1 receptor

(Murasawa et al., 1993). The AT_{1A} receptor seems to be the predominant form in most AT_1 receptor positive tissues, except the anterior pituitary, adrenal glands and uterus which have the AT_{1B} receptor (Kakar et al., 1992). In contrast, others, such as the pancreas and ovarian granulosa cells, express mainly AT_2 receptors. Both AT_1 and AT_2 receptors are expressed in the adrenal gland, heart, renal arteries, uterus and central nervous system (Unger et al., 1988; Chang and Lotti, 1991).

I.1.4.1.1. Angiotensin II Type I Receptor (AT_1 Receptor)

The AT_1 receptor has been cloned and its gene is located on chromosome 3 in humans and on chromosomes 2 and 7 in rodents (Inagami et al., 1994). Analysis of the cloned AT_1 receptor cDNA indicates that the AT_1 receptor consists of 359 amino acids with a predicted molecular weight of 40.9 kDa. There are seven transmembrane domains (Sasaki et al., 1991). The genomic DNA sequence of the rat AT_1 receptor is composed 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 5'-flanking region has revealed a number of typical DNA sequences found in many eukaryotic promoters. A TATAA box (5'-CTATAAATA-3') and a GC box (5'-AGGGCGGGGCGGGG-3') are located 36 bp and 96 bp upstream of the start site. Several important regulatory elements also exist, including GREs, CRE, SP_1, AP_1, $GATA_4$, and PEA_3. Some hormones, second messengers and growth factors, such as glucocorticoids, cAMP, and EGF, upregulate AT_1 receptor expression. A negative regulatory element (NRE) is located between -456 to -442 (5'-TAATCTTTTATTTTA-3'). A 53 kDa nuclear protein binds to this element in PC 12 cells (a neuron like cell line), and inhibits the expression of the AT_1 receptor gene (Murasawa et al., 1993; 1995). This 53 kDa nuclear protein is not found in vascular smooth muscle (VSM) and glial cells. It has been suggested that the 53 kDa nuclear protein may be a transacting factor that inhibits AT_1 receptor gene transcription in neurons and confers the cell-specificity on AT_1 receptor gene expression (Murasawa et al., 1993; 1995). A short open reading

frame (sORF) is detected upstream of the translational initiation site of the AT_1 receptor gene (+21 to +57 relative to the cap site). Mutation of this sORF increases AT_1 receptor expression without increasing the AT_1 receptor mRNA level, indicating that rat AT_1 receptor expression is negatively regulated by the sORF at the translational level (Mori et al., 1996). The coding strand from positions 274 to 417 is composed of virtually all pyrimidines. Such DNA sequence has been reported to bind abnormally to histones in the formation of nucleosomes. Whether this region influences the transcriptional activity of the AT_1 gene is unknown. Another noteworthy feature is the sequence from positions 2116 to 2147, where the DNA is composed of 16 repeats of the dinucleotide AG. This sequence could be polymorphic among different rat strains; such microsatellite DNA has been used to develop markers for linkage analysis (Langford et al., 1992).

The human AT_1 receptor gene has been cloned (Guo et al., 1994; Su et al., 1994). It consists of 5 exons and spans greater than 55 kbp. The introns are 8 to 10 kbp or longer. Exons 1 through 4 encode the 5'-untranslated region and exon 5 encodes the entire coding region (Guo et al., 1994; Su et al., 1994).

I.1.4.1.2. Angiotensin II Type 2 Receptor (AT_2 Receptor)

The human AT_2 receptor has been cloned (Kambayashi et al., 1993; Mukoyama et al., 1993), and its gene has been located on the X-chromosone (Inagami et al., 1994). The putative human AT_2 receptor is comprised of 363 amino acid residues and has a molecular weight of 41kDa. Hydropathy analysis of the deduced amino acid sequence revealed the presence of seven putative transmembrane domains (Tsuzuki et al., 1994; Koike et al., 1994). Sequence analysis showed that the human AT_2 receptor gene is composed of three exons and spans at least 5 kbp. Exons 1 and 2 encode the 5'-untranslated mRNA sequence and exon 3 encodes the entire uninterrupted open reading frame of the human AT_2 receptor. This open reading frame is highly homologous to the coding regions of AT_2 receptor cDNAs for the rat (90%) (Mukoyama et al., 1993) and mouse (90%) (Nakajima et al., 1993). AT_2 receptor expression is influenced by multiple factors. It has

been reported that increased intracellular calcium levels, phorbol ester and G-protein-coupled receptor stimulation, such as elicited by norepinephrine and AII, down-regulate AT_2 receptor gene transcription (Kizima et al., 1996). Growth factors (EGF and NGF), glucocorticoids (Kizima et al., 1995), and cAMP analogs also decrease AT_2 receptor expression (Murasawa et al., 1996). Sequence analysis of the 5'-flanking region of the human AT_2 receptor gene revealed that it contains the typical DNA elements found in many eukaryotic promoters. There is a TATAA box (at -33 bp), a putative C/EBP (at -1434) (Akira et al., 1990), SP1, AP-3, NF-1 and GRE sites. This promoter region also includes an interferon consensus sequence binding protein site (ICSBP) and a putative embryonal, long terminal repeat binding protein (ELP) site. The presence of these novel putative transcription factor binding sites suggests that this gene may be regulated by transcription factors known to play a role in embryogenesis.

I.1.4.1.3. Angiotensin II Receptor at Sites Other than the Plasma Membrane

The cytosolic AII receptor has been isolated and purified (Hagiwara et al., 1989). The cytosolic AII receptor is not a classical AT_1 or AT_2 receptor, since no affinity for either losartan or PD123177 has been found (Bumpus et al., 1991). It has been reported that the AII receptor complex is internalized within cells (Bianchi et al., 1986; Ullian et al., 1989). Once inside cells, AII is released from the receptor, which is then recycled to the plasma membrane. The cytosolic AII receptor, therefore, may represent a means of transporting AII to the nucleus, or alternatively, may serve as the means for destroying the peptide. As AII can be formed intracellularly (De Mello, 1995), another function of the cytosolic AII receptor may be to transport AII from the site of formation within the cell to the plasma membrane for release, while protecting the peptide from intracellular degradation.

The existence of the AII receptor in liver nuclei has been reported (Re et al., 1981; Tang et al., 1992). This receptor has been classified as an AT_1 receptor. Binding of AII to the nuclear receptor not only increases renin and ANG mRNAs, but also the mRNAs of growth-related factors such as PDGF and the oncogene c-myc (Eggena et al., 1996).

I.1.4.2. Signal Transduction Mechanisms and Second Messengers of AII Receptors

I.1.4.2.1. Signaling Mechanism of the AT_1 Receptor

I.1.4.2.1.1. Role of G-proteins

The AT_1 Receptor belongs to the superfamily of seven transmembrane domain, G-protein - coupled receptors. It interacts with various G-proteins and is coupled to one of the two heterotrimeric G-proteins, Gq or Gi (Unger et al., 1996).

I.1.4.2.1.2. Stimulation of Phospholipase Cβ via G-Protein

Binding of AII to the AT_1 receptor results in the release of the α-subunit of the G-protein and the subsequent stimulation of phopholipase Cβ1 via Gq or inhibition of adenylate cyclase via Gi, respectively (Unger et al., 1996). The activation of phospholipase Cβ results in the generation of 1,4,5,-inositoltrisphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases calcium from intracellular stores; DAG stimulates protein kinase C (PKC), leading to an influx of extracellular calcium via L-type calcium channels. Thus, both pathways contribute to an increase in intracellular calcium concentration (Schieffer et al., 1996; Matsubara and Inada, 1998).

I.1.4.2.1.3. Stimulation of Phospholipase Cγ via Tyrosine Kinase

AII induces a rapid phosphorylation of tyrosine residues of PLCγ1 via Src tyrosine kinase and subsequent increase of intracellular IP_3 (Morrero et al., 1994) in both vascular smooth muscle (VSM) (Schieffer et al., 1996) and glomerular mesangial cells (Zhou et al., 1991). This effect of AII is blocked by inhibitors of tyrosine phosphorylation (Schieffer et al., 1996).

I.1.4.2.1.4. Stimulation of JAK-STAT Pathway

Activition of both the cytokine receptor and the AT_1 receptor leads to a rapid increase of c-fos, c-myc and c-jun (Unger et al., 1996), all of which are growth-related transcription factors. Their

increase is an important initial step in the series of molecular events leading to AII induced cell proliferation (Unger et al., 1996). It has been reported that this process involves the induction of the JAK-STAT pathway by AII (Marrero et al., 1995). AII induces a rapid phosphorylation of tyrosine residues of the intracellular kinases JAK2 and TYK2. The phosphorylation is associated with increased enzyme activity of JAK2. In response to JAK2 activation, STAT1 and STAT2 rapidly undergo phosphorylation of their tyrosines and are translocated into the nucleus, where they initiate transcription of c-fos, c-jun and c-myc (Schieffer et al., 1996).

I.1.4.2.1.5. Stimulation of Phospholipase A_2

AII stimulates phospholipase A_2 (PLA_2) through a pertussis toxin-sensitive G-protein (Pfeilschifter and Bauer, 1986). In renal mesangial cells, AII activates PLA_2 with the release of arachidonic acid from the mesangial cell plasma membrane and consequent production of prostaglandins (Scharschmidt and Dunn, 1983; Scharschmidt et al., 1983; Schlondorff et al., 1987).

I.1.4.2.1.6. Modulation of Adenylate Cyclase

The role of adenylate cyclase in the mechanism of action of AII is still enigmatic. The majority of reports suggest that AII inhibits adenylate cyclase by interacting with the Gi-protein (Jard et al., 1981). Recent work suggests that the activation of the AT_1 receptor can either stimulate Na^+, K^+ - ATPase activity via a pertussis toxin sensitive G-protein-linked inhibition of the adenylyl cyclase pathway, or lead to inhibit Na^+, K^+ - ATPase activity through multiple signaling pathways which may include stimulation of adenylyl cyclase and PLA_2 (Bharatula et al., 1998).

I.1.4.2.1.7. Regulation of Ca^{++}, K^+, Na^+ and Cl^- Channels

AII has been shown to stimulate voltage-gated Ca^{++} channels and block K^+ channels in adrenal glomerulosa cells (Hescheler et al., 1988; Brauneis et al., 1991). Na^+ channels are also modulated by AII. AII increases the frequency of opening of Na^+ channels and enhances the rate

of activation of single-channel Na^+ currents. The effect of AII on Na^+ movements is mimicked by phorbol esters suggesting the involvement of PKC as the second messenger. AII also increases Ca^{++} -activated Cl^--conductance. The effect of AII on Cl^--conductance is thought to be mediated by an increase in the concentration of intracellular Ca^{++} secondary to IP_3 generation and the activation of PKC (Kurokawa and Okuda, 1990).

I.1.4.2.2. Signaling Mechanisms of the AT_2 Receptor

In the traditional view almost all of the functions of AII which have been described are mediated by AT_1 receptors (Timmermans et al., 1993). Recently, some experiments have demonstrated that AT_2 receptors also mediate some important physiologic functions of AII (Matsubara and Inada, 1998). The AT_2 receptor of brain was found to inhibit vasopressin release and drinking that was mediated by the AT_1 receptor (Hohle et al., 1995). Like AT_1 receptors, AT_2 receptors also mediate the contraction of portal vein smooth muscles (Pelet et al., 1995). As the AT_2 receptor is highly and transiently expressed in fetal tissues followed by a dramatic drop in most organs just before birth (Grady et al., 1991), it has been proposed that this receptor plays a role in physiological processes involving cellular growth, differentiation and adhesion (Unger et al., 1996).

I.1.4.2.2.1. Role of G-Proteins

In contrast to the AT_1 receptor, the AT_2 receptor signal transduction pathway is less characterized. Although the AT_2 receptor was once thought not to act through G-proteins and therefore probably did not belong to the seven transmembrane domain, G-protein coupled receptor superfamily (Bottari et al., 1991), more and more evidence has indicated that the AT_2 receptor is involved in a Gi or Go mediated signal transduction pathway (Kang et al., 1994; Buisson et al., 1995). An immunoprecipitation study has demonstrated a direct binding of the AT_2 receptor to $Gi\alpha_2$ and $Gi\alpha_3$ proteins in the rat fetus (Zhang and Pratt., 1996).

I.1.4.2.2.2. Stimulation of Phosphotyrosine Phosphatase

It has been reported that the AT_2 receptor is involved in Gi or Go mediated activation of phosphotyrosine phosphatase (PTP) in both PC12W cells (Bottari et al., 1992) and mouse R3T3 cells (Tsuzuki et al., 1996). The AT_2 receptor inhibits proliferation induced by the AT_1 receptor in coronary endothelial cells (Stoll et al., 1995), VSMC (Nakajima et al., 1995), cardiac fibroblasts (Ohkubo et al., 1997), and N1E-115 neuroblastoma cells (Mahmias et al., 1995). It appears to be involved in the activation of the extracellular signal-regulated kinase (ERK) phosphatase 1, a protein tyrosine phosphatase that inactivates ERK by dephosphorylating a tyrosine phosphate moiety in ERK. This effect of the AT_2 receptor can be reversed by pertussis toxin (PTX) and orthovanadate (Matsubara and Inada., 1998). A synthetic peptide containing a 22-residue sequence from the third cytosolic loop of the rat AT_2 receptor suppresses ERK activity in VSMC, and PTX or orthovanadate reverses this inhibition. This suggests that the third cytosolic loop plays a role in the activation of a Gi-mediated PTP that can inhibit ERK (Hayashida et al., 1996).

I.1.4.2.2.3. Stimulation of Phosphoserine/Phosphothreonine Phosphatase

Activation of the AT_2 receptor stimulates the outward K^+ current in primary cultured neurons (Kang et al., 1993; 1994) and inhibits the T-type calcium current in non-differentiated NG 108-15 cells (Buisson et al., 1995). These effects are mediated by activation of phosphoserine/phosphothreonine phosphatase in a Gi-protein dependent manner (Buisson et al., 1995).

I.1.4.2.2.4. Inhibition of Particulate Guanylate Cyclase Activity

Some data have shown that AT_2 receptors mediate the AII-induced decrease of intracellular cGMP in neurons and astrocytes in primary cultures prepared from rat brain (Sumners et al., 1990). AII induces a decrease in both basal and atrial natriuretic factor (ANF)-stimulated intracellular cGMP levels by inhibiting the activity of particulate guanylate cyclase (pGC). This effect is not inhibited by losartan and, surprisingly, it is mimicked by CGP 42112, which thus

behaves as an agonist in this system. It is, however, not affected by the nonselective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, indicating that this AT_2 receptor mediated effect does not involve a phosphodiesterase (Bottari et al., 1992).

I.1.4.3. Mechanism of AII Effects on Important Targets

I.1.4.3.1. Vascular Smooth Muscle

The cellular mechanisms by which the interaction between AII and its receptors on vascular smooth muscle cells (VSMC) evoke a cellular response involve two pathways: the Ca^{++} - calmodulin branch, which initiates the contractile response of VSMC, and the protein kinase C (PKC) branch, which sustains the contractile response of VSMC (Rasmussen, 1986; Alkon and Rasmussen, 1988).

The AT_1 receptor is primarily expressed in VSMC (Kakar et al., 1992). The density of AII receptors on cultured VSMC is about $4.5x10^4$ receptors per cell (Gunther et al., 1980). The Kd of 0.5-2 nM in VSMC indicates an affinity of AII receptors in VSMC that would allow interaction of the receptor with AII at prevailing circulating hormone concentrations. It was demonstrated that VSMC contraction and increased intracellular Ca^{++} concentrations were concomitant responses to AII (Morgan and Morgan, 1982). AII induced a rapid concentration-dependent increase in cytosolic Ca^{++} in the cultured VSMC. The threshold for the AII response was 10 pM, the half-maximal response was at 500 pM, and maximal response occurred at 10 nM (Gunther et al., 1980).

The binding of AII to VSMC AT_1 receptors activates phospholipase C (PLC) and releases the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Volpe et al., 1988). Water-soluble IP_3 binds to specific binding sites on intracellular calcium storage organelles (calciosomes) leading to the release of calcium. DAG remains membrane-associated, where it activates membrane-bound protein kinase C (Nishizuka 1988). Influx of calcium from the extracellular space is modified by PKC and IP_3 (Griendling et al., 1987). The rise in intracellular

Ca^{++} serves to activate Ca^{++}-calmodulin-sensitive myosin light-chain kinase (MLCK) and thus stimulates phosphorylation of myosin light-chains in a concentration-dependent manner (Anderson et al., 1981). In the presence of actin, phosphorylated myosin light chains cleave myosin-bound ATP, and cross-bridge formation between actin and myosin occurs, resulting in the initiation of VSMC contraction. The actin-myosin bond is maintained when ADP and Pi are released from myosin, and the binding of new ATP to myosin results in dissociation of cross-bridges and the relaxation of VSMC.

Angiotensin II stimulates the synthesis of prostaglandins (Gimbrone and Alexander, 1982; Morgan et al., 1983) through the activation of PLA_2, and the release of endothelium-derived relaxation factor (EDRF) (Griffith et al., 1984) in both endothelial cells and VSMC (Furchgott, 1983; Palmer et al., 1987). At least a portion of the EDRF activity has been identified as nitric oxide (NO) (Saito et al., 1996). AII also induces the release of ANF. Both NO and ANF increase guanylate cyclase activity and the formation of cGMP, which mediates vasorelaxation. Thus, the vasoconstrictor AII and the vasodilator ANF have physiologically opposing actions in VSMC that are due, at least in part, to modulation of opposing intracellular signals (Palmer et al., 1987).

AII increases intracellular Na^+ and pHi in cultured VSMC from rat aorta. This effect is due to the stimulation of the Na^+/H^+ antiporter mediated by the AT_1 receptor (Ye et al., 1996).

I.1.4.3.2. Adrenal Cortex

As in all other target tissues, the effect of AII on adrenal cells is mediated through specific cell surface receptors. Both AT_1 and AT_2 receptors are present in adrenal cells (Andrews, 1981). The AT_1 receptor of the human adrenocortical H295R cell line was downregulated by forskolin, dibutyryl-cAMP, AII (Bird et al., 1995) and potassium (Bird et al., 1995). The AT_{1A} receptor has been localized by *in situ* hybridization to the zona glomerulosa and zona fasciculata, while the AT_{1B} receptor has only been localized to the zona glomerulosa (Naruse et al., 1998).

In the adrenal zona glomerulosa, the cellular responses to AII depend on distinct pertussis-toxin-sensitive and insensitive G-proteins. A pertussis-toxin-insensitive G-protein is involved in stimulating PLC-induced PIP_2 hydrolysis, and a pertussis-toxin-sensitive G-protein may directly regulate plasma membrane Ca^{++} channels (Kojima et al., 1986; Brown and Birnbaumer, 1988). Pertussis-toxin-sensitive Gi mediates the AII-induced inhibition of adenylyl cyclase. AII directly or indirectly stimulates aldosterone release from the adrenal glomerulosa (Foster et al., 1997). The stimulation of aldosterone release by AII is initiated by Ca^{++} influx through hormone-operated Ca^{++} channels and G-protein- and PLC-dependent hydrolysis of phosphoinositides leading to the generation of IP_3 and DAG. These induce intracellular Ca^{++} release and PKC activation, respectively (Kojima et al., 1984; Foster et al., 1997). AII also enhances the release of adrenocorticotrophic hormone (ACTH) (Bottari et al., 1993). ACTH stimulates aldosterone secretion in a cAMP- and Ca^{++}-dependent pathway (Foster et al., 1997).

Some differences in the binding characteristics of adrenal angiotensin receptors have been noted when compared to those in other tissues. In adrenal glomerulosa cells, AII and AIII are nearly equipotential in increasing aldosterone release, whereas AIII is much less potent than AII in eliciting VSMC contraction (Bumpus et al., 1964; Capponi and Catt, 1979). Both AII and AIII stimulate aldosterone biosynthesis in several different ways. They act at both an early step (cholesterol to pregnenolone) and a later step (corticosterone to aldosterone) through enhanced desmolase activity and augmented conversion of corticosterone to aldosterone in the biosynthetic pathway (Biron et al., 1961; Fraser et al., 1979). The early step was shown to be calcium-dependent (Fraser et al., 1979). It has been reported that AII stimulates aldosterone secretion via both AT_{1A} and AT_{1B} subtypes in the zona glomerulosa (Naruse et al., 1998). Another effect of AII on the adrenal gland is that it stimulates granulosa cell multiplication and differentiation. AII stimulates rat zona glomerulosa cell proliferation via the AT_1 receptor coupled with phospholipase C, which activates both PKC and tyrosine kinase signalling systems. This effect of AII is partially counteracted by the activation of the AT_2 receptor (Mazzocchi et al., 1997). Thus, the acute effect of angiotensin is to stimulate the enzymes in the steroid biosynthetic pathway, but under conditions

of chronic stimulation, this may be accompanied by an increase in receptor number and by a trophic effect on the zona glomerulosa (Blair-West, 1962). Furthermore, AII also augments the production and secretion of corticosterone via the AT_{1A} subtype in the zona fasciculata (Naruse et al., 1998). These results clearly indicated differential roles of AT_{1A} and AT_{1B} in the adrenal. In adrenal medulla, AII stimulates the release of catecholamines (Peach et al., 1966; Harrison et al., 1973; Feuerstein et al., 1977; Douglas et al., 1984).

I.1.4.3.3. Kidney

Both AT_1 and AT_2 receptors are present in the kidney, but they are not uniformly distributed (Gibson et al., 1991). Mesangial cells express only AT_1 receptors, whereas the renal arteries express both types of receptors. After birth, renal AT_1 receptor density increased rapidly in association with an increase of mRNA levels of both AT_{1A} and AT_{1B} receptors. As the predominant receptor isoform in adult kidney, the AT_1 receptor may account for the majority of the effects of AII on glomerular and tubular function (Griffin et al., 1997). AII enhances renal sodium reabsorption and therefore, fluid reabsorption. Hence, it is essential to the conservation of extracellular fluid volume. The renal actions of AII to conserve sodium are due to its direct hemodynamic and tubular effects (Lohmeier et al., 1977).

The renal vasculature is highly sensitive to AII. Intrarenal infusion of low doses of AII elicits an increase in renal vascular resistance and consequently leads to a decrease in renal plasma flow (RPF), and, to a lesser degree, to a decrease in glomerular filtration rate (GFR) (Fagard et al., 1976; Lohmeier et al., 1977). Abundant AII binding sites have been found in the medulla of the kidney (Burns et al., 1993). Intrarenal infusion of AII reduces renal medullary blood flow (Arendshort and Finn, 1977). Since renal solute and water excretion rates are, in part, determined by medullary blood flow rates, it has been suggested that the hemodynamic actions of AII at this site may also participate in the regulation of urinary salt and water excretion (Spitalewitz et al., 1982).

A single class of high-affinity AII receptors is present in glomeruli and has been localized to mesangial cells (Osborne et al., 1975). Glomerular AII receptors appear to resemble adrenal AII receptors more than they resemble VSMC AII receptors, in that AIII competes rather effectively for AII binding in glomeruli and adrenal glomerulosa cells but is ineffective in competing for VSMC AII receptors (Brown et al., 1980; Skorecki et al., 1983). After the binding of AII to its receptors in mesangial cells, it activates not only phospholipase C (PLC), but also phospholipase A_2 (PLA_2) with the release of arachidonic acid from the mesangial cell plasma membrane and consequent production of prostaglandins (Scharschmidt and Dunn, 1983; Scharschmidt et al., 1983; Schlondorff et al., 1987). Mesangial cells produce predominantly PGE_2 which modulates the cell contractile response to the stimulation of AII.

AII not only augments the release of aldosterone from the adrenal gland which subsequently enhances renal sodium reabsorption in the collecting duct (Aguilera and Marusic, 1971), but also exerts a direct effect on the proximal tubular sodium transport (Harris and Young., 1977). The AT_{1A} receptor is presented on both apical and basolateral membranes prepared from rat kidney (Brown and Douglas, 1982; Cox et al., 1984; Linas et al., 1997). Until recently, it had been assumed that the basolateral receptor was functional while the apical receptor was not physiologically important because: first, it had been felt that urine AII concentrations were low; second, most of the well studied signaling enzymes transducing AII actions were located basolaterally; and third, apical receptor signaling in the proximal tubule has not been reported (Linas, 1997). However, numerous studies indicate that the apical AT_{1A} receptor is functional: first, urine AII levels were 10-fold higher than circulating AII levels (Seikali et al., 1990; Navar et al., 1994); second, micropuncture studies revealed comparable increase in bicarbonate and volume transport when AII was added to urine or plasma; and third, some functions such as phospholipase A_2 are only activated by the apical receptor (Morduchowicz et al., 1991). Luminal infusion of AII resulted in increased phospholipase C activity as indicated by increased IP_3 (Schelling et al., 1992), and decreased adenylyl cyclase activity as indicated by decreased cAMP (Schelling et al., 1994a). As the agents known to inhibit receptor mediated endocytosis inhibited the apical AT_{1A}

receptor effect, the apical AT_{1A} receptor function in proximal tubule cells is apparently mediated by receptor mediated endocytosis. In contrast to the apical AT_{1A} receptor, the formation of IP_3 and decrease of cAMP that were induced by the basolateral AT_{1A} receptor were not inhibited by these agents (Linas, 1997). Selective activation of either apical or basolateral AT_{1A} receptor resulted in increases in transcellular sodium transport. Proximal tubule sodium reabsorption is mediated by the apical sodium/hydrogen exchanger and the basolateral Na^+,K^+-ATPase (Schelling et al., 1994b). AII increases the Na^+ affinity of the Na^+/H^+ exchanger and controls 30% of all renal acidification by stimulating HCO_3^- reabsorption. Perfusion with 1 nM AII at the basolateral side of the rabbit proximal tubule not only increases the rate of apical Na^+/H^+ exchange, but also directly increases basolateral Na^+/HCO_3^- cotransport (Geibet et al., 1990). This is mediated by the pertussis toxin-sensitive Gi-protein and the inhibition of adenylate cyclase (Jard et al, 1981; Liu and Cogan, 1989). Since cAMP inhibits proximal tubule Na^+/H^+ exchange, it can decrease bicarbonate reabsorption. Lowering cAMP levels appears to be one mechanism by which AII stimulates proximal tubule bicarbonate reabsorption (Liu and Cogan, 1989), because this reabsorption is stimulated by increased electroneutral Na^+/H^+ exchange (Schuster et al., 1984; Liu and Cogan, 1988). AII has a bimodal dosage effect, as levels of AII in the range of 1-100 pM stimulate Na^+ reabsorption, whereas Na^+ transport is inhibited with >100 nM AII (Harris and Young, 1977; Schuster et al., 1984). AII modulates at least 15% of all Na^+ reabsorbed by the kidney (Liu and Cogan, 1987).

The direct effect of AII on stimulation of water reabsorption in the proximal tubules (PT) is mediated by the enhanced Na^+/H^+ exchanger in the apical membrane. AII also stimulates water reabsorption by stimulating the insertion of antiporters into the plasma membrane (Bloch et al., 1992). The effect of AII on proximal tubular water reabsorption is significantly reduced following renal denervation. These observations suggest that AII has an important presynaptic action on renal sympathetic nerve terminals on renal tubular epithelial cells to facilitate the release of norepinephrine (NE). NE has a greater effect on renal tubular sodium reabsorption and urinary

sodium excretion when AII is present in normal (but not increased) amounts, and a lesser effect when AII is decreased or absent (DiBona and Kopp, 1997). In addition to the effects of AII on PT transport, AII has direct effects on PT cell growth. AII induces PT cell hypertrophy, as measured by the increase in cell size and total protein content (Wolf and Neilson, 1990; Burns et al., 1993).

The classification, signal transduction pathway and functions of the major AII receptor classes are summarized in Table 1-2.

I.1.5. Intrarenal Renin-Angiotensin System

Traditionally, AII has been viewed as a hormone synthesized solely in the plasma by the pathway described above (section I.1.). The discovery of components of the renin-angiotensin system in many tissues of the body has led to the hypothesis that, in addition to being a circulating hormone, AII may be formed locally and therefore possess additional autocrine or paracrine actions (Burns et al., 1993).

The existence of an intrarenal renin-angiotensin system has now been generally accepted (Levens et al., 1981c). The local renal RAS may play an important role in renal development. All the components of the RAS are highly expressed in the developing kidney and are associated with nephrogenesis, vascularization, and the proper architectural and functional development of this organ (Hilgers et al., 1997). Both ANG gene knockout and ACE gene knockout mice show structural and functional developmental abnormalities of the kidney (Kim et al., 1995). In the fetal kidney the AT_2 receptor is predominant, whereas in the adult kidney AT_1 is the major AII receptor subtype (Grady, et al., 1991).

By employing 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 content is estimated to be 5% of

Table 1-2. Pharmacology, Regulation and Physiological Function of AT_1 Receptor and AT_2 Receptor

	AT_1 Receptor	AT_2 Receptor
Distribution	artery, liver, kidney, adrenal gland, heart, brain (glial cell)	fetus, brain (neuron), myometrium, kidney, lung, heart
Ligand	losartan, TCV116, valsartan	PD123319, CGP42112A
Size	359aa	363aa
Chromosome	#3 (human), #17 (rat)	X (human, rat)
Exons	5 (human), 3 (rat)	3 (human, rat)
Signaling	Gq/Gi coupling, PLC activition (Ca^{++}/IP3), Ras/ERK, JAK2/STATS	Gi coupling (?) tyrosine phosphatase, serine/threonine phosphatase
Function	vasoconstrictor, aldosterone secretion, catecholamine release, cell hypertrophy	voltage-gated Ik/Ito K^+ channel activation, T-type Ca^{++} channel activation, growth inhibition, apoptosis induction
Null mice	decrease in blood pressure; normal development, hypertrophy of renin-producing cells	increase in basal blood pressure, hyperresponse to AII pressor action, exploratory behavior
Overexpression	severe bradycardia, fetal death	negative chronotropic action
Regulation	upregulation by glucocorticoids, cAMP, cytokines, cardiac hypertrophy, myocardial infarction	downregulation by glucocorticoids, growth factors, phorbol ester, Ca^{++} ionophore; upregulation by cytokines, cardiac hypertrophy, myocardial infarction

Modified from Matsubara H, and Inada M., (1998) Molecular insights into angiotensin II type 1 and type 2 receptors: expression, signaling and physiological function and clinical application of its antagonists. Endocrine Journal 45: 137-150.

the quantity of rat liver ANG mRNA (Dzau et al., 1987). In mice, the amount is 20% of liver ANG mRNA (Dzau et al., 1987). ACE mRNA 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 concentrations are found in the brush border (Defendini et al., 1983). Although AT_1 receptors are predominant in the adult kidney, AT_2 receptors are present in large preglomerular vessels.

The intrarenal RAS plays a critical role in the paracrine regulation of renal hemodynamics and tubular transport function. Intrarenal AII is formed from systemically delivered AI and from intrarenally formed AI derived from systemically delivered ANG as well as locally synthesized ANG (Navar et al., 1997). Proximal tubule cells contain all the components of the RAS for synthesis and secretion of AII (Burns et al., 1993). The intratubular concentration of AII is in the nanomolar (10^{-9} M) range (Seikaly et al., 1990), indicating a substantial capability to influence luminal AII receptors on the tubular cell membranes. Many of the AII-dependent actions on tubular transport could be due to specific effects of locally synthesized AII on luminal AII receptors. Experimental evidence shows that the intratubular AII concentrations are regulated independently of the circulating concentration. For instance, with the use of renal interstitial macrodialysis in conscious dogs, it was found that sodium restriction or renal interstitial administration of epinephrine produced increases in renal interstitial AII concentration to levels 1000 times higher than those in plasma (Siragy et al., 1995). These studies indicate that the intrarenal RAS may play an important role in the local regulation of renal function.

I.2.REGULATION OF RENIN-ANGIOTENSIN SYSTEM GENE EXPRESSION

Many eukaryotic genes are under the control of multiple hormones and environmental factors. Consequently, hormone response elements (HREs) are usually found in multiple copies or clustered with other *cis* -acting elements. If either one of the HREs or the adjacent *cis* -acting elements is mutated, the promoter activity will be dramatically changed (Strahle et al., 1988). For

the sake of continuity, specific aspects of regulation of the constituents of the renin-angiotensin system are presented now. More general aspects of basal transcription and of regulation by glucocorticoids and catecholamines is presented later in the Introduction (Sections I.3-I.5).

I.2.1. Regulation of Angiotensinogen Gene Expression

I.2.1.1. Steroid Hormone Action on ANG Gene Expression

I.2.1.1.1. Glucocorticoids

Many studies have indicated that glucocorticoids stimulate the production of ANG. This augmentation has been known clinically for a long time, since an increase in serum renin substrate levels is observed during hypercortisolism (Cushing's syndrome) (Krakoff, 1973) and a decrease in these levels is observed during adrenal insufficiency (Addison's disease) (Stockigt et al., 1979). Animal experiments showed that administration of cortisol increases the liver ANG synthesis and the plasma ANG level (Freeman and Rostorfer, 1972; Reid, 1977; Krakoff and Eisenfeld, 1977). In addition, ANG secretion by liver slices and hepatocytes as well as hepatoma cell lines can be stimulated by incubation with the cortisol analog-dexamethasone (DEX) or by hydrocortisone (Hasegawa et al., 1973; Dzau and Herrmann, 1982; Clauser et al., 1983; Stuzmann et al., 1986).

Adrenalectomy results in diminished liver ANG synthesis, but this deficit is reversed by glucocorticoid administration. There is increased liver ANG mRNA accumulation in response to glucocorticoids applied to intact animals (Kalinyak and Perlman, 1987). Such increases are also observed for dispersed hepatocytes (Ben-Ari and Garrison, 1988), and cultured rat hepatoma cell lines, including Reuber H35 (H4IIE) and FTO-2B cells (Chang and Perlman, 1987; Klett et al., 1992). The increase in ANG mRNA accumulation occurs rapidly. In isolated hepatocytes exposed to DEX, the ANG mRNA began to increase within less than 1 h, followed, with a time lag of about 2 h, by an increase in the secretion rate of ANG (Klett et al., 1992). The ANG mRNA accumulation was insensitive to protein synthesis inhibitors, and was blocked by the glucocorticoid antagonist RU486 (Ben-Ari and Garrision, 1988). These observations, along with

the existence of glucocorticoid response elements in the ANG gene, suggest that the activated glucocorticoid receptor interacts directly with this gene.

The increased ANG mRNA accumulation induced by glucocorticoids may be explained as follows: glucocorticoids cause an increased transcription rate, since glucocorticoid response elements have been identified in the promoter region of the ANG gene (Coezy et al., 1984); glucocorticoids also induce the accumulation of novel transcripts of the ANG gene both in isolated hepatocytes and in the intact liver. The novel transcripts are generated by the use of two new transcription initiation sites in the ANG gene, and they are absolutely dependent on the presence of glucocorticoids (Ben-Ari et al., 1989). Glucocorticoids also decrease the degradation of ANG mRNA. Finally, studies have shown that DEX may stabilize the ANG mRNA (Brasier et al., 1986).

I.2.1.1.2. Estrogen

Putative estrogen response elements have been identified in the promoter regions of the human, rat and mouse ANG genes (Fukamizu et al., 1986; Campbell and Habener, 1986; Clouston et al., 1988).

Administration of an oral contraceptive containing estrogen increases plasma ANG in both adult men and women (Newton et al., 1968; Oelkers et al., 1976). In the steady state, a high plasma level of ANG produces only a very small increase of AII and plasma renin activity, but they are sufficient to elicit a slight reduction in renal blood flow, and a slight increase in exchangeable sodium and blood pressure (Oelkers et al., 1996). In susceptible cases, blood pressure may rise considerably (Oelkers et al., 1995). A significant positive correlation between estradiol and ANG has been noted in normal human pregnancy (Immonen et al., 1983). The large amount of estrogen secreted by the placenta during pregnancy leads to a massive increase of plasma ANG (four to five-fold), due to the activated estrogen receptor-mediated stimulation of transcription of the ANG gene in the liver (Gordon et al., 1992). Estrogen administration causes a proportionally greater increase in HMrA concentration than in LMrA concentration (Tewksbury and Dart, 1982).

Some animal experiments have also shown that administration of estrogen causes a rise in both the cellular ANG mRNA level and the plasma ANG concentration (Helmer and Griffith, 1952; Menard et al., 1970; Saruta et al., 1973; Nasjletti and Masson, 1972; Dzau and Herrmann, 1982; Clauser et al., 1983; Coezy et al., 1987; Gordon et al., 1992; Klett et al., 1992). However, 17β–estradiol did not increase ANG mRNA in cultured rat hepatoma cells (FTO-2B) (Fatigati et al., 1987). The reason for this conflict may be that the cultured cells had some alteration in their receptors which made them refractory to estrogen treatment, because in another rat hepatoma cell line, Fe33, a subclone of FTO-2B which had been stably transfected with an estrogen receptor expression plasmid, estrogen treatment increased ANG mRNA as well as ANG secretion to more than 2- fold (Klett et al., 1992).

I.2.1.1.3. Androgen

Renal ANG mRNA levels in male rats increased significantly during puberty (Ellison et al., 1989), and castration lowered ANG mRNA level in male kidneys by >60% compared with controls. Whereas the renal ANG mRNA levels in adult female rats are the same as that before puberty, they are considerably lower than that in adult male rats (Ellison et al., 1989). Moreover, male rats castrated as weanlings and normal adult female rats implanted with testosterone displayed significant increases in renal ANG mRNA levels. These results suggest that androgen could be involved in the regulation of the renal ANG gene .

It has been reported that dihydrotestosterone induced a rapid increase in total hepatocyte RNA and ANG mRNA with a peak at 2 hours (Klett et al., 1992). However, this increase in ANG mRNA was not followed by an increased translation of ANG protein. No convincing explanation can be given for these observations. One possible interpretation is that dihydrotestosterone induces transient sequestration of ANG mRNA into a nondegradable, and at the same time, untranslatable form or compartment (Klett et al., 1992).

I.2.1.1.4. Thyroid Hormone

Thyroid hormones play a role in the regulation of ANG synthesis. Thyroidectomized rats exhibited a significant decrease in plasma ANG levels which could be corrected by the administration of triiodothyronine (T_3) or thyroxine (T_4) (Bouhnik et al., 1981; Clauser et al., 1983). Measurement of plasma ANG in thyroidectomized rats by direct radioimmunoassay and the indirect enzymatic assay gave similar results, indicating that the decrease was due to decreased production and not to increased turnover (Bouhnik et al., 1982). Chronic hypothyroidism resulted in an approximately 50% decrease in plasma ANG and ANG mRNA levels in liver. In contrast, plasma ANG and liver ANG mRNA levels were elevated by about 75% during hyperthyroidism (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).

I.2.1.2. Angiotensin II

There is a positive-feedback loop in which, as renin consumes ANG, the production and release of ANG is stimulated by the active product, AII. Plasma AII increases both liver ANG mRNA (Kohara et al., 1992) and the plasma ANG levels in rats (Nasjletti and Masson, 1973; Khayyall et al., 1973) and dogs (Blair-West et al., 1974). The addition of AII to the perfusion medium increased the rate of ANG synthesis in isolated rat liver preparations (Nasjletti and Masson, 1973), whereas the administration of ACE inhibitors decreased plasma ANG levels (Herrmann and Dzau, 1983; Radziwill et al., 1986).

Three hypotheses for AII stimulation of ANG synthesis and secretion in liver parenchymal cells have been proposed. First, AII may act as a transcriptional enhancer. Using transcription assays in AT_1 receptor-complemented human hepatocytes, a multihormonal response element, spanning nucleotides -615 to -470 in the ANG gene, has been shown to be an AII-inducible enhancer (Brasier and Li, 1996). Mutations in this region did not only abolish AII induction of the

transfected ANG transgenes, but also blocked NF-κB binding. These results suggested that the acute-phase response element (APRE) may be an AII-inducible enhancer. This hypothesis was supported by the observation that APRE conferred AII inducibility onto an inert promoter driving the luciferase reporter, and AII stimulation depended on the binding between APRE and NF-κB (Brasier and Li, 1996). Second, AII may act via inhibition of adenylyl cyclase which is linked by a Gi protein to the low-affinity AII receptor and not on the stimulation of phospholipase C. The agents known to affect intracellular Ca^{++} concentrations (i.e., Bay K 8644, calcimycin, or methoxamine) failed to influence the synthesis of ANG (Klett et al., 1993). Finally, AII may increase ANG synthesis and secretion by stabilizing its mRNA. It has been demonstrated that AII activates a 12 kDa protein which may interact with the 3'- untranslated ANG mRNA and increases the half-life of endogenous as well as exogenous 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 upstream of it. In the latter 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 demonstrated that in addition to the effect of transcriptional factors, ANG was also regulated by protein stabilization. Since AII induced ANG mRNA increases very rapidly, it is conceivable that this effect of AII cannot be attributed to *de novo* synthesis of a putative stabilizing factor but rather to a posttranslational modification of ANG, such as phosphorylation or dephosphorylation (Klett et al., 1993). This would also be in accordance with the transient change in cAMP, since such a mechanism has been proposed to be responsible for the stabilization of mRNAs coding for cytokines (Gillis and Malter, 1991; Malter and Hong, 1991).

I.2.1.3. Inflammation

ANG is an acute-phase response protein (Kageyama et al., 1985). Elevated plasma ANG

levels are observed in injuries (Bing, 1972), and ANG mRNA in livers and brains increases 5-fold within 5 hours of injection of *E. coli* lipopolysaccharide (LPS) in rats (Kageyama et al., 1985). Nielsen (1987) measured ANG in a group of age- and sex-matched human individuals with proven acute inflammatory disease and in an uninfected control group. The median value of the plasma concentration of ANG was increased 70% in the group with inflammatory disease compared to the control group.

A *cis*-acting DNA element, centered on a 16-base-pair palindrome (5'-GTTGGGATTTCCCAAC-3'; N-552/-537 from the transcription start site) in the 5'-flanking region of the rat 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). This element, when fused upstream of a cytokine-unresponsive gene, confers a cytokine-responsiveness on that gene. The acute-phase response element (APRE) of the ANG gene was flanked on both sides by classic GREs (GRE I, GRE II), which were centered on -582 and -475, respectively. Several experiments have shown that glucocorticoids are necessary for the increased level of ANG mRNA that is induced by cytokines (Ron et al., 1990b; Ohtani et al., 1992). The 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 (Ron et al., 1990c).

I.2.2. Regulation of Renin Gene Expression

In the rat kidney, renin gene expression is under tonic influence of the renal sympathetic nerves (Holmer et al 1993; Holmer et al 1994). Graded frequency stimulation of the renal sympathetic nerves results in graded increases of arterial plasma renin activity (PRA) and renal renin mRNA levels (Nakamura and Johns, 1994). Renal denervation reduces renal renin content as well as both renin mRNA and renal venous PRA. This suggests that the renal sympathetic nerves may influence renin secretion rate not only at a posttranslational step but also at the

transcriptional level. There is a cAMP response element (CRE) in the renin gene, and 8-Bromo-cAMP may stimulate renin gene expression (Burt et al., 1989). Beta adrenergic agonists stimulate renin secretion, through mediation by cAMP. This stimulatory action is receptor-dependent, since it can be blocked by β_1-adrenergic specific antagonists (Pfeifer et al., 1995). Dietary sodium restriction increases renin gene expression due to increased RAS activity (Miller et al., 1989). AII feedback can act to inhibit renin gene expression.

I.2.3. Regulation of ACE Gene Expression

The mechanism of regulation of ACE gene expression remains largely unknown. AII infusion decreases ACE mRNA levels to 50% in the lung and testis, two major sites of ACE synthesis (Schunkert, et al., 1993). The synthesis of somatic ACE is induced by glucocorticoids and thyroid hormone (Krulewitz, 1984). Serum ACE activities are elevated in hyperthyroidism and normalized after therapy (antithyroid drugs, iodine 131, or surgery), while ACE activities are reduced in hypothyroidism and normalized after receiving exogenous thyroid hormone (Smallridge et al., 1983). ACE activity increases in patients on long-term captopril treatment (Boomsma et al., 1981). Furthermore, the expression of the two ACE isoenzymes is regulated in a tissue-specific fashion. The expression of testicular ACE is developmentally regulated and is under hormonal control. The enzyme activity is low in the testis of immature rats and rises with puberty, but not in prepubertally hypophysectomized animals. Hypophysectomy of mature animals results in depletion of ACE activity from the testis but not from the lung (Hohlbrugger et al., 1982; Strittmatter et al., 1985; Velletri et al., 1985); this loss can be prevented by the early administration of follicle stimulating hormone/luteinizing hormone (FSH/LH), human chorionic gonadotropin (HCG), or testosterone.

I.2.4. Regulation of AII Receptor Gene Expression

The expression of the AII receptor gene is developmentally regulated. Studies using autoradiography have described the presence of both AII receptor types in fetal tissues (skin, brain,

kidney, liver and aorta), but a 10-fold greater quantity of AT_2 than AT_1 receptors appears to be present in many tissues during fetal development (Grady et al., 1991; Tsutsumi et al., 1991; Tsutsumi and Saavedra., 1992). However, the AT_1 receptor predominates in adults.

Studies have shown that in rat heart, DEX increased AT_1 mRNA, whereas deoxycorticosterone acetate decreased AT_1 mRNA (Della et al., 1995). Rat proximal tubule AII receptor protein density was increased with sodium depletion and decreased with sodium loading or mineralocorticoid administration (Douglas,1987). Treatment with an ACE inhibitor may up-regulate the density of the renal vascular AT_1 receptor, with no effect on the glomerular AT_1 receptor (Haddad and Garcia., 1997).

Forskolin or dibutyryl–cAMP, which can activate the PKA pathway, caused a rapid and sustained decrease in AT_1 receptor mRNA, followed by a time-dependent and concentration-dependent loss of AII binding and decreased phosphoinositidase C activity (Bird et al., 1994; Bird et al., 1995a; 1995b). All treatments that activated the Ca^{++} /PKC pathway also caused a rapid decrease of AT_1 receptor mRNA.

More recently, a study has indicated that renal AT_{1A} gene expression is enhanced by sodium restriction, extracellular fluid volume depletion and AT_{1A} protein depletion (Nishimura et al., 1997).

The AT_2 receptor is abundant in mesenchymal tissues of the developing fetus, but its level drops dramatically in most organs just before birth (Grady et al., 1991). Under pathophysiological conditions, such as cardiac failure, post-infarction repair or skin lesions, a transiently increased AT_2 receptor expression has been reported (Kimura et al., 1992; Nio et al., 1995; Regitz-Zagrosek et al., 1995).

1.3. MECHANISM OF BASAL TRANSCRIPTION

Genes consist of DNA segments which encode information for functional products, either

RNA molecules or proteins used for various cellular functions. The base pairs of the encoding segments specify the amino acid residues to be linked together into a protein chain. As shown in Figure 1-3, the genes contain exons and introns. Exons start 5' from the transcription start site, or the first nucleotide of the mRNA sequence, and include the entire sequences corresponding to the mature mRNA (including 5'-and 3'- untranslated regions). Introns are stretches of noncoding DNA interrupting the exons (Rosenthal, 1994a; Rosenthal, 1994b). For instance, the rat ANG gene contains 5 exons and 4 introns (cf., Figure 1-1). In addition to the coding region, genes also include regulatory elements (nucleotide sequences) that influence the rate of transcription. Usually the regulatory elements lie in the region upsteam of the transcription start site. They include the promoter and enhancers/silencers. The promoter is located within about 100 bp of the initiation site (Figure 1-3). Its position is relatively inflexible. The promoter often includes an element, rich in adenine and thymine, known as the TATAA box, and other sequence motifs, such as the CAAT box, and the GC box. The promoter comprises binding sites for RNA polymerase and its numerous cofactors (Mcknight, 1991; Rosenthal, 1994a; Rosenthal, 1994b).

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 from the gene promoter (Figure 1-3). Like promoters, enhancers form binding sites for regulatory proteins, but unlike promoters, the position and orientation of an enhancer are flexible with regard to the gene. 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 promoter or enhancer sequences that interact with specific transcription factors consist of small stretches of DNA (< 30 bp) called *cis* elements (such as CRE). The corresponding transcription factors are known as *trans* factors (such as CREB).

Transcription is DNA-directed RNA synthesis, the first and usually most important step in the control of gene expression. There are three classes of transcription mediated by different RNA polymerases (Pol I-III). Pol I transcribes ribosomal pre-RNAs; Pol II is the enzyme which directs

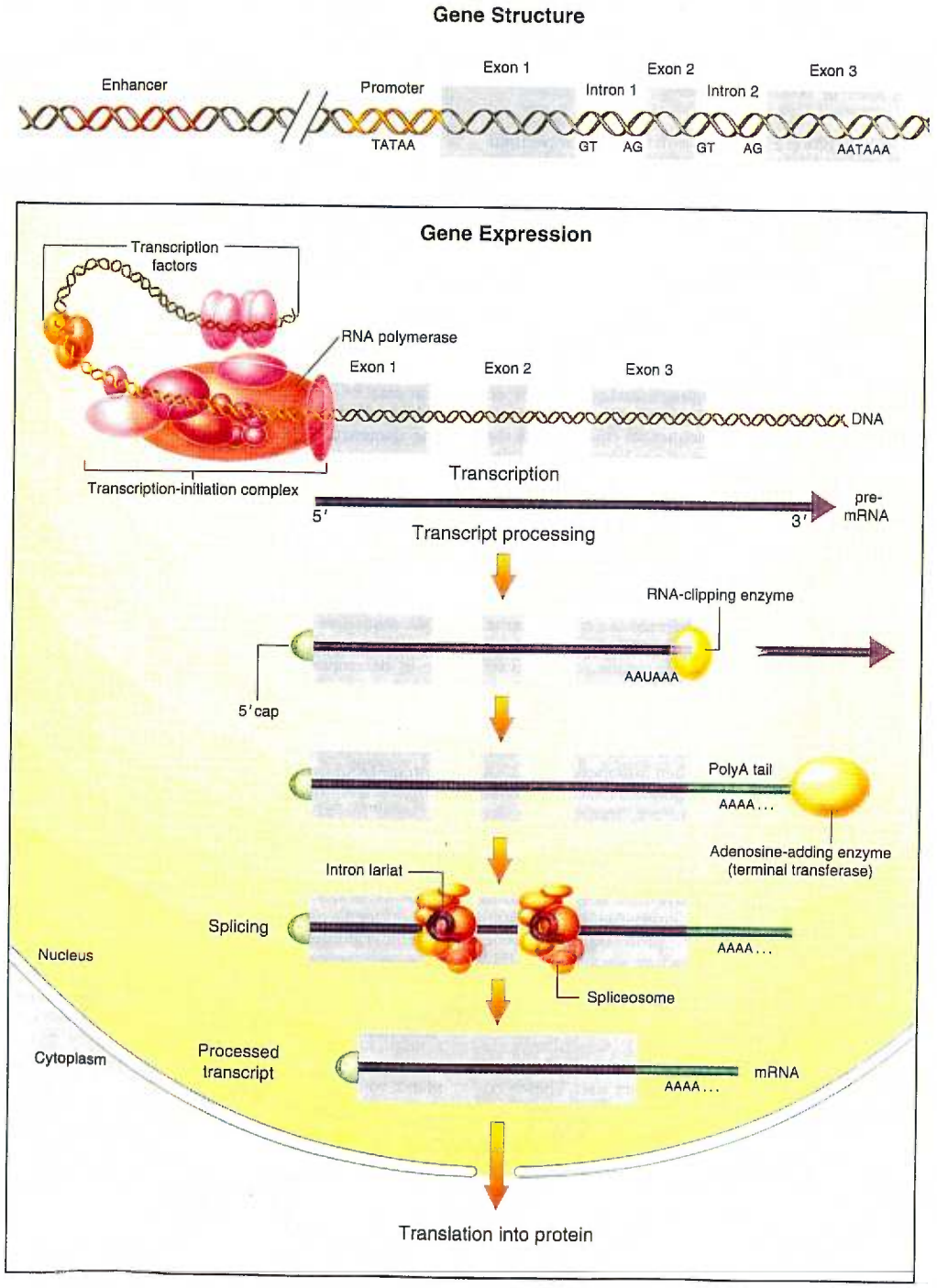


Figure 1-3. Schematic Structure of a Gene and the Process of Gene Expression.

Reproduced from Rosenthal N: (1994b) Regulation of gene expression. New Eng J Med 331: 931-933.

synthesis of the mRNA and its encoded proteins; Pol III directs the synthesis of 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, nondenaturing gel electrophoresis, and nuclease protein assays (Figure 1-4). An initial committed complex is formed by TATAA box binding protein (TBP) binding to the TATAA box of a promoter. TBP is a small protein (~ 30 kDa) which is sufficient for recognition of a TATAA box and subsequent incorporation of other TBP-associated factors (TAFs). However, TBP is not sufficient to mediate transcriptional regulation by upstream regulators, which requires the entire transcription factor IID (TFIID) complex, consisting of TBP and the TAFs. As a central piece of the basal transcription machinery, TBP has been highly conserved during eukaryotic evolution (Buratowski, 1994).

Transcription factor IIA (TFIIA) links the complex and may activate the TBP by relieving a repression caused by the TAFs. TFIIA contains three subunits and can associate with TBP or TFIID even in the absence of target DNA.

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

TFIIF contains 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 contacts the core polymerase, binds tightly to Pol II. TFIIF 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 TFIIF-polymerase joins the complex. The yeast RNA polymerase II consists of 12 subunits and their genes have been cloned (Young, 1991). The mammalian counterparts of many of the subunit genes remain to be cloned.

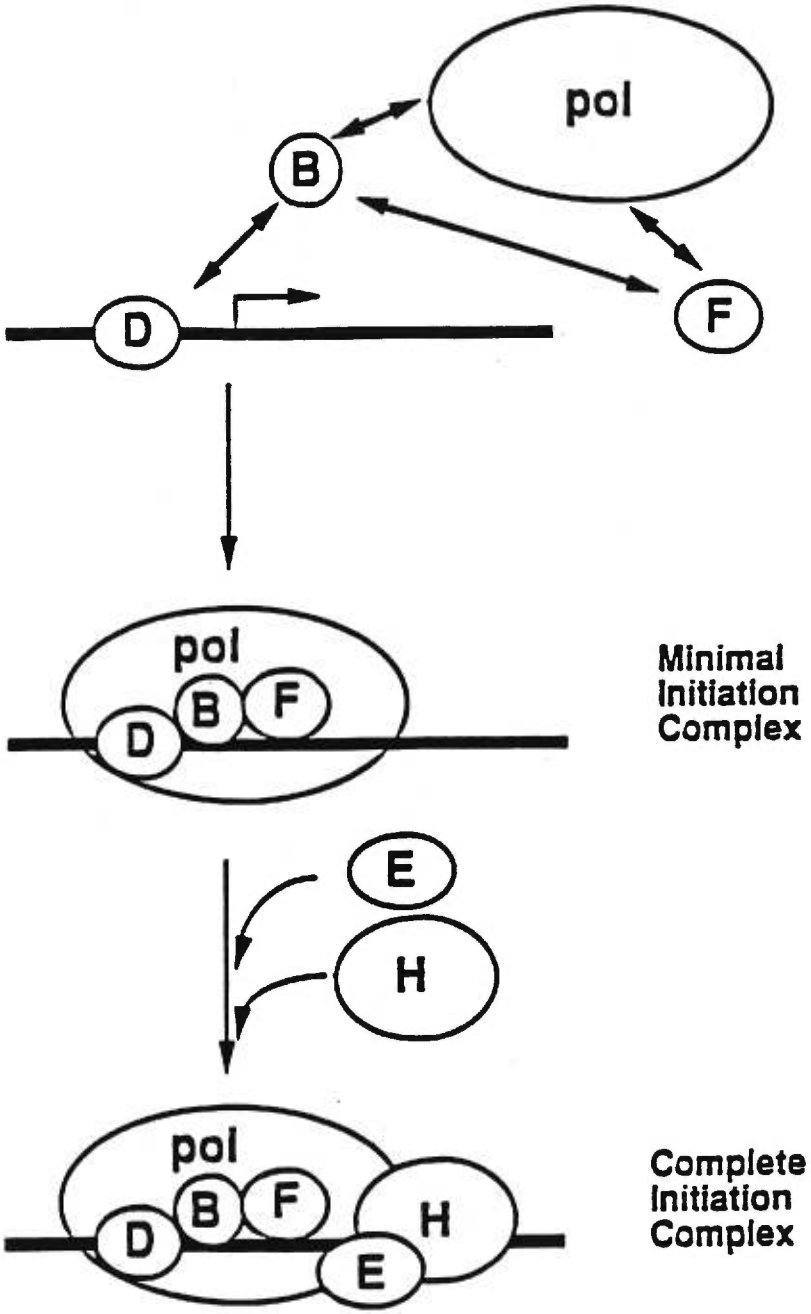


Figure 1-4. Assembly of Transcription Initiation Complex.

Pol: RNA polymerase II; B, D, E, F, H: TFIIB, D, E, F, H.

Adapted from Buratowski S: (1994) The basics of basal transcription by RNA polymerase II. Cell 77; 1-3.

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 subunits of each type. Its incorporation appears necessary for subsequent recruitment 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 Pol II, which alone has 12 subunits with a mass of 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 TAF 110), or even more indirectly with a coactivator (such as CREB with CBP, which then interacts with TFIIB), as will be discussed later (section I.5.2.3.).

I.4.MECHANISM OF STEROID HORMONE ACTIONS ON GENE EXPRESSION

Steroid hormones include mineralocorticoids (aldosterone), glucocorticoids (cortisol/corticosterone), and sex hormones (androgen, estradiol and progesterone). In addition, vitamin D can be converted to the steroid hormone, dihydroxy-vitamin D_3. The steroid hormone superfamily also includes thyroid hormone and retinoic acid. Althrough they do not closely resemble steroid hormones in their structures, they do have a six-membered ring that is thought to resemble the A ring of a steroid, and their receptors are included in the steroid receptor superfamily (Tsai and O' Malley, 1994).

A DNA sequence responsive to glucocorticoids was first identified by mutational analysis of the long terminal repeat (LTR) of the mouse mammary tumor virus (Yamamoto, 1985). The glucocorticoid response element (GRE) consists of two short, imperfect inverted repeats separated by three nucleotides (G/TG/TTACA/CnnnTGTT/CCT). Later, response elements for progesterone, mineralocorticoid, and androgen receptors were shown to be identical to that of the glucocorticoid

receptor (Tsai and O' Malley, 1994).

In contrast to polypeptide hormone receptors that are generally located on the cell surface, steroid hormone receptors are located either in the cytoplasm or in the nucleoplasm (Figure 1-5) (Tsai and O' Malley, 1994). Among the steroid receptors, there are some differences in the subcellular locations of their non-DNA binding forms. The glucocorticoid receptor and possibly the mineralocorticoid receptor appear to reside in the cytoplasm, whereas other receptors may be located within the nucleus, presumably in association with DNA, although not necessarily at acceptor sites on the DNA. The free form of a steroid hormone may enter the cell by a process of free diffusion. In the case of glucocorticoids, e.g., cortisol, the steroid would bind to an intracellular unactivated nontransformed receptor with an open ligand binding site (Burnstein and Cidlowski, 1989). The binding constant is of the order of 1 nM for this reaction. The unactivated receptor is associated with heat shock proteins (HSP70 and HSP90) which block the DNA binding domain of the receptor (Truss and Beato, 1993). Activation or transformation to the DNA binding form is accomplished by releasing the HSPs. The binding of the steroid ligand is important for this process. Following activation and exposure of the DNA binding domain, the receptor translocates to the nucleus, possibly through the nuclear pores, searches the DNA for a high-affinity acceptor site and binds to it. At this site, the bound hormone receptor complex acts as a transactivation factor, which together with other transactivators allows the binding of RNA polymerase and stimulates RNA transcription. New mRNAs are translocated to the cytoplasm and assembled into translation complexes for the synthesis of proteins that alter the metabolism and function of the cell (Burnstein and Cidlowski, 1989).

When the unoccupied (nonliganded) steroid hormone receptor is located in the nucleus, as may be the case with the estradiol receptor, the progesterone receptor, the androgen receptor, and the vitamin D_3 receptor (Tsai and O' Mailey, 1994), the steroid must travel through the cytoplasm and cross the perinuclear membrane. Once inside the nucleus, the steroid can bind to the high-affinity, unoccupied receptor, presumably already on DNA, and cause it to be activated to a form

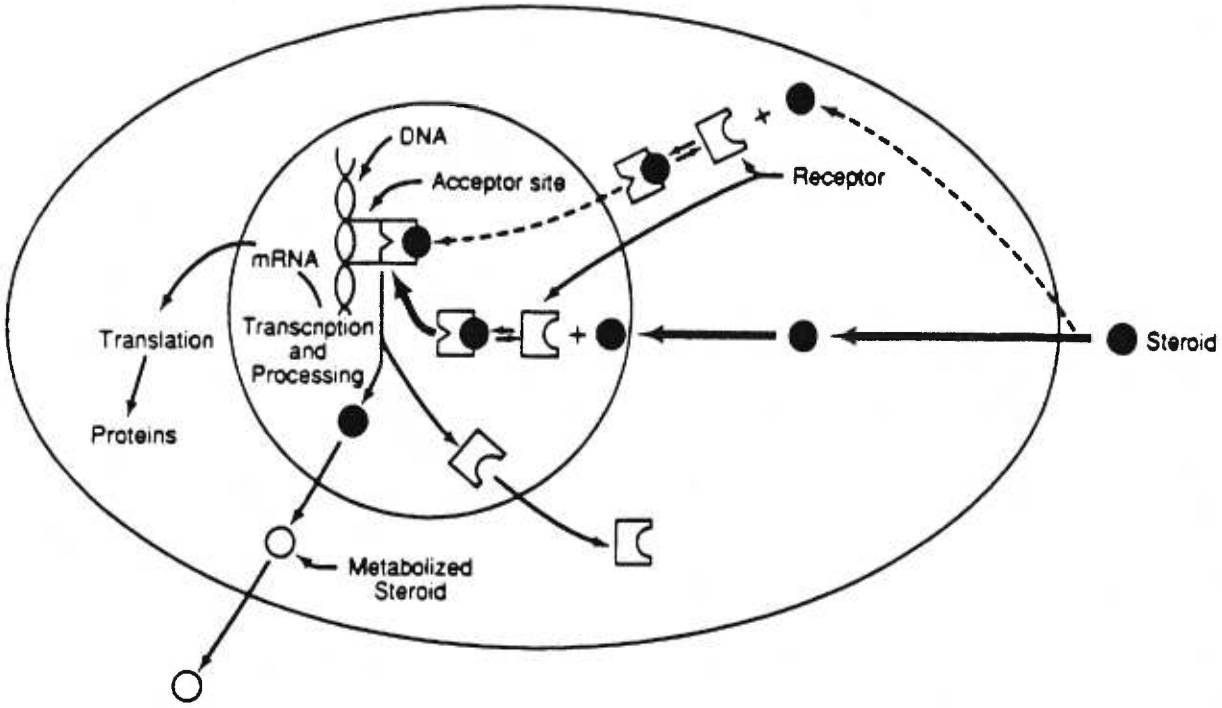


Figure 1-5. General Model of Steroid Hormone Action.

The hormone binds to intracellular receptors in the cytoplasm or the nucleus and causes a conformational change. The hormone-receptor complex then binds to a specific region of DNA (the hormone response element). This interaction results in the activation or repression of a restricted number of genes.

Adapted from Granner DK: (1995) Hormonal action. In: Principle and practice of endocrinology and metabolism. Second edition, edited by Becker KL et al., J.B.Lippincott Company. Philadelphia. 27.

bound to the acceptor site. The ligand might promote a conformation that decreases the off-rate of the receptor from its acceptor if it is located on or near its acceptor site, or might cause the receptor to initiate searching if the unoccupied receptor is on DNA at a locus remote from the acceptor site. After binding of the activated receptor complex to the DNA acceptor site, the same steps as described above may lead to the enhancement or repression of transcription (Tsai and O' Mailey, 1994).

Early studies indicated that the chicken ovalbumin upstream promoter transcription factor (COUP-TF), a member of the steroid/thyroid hormone receptor superfamily, interacted directly with TFIIB without the need for a cofactor (Tsai et al., 1987; Ing et al., 1992). Similar observations were also made for the receptors for progesterone, estrogen, thyroid hormone, and retinoic acid (RAR) (Ing et al., 1992; Baniahmad et al., 1993). It has been found that the N-terminal end of the thyroid hormone receptor interacts specifically with TFIIB (Baniahmad et al., 1993). Since binding of TFIIB to the TFIID-DNA complex is one of the rate-limiting steps in pre-initiation complex formation, it is possible that the thyroid hormone receptor enhances the formation of the complex through such an interaction.

Ptashne (1988) proposed two models to explain the synergism that is relevant to the steroid/thyroid hormone receptor superfamily members. In the cooperative binding model, binding of one receptor complex facilitates the binding of the second. This cooperative interaction allows both complexes to bind with higher affinity and consequently results in higher occupancy of the *cis*-acting elements, thus promoting higher transcriptional activity. Indeed, cooperative binding of receptors to multiple response elements has been observed with the receptors for progesterone, glucocorticoids, and estrogen (Tsai et al., 1989; Schmid et al., 1989; Martinez and Wahli, 1989). Protein-protein interactions between the two dimers probably facilitates cooperativity. Two molecules of the *E.coli* -expressed DNA binding domain (DBD) of the glucocorticoid receptor were able to bind to a single GRE in a cooperative manner (Tsai et al., 1988). However, two dimers of the DBD were unable to bind cooperatively to two adjacent GREs, although the full-

length receptor could do so (Argente et al., 1990). Since both the DBD and the full-length receptor can bind DNA, cooperative binding between two receptors appears to require protein-protein interactions involving regions outside the DNA binding domains. Although cooperative binding is an attractive model, it is unlikely to account for the total level of transcriptional synergism observed at target genes, especially when different HREs are present. For example, a GRE and an ERE can activate transcription of a linked target gene synergistically, but very little cooperative binding between progesterone and estrogen receptors can be observed (Bradshaw et al., 1991). Therefore Ptashne proposed a second, protein-protein interaction model in which cooperative interactions of receptors or transcription factors with multiple target sites (presumably components of the aggregate transcriptional machinery) may play a role in synergism. It has now been developed as the transcriptional cross-talk model (Gottlicher et al., 1998). A particular well-studied system is the cross-talk between the steroid hormone receptor superfamily and the AP-1 transcription factors. Steroid hormones and related nuclear receptors act as ligand-dependent transcription factors in response to low molecular weight lipophilic ligands (Beato et al., 1995; Mangelsdorf et al., 1995). AP-1 transcription factors translate signals that are generated by cell membrane receptors and are transduced through protein kinase signaling into altered expression of target gene cascades (Cano and Mahadevan, 1995; Treisman, 1996). The paradigm of transcriptional cross-talk refers to the observation that members of these two families can interfere (usually negatively, but under certain conditions also synergistically) with each other's functions (Jonet et al., 1990, Schule et al., 1990). The cross-talk factor can modulate the activity of the DNA-binding factor only through protein-protein interaction (Yang-Yen et al., 1990).

I.5.MECHANISM OF CATECHOLAMINE ACTIONS ON GENE EXPRESSION

The most important neurotransmitters of the sympathetic nervous system are the catecholamines which are derivatives of tyrosine (Ganong, 1991a). Catecholamines, like most of the hormones derived from amino acids and polypeptides, can not diffuse into cells. They must combine with their membrane receptors (adrenoceptors or dopaminergic receptors) and exert their

intracellular functions via activation of second messengers.

I.5.1. Signal Transduction Pathway

The binding of catecholamines with their membrane receptors activates G-proteins, the cellular transducers of the hormone signal, then G-proteins stimulate either adenylate cyclase to form cAMP or phospholipase C (PLC) to form diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). The important second messengers cAMP, DAG, and IP_3 can activate protein kinase A (PKA), protein kinase C (PKC) or increase the release of calcium ions from intracellular stores, respectively. Activated PKA, PKC, and calcium-calmodulin dependent kinase which is activated by increased intracellular Ca^{++}, can phosphorylate other proteins. Phosphorylation and dephosphorylation are very important mechanisms that modulate the protein activity and therefore the physiological functions of the cell (Ganong, 1991).

I.5.1.1. Catecholamine Receptors

Ahlquist (1948) first suggested that there are two types of adrenoceptors: α- and β-adrenoceptors. Isoproterenol is generally viewed as a pure β-adrenoceptor agonist, epinephrine is viewed as a combined α- and β-adrenoceptor agonist, and norepinephrine is viewed as a preferentially α -adrenoceptor agonist. Up to now, α adrenoceptors have been subclassified into α_1 (Schwinn et al., 1992) and α_2 (Ruffolo et al., 1993), and β- adrenoceptors have been subclassified into β_1, β_2, and β_3 (Deighton et al., 1992). Both α_1 and α_2 include three different subtypes: α_{1A} (Encloh et al., 1992), α_{1B} (Williamson et al., 1993), α_{1D}, and α_{2A} (Millan, 1992), α_{2B}, α_{2C} (Watson and Girdlestone, 1995).

The dopaminergic receptors have been subclassified into D_1, D_2, D_3, D_4, and D_5 (Kebabian and Neumeyer, 1994), and their natural agonist is dopamine.

The subtypes and the signal transduction pathways of the catecholamine receptors are shown in Table 1-3.

Table1-3. Subtypes and Signal Transduction Pathways of Catecholamine Receptors

α1-Adrenoceptors			
Currently Accepted Name	α_{1A}	α_{1B}	α_{1D}
Alternate Name	α_{1a}	α_{1b}	$\alpha_{1a/d}$
Signal Transduction mechanism	IP_3/DAG	IP_3/DAG	IP_3/DAG
α2-Adrenoceptors			
Currently Accepted Name	α_{2A}	α_{2B}	α_{2C}
Alternate Name	–	–	–
Signal Transduction mechanism	cAMP↓	cAMP↓	cAMP↓
	K^+ ↑	Ca^{++}↓	Ca^{++}↓
β-Adrenoceptors			
Currently Accepted Name	β_1	β_2	β_3
Alternate Name	–	–	–
Signal Transduction mechanism	cAMP↑	cAMP↑	cAMP↑
Dopamine D1-Like Receptors			
Currently Accepted Name	D_1	D_5	
Alternate Name	D-1, D_{1A}	D-5, D_{1B}	
Signal Transduction mechanism	cAMP↑	cAMP↑	
Dopamine D2-Like Receptors			
Currently Accepted Name	D_2	D_3	D_4
Alternate Name	D_{2A}	D_{2B}	D_{2C}
Signal Transduction mechanism	cAMP↓	?	cAMP↓
	Ca^{++} ↓		
	K^+ ↓		

Adapted from Watson, S., and Girdlestone, D.: (1995) Trends in Pharmacological Science, Receptor and Ion Channel Nomenclature Supplement (6th ed) p 1-73.

Some adrenoceptors have been cloned and structural investigation indicates that the adrenoceptors consist of a single polypeptide chain which contains seven transmembrane domains (regions of hydrophobic amino acids) (Kobilka et al., 1987; Litwack, 1992) interconnected by hydrophilic segments which form extramembranous extracellular and cytoplasmic loops. A polypeptide sequence from the first transmembrane domain extends to the extracellular space. A long extended chain from the last transmembrane domain contains phosphorylation sites (serine residues) of the receptor, which are part of the receptor regulation process involving receptor desensitization. The ligand binding occurs in a pocket formed by the location of membrane-spanning domains I-VII (Kobilka et al., 1987; Litwack, 1992).

I.5.1.2. G-Proteins

Most transducers of receptors in the cell membrane are GTP binding proteins, G-proteins. G-proteins consist of three types of subunits, α, β, and γ. The α subunit binds and hydrolyzes GTP. The β and γ subunits form a dimer that only dissociates when it is denatured (Neer, 1994). Both α and $\beta\gamma$ subunits can bind to the receptor. Monomeric, GDP-liganded α subunits can interact with receptors, but the association is greatly enhanced by $\beta\gamma$. When a signal stimulates the receptor, it becomes activated and changes its conformation. The GDP-liganded α subunit responds with a conformational change that decreases GDP affinity, so that GDP leaves the active site. Once GTP is bound, the α subunit assumes its activated conformation and dissociates both from the receptor and from $\beta\gamma$ subunits. The activated state lasts until the GTP is hydrolyzed to GDP by the intrinsic GTPase activity of the α subunit. Once GTP is cleaved to GDP, the α and $\beta\gamma$ subunits reassociate, become inactive, and return to the receptor.

The prevalent hypothesis for the mechanism of G protein mediated signal transduction was that the GTP- liganded α subunit activated effectors, while the $\beta\gamma$ subunit was only a negative regulator (Gilman, 1987). This paradigm, however, changed fundamentally with the discovery that the $\beta\gamma$ subunit could activate the muscarinic K^+ channel and the realization that both α and $\beta\gamma$

subunits positively regulate effectors (Logothetis et al., 1987). Subsequently, the βγ subunit was shown to be a positive regulator of a large number of effectors in addition to this K^+ channel, including adenylate cyclase, phospholipase Cβ (PLCβ), phospholipase A_2 (PLA_2), phosphoinositide 3-kinase (PI_3-kinase), and β–adrenoceptor kinase (Clapham and Neer, 1993). The βγ subunit may also act through *ras* to activate the mitogen-activated protein (MAP) kinase pathway (Crespo, 1994). It has become clear that many effectors are regulated both by α and βγ subunits.

Mammals have over 20 different G protein α subunits (16 gene products, some with alternatively spliced isoforms) (Kaziro et al., 1991). These proteins can be divided into four major classes according to the similarity (56%-95% homology) of their amino acid sequences (Table 1-4). Most α subunits are widely expressed. Individual cells usually contain at least four or five types of α subunits (Neer, 1994).

The five known mammalian β subunits share between 53% and 90% identical amino acid sequences (Simon et al., 1991). In contrast, the six γ subunits are much more varied in sequence than the β subunits or the α subunits (Cali et al., 1992). Five different β subunits and six γ subunits could produce 30 different combinations. However, not all the possible pairs can form.

I.5.1.3. Effectors

Adenylate cyclase, one of the most important effectors, catalyzes the generation of cAMP from ATP. The increased intracellular cAMP binds to protein kinase A, which is a tetramer containing two regulatory and two catalytic subunits. The binding of four cAMP molecules with the two regulatory subunits releases the catalytic subunits. The liberated catalytic subunits are able to phosphorylate proteins to produce cellular effects (Ganong, 1991 a) (Figure 1-6).

Phospholipase C, another important effector, is also coupled to the G protein. A hormone operating through this system binds to a specific cell membrane receptor, which interacts with a G-

Table 1-4. Classes of Gα Subunit

Class	Members	Modifying Toxin	Some Functions
α_s	α_s, α_{olf}	cholera	Stimulate adenylyl cyclase, regulate Ca^{++} channel
α_i	α_{i-1}, α_{i-2}, α_{i-3}, α_0, α_{t-1}, α_{t-2}, α_{gust}, α_z	pertussis (except α_z)	Inhibit adenylyl cyclase, regulate K^+ and Ca^{++}channels, activate cGMP phosphodiesterase
α_q	α_q,α_{11},α_{14},α_{15},α_{16}		Activate PL
α_{12}	α_{12}, α_{13}		Regulate Na^+/K^+ exchange

Reproduced from Neer EJ: (1995) Heterotrimeric G proteins: organizers of transmembrane signals. Cell 80: 249-257.

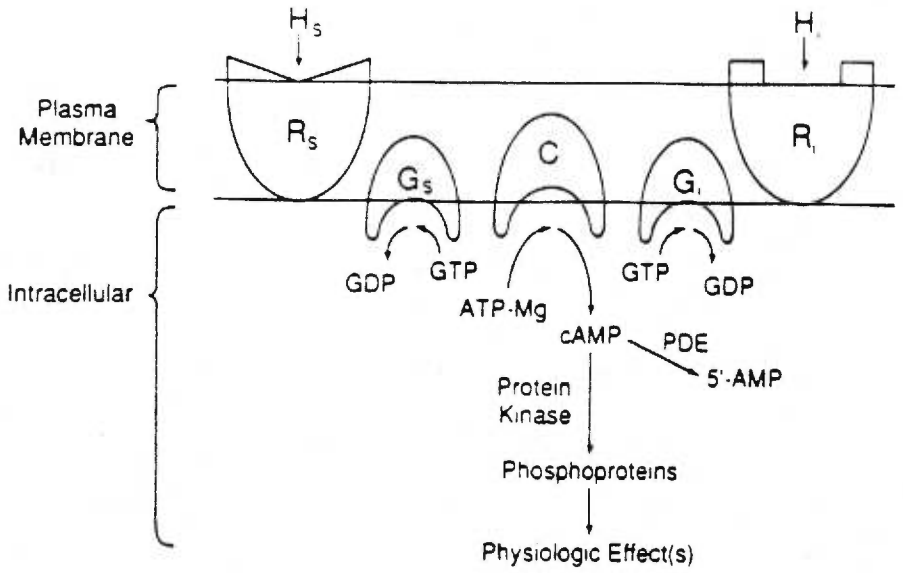


Figure 1-6. Protein kinase A (PKA) pathway.

Activated catalytic unit (Cat) of adenylate cyclase catalyzes the conversion of ATP to cAMP. cAMP activates protein kinase A, which phosphorylates proteins, producing physiologic effects. Stimulatory ligands bind to the stimulatory receptor (Rs) and activate Cat via the stimulatory G- protein (Gs). Inhibitory ligands inhibit Cat via the inhibitory receptor (Ri) and the inhibitory G-protein (Gi).

Reproduced from Ganong WF: (1991) Review of Medical Physiology. Fifteen Edition. Lange Medical Publication. 39.

protein and transduces the signal resulting in stimulation of phospholipase C. This enzyme catalyzes the hydrolysis of phosphatidylinositol-4,5- bisphosphate (PIP_2) to form two second messengers, diacylglycerol (DAG), and inositol-1,4,5-trisphosphate (IP_3). IP_3 diffuses through the cytoplasm and binds to the IP_3 receptor on the membrane of a particular calcium storage organelle (calciosome). Its binding results in the release of calcium ions and the increase of cytoplasmic Ca^{++}. Calcium ions may be important to some cellular functions, e.g., the process of exocytosis. The increased intracellular [Ca^{++}] also activates the Ca-calmodulin dependent protein kinase which may phosphorylate other proteins (Ganong, 1991b).

Diacylglycerol binds to a regulatory site on protein kinase C in the cell membrane; some of the enzyme is in the cytoplasm either with its regulatory site intact or with it cleaved off. In the latter form, it is called protein kinase M. Binding of DAG stimulates the activity of PKC in the presence of phosphatidylserine and Ca^{++}, which then phosphorylates specific proteins in the cytoplasm, or in some cases, in the membrane (Figure 1-7).

The phosphorylated proteins perform specific functions that they could not carry out in the nonphosphorylated state. For example, a certain phosphorylated protein might migrate to the nucleus and increase mitosis and growth.

I.5.2. Role of cAMP in the Signal Transduction Pathway of Catecholamines

I.5.2.1. cAMP Response Element (CRE)

The DNA enhancer element responsive to the cAMP/PKA signal pathway consists of the core octamer motif, 5'-TGACGTCA-3' which is designated a cAMP response element (CRE) (Montminy et al., 1986). This element displays dyad symmetry. Observations have indicated that not only the consensus CRE, but other sequences, that are similar to the consensus CRE, may mediate the cAMP effect (Table 1-5).

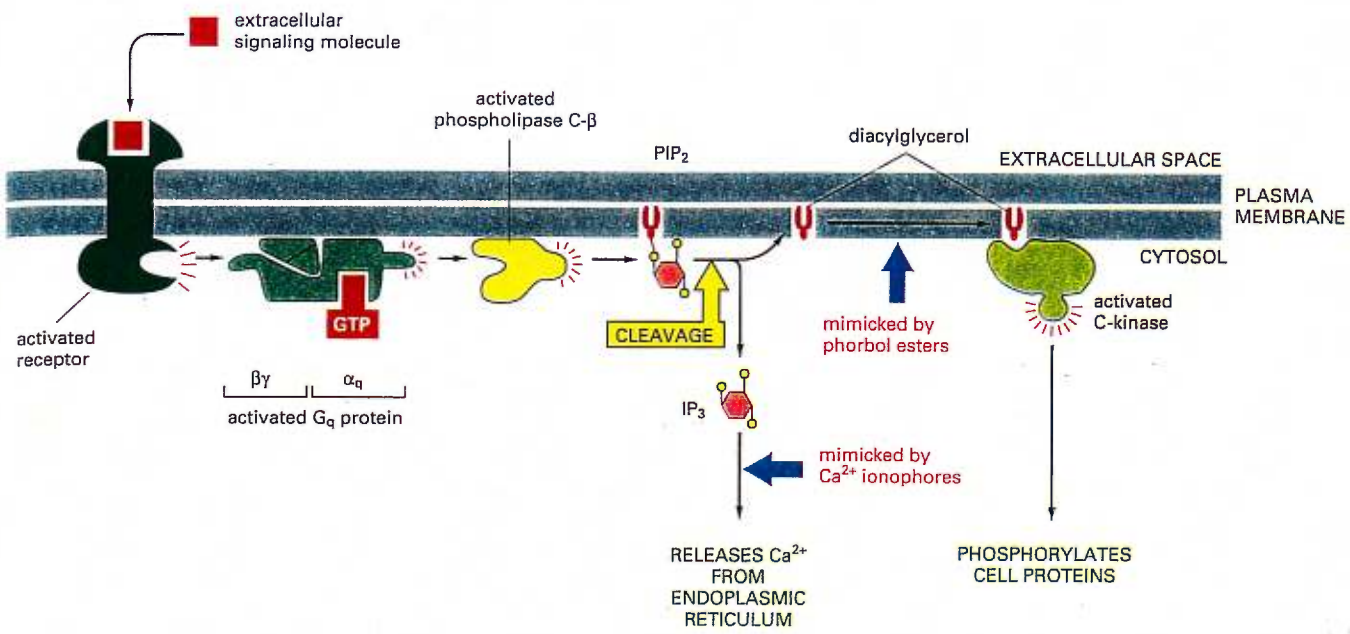


Figure 1-7. Phospholipase C (PLC) Pathway.

Diagrammatic representation of release of inositol trisphosphate (IP_3) and diacylglycerol (DAG) as second messengers. Binding of ligand to receptor activates phospholipase C (PLC) via a G-protein. The resulting hydrolysis of PIP_2 produces IP_3, which releases Ca^{++} from the endoplasmic reticulum (ER), and DAG, which activates protein kinase C (PKC).

Adapted from Albert B et al: (1994) Molecular Biology of The Cell. Third Edition. Garland Publishing, Inc. 749.

Table 1-5. DNA Sequence of CRE in Some Genes

	5'-	CRE-Core	-3'		
SMS CRE	CTGGGGGCGCCTCCTTGGC	TGACGTCA	GAGAGAG	(N-32)	(1)
α-CG CRE	AACAAGATAAGATCATACTG	TGACGTCA	TGGTAATTA	(N-111)	(2)
VIP CRE	TCCCATGGCCGTCATACTG	TGACGTCT	TTCAGAGCA	(N-60)	(3)
PEPCK CRE	TGATCCAAAGGCCGGCCCC	TTACGTCA	GAGGCGAGC	(N-74)	(4)
ANG CRE-like	CCATGGCCAAGAGATTACT	TGACGTAC	GAGGCGAGC	(N-779)	(5)

(1). Montminy MR, Sevarino KA, Wagner JA, Mandel G, and Goodman RH: (1986) Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc Natl Acad Sci USA 83; 6682-6686.

(2). Silver BJ, Boker JA, Virgin JB, Vallen EA, Milsted A, and Nilson JH: (1987) Cyclic AMP regulation of the human glycoprotein hormone a-subunit gene is mediated by an 18-base pair element. Proc Natl Acad Sci USA 84: 2198-2202.

(3). Tsukada T, Fink JS, Mandel G, and Goodman RH: (1987) Identification of a region in the human vasoactive intestinal polypeptide gene responsible for regulation by cyclic AMP. J Biol Chem 262: 8743-8747.

(4). Wynshaw-Boris A, Lugo TG, Short JM, Fournier EK, and Hanson RW: (1984) Identification of a cAMP regulatory region in the gene for rat cytosolic phosphoenolpyruvate carboxykinase (GTP). Use of chimeric genes transfected into hepatoma cells. J Giol Chem 259: 12161-12169.

(5). Chan JSD, Chan AHH, Jiang Q, Nie Z-R, Lachance S, and Carriere S: (1990) Molecular cloning and expression of the rat angiotensinogen gene. Pediatr Nephrol 4: 429-435.

I.5.2.2. cAMP Response Element Binding Protein (CREB)

The CRE binds with its transacting factor, cAMP response element binding protein (CREB) and subsequently regulates gene expression (Montminy and Bilezikjian, 1987). CREB, CREM (cAMP response element modulator) and TAF-1(transcription activation factor-1) form a distinct subfamily of transcription factors characterized by their participation in the final communicative link in the regulation of gene expression in response to activation of the cAMP-dependent signaling pathway.

The human CREB gene (*creb*) which encodes the CREB protein, has been mapped to position 2q32.3-q34 on the long arm of chomosome 2 (Taylor et al., 1990). The mouse CREB gene has been localized to the proximal region of chomosome 1 (Cole et al., 1992). It consists of multiple exons (at least 11 exons have been identified in the human CREB gene) across a span of more than 80 kbp (Hoeffler et al., 1988). The DNA sequence of the 5'-untranslated region and the promoter region is highly enriched in guanines and cytosines, and contains the binding sites for the transcription factors AP2 (Imagawa et al., 1987; Williams et al., 1988), C/EBP (Landschultz et al., 1988), SP1 (Kadonaga et al., 1987), GCF (Kageyama and Pastan, 1989), and the CREB protein itself. There are three binding sites (CRE1, CRE2, and CRE3) for CREB, but a notable absence of TATAA and CCAAT sequence motifs (Cole et al., 1992). It appears that multiple transcription start sites are present in the CREB gene. A major start site, however, occurs within an initiator (InR) sequence, CCTCA (Cole et al., 1992).

The CREB mRNA is particularly difficult to detect by standard Northern blotting techniques, whereas the CREB protein is easily detected by antibodies (Meyer and Habener, 1993). This circumstance suggests that the CREB mRNA is expressed at a relatively low basal level and/or is rapidly degraded (Meyer and Habener, 1993).

CREB is a 43 kDa nuclear protein, which has a binding specificity for the CRE. Comparison of the cDNAs from humans and mice reveals the presence of two primary CREB protein isoforms, CREB327 (327 amino acids) and CREB341 (341 amino acids), that are encoded by the same gene using two alternatively spliced mRNAs. The amino terminal three-fourths of CREB comprises the transcriptional transactivation domain, and the carboxy-terminal one-fourth of the sequence contains the DNA binding and dimerization domains. Dimerization and phosphorylation are two important posttranslational modifications; the phosphorylation especially plays a major and critical role in the regulation of transactivational functions of CREB. Upon activation of the adenylyl cyclase pathway, a serine residue at position 133 of CREB is phosphorylated by PKA (Gonzalez and Montminy, 1989). The phosphorylation appears indispensable for activation, and the phosphoserine cannot be substituted for by other negatively charged residues (Gonzalez and Montminy, 1989).

Phosphorylation of CREB by PKA causes a modest increase in binding to high affinity CRE sites, but a stronger enhancement of binding to low affinity CREs (Nichols et al., 1992). By contrast, other experiments indicated that PKA mediated phosphorylation of CREB did not affect DNA binding (Yamamoto et al., 1988). In any case, the major effect of phosphorylation seems to occur at the level of the trans-activation function of CREB. It has been proposed that this could happen by inducing a conformational change in the protein (Yamamoto et al., 1988).

I.5.2.3. Mechanisms of CREB-Induced Transactivation

There is evidence for direct (Chrivia et al., 1993; Ferreri et al., 1994) and indirect (Kowk et al., 1994) interaction of CREB with the basal transcription machinery.

I.5.2.3.1. Direct CREB-TAF110 Interaction

Site-directed mutagenesis studies in a cell free system(s) have revealed that the transactivation domain of CREB directly interacts with TAF110 (a TATAA binding protein associated factor) (Chrivia et al., 1993; Ferreri et al., 1994). Ferreri and Gill (1994) tested the interactions between

CREB and ATF110. They prepared CRE oligonucleotide affinity resin bound with purified CREB or the mutant CREB which had been modified in the transactivation domain. The TAF110 protein could indeed bind to CRE resin containing wild-type CREB, but not to mutant CREB, indicating a direct interaction of CREB (through the transactivation domain) with the basal transcriptional complex (through TAF110).

I.5.2.3.2. CREB Acting through a Coactivator CBP and TFIIB

Activated CREB may also function through coactivator molecules. A CREB binding protein (CBP) has been identified which binds specifically to phosphorylated CREB. Evidence has been provided that the activation domain of CBP interacts with the basal transcriptional factor TFIIB through a domain that is conserved in the yeast coactivator ADA-1 (Kowk et al., 1994). In CREB-deficient F9 teratocarcinoma cells, in the absence of endogenous CBP, the combination of CREB and PKA could only activate expression of a reporter gene 15-fold; addition of CBP increased transcription in a dose-dependent manner up to 90-fold. CBP did not activate gene expression in the absence of CREB, even in the presence of PKA. Furthermore, CREB mutated at the PKA phosphorylation site did not allow CBP to activate expression. These findings suggest that CBP induces gene expression only if CREB and PKA are both present, implying that CBP is the mediator which is responsible for cAMP-activated gene transcription (Kowk et al., 1994).

The binding of CBP to TFIIB was tested by using glutathione-S-transferase (GST)-TFIIB fusion proteins linked to glutathione agarose beads (Nordheim, 1994). Specific binding was observed with a minimum CBP fragment containing a zinc finger structure. Binding between CBP and deletion mutants of TFIIB suggested that CBP may require the amino-terminal region of TFIIB. These data support the model that CBP serves as a coactivator for phosphorylated CREB. Since TFIIB interacts with TBP and helps to recruit RNA polymerase II (Pol II) to the promoter, a continuous chain of physical contacts is now established, linking the stimulus-activated and phosphorylated CREB, bound distal to the promoter, with the Pol II complex that initiates transcription (Nordheim, 1994). Thus CBP may play a critical role in the transmission of

inductive signals from the cell surface receptor to the transcriptional apparatus (Kowk, 1994; Arias et al., 1994; Nordheim, 1994) (Figure 1-8).

I.6. RATIONALE AND OBJECTIVES OF PRESENT STUDIES

Hypertension in humans and in experimental animals has been linked to either increased glucocorticoid levels or increased catecholamine levels. For instance, moderate to severe elevation of arterial blood pressure is associated with endogenous cortisol excess in Cushing's syndrome (hypercortisolism) (Krakoff and Mendlowitz, 1973). Increased norepinephrine levels in cerebrospinal fluid (Eide et al., 1979; Lake et al., 1981; Ziegler et al., 1982) and increased epinephrine levels in plasma (Goldstein, 1983a; Goldstein, 1983b) have been found in patients with essential hypertension. Both glucocorticoids and catecholamines can enhance total peripheral vascular resistance and cardiac output which are involved in the development of hypertension (Krakoff and Mendlowitz, 1973; Wyss et al., 1990). A positive correlation between blood pressure and plasma ANG levels in a wide variety of clinical conditions and animal experiments has indicated that high ANG can be a causal factor in hypertension (Gordon, 1983). To learn whether glucocorticoids or catecholamines can induce ANG gene expression, and whether there is a cooperation between them is important for understanding the mechanism of ANG gene regulation and to provide further insight into the pathogenesis of hypertension.

Two glucocorticoid response elements, GRE I and GRE II, have been identified at positions -585/-570 and -477/-472, respectively, in the rat ANG gene. Previous studies in our laboratory have also found a DNA sequence 5'-TGACGTAC-3' located between -795 and -788 (Chan et al., 1990). Comparison of this sequence with the known consensus sequence of CRE, 5'-TGACGTCA-3', showed that only the last two basepairs were reversed. Whether it may mediate the responses to cAMP and furthermore to catecholamine β-adrenergic agonist(s) on ANG gene expression is unclear.

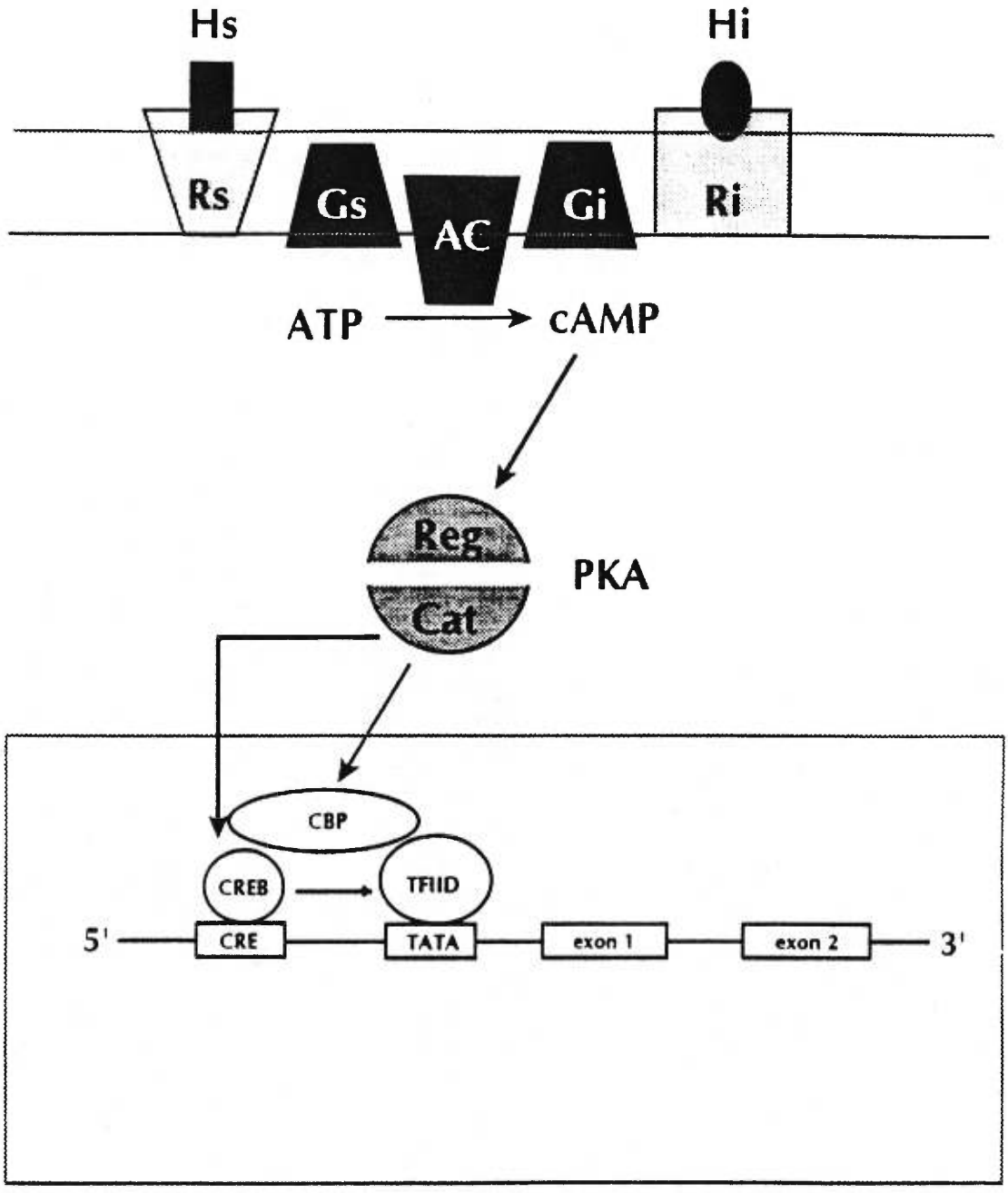


Figure 1-8. Schematic Model of cAMP-Mediated Target Gene Regulation.

cAMP binds to the regulatory subunit (Reg) of PKA, leading to the dissociation and activation of the catalytic (Cat) subunit. It then translocates to the nucleus where it phosphorylates CREB and CBP. CREB activates target genes either through direct interaction with TAFs-TFIID, or indirectly through CBP to activate TFIIB and TFIID.

Therefore, our hypothesis in the present studies " Regulation of Expression of ANG Gene in mouse hepatoma and opossum kidney cell lines " is that the addition of glucocorticoids (DEX) and catecholamines (isoproterenol) may act synergistically to stimulate the expression of the ANG gene in both cell lines *in vitro*. This hypothesis is based on clinical observation of a cooperation between glucocorticoids and catecholamines in stress-induced hypertension.

To test the hypothesis, two theoretical models are proposed to explain the possibly synergistic effects of DEX and isoproterenol on the expression of the ANG gene.

Model I

In model I, the binding of isoproterenol with its membrane receptors in the hepatoma cell line (Hepa 1-6) or kidney-derived cell line (OK) would activate G-proteins, the cellular transducers of the hormone signal. The activated G-proteins can stimulate adenylate cyclase to catalyze the generation of cAMP from ATP. The increased intracellular cAMP can bind to the regulatory subunits of PKA and release the catalytic subunits. The liberated catalytic subunits are able to translocate into the nucleus and phosphorylate the cAMP response element binding protein (CREB). The phosphorylation of CREB may increase the binding affinity of CREB with CRE, and stimulate ANG gene transcription.

After diffusion into the cytosol, DEX would bind with glucocorticoid receptors and cause the release of heat shock proteins (HSPs) and exposure of DNA binding sites. The activated receptors can translocate into the nucleus and bind to GRE(s) and stimulate ANG gene transcription.

Either isoproterenol or DEX alone may stimulate ANG gene expression and the two in combination may act synergistically. This would be achieved by protein-protein interaction between the phosphorylated CREB bound to CRE, and the activated glucocorticoid receptor bound to GRE(s). The interaction between them may further increase ANG gene transcription (Figure 1-9).

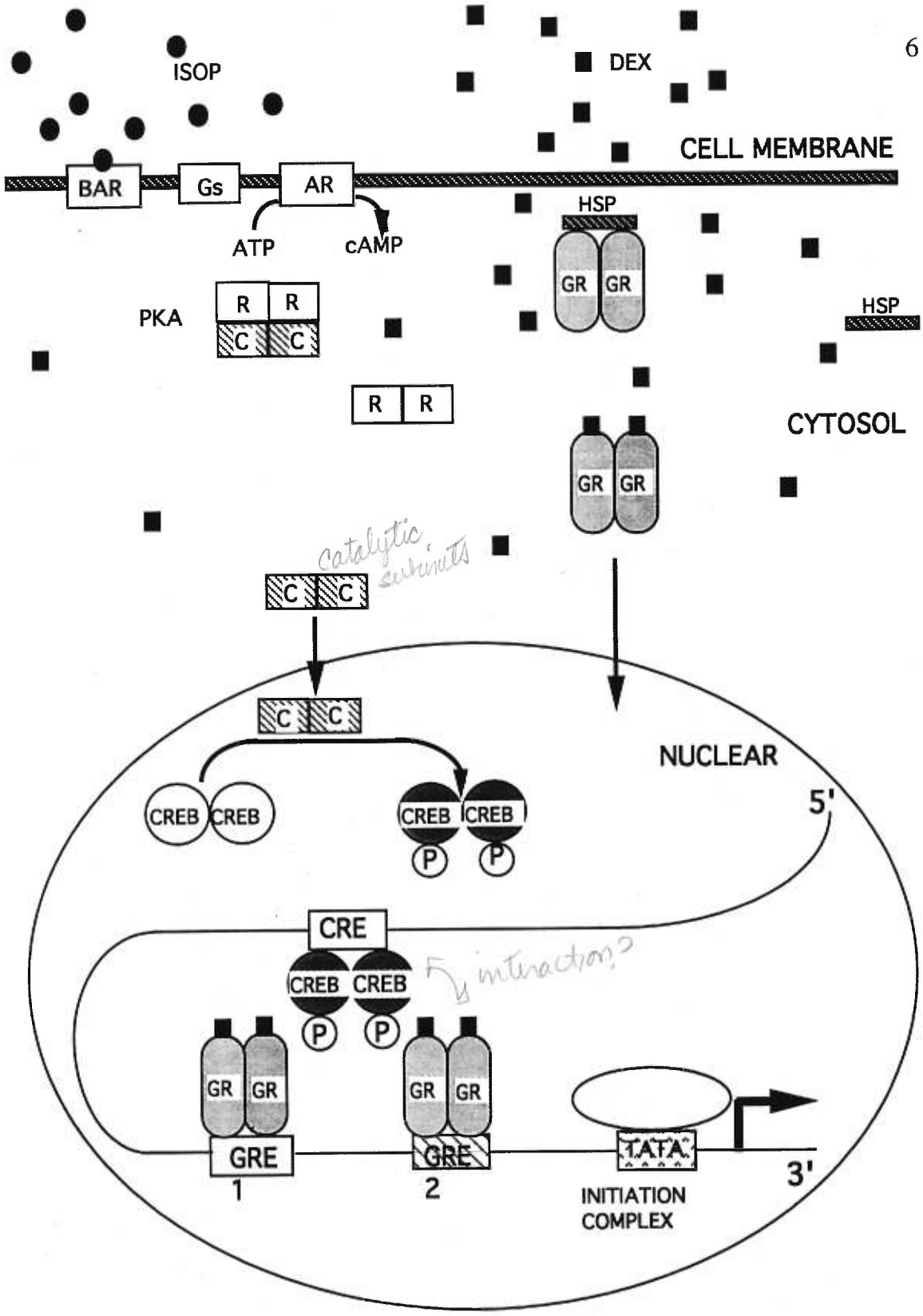


Figure 1-9 Model I

Model II

In model II, after binding DEX, glucocorticoid receptors in the cytoplasm of Hepa 1-6 or OK cells would be activated. Following release of HSPs, the DNA binding sites would then be exposed. The binding of activated receptors with GRE(s) may stimulate the formation of a pre-initiation complex and increase ANG gene transcription.

Isoproterenol would bind to its membrane receptors and activate the G proteins which can then stimulate the adenylate cyclase to synthesize cAMP. The increased intracellular cAMP would bind to the regulatory subunits of PKA and cause the release of catalytic subunits. The activated catalytic subunits can translocate into the nucleus and phosphorylate glucocorticoid receptors which have bound to the GRE(s) after being activated by DEX. The phosphorylation may increase the binding affinity and/or stabilize the binding between the glucocorticoid receptors and GRE(s) to further enhance ANG gene transcription (Fig 1-10).

Model II differs from model I in that there is no direct interaction between CREB and glucocorticoid receptor. This model is supported by the studies showing that PKA can enhance the DNA binding activity of the glucocorticoid receptor thereby activating gene transcription in F9 embryonal carcinoma cells, without the need for endogenous CREB (Rangarajan et al., 1992).

The objective of my Ph.D research project is to study the interaction of β-adrenoceptor agonists (focusing on isoproterenol and its second messenger cAMP) with glucocorticoids (DEX) on the expression of the ANG gene in both the mouse hepatoma cell line (Hepa 1-6) and the opossum renal proximal tubular cell line (OK) to elucidate the underlying molecular mechanism(s) involved in its regulation.

The specific aims of my project are:

(1) to study the possible stimulatory effects of various steroid hormones (dexamethasone, β-estradiol, dihydrotestosterone, progesterone), thyroid hormone (L-T3), and β-adrenoceptor

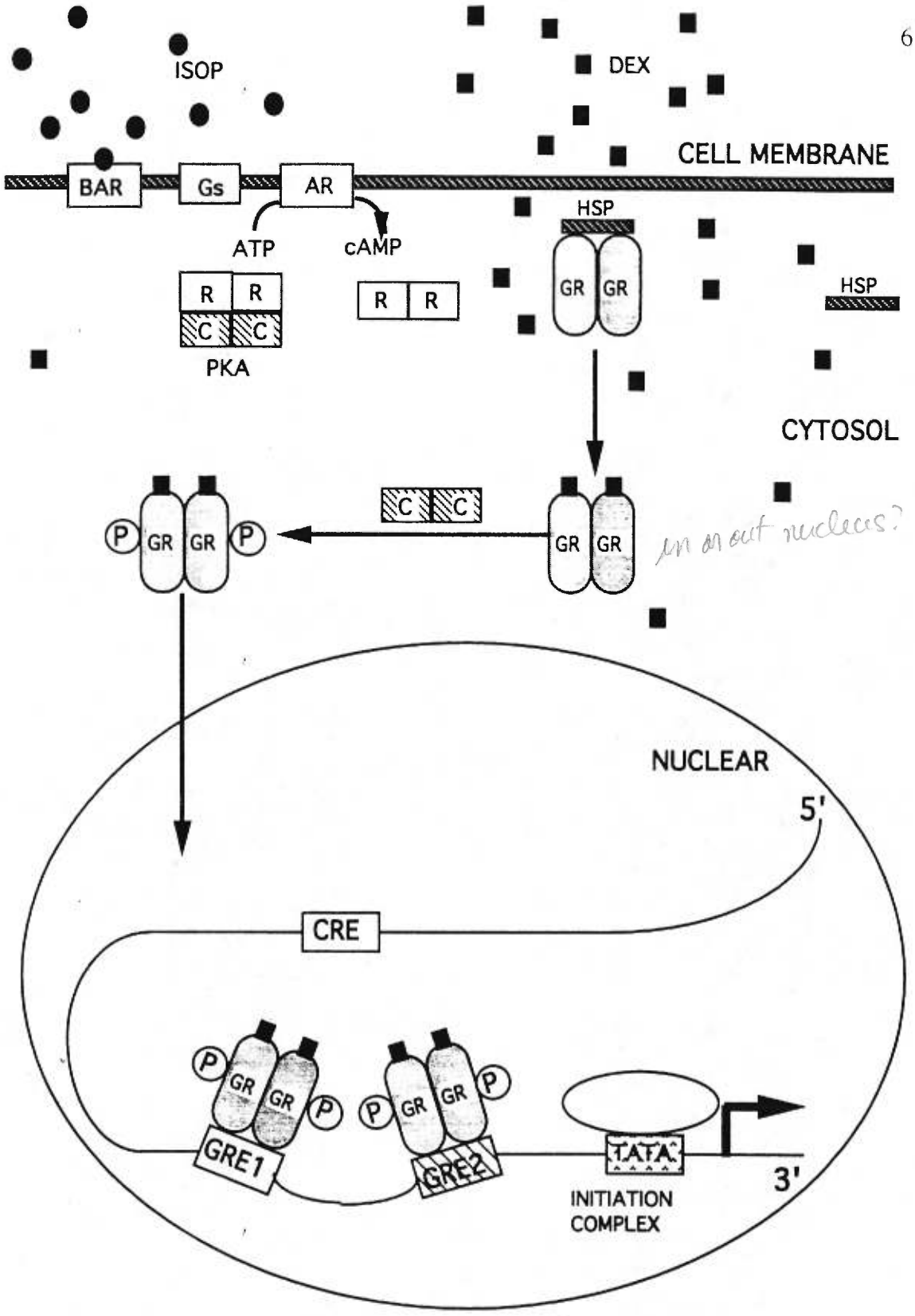


Figure 1-10 Model II

agonists (focused on isoproterenol and its second messenger, 8-Bromo-cAMP) on the expression of the rat ANG gene in Hepa 1-6 and OK cell lines.

(2) to identify the DNA *cis* -regulatory elements in the 5'-flanking region of the rat ANG gene which mediate the effect of cAMP and isoproterenol on the expression of the ANG gene.

(3) to identify the DNA *cis* -regulatory elements in the 5'-flanking region of the rat ANG gene which mediate the cooperation between glucocorticoids and cAMP or isoproterenol on the expression of the ANG gene.

Various ANG-CAT fusion genes containing different lengths of the 5'-flanking region of the rat ANG gene with or without CRE, GRE(s), or both were transfected into Hepa 1-6 or OK cells. DEX, 8-Bromo-cAMP, isoproterenol, or the combination of DEX with either 8-Bromo-cAMP or isoproterenol, were added to test their effects on the expression of various ANG-CAT fusion genes, in an attempt to obtain evidence supporting either model I or model II proposed above.

CHAPTER II MATERIALS AND METHODS

II.1. MATERIALS

II.1.1. Restriction Enzymes

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

II.1.2. Vectors

The expression vectors, a plasmid containing the coding sequence for chloramphenicol acetyltransferase (CAT) without promoter (pOCAT) or with the Rous sarcoma virus enhancer/promoter (pRSVCAT) or the thymidine kinase promoter sequence (pTKCAT) fused to the 5'-end of the CAT coding sequence, were gifts from Dr. Joel F. Habener (Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Boston, MA). The pGEM-3 vector was purchased from Promega-Fisher Inc. (Montreal, Canada). Both pTKGH and pOGH vectors containing the coding sequence of the human growth hormone (hGH) gene (from the start of transcription at the BamHI site to 526 base pairs past the poly (A) addition site) with or without a thymidine kinase enhancer/promoter sequence fused to the 5'-end of the hGH gene, respectively, were purchased from Nichols Diagnostics Institute (La Jolla, CA, USA). The mammalian expression vectors, pBC-BC12 containing the cDNA for human β_1-adrenoceptor (designated as pBC-β_1AR) or the β_2-adrenoceptor (designated as pBC-β_2AR) were gifts from Dr. Michel Bouvier (Department of Biochemistry, University of Montreal, Montreal, Quebec).

II.1.3. Chemical Reagents

II.1.3.1. Isotopes

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 (Boston, MA). Na-^{125}I was purchased from ICN Biochemicals (Montreal, Quebec, Canada). The radioimmunoassay (RIA) kit for hGH was a gift from NIADDK, NIH, USA.

II.1.3.2. Hormones and Drugs

Dexamethasone (Catalog No. D-4902), β-estradiol (Catalog No. E-2758), dihydrotestosterone (Catalog No. D-5027), progesterone (Catalog No. P-0130), 3,3',5-triiodo-l-thyronine (L-T3) (Catalog No. T-2752), forskolin (Catalog No. F-6886) (a post-receptor activator of adenylate cyclase), glucagon (Catalog No. G-9154) (a receptor mediated activator of adenylate cyclase), 8-bromo-cyclic-adenosine-3':5'-monophosphate, (8-Bromo-cAMP) (Catalog No. B-7880), and insulin (Catalog No. I-5500), were purchased from Sigma (St. Louis MO). Insulin like growth factor I (IGF-I) (Catalog No. 1048058) and insulin like growth factor II (IGF-II) (Catalog 1183141) were purchased from Boehringer Manheim (Doval, Quebec, Canada). R(-)-isoproterenol(+)-bitartrate salt (Catalog No. I-102), S(-)-propranolol hydrochloride (Catalog No. P-110), S(-)-atenolol (Catalog No. A-143), ICI-118,551 HCl (Catalog No. I-127) and Rp-cAMP (Catalog No. A-165) (an inhibitor of the cAMP-dependent protein kinases AI and II) (Landsberg and Jastorff, 1985) were all purchased from Research Biochemicals Inc (RBI, Natick, MA, USA). RU486 (a glucocorticoid antagonist) was a gift from Dr. Alain Belanger (Laval University, Quebec, Canada).

All hormones and drugs were dissolved in sterile double distilled water except dexamethasone, β-estradiol, dihydrotestosterone, progesterone, and RU486 which were dissolved in 95% ethanol. The final ethanol concentration in the incubation medium was 0.095%. Other reagents were molecular biology grade and were obtained from Sigma Chemical Co, Bethesda

Research Laboratories, Boehringer-Mannheim, or Pharmacia Inc.

II.1.4. Oligonucleotides

Oligonucleotides which contain the rat ANG gene cAMP response element (CRE, ANG N-795 to N-788; 5'-AGCTTAAGAGATTACTTGACGTACTGGATGCAAT-3'), and glucocorticoid response element I (GRE I, ANG N-585 to N-570; 5'-AAGCCCAGAACATTTTGTTTCAATATGG-3'), and II (GRE II, ANG N-477 to N-472; 5'-CCCTGTGCAGAACAGACAACT-3'), and their complementary strands were synthesized by Bio-Synthesis Inc. (Lewisville, TX).

II.1.5. Culture Media

Bactotryptone (Catalog No. 30391-039) and yeast extract (Catalog No. 30393-029) were purchased from GIBCO BRL (Grand Island, N.Y.) The LB-medium was prepared by dissolving 10g of bactotryptone, 5g of yeast extract, and 10g of NaCl (Catalog No.11830-049) in 1L of distilled water.

Dulbecco's modified Eagle medium (DMEM) (Catalog No.12800-082), heat inactivated fetal bovine serum (FBS) (Catalog No.16140-071), Dulbecco's phosphate-buffered saline (PBS) (Catalog No.14200-075), trypsin-EDTA (Catalog No.25300-062), and other tissue culture reagents were also purchased from GIBCO BRL.

II.1.6. Others

II.1.6.1. DNA Purification Kit

Mermaid and Gene Clean II DNA purification kits (Catalog No. 1005-200, 1001-400, respectively) were purchursed from BIO101 (La Jolla, CA).

II.1.6.2. TLC Plates

Thin-layer chromatography (TLC) plates (Catalog No. 05-719-800) were purchased from Fisher Scientific Ltd. (Montreal, Quebec, Canada).

II.1.7. Equipment

II.1.7.1. DNA Construction

The equipment used for constructing various fusion genes were as follows: DNA thermal cycler (Perkin Elmer Cetus), thermostatic circulator (2219 Multitemp II; LKB, Bromma), DU-6 spectrophotometer (Beckman), incubator shaker (Series 25; New Brunswick Scientific Co., Inc.), refrigerated superspeed centrifuge (Sorvall RC-5B; Dupont Instruments), isotemp vacuum oven (model 281A; Fisher Scientific), micro-centrifuge (model 235 C; Fisher Scientific), Speed Vac concentrator (Savant), vacuum pump (Bio-Rad), hybridization oven (Tek*Star Bio/Can Scientific), water bath (B 6990; American), Polaroid MP4 camera (Fotodyne Incorporated), UV transilluminator box (Fotodyne Incorporated), DNA sequencing gel box (Model S2; BRL), power supply (2197; LKB), gel dryer (Model 583; Bio-Rad), and freezer (-20°C, -80°C; Kenmore).

II.1.7.2. Cell Culture

The equipment used for cell culture were as follows: Biohood (Contamination Control Inc (CCI); Kulpsville, Penna), CO_2 incubator (Moquin Premier; Par/by Revco), centrifuge (Sorvall RT 6000D; Dupont), refrigerator (Kenmore), liquid nitrogen tank (Locator 4; Thermolyne Cryo Biological Storage System), and microscope (Diavert; Leitz Wetzlar Germany).

II.1.7.3. Assays

The equipment used for assays were as follows: Protein assay: Microplate reader (model 3550; Bio-Rad). CAT assay: liquid scintillation counter (Nuclear-Chicago Instrumentation Inc) and TLC tank (Fisher Scientific). Radioimmunoassay: Automatic gamma counter (1272

Clinigamma; LKB).

II.2. METHODS

II.2.1. Construction of Fusion Genes

The construction of fusion genes pOCAT (ANG N-1498/+18), pOCAT (ANG N-1138/+18), pOCAT (ANG N-960/+18), pOCAT (ANG N-814/+18), pOCAT (ANG N-688/+18), pOCAT (ANG N-280/+18), pOCAT (ANG N-196/+18), pOCAT (ANG N-110/+18), pOCAT (ANG N-53/+18), and pOCAT (ANG N-35/+18), were described previously (Chan et al., 1992a; Ming et al., 1995a). Briefly, pGEM-3 (Promega-Fisher, Inc.) was cut with *Nhe* I, repaired with DNA polymerase I (Klenow fragment) and digested with *BamH* I. The DNA fragment (279 base pairs) containing a SP6 promoter sequence and eight polylinker sites (*Hind* III, *Sph* I, *Pst* I, *Sal* I, *Acc* I, *Hinc* II, *Xba* I, and *BamH* I) was isolated from a low melting point agarose gel and inserted into pOCAT 5' to the CAT coding sequence via polylinker sites (blunted *Sac* I and *BamH* I). This modified pOCAT was then used for all subsequent ANG-CAT fusion gene constructions. The pOCAT (ANG N-53/+18) and pOCAT (ANG N-35/+18) constructs were created by isolating the DNA fragments (*Hind* III/*BamH* I) from pOGH (ANG N-53/+18) and pOGH (ANG N-35/+18), respectively, which were inserted into the modified pOCAT, previously digested with restriction enzymes *Hind* III/*BamH* I. The pOCAT (ANG N-1498/+18), pOCAT (ANG N-1138/+18), pOCAT (ANG N-960/+18), pOCAT (ANG N-814/+18), pOCAT (ANG N-688/+18), pOCAT (ANG N-280/+18), pOCAT (ANG N-196/+18), and pOCAT (ANG N-110/+18) constructs were produced by isolating the DNA fragments (*Hind* III/*Xho* I) from pOGH (ANG N-1498/+18), pOGH (ANG N-1138/+18), pOGH (ANG N-960/+18), pOGH (ANG N-814/+18), pOGH (ANG N-688/+18), pOGH (ANG N-280/+18), pOGH (ANG N-196/+18), and pOGH (ANG N-110/+18), respectively, and by inserting them into pOCAT (ANG N-53/+18), which had been previously digested with the restriction enzymes *Hind* III/*Xho* I.

The fusion genes, pTKCAT containing various lengths of the 5'-flanking region of the rat

angiotensinogen gene pTKCAT (ANG N-814/-689), pTKCAT (ANG N-814/-761), and pTKCAT (ANG N-760/-689) were constructed by Dr. Chan, and the method was also described previously (Ming et al., 1993). Briefly, various DNA fragments of the rat ANG gene (ANG N-814/-689, ANG N-814/-761, and ANG N-760/-689) were transferred into the modified pTKCAT vector. The modified pTKCAT vector was constructed by the following procedures. pGEM-3 (Promega-Fisher, Inc.) was cut with *Nhe* I, repaired with DNA polymerase I (Klenow fragment) and digested with *BamH* I. The DNA fragment (279 base pairs) containing a SP6 promoter sequence and eight polylinker sites (*Hind* III, *Sph* I, *Pst* I, *Sal* I, *Acc* I, *Hinc* II, *Xba* I, and *BamH* I) was isolated from low melting point agarose gels and inserted into pTKCAT 5' adjacent to the TK promoter via polylinker sites (blunted *Sac* I and *BamH* I). This modified pTKCAT was then used for all subsequent ANG-TK-CAT fusion gene constructions. The pTKCAT (ANG N-814/-689) construct was created by isolating the DNA fragment (ANG N-814/-689) from pGEM (ANG N-814/+18) and then inserting the fragment into the modified pTKCAT, which had previously been digested with *Hind* III/*BamH* I. The pTKCAT (ANG N-814/-761) and pTKCAT (ANG N-760/-689) constructs were made by cutting the DNA fragment ANG N-814/-689 with *Nco* I to yield ANG N-814/-761 and ANG N-760/-689, repaired with the Klenow fragment of DNA polymerase I, and finally inserted into the modified pTKCAT, which had previously been digested with *Xba* I, repaired with Klenow, and treated with alkaline phosphatase.

The procedure to construct pTKCAT (ANG N-806/-779, ANG CRE), pTKCAT (ANG N-591/-563, ANG GREI), and pTKCAT (ANG N-485/-465, ANG GREII) is depicted schematically in Figure 2-1. Both the ANG CRE and the ANG GREI as well as the ANG GREII were synthesized by Bio-Synthesis Inc. For ANG-CRE, the fragment between bases N-806 and N-779 was chosen. It contains the CRE core sequence: 5'-<u>TGACGTAC</u>-3', (ANG N-795/-788); *Hind* III and *Xba* I sites were linked to the 5'- and 3'- ends, respectively. For GRE I, the fragment between bases N-591 and N-563 was chosen. It contains the GRE I core sequence: 5'-<u>AGAACATTTTGTTTC</u>-3', (ANG N-585/-570). For GRE II, the fragment between bases N-485 and N-465 was chosen. It contains the GRE II core sequence: 5'-<u>AGAACA</u>-3', (ANG N-477/

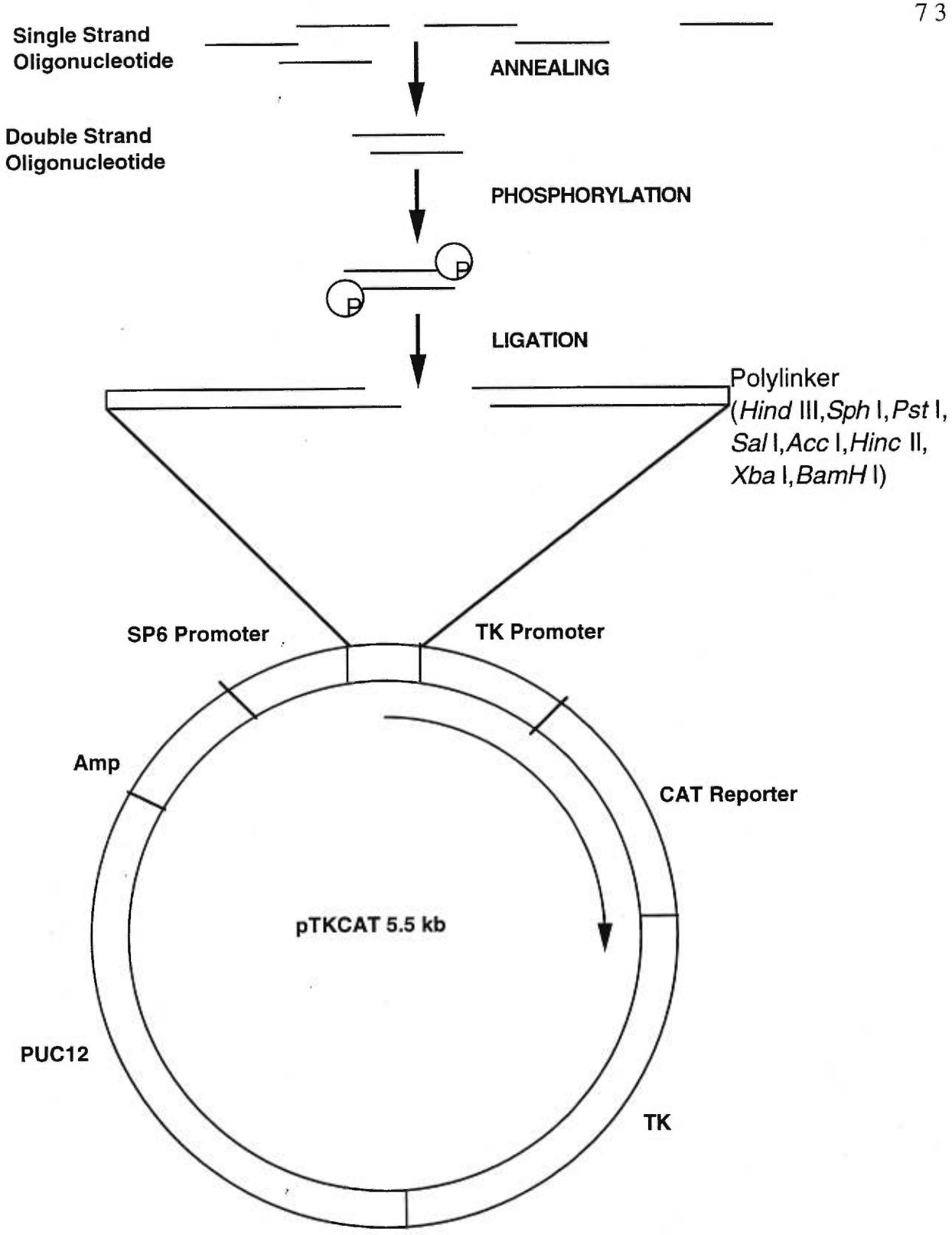


Figure 2-1 Schematic Diagram of Procedure to Construct pTKCAT (ANG CRE), pTKCAT (ANG GREI), and pTKCAT (ANG GREII)

-472). *Xba* I sites were linked to both the 5'- and 3'- ends of the fragments which contain the GRE I or GRE II.

The sequences of the synthesized oligonucleotides, which contain the ANG-CRE, ANG GREI, and ANG GREII motifs, are as follows:

ANG CRE:

5' AGCTTAAGAGATTACTTGACGTACTGGATGCAAT 3'	(-806/-779)
3' ATTCTC TAATGAACTGCATGACCTACGTTAGATC 5'	(-779/-806)

ANG GREI:

5' CTAGAAAGCCCAGAACATTTTGTTTCAATATGGT 3'	(-591/-563)
3' TTTCGGGTCTTGTAAAACAAAGTTATACCAGATC 5'	(-563/-591)

ANG GREII:

5' CTAGACCCTGTGCAGAACAGACAACTT 3'	(-485/-465)
3' TGGGACACGTCTTGTCTGTTGAAGATC 5'	(-465/-485)

II.2.1.1. Annealing

The synthetic single-stranded oligonucleotides were annealed to form the complementary DNA fragments of ANG CRE, ANG GREI and ANG GREII. The oligonucleotides were annealed as follows: Oligonucleotides (20 pmol, equal quantity of complementary single strand oligonucleotides), 2 µl of 10 x annealing buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), and DDW (double distilled water) were added to a total volume of 20 µl. The reaction mixtures were incubated for 10 minutes at 75°C, then slowly cooled down over 2 h at room temperature.

II.2.1.2. Phosphorylation

Double-stranded oligonucleotides (annealed, 20 pmol), 2 µl of 10 x kinase buffer [700 mM Tris-HCl (pH 7.6), 100 mM $MgCl_2$, 1M KCl, 10 mM 2-mercaptoethanol], 1 µl of T_4 DNA kinase (1U), 3 µl of 10 mM ATP, and DDW were combined in a total volume of 20 µl. The reaction mixtures were incubated for 1 h at 37°C, extracted once with phenol/chloroform, precipitated with

alcohol, and dried in a Speed-Vac centrifuge. The pellets were resuspended in 20 µl of DDW.

The procedures to construct pTKCAT containing various lengths of the 5'-flanking region of the rat ANG gene, pTKCAT (ANG N-806/-465, CRE/GREI/GREII), pTKCAT (ANG N-806/-563, CRE/GRE I), and pTKCAT (ANG N-591/-465, GREI/GREII) are schematically depicted in Figure 2-2. All three fragments of the ANG gene were synthesized using the polymerase chain reaction (PCR).

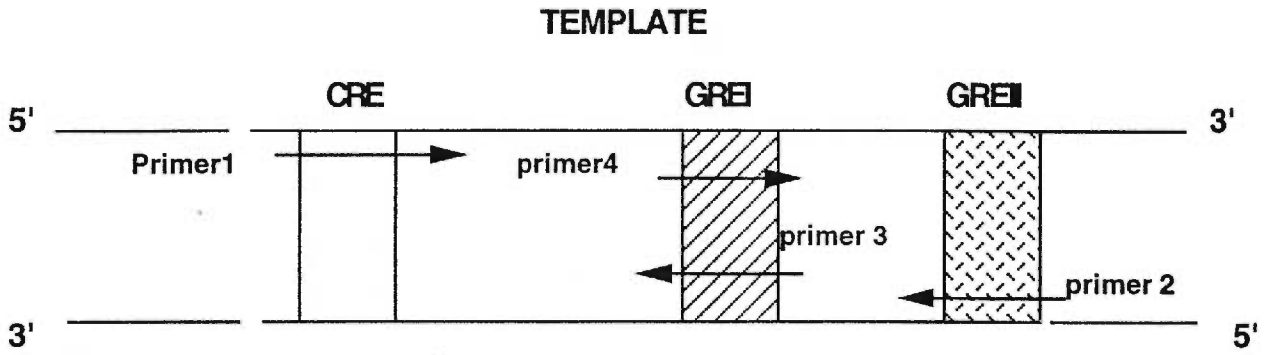
II.2.1.3. Polymerase Chain Reaction (PCR)

The DNA fragment ANG N-814/+18 was isolated from pOCAT (ANG N-814/+18) with the restriction enzymes *Hind* III and *Xho* I and was used as the template for synthesizing the ANG N-806/-465 and ANG N-806/-563 constructs. The DNA fragment ANG N-688/+18 was isolated from pOCAT (ANG N-688/+18) with *Hind* III and *Xho* I and was used as the template for synthesizing ANG N-591/-465.

The synthesized oligonucleotides were used as primers; their sequences have been shown in Figure 2-2. The pair of primers for PCR are shown in Table 2-1.

Table 2-1. Primers for the Synthesis of Various Fragments of The ANG Gene by PCR

oligonucleotide	primer location	length (bp)
CRE/GREI /GREII	primer 1 -806/-779	33 bp (plus *Hind* III site)
	primer 2 -465/-485	27 bp (plus *Xba* I site)
CRE /GREI	primer 1 -806/-779	33 bp (plus *Hind* III site)
	primer 3 -563/-591	34 bp (plus *Xba* I site)
GREI /GREII	primer 4 -591 /-563	34 bp (plus *Xba* I site)
	primer 2 -465/-485	27 bp (plus *Xba* I site)



PRIMER 1: ANG N-806/-779 (ANG CRE)
5'-AGCTTAAGAGATTACTTGACGTACTGGATGGATGCAAT-3'

PRIMER 2: ANG N-465/-485 (ANG GREI)
5'-CTAGAAGTTGTCTGTTCTGCACAGGGT-3'

PRIMER 3: ANG N-563/-591 (ANG GREII)
5'-CTAGACCATATTGAA ACAAAATGTTCTGGGCTTT-3'

PRIMER 4: ANG N-591/-563 (ANG GREI)
5'-CTAGAAAGCCCAGAACATTTTGTTTCAATATGGT-3'

Figure 2-2: Schematic Principle of PCR Synthesis of ANG (CRE/GREI/GREII), ANG(CRE/GRE I), and ANG(GRE I/GRE II)

Target sequences were amplified in a total reaction volume of 100 µl containing 200 µM of each of the four deoxynucleotide triphosphates, 100 pmol of each oligonucleotide primer, 0.5 µl (2.5 u) of Taq-polymerase (Perkin-Elmer Cetus, Norwalk, CT. USA), 1 x reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $NaCl_2$, 0.01% W/V gelatin) and 1-3 µM DNA template. After heating for 5 minutes at 95°C, the samples were subjected to 35 cycles of amplification using the following conditions: 1 minute at 94°C for denaturing the DNA template, 1 minute at 55 °C for annealing of the primer with the template, and 2 minutes at 72°C for sequence extension. The PCR products were purified with the Gene Clean II kit and inserted into the PCR vector, pCR II (Figure 2-3). The fusion genes were digested with the restriction enzymes *Hind* III and *Xba* I for fragments ANG N-806/-465 and ANG N-806/-563 or *Xba* I for fragment ANG N-591/-465. The inserts were separated on a 1.2% agarose gel and isolated by Gene Clean II kits. Subsequently, the DNA fragments were inserted into dephosphorylated pTKCAT.

pTKCAT was digested with *Hind* III and *Xba* I, or only *Xba* I for inserting the different fragments of the ANG gene. pTKCAT (100 µg), 20 µl of 10 x enzyme buffer #2, 5 µl of enzyme (50u/ml), and DDW were mixed to obtain 200 µl of total volume. The digestion reactions were incubated for 3 h at 37°C. The linearized pTKCAT was isolated with the Gene Clean II kit and resuspended in 50 µl of DDW.

II.2.1.4. Dephosphorylation

The linearized pTKCAT (digested with *Xba* I) was dephosphorylated. Digested pTKCAT (10 pmol), 5 µl of 10 x CIP buffer [500 mM Tris-HCl (pH 8.5), 1mM EDTA], 3 µl of CIP (calf intestinal alkaline phosphatase), and DDW were mixed to achieve a final volume of 50 µl. The reactions were incubated 30 minutes at 37°C, then diluted with 1 x CIP buffer to 75 µl when another 3 µl of CIP was added, and incubation was continued for another 30 minutes at 37°C. The samples were incubated for 15 minutes at 65°C to stop the reaction, extracted once with phenol/chloroform (1:1,vol/vol), extracted once with chloroform, precipitated with ethanol,

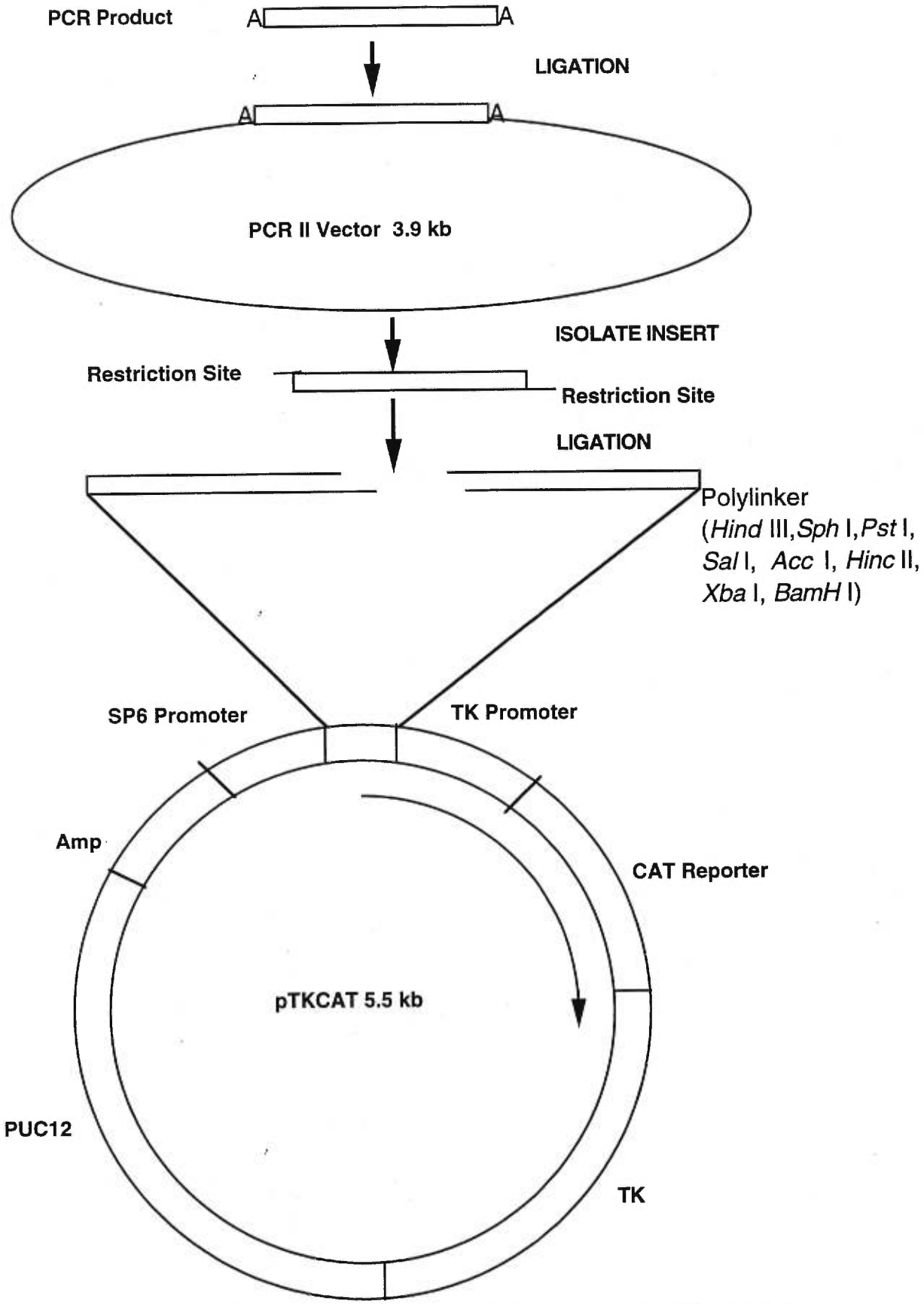


Figure 2-3 Schematic Diagram of Procedure to Construct pTKCAT (ANG CRE/GREI/GREII), pTKCAT (ANG CRE/GREI), and pTKCAT (ANG GREI/GREII)

decanted and dried in a Speed-Vac centrifuge. The pellets were resuspended in 20 µl of DDW.

II.2.1.5. Ligation

The phosphorylated oligonucleotides (inserts) were ligated to the dephosphorylated pTKCAT as follows: 0.5 µl of plasmid pTKCAT (0.1µg), 2 µl of double stranded oligonucleotide, 2 µl of 10 x ligase buffer [500 mM Tris-HCl (pH 7.6), 100 mM $MgCl_2$, 10 mM ATP, 10 mM DTT, 50% (W/V) polyethylene glycol-800], 1 µl of T_4 DNA ligase (5U/µl), and DDW were mixed in a 20 µl final volume. The reactions were incubated overnight at 15°C. Linearized and dephosphorylated pTKCAT without insert was used as a negative control and linearized, non-dephosphorylated pTKCAT without insert was used as the positive control for ligation. The ligated fusion genes were transformed into competent HB101, or DH 5α cells.

II.2.1.6. Preparation of the Competent HB 101 Cells

A single colony of HB 101 cells was picked up and cultured in 10 ml LB medium overnight at 37°C. The cultured HB 101 cells were diluted 1:100 with SOB (20g bacto-tryptone, 5g yeast-extract, 0.581g NaCl, 0.1864g KCl, DDW added to 1 liter), or SOC (SOB with 0.01 M $MgSO_4$, 0.01 M MgCl, and 0.02 M glucose), and incubated at 37°C with shaking until the OD_{650} of the culture was 0.3 - 0.4. They were transferred to pre-chilled tubes (2 x 50 ml), and centrifuged (2500 rpm) for 12 minutes at 4°C. The pellet was resuspended in 1/3 volume (16.5 ml) of FSB [10 mM KOAc (pH 7.0), 100 mM KCl , 45 mM $MnCl_2$, 10 mM $CaCl_2$, 3 mM $HACoCl_3$, 10% redistilled glycerol], incubated on ice for 10-15 minutes, then centrifuged (2500 rpm) for 10 minutes at 4°C. The pellet was resuspended in 1/12.5 volume (4 ml) of FSB, 140 µl of DMSO was added, followed by incubation on ice for 5 minutes and another 140 µl of DMSO was added with further incubation on ice for 10 minutes. The competent cells were aliquoted (200 µl/vial), and stored at -80°C.

II.2.1.7. Transformation

The HB101 (200 µl) or DH 5α (50 µl) cells were transferred to pre-chilled tubes and the ligated DNA was added. The tubes were incubated on ice for 30 minutes, heated for 90 seconds at 42°C (HB 101) or for 30 seconds at 37°C (DH 5α), and placed on ice for 1-2 minutes. SOC solution (800 µl) was added to HB 101 or (400 µl) to DH 5α cells, then the reaction mixtures were incubated for 1 h at 37°C with shaking. After incubation, the transformed cells were spread onto pre-warmed LB agar plates (10 g bacto-tryptone, 5 g yeast-extract, 10 g NaCl, 15 g agar, DDW to 1 liter) with ampicillin (100 u/ml). The plates were dried in air for 20 minutes, and incubated overnight at 37°C.

II.2.1.8. Assay for Positive Colonies

The growing colonies were transferred from the plates to nitrocellulose membrane filters (S & S). The filters were denatured in denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 15 minutes, neutralized in neutralizing solution (0.5 M Tris-HCl, 3 M NaCl) for 20 minutes, dried in air and baked for 2 h in an oven at 80°C. Then the filters were pre-hybridized with hybridization buffer [5 x SSPE, 5 x Dendhart, 1% SDS, and salmon sperm DNA (100 µg/ml)] for 1 h at 37°C.

II.2.1.8.1. Preparation of the Probes

Single-stranded oligonucleotide (1 µl, 10 pmol), 2 µl of 10 x kinase buffer, 1 µl of T_4 kinase, 3 µl of γ-^{32}P-ATP, and DDW were added to a 20 µl total volume. The reactions were incubated for 1 h at 37°C. The labeled oligonucleotides were separated from free γ-^{32}P-ATP by chromatography on Sephadex G50, and blue dextran 2000 (Pharmacia catalog no. C064) was used as an indicator for the fractions containing the labeled oligonucleotides.

II.2.1.8.2. Hybridization

The probes were added to the pre-hybridized filters. The reactions were performed overnight at 37°C with shaking. After overnight hybridization, the filters were washed twice with 2 x SSC

(0.6 M NaCl, 0.06 M Na Citrate) + 0.1% SDS for 20 minutes at room temperature with shaking, then washed twice with 0.2 x SSC + 0.1% SDS for 20 minutes at room temperature with shaking. Radioactivity was checked with a Geiger Counter after each washing, until it registered from 200 to 2,000 cpm. The filters were air dried and exposed to X-ray films overnight at -80°C. Positive colonies were selected based on positive hybridization signals, then cultured in 10 ml of LB medium overnight for DNA isolation and DNA sequencing.

II.2.1.9. Small Scale DNA Preparation (Mini-prep)

After overnight incubation at 37°C, the positive colony cultures were transferred to Eppendorf vials (1.5 ml), centrifuged, and the supernatants were decanted. The pellets were resuspended in 100 µl of solution I (0.1 M glucose, 0.25 M Tris-HCl, 0.05 M EDTA) and incubated for 5 minutes at room temperature. After addition of 200 µl of solution II (0.2 N NaOH, 10% SDS) and incubation on ice for 5 minutes, 150 µl of solution III (3M KOAC, 11.5% glacial acetic acid) was added. The samples were incubated on ice for another 5 minutes, then were centrifuged for 15 minutes at 4°C. The supernatants were transferred to new Eppendorf vials, and extracted once with an equal volume of phenol/chloroform. The aqueous phases were transferred to new Eppendorf vials, 2 volumes of 95% ethanol were added, then incubated for 5 minutes at -80°C, and centrifuged for 5 minutes at 4°C. The pellets were washed once with 70% ethanol, dried in a Speed-Vac centrifuge and resuspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0), then digested for 1 h at 37°C with RNase A and RNase T1 (10 u/µl each). Proteinase K (10 u/µl) was added, and the samples were further incubated for 1 h at 37°C. The digestion mixtures were extracted once with phenol/chloroform. The aqueous phases were transferred to new Eppendorf vials and a half volume of 7.5 N NH_4OAC and 2 volumes of 95% ethanol were added. The mixtures were incubated for 30 minutes at -80°C, then centrifuged for 5 minutes at 4°C. The pellets were washed once with 70% ethanol, dried in the Speed-Vac and resuspended in 6 µl of TE buffer for sequencing.

II.2.1.10. DNA Sequencing

The sequences and orientations for all fusion genes were determined by the dideoxy nucleotide chain-termination reaction (Sanger et al., 1977) with SP6 primers by using T7 DNA polymerase according to the protocol supplied with the kit from Pharmacia Inc. DNA (2 to 3 μg) was diluted to 8 μl with DDW, 2 μl of NaOH (2N) was added and incubated for 10 minutes at room temperature, then 3 μl of NaOAC (pH 5.4) and 7 μl of DDW were added with mixing. The reaction mixture was precipitated with 60 μl of 100% ethanol and incubated for 30 minutes at -80°C, then centrifuged for 10 minutes at 4°C. The pellets were washed with 70% ethanol and dried in a Speed-Vac, then redissolved in 10 μl of DDW, followed by the addition of 2 μl of annealing buffer and 2 μl of primer (SP6 or T7) solution, incubation for 20 minutes at 37°C and for at least 10 minutes at room temperature. After incubation, 3 μl of labeling mixtures (dATP, dGTP, dCTP), 1 μl of ^{35}S-dATP, and 2 μl of diluted DNA polymerase enzyme (the final concentration 1.5 U/μl) were added and incubated for 5 minutes at room temperature. dATP, dGTP, dCTP and dTTP (short or long) mixtures were added to Eppendorf vials (2.5 μl/vial), respectively, incubated for 3 minutes at 37°C, followed by the addition of 4.5 μl of labeling mixture, and further incubation for 5 minutes at 37°C. At the end of the incubation, the reactions were stopped by the addition of 5 μl of stop buffer, then heated at 75-80°C for 2-3 minutes and loaded onto a sequencing gel. After electrophoresis the gel was dried in a gel drier and exposed to X-ray film for 24 hours at room temperature. The colonies that contained the correct sequences of the fusion genes were selected for DNA preparation in a large quantity (maxi-prep) for transfections.

II.2.1.11. Large Scale DNA Preparation (Maxi-prep)

The selected colonies were cultured in LB medium (250 ml) overnight at 37 °C with shaking. The bacteria were collected by centrifugation (4000 rpm) for 20 minutes at 4 °C. The pellets were resuspended in 15 ml of solution I, mixed well, then 30 ml of solution II was added and incubated on ice for 10 minutes, followed by addition of 22.5 ml of solution III, and further incubated on ice

for 15 minutes. The reaction mixtures were centrifuged (7000 rpm) for 30 minutes at 4 °C. The supernatants were filtered through two layers of gauze and transferred into new bottles, 2 volumes of 95% ethanol were added and incubated for 10 minutes at room temperature, then the mixtures were centrifuged (8000 rpm) for 30 minutes at room temperature. The pellets were resuspended in 4.5 ml of TE buffer and transferred to Eppendorf vials (1ml/vial), 500 µl of 7.5 M NH_4OAC was added and incubated on ice for 30 minutes, then centrifuged for 10 minutes at 4°C. The supernatants were transferred to SS-34 tubes, 15 ml of 95% ethanol was added and the mixtures were incubated overnight at -80 °C, then centrifuged (11,000 rpm) for 30 minutes at 4°C. The pellets were resuspended in 1 ml of TE buffer, transferred to Eppendorf vials (200 µl/vial), digested with 10U/ml RNase A and 10U/ml RNase T1 (2µl of 5 M NaCl, 15 µl of RNase A and 15 µl of RNase T1), incubated for 1 h at 37°C, then treated with 2 µl of 20% SDS, and 15 µl of proteinase K, and further incubated for 1 h at 37°C and extracted twice with an equal volume of phenol/chloroform. The DNA was precipitated with 95% ethanol, frozen for 2 h at -80°C, then centrifuged for 10 minutes at 4°C. The pellets were washed once with 70% ethanol, dried in the Speed-Vac and resuspended in 50 µl of TE buffer. The concentration of DNA was measured in a DU-6 spectrophotometer at wave-lengths of 260 nm and 280 nm. The purity of the DNA was analyzed by mini-gel electrophoresis. DNA which showed no degradation and no contamination with RNA, was used for subsequent transfection experiments.

II.2.2. Cell Cultures

Both the mouse hepatoma cell line (Hepa 1-6) and the opossum kidney cell line (OK) were obtained from the American Tissue Type Culture Collection (ATCC). Approximately three to five passages after either cell type was obtained from ATCC, they were harvested and frozen in aliquots in liquid nitrogen for future use. For the present studies, aliquots of cells were thawed and passaged an additional three to five times before use. The cells were grown in plastic Petri dishes (100 x 20 mm) (Gibco, Burlington, Ontario, Canada) in Dulbecco's modified Eagle's medium (DMEM) (pH7.2), supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin,

and 50 μg/mL streptomycin, and were cultured in a humidified atmosphere in 95% air/ 5% CO_2 at 37°C.

II.2.2.1. Hepa 1-6 Cell Line

We have attempted to transfect a rat hepatoma cell line (Reuber H35, H-4-II-E) with the ANG-CAT fusion genes. The cell line Reuber H35 has been used extensively by other investigators to study the regulation of the release of ANG and the expression of the ANG mRNA *in vitro* (Bouhnik et al., 1983; Chang and Perlman, 1987). Unfortunately, due to the low efficiency of transfection (less than 1% of CAT conversion activity), this cell line was abandoned. The poor transfectability of H35 has also been observed by other investigators (Ron et al., 1990b). The human hepatoma cell line HEP G2 has also been considered. It constitutively expresses ANG and is readily transfectable. However, this cell line is deficient in functional glucocorticoid receptors (Ron et al., 1990b). Thus, it is not very well suited to the studies on the regulation of ANG gene expression by glucocorticoids.

The hepatoma cell line (Hepa 1-6) is derived from the transplantable BW 7756 tumor carried in a C57L mouse. It was originally adapted to *in vitro* growth from BW7756, a tumor passaged subcutaneouly in mice, by dissociating tumor pieces with trypsin and plating the cells *in vitro*. After varying periods in culture, the cells were reinoculated into the animal. The resulting tumor was processed in a fashion similar to the original tumor to reestablish the cells in culture. Alternate *in vivo - in vitro* passage resulted in the growth of a cell line which was capable of proliferation under standard conditions of cell culture or could be propagated in serum-free medium (Darlington et al., 1980; Darlington, 1987). The Hepa 1-6 cell line has been extensively characterized. It secretes several liver-specific proteins (albumin, α-fetoprotein, α_1-antitrypsin and amylase), expresses low levels of ANG mRNA (Ming et al., 1993), and is readily transfectable. This cell line also respond to the stimulation with glucocorticoids on ANG gene expression in a concentration-dependent manner (Ming et al., 1994). For these reasons, Hepa 1-6 cells were

chosen for the present studies. They were used between passages 7 and 70, where we observed stable responses.

II.2.2.2. OK Cell Line

The OK cell line is derived from renal tissue of an adult female American opossum and retains several properties of proximal tubular epithelial cells in culture (Koyama et al., 1978), including a parathyroid hormone (PTH)-inhibitable Na^+-dependent phosphate transport system (Pollock et al., 1986; Caverzazio et al., 1986; Malstrom and Murrer, 1986) and a PTH-stimulatable adenylate cyclase system (Teitelbaum and Strewler, 1984). These cells also express α_2-adrenergic receptors (Murphy and Bylund, 1988a), serotonin-1 receptors (Murphy and Bylund, 1988b), dopaminergic receptors (DA_1) (Cheng et al., 1990), insulin receptors (Nagil et al., 1988) and endogenous glucocorticoid receptors (Vrtovsnick et al., 1994). The OK cell line expresses low levels of ANG mRNA and the angiotensin-converting enzyme (Chan et al., 1991; Ingelfinger et al., 1991), which make it an excellent model to investigate the mechanism of ANG gene regulation in proximal tubule cells. In the present study, OK cells were used between passages 7 to 40. Beyond passage 40, their response to DEX was decreased.

Both Hepa 1-6 and OK cells have low levels of ANG mRNA, indicating that the trans-acting factors regulating ANG gene expression are likely to be present in these cells. Hence, they become very useful cell models for transfection of ANG-CAT fusion genes and study of their regulation.

II.2.3. DNA Transfection

II.2.3.1. Selected Reporter Genes

II.2.3.1.1. Chloramphenicol Acetyltransferase (CAT) Reporter System

The CAT gene is the most widely used reporter gene, and encodes chloramphenicol acetyltransferase (CAT) (Gorman et al., 1982). This gene is of prokaryotic origin, and is not found in eukaryotic cells. In the present study, CAT activity has been used to analyze the effects

of various hormones on ANG gene expression. This is based on the fact that, using our constructs, the amount of accumulated chloramphenicol acetyltransferase in transfected cells is under the control of the promoter/enhancer of the ANG gene. Intracellular CAT levels can be easily quantified using commercially available reagents. The detailed procedure is described below (in section II.2.3.2.).

It has been demonstrated that the secretion of ANG from hepatocytes is constitutive in nature (Tewksbury, 1990; Klett et al., 1992). The ANG secretion rate correlates well with an increase in intracellular ANG mRNA concentration (Klett et al., 1992). Our experiments have demonstrated that the CAT enzyme that is coded by the ANG-CAT fusion genes is predominantly or exclusively stored in the cells and is not secreted into the medium (c.f., Figure 3-1). There is a signal peptide in the N-terminus of the pre-ANG protein, which is encoded by exon 2 of the ANG gene. It consists of 25 or 34 amino acid residues and dictates the secretion of ANG from the hepatocytes into the circulation (Ohkubo et al., 1983; Kageyama et al., 1984). However, the various ANG-CAT fusion genes contain only the untranslated 5'-flanking region with or without a partial sequence of exon I, but lack the coding sequence for the signal peptide. This may explain why the CAT enzyme tends not to be secreted from the cells transfected with these constructs.

II.2.3.1.2. Human Growth Hormone (hGH) Reporter System

Human growth hormone (hGH) has been used as a reporter gene in studies of gene regulation (Selden et al., 1986). The advantages of the hGH system are: (a) the extreme sensitivity (10-100 -fold greater accumulation of hGH protein in the medium than CAT protein in the cells), (b) ease of assessment (radioimmunoassay, RIA), and (c) the unique feature of this transient expression system, secretion of the protein, permitting repetitive assays of the media, and obviating the need to prepare RNA or enzyme from the cells. It is also used as an internal control to monitor the transfection efficiency along with any other transient expression system. The potential disadvantage of this system is the possibility that any hGH secreted into the medium may be biologically active leading to the expression of other genes (Selden et al., 1986). Another

disadvantage of the hGH system is that intron 1 of the hGH gene contains sequences that render it glucocorticoid-responsive (Beato, 1985). This intrinsic effect must be considered when the hGH system is used to study the regulation of gene expression by glucocorticoids. Hence, the transcriptional activity as measured by RIA-hGH may be due to the response of the endogenous glucocorticoid response element(s) residing within the 5'-flanking region of the ANG gene, the response of the GRE residing within the first intron of the hGH gene, or both. For this reason, we used CAT instead of hGH as reporter when studying the effect of DEX.

To normalize transfection efficiency between various ANG-CAT fusion genes, we cotransfected cells with pTKGH and various ANG-CAT fusion genes. Immunoreactive hGH (IRA-hGH) levels were used as an internal control to monitor the efficiency of different transfections. The levels of IRA-hGH in the medium of the control cells (without the addition of hormones) were compared among the different groups. Then the CAT activities of the experimental samples (with hormones) were normalized by the transfection efficiency of pTKGH as assessed by the control.

IRA-hGH levels were undetectable in the cultured media of either OK or Hepa 1-6 cells which were not transfected with pTKGH. In the present study, the range of IRA-hGH levels was between 0.3 - 0.6 ng/ml when cotransfecting 1 or 2 μg of pTKGH into OK or Hepa 1-6 cells respectively.

II.2.3.2. DNA Transfection Procedure

Plasmids or ANG-CAT fusion genes were transfected into OK or Hepa 1-6 cells utilizing calcium phosphate-mediated endocytosis (Davis et al., 1986; Chen and Okayama, 1987). 10 μg of supercoiled DNA was routinely used in the OK cell transfection and 20 μg of supercoiled DNA was routinely used in the Hepa 1-6 cell transfection. Approximately 16 to 24 hours before transfection, the adherent cells were harvested with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA), and plated in 6-well-plates (Nunclon; Denmark) at 3.5 x 10^4 cells/cm^2 for OK cells and 5

x 10^4 cells/cm^2 for Hepa 1-6 cells in DMEM containing 10% FBS, then incubated at 37°C. Just 1 to 2 h prior to transfection, the media was aspirated from the plates, 5 ml of fresh 10% FBS DMEM was added and the incubation continued at 37°C.

The $CaPO_4$/DNA mixture for one well was prepared by combining in order: DDW (enough to bring the final volume to 1000 µl), 10-20 µg plasmid DNA, 62 µl of 2M $CaCl_2$ (0.125 M), and 500 µl of 2 x HBSP (1.5 mM Na_2HPO_4, 10 mM KCl, 280 mM NaCl, 12 mM glucose, 50 mM Hepes, and DDW to 500 mL, adjusted to pH 7.0). To form a precipitate, DDW was added to the bottom of a sterile 15 ml or 50 ml conical tube, DNA was added gently into DDW (without mixing), then 2M $CaCl_2$ was added (without mixing). The 2 x HBSP buffer was added by putting an autopipette inside the tube, touching the bottom, then the buffer was released slowly and 10 bubbles blown with the pipette to achieve a gentle mixing of reagents. The reaction mixture was incubated for 15 minutes at room temperature (the solution appeared slightly cloudy). At the end of the incubation, the precipitate was mixed by pipetting up and down once. Finally, the reaction mixture (1 ml) was added slowly to the plated cells. The plates were gently rocked a few times (the medium turned yellow and became turbid) and incubated for 16 to 24 h at 37°C in a humidified CO_2 incubator. To verify the formation of particles, the plates were removed from the incubator after the first 10 minutes of incubation, and examined under the microscope. A fine precipitate could be seen on the top of the cells. pOCAT (containing the coding sequence for CAT without Rous sarcoma virus enhancer/promoter sequence fused to the 5'-end of the CAT sequence) was used as negative control, and pRSVCAT (containing the coding sequence for CAT with Rous sarcoma virus enhancer/promoter sequence fused to the 5'-end of the CAT sequence) was used as positive control.

Twenty-four hours after transfection, the cells were washed once with serum-free DMEM. Cells were re-incubated in DMEM containing 1% depleted FBS (dFBS) or serum-free DMEM. Then a variety of hormones (including dexamethasone, β-estradiol, dihydrotestosterone, progesterone, and 3,3',5-triiodo-l-thyronine (L-T_3)), or drugs (including 8-Bromo-cAMP,

forskolin, isoproterenol, propranolol, atenolol, ICI-118,551, Rp-cAMP, and RU486) were added. After an additional 24 h of incubation the cells were harvested and assayed for protein concentration as well as CAT activity.

The dFBS was prepared by incubation with 1% activated charcoal and 1% analytical grade 1 x 8 ion-exchange resin (Bio-Rad, Richmond, CA) for >16 h at room temperature (Samuels et al., 1979). This procedure removed endogenous steroid and thyroid hormones from the FBS.

To normalize the efficiency of transfection of various fusion genes, 1 to 2 μg of pTKGH (a vector with the TK enhancer/promoter fused to the 5'-human growth hormone gene) was cotransfected with each ANG-CAT fusion gene (10-20 μg DNA). Subsequently, the levels of IRA-hGH secreted into the media were measured and used to normalize the transfection efficiency of various ANG-CAT fusion genes. The transfection efficiency of pOCAT (ANG N-1498/+18) ranged from 35% to 55% and 10% to 20% compared to pRSVCAT in OK cells and Hepa 1-6 cells respectively. The inter- and intra- assay coefficient of variation of transfection for pOCAT (ANG N-1498/+18) in OK cells was 25% (n = 10) and 8% (n = 10) respectively. In Hepa 1-6 cells, the inter- and intra- assay coefficient of variation of transfection for pOCAT (ANG N-1498/+18) was 22% (n = 10) and 7% (n = 10) respectively.

The results presented throughout the thesis were normalized to the efficiency of transfection of pTKGH. The radioimmunoassay for hGH was performed according to the method described later (section II.2.4.3).

II.2.4. Assays

II.2.4.1. Cell Extract and Protein Assay

The cells were washed twice with 1 x PBS (pH 7.2), then 0.5 ml of Trypsin-EDTA was added to each well followed by incubation for 1-2 minutes at 37°C, with an addition of 0.5 ml of 1 x PBS and transfer to Eppendorf vials. The cells were centrifuged for 2 minutes at 4°C, then the

pellets were resuspended in 100 µl of 0.25 M Tris-HCl pH 7.8. Cell extracts were prepared by 3 cycles of freezing and thawing (5 minutes in a dry ice bath, 5 minutes at 37°C), then centrifuged at 10,000 x g for 10 min at 4°C. The supernatants were transferred to new Eppendorf vials. A volume of 8 or 12 µl of the supernatant was assayed for protein concentration by the method of Bradford (1976). Bovine serum albumin (BSA) was used as a standard. Various concentrations (i.e. 0, 1.25, 2.5, 5, 10, and 20 µg) of BSA in 800 µl of 0.25 M Tris-HCl pH 7.8 were mixed with 200 µl of Bio-Rad protein dye (Bio-Rad Laboratories, Richmond, CA). The final standard concentrations were 0 µg/ml, 1.25 µg/ml, 2.5 µg/ml, 5 µg/ml, 10 µg/ml, and 20 µg/ml. After 5 minutes (but no more than 1 h) incubation at room temperature, the optical density was read at 595 nm. For cell extracts, 8 to 12 µl of samples in 800 µl of 0.25 M Tris-HCl pH 7.8 were mixed with 200 µl of Bio-Rad protein dye. The cell extracts were read under the same conditions.

II.2.4.2. Chloramphenicol Acetyl Transferase (CAT) Assay

CAT activity of the cell extract was measured by the method of Gorman et al. (1982). Twenty to fifty micrograms of protein from cell extracts, 20 µl of 4 mM acetyl coenzyme A, 71 µl of diluted [^{14}C] chloramphenicol solution (70 µl of 1M Tris-HCl pH 7.6 + 0.25 µl of [^{14}C] chloramphenicol for each sample), and 0.25 M Tris-HCl buffer (pH 7.8) were mixed to bring the final volume to 146 µl. OK cell extracts were incubated for 30 to 60 minutes at 37°C. Hepa 1-6 cell extracts were incubated initially for 1 h, then 10 µl of 4 mM acetyl coenzyme A was added and the samples were incubated for another 15 h at 37°C. At the end of the incubation, the reaction mixtures were extracted with 1ml of 100% ethyl acetate (vortexed 1 minute, then centrifuged for 1 minute at room temperature). The organic phase was collected and dried in a Speed-Vac. The final samples were resuspended in 10 µl of ethyl acetate and loaded onto thin layer chromatography (TLC) plates. The TLC plates were developed with a saturated mixture of 190 ml of chloroform and 10 ml methanol (19:1) for 1 h in a TLC tank. The plates were then air dried, and subjected to autoradiography for 1-2 days at room temperature. The bands corresponding to acetylated and nonacetylated forms of [^{14}C] chloramphenicol were scraped from the TLC plates,

and mixed individually with 10 ml of scintillation fluid (950 ml toluene + 50 ml liquifluor).

CAT activity was quantified by counting the radioactivity of the bands containing either the acetylated or nonacetylated forms of [^{14}C] chloramphenicol in a liquid scintillation counter. The results are expressed as the percent of [^{14}C] chloramphenicol converted to the acetylated forms according to the following equation:

$$(\text{CAT Activity})\% = \frac{\text{acetylated } [^{14}\text{C}] \text{ chloramphenicol}}{\text{acetylated+nonacetylated } [^{14}\text{C}] \text{ chloramphenicol}} \times 100\%.$$

II.2.4.3. Radioimmunoassay (RIA) for Human Growth Hormone (hGH)

The amount of IRA-hGH in the medium was measured by the method of double-antibody radioimmunoassay. The RIA procedure was similar to that previously described for the radioimmunoassay of ovine placental lactogen (RIA-oPL) (Chan et al., 1978). Human [^{125}I]GH was prepared by using a slight modification of the lactoperoxidase method (Thorell and Johannsson, 1971). Human GH was iodinated and used as a hormone standard. The sensitivity of the assay was 0.1ng/ml. The inter- and intra-assay coefficient of variation were 12% (n=10) and 10% (n=10) respectively.

II.2.4.3.1. Iodination of hGH

Iodination reactions included 25 µl of 0.5M phosphate buffer (pH 7.4), 10 µl of hGH (10 µg), 10 µl of Na^{125}I, 50 µl of Lactoperoxidase (10 µg), and 10 µl of H_2O_2 (50 µl of 33% H_2O_2 diluted to 750 ml with DDW; final H_2O_2 concentration 0.0022%). The initial reaction mixture was incubated for 5 minutes at room temperature, after which an additional 10 µl of H_2O_2 was added and the mixture was incubated for another 5 minutes at room temperature. One ml of phosphate

buffer was added to stop the reaction. The reaction mixture was loaded on a Sephadex G100 column to separate the labeled hGH from the free iodine. The specific activity of the iodinated hGH was 40-80 μCi/μg.

II.2.4.3.2. RIA for hGH

To prepare the standard curve, 0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, and 100 ng of hGH were each added to 1ml of RIA buffer. The final concentrations of the standard curve were 0 ng/ml, 0.1 ng/ml, 0.2 ng/ml, 0.5 ng/ml, 1 ng/ml, 2 ng/ml, 5 ng/ml, 10 ng/ml, 20 ng/ml, 50 ng/ml, and 100 ng/ml. The RIA procedure was performed as follows:

	TC[1]	NSB[2]	B_0[3]	SS[4]
RIA buffer (μl)	0	300	200	100
SS (μl)	0	0	0	100
First Antibody (μl)[5]	0	0	100	100

[1] Total Counts
[2] Non-specific Binding
[3] Maximum Binding
[4] Standard or Sample
[5] Rabbit anti-human GH (1:5000 dilution)

The reaction mixtures were incubated for 24 h at 4°C, 100 μl of tracer (^{125}I-hGH; 20,000-25,000 cpm) was then added, the mixture was incubated for another 24 h at 4°C, then 50 μl second antibody (1:10 dilution of goat anti rabbit serum) and 50 μl of normal rabbit serum (1:100 dilution) were added and further incubated for 24 h at 4°C. At the end of the incubation, the reaction mixtures were centrifuged (3500 rpm) for 30 minutes at 4°C. The supernatants were discarded and radioactivity of the pellets was determined in a scintillation counter.

II.3. STATISTICAL ANALYSIS

The experiments were performed at least three times in triplicate. The data are shown as mean±SD. The software package SAS 6.12 was used for all statistical measurements. When it was important to compare within and among groups, analysis of variance (ANOVA) was used; otherwise, the Student's *t*- test was the method chosen. For the former, *post-hoc* comparisons were made using Tukey's procedure. $P< 0.05$ was significant.

CHAPTER III RESULTS

III. 1. EXPRESSION OF THE ANG-CAT FUSION GENES IN HEPA 1-6 CELLS

III.1.1. Preliminary Experiments

In order to investigate the hormonal effects on the expression of the angiotensinogen gene in Hepa 1-6 cells, preliminary experiments were performed to optimize the conditions for the basal expression of the ANG-CAT fusion gene. In the present study, the expression vectors, pRSVCAT and pOCAT containing the coding sequence for CAT with or without the Rous sarcoma virus enhancer/promoter sequence fused to the 5' end of the CAT coding sequence, were used as positive and negative controls, respectively .

III.1.1.1. Time Course of CAT Activity in Cell Extract and Medium

Figure 3-1 shows the relationship between the incubation time and the level of CAT expression in Hepa 1-6 cellular extracts and in the incubation medium. Twenty four hours after DNA (20 μg) transfection (i.e., pOCAT (ANG N-1498/+18)), the medium was changed to DMEM without fetal bovine serum (FBS). Then, the cells were further incubated at 37°C. Subsequently, the cells and media were harvested after various time periods. Figure 3-1 shows that the amount of CAT activity in the cell extract increased with the incubation time during a 24 to 48 hour period following DNA transfection. The intracellular CAT activity decreased between 48 and 72 hours. The maximal conversion rate was 13±3 % per 20 μg of cellular protein at 48 hours and there was a non-significant decrease to 7.8±1.0 % at 72 hours. The amount of CAT activity in the medium was undetectable for the first 27 hours. The maximal CAT activity in the medium was 2.0±0.3% (30 hours) which then decreased to 1% after 36 hours of incubation. These studies indicated that the CAT protein was predominantly stored in the cells. In subsequent experiments, cells were harvested at 48 hours (unless otherwise stated) after transfection, and then assayed for intracellular CAT activity.

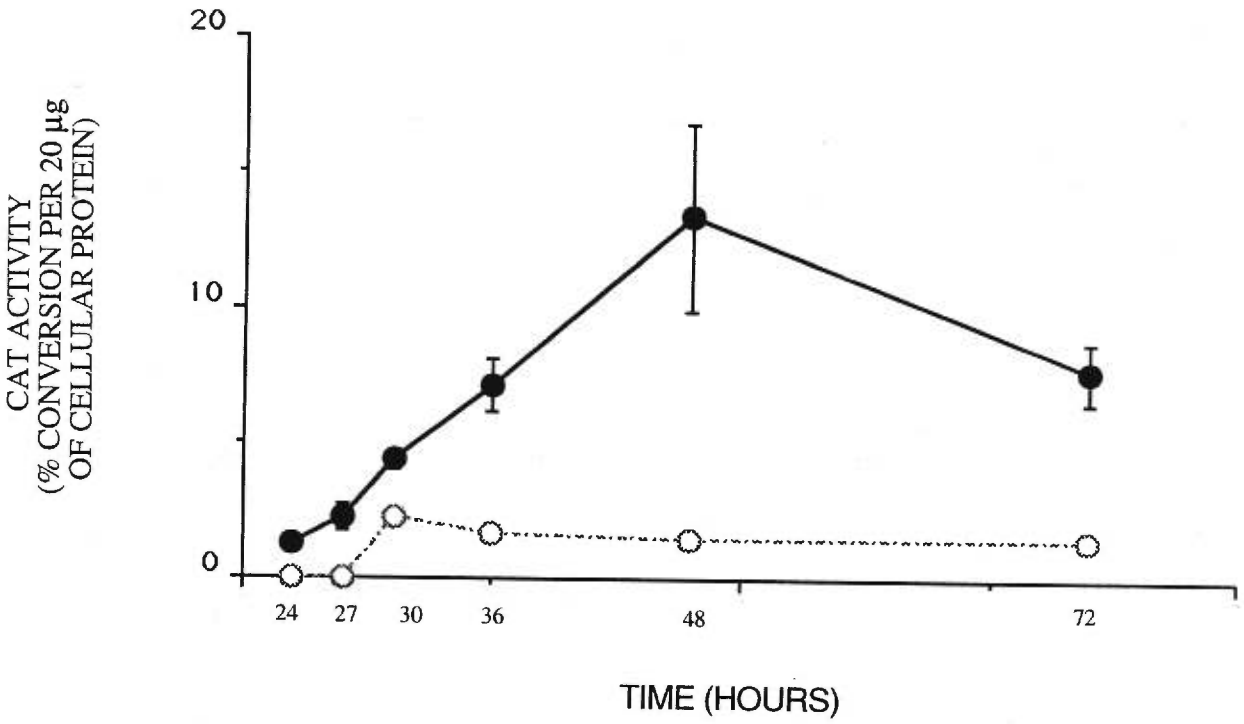


Figure 3-1. Time-course study of CAT activity in Hepa 1-6 cell extracts and in incubation medium. Cells were transfected with pOCAT (ANG N-1498/+18). Twenty-four hours after DNA transfection, cells were incubated in DMEM without FBS for the time indicated, then cells were harvested and medium was collected for CAT assay. Results are expressed as percentage of conversion per 20 µg of cellular protein. ●, cell extract; ○, medium. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). (Note: For medium measurements, SD's were smaller than the radius of the open circles.)

III.1.1.2. Concentration-Response Studies

Figure 3-2 shows the relationship between the amount of transfected pOCAT (ANG N-1498/+18) and the level of CAT expression in Hepa 1-6 cells. The amount of CAT assayed in the cell extract was proportional to the amount of transfected pOCAT (ANG N-1498/+18) over the range of 5-20 μg of DNA. The maximal conversion rate was 36.9±1.9% per 50 μg of cellular protein, when 20 μg of DNA was transfected. The conversion rate was decreased to 18±3.4% ($P<0.01$) and 13±1.2% ($P<0.005$) after transfection of 30 μg and 50 μg of DNA, respectively. In subsequent studies, we routinely used 20 μg of various fusion gene constructs for transfection into mouse Hepa 1-6 cells.

III.1.1.3. Effect of hGH, Insulin-Like Growth Factor I (IGF-I) and Insulin-Like Growth Factor II (IGF-II) on the Expression of the ANG-CAT Fusion Gene in Hepa 1-6 Cells

To normalize the transfection efficiency of the various ANG-CAT fusion genes, we cotransfected pTKGH with various ANG-CAT fusion genes. pTKGH is a gene construct containing the coding sequence of the human growth hormone (hGH) gene fused to the thymidine kinase enhancer/promoter sequence at the 5'-end of the hGH reporter. The cells which were transfected with pTKGH could secrete hGH into the medium. The amount of radioimmunoassayable hGH in the medium was used as an internal control to normalize the transfection efficiency of the various ANG-CAT fusion genes. In order to ensure that hGH had no stimulatory effect on the expression of the ANG gene in Hepa 1-6 cells, we examined the addition of hGH on the expression of the ANG-CAT fusion gene in Hepa 1-6 cells. Figure 3-3 shows the effect of the hGH on the expression of pOCAT (ANG N-1498/+18). The addition of various concentrations of hGH (0.1-100 ng/ml) had no significantly stimulatory or inhibitory effect (NS) on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells.

Studies have shown that GH stimulates hepatocytes to synthesize and secrete insulin-like

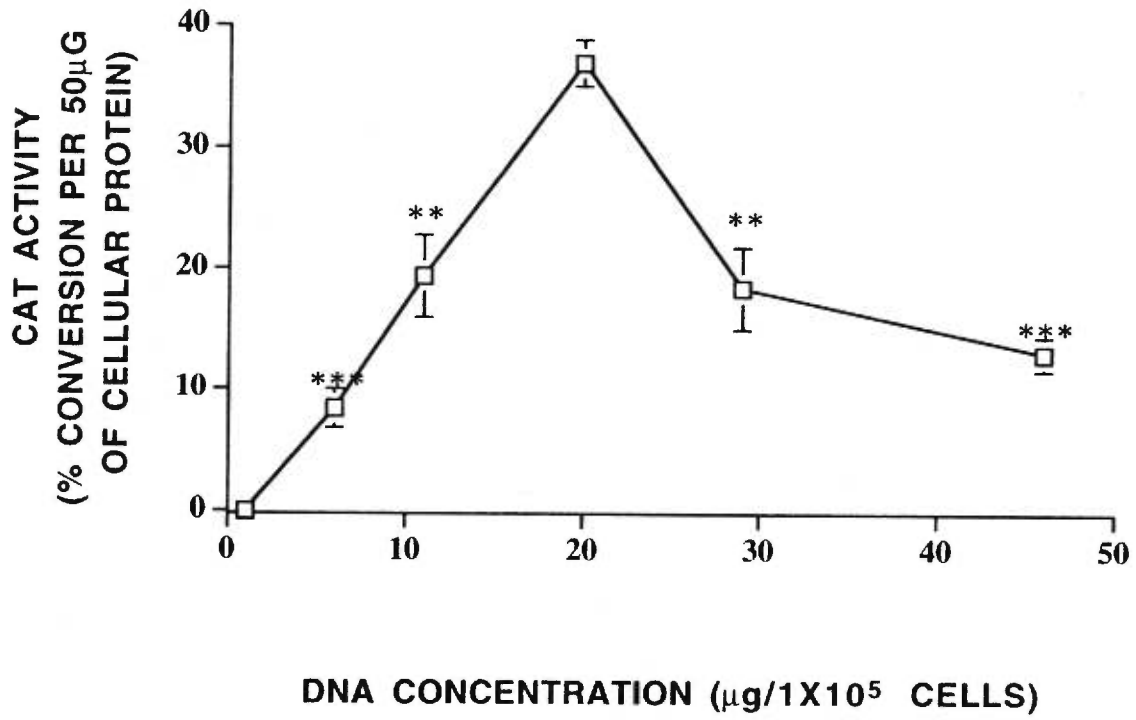


Figure 3-2. Concentration-dependent expression of the ANG-CAT fusion gene pOCAT (ANG N -1498/+18) in Hepa 1-6 cells on day 4 after DNA transfection. The levels of transcription were quantified by CAT activity, and transfection efficiency has been normalized by the cotransfection of pTKGH. Results are expressed as percentage of conversion per 50 µg cellular protein. Comparison was made between transfection with 20 µg of DNA and other amouts of DNA. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3).

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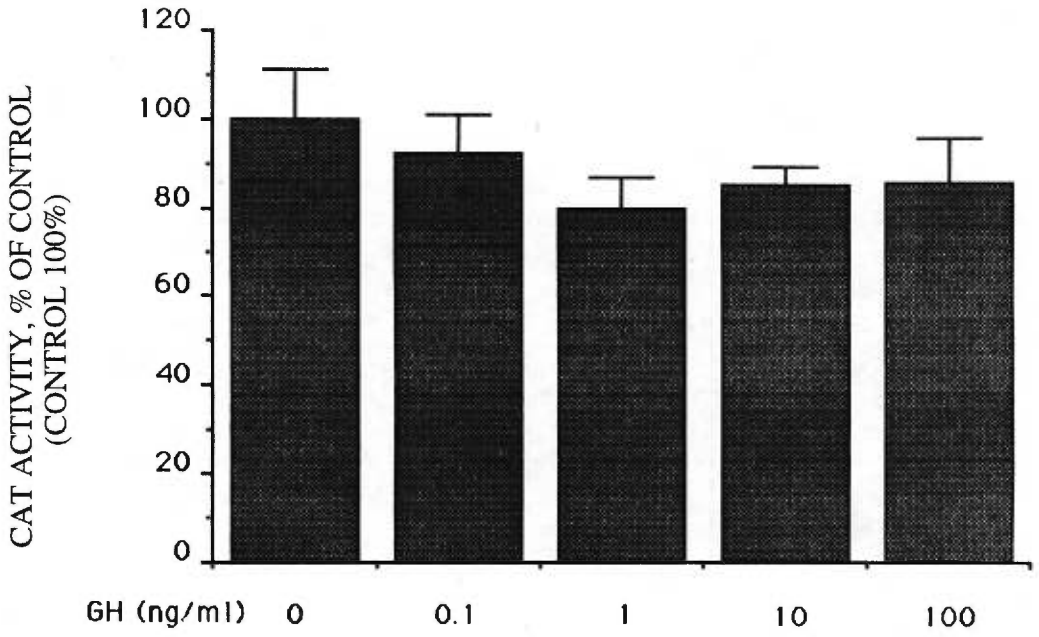

Figure 3-3. Relationship between hGH concentrations and the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells. Cells were incubated for 24 hours in the presence of various concentrations of hGH (0.1 - 100 ng/ml). The CAT activity of pOCAT (ANG N-1498/+18) in the absence of hGH is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). ANOVA was used for comparison.

Reproduced from Ming, M., Sikstrom, R., Lachance, S., Delalandre, A., Carrière, S. and Chan, J.S.D.: Insulin and insulin-like growth factor (IGF-1) have no effect on the transcription of the angiotensinogen gene in mouse hepatoma cells. 61st Annual Meeting, Can. Soc. Clin. Invest., Ottawa, Canada, September 11-14, 1992.

growth factors I and II (IGF-I, IGF-II). Both of them have various physiological functions on target cells (Ganong, 1991d). In this regard, we have examined whether IGF-I and IGF-II had any effect on the expression of the ANG gene. Figure 3-4 shows the effect of various concentrations of IGF-I (5-100 ng/ml) on the expression of pOCAT (ANG N-1498/+18). While there appeared to be a slight increase (30%-40%) of CAT activity with the different concentrations of IGF-I, it was not significant compared with the control. A similar result was obtained when using corresponding concentrations of IGF-II (5-100 ng/ml) to induce the expression of pOCAT (ANG N-1498/+18) as shown in Figure 3-5.

From these studies, it appears that hGH, IGF-I, and IGF-II did not significantly stimulate the expression of the ANG-CAT fusion gene in Hepa 1-6 cells. Therefore, the Hepa 1-6 cells were co-transfected with various ANG-CAT fusion genes (20 μg) and pTKGH (2μg) in the experiments. Subsequently, the CAT activity in the cell extract and the amount of immunoreactive (IRA)-hGH secreted into the medium were measured. The levels of IRA-hGH were used to normalize the transfection efficiencies of various ANG-CAT fusion genes.

III.1.1.5. Transient Expression of Fusion Gene Constructs

Figure 3-6 shows the result of the transfection of various fusion gene constructs into Hepa 1-6 cells. The plasmid containing the Rous sarcoma virus enhancer/promoter (RSV) fused to the CAT gene was used as a positive control, whereas the plasmid containing no promoter, pOCAT, was used as a negative control. The pOCAT itself is minimally expressed; the conversion rate is only 6.5±0.5 % (in figure 3-6, its CAT activity is shown as 100%). It is apparent that pRSVCAT is highly expressed, the conversion rate is 78.2±3.4%. The conversion rates of the various ANG-CAT fusion genes, pOCAT (ANG N-1498/+18), pOCAT (ANG N-688/+18), pOCAT (ANG N-110/+18), pOCAT (ANG N-53/+18), and pOCAT (ANG N-35/+18) are 23.8±3.6% ($P<0.005$), 26.0±3.6% ($P<0.005$), 7.8±4.4% (NS), 26.0±4.2% ($P<0.005$), and 21.5±3.3% ($P<0.005$), respectively. The conversion rates have been normalized for the efficiencies of transfection with the amount of the IRA-hGH expressed by pTKGH. In contrast, no significant expression of any

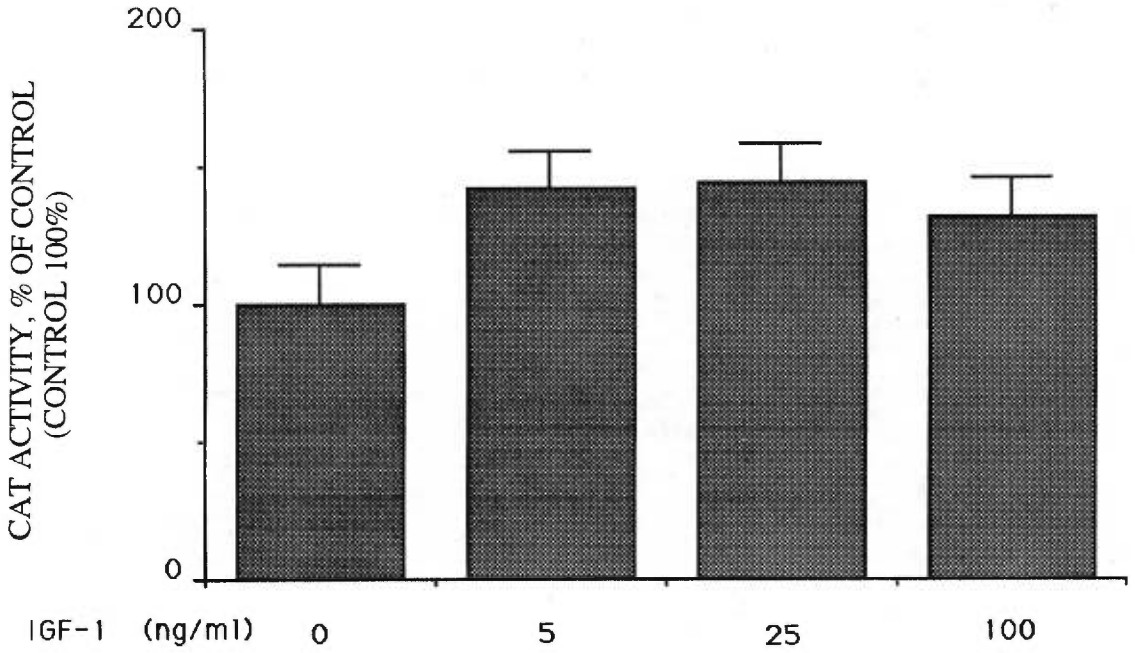

Figure 3-4. Relationship between IGF-I concentrations and the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells. Cells were incubated for 24 hours in the presence of various concentrations of IGF-I (5 - 100 ng/ml). The CAT activity of pOCAT (ANG N-1498/+18) in the absence of IGF-I is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). ANOVA was used for comparison.

Reproduced from Ming, M., Sikstrom, R., Lachance, S., Delalandre, A., Carrière, S. and Chan, J.S.D.: Insulin and insulin-like growth factor (IGF-1) have no effect on the transcription of the angiotensinogen gene in mouse hepatoma cells. 61st Annual Meeting, Can. Soc. Clin. Invest., Ottawa, Canada, September 11-14, 1992.

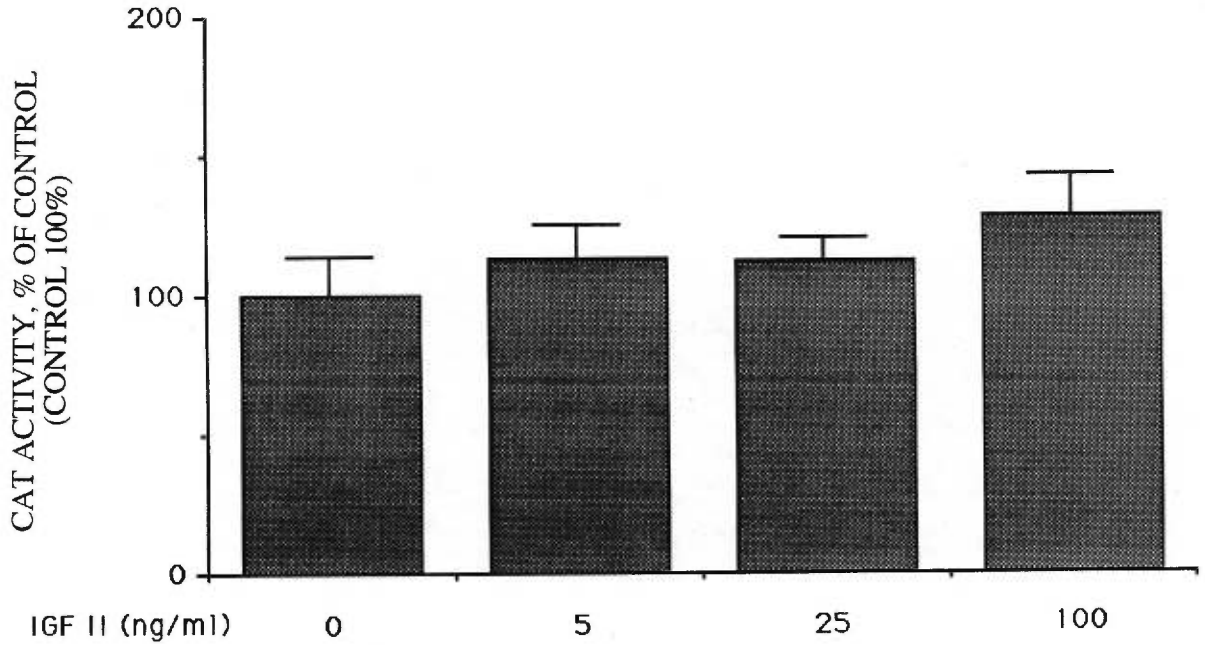

Figure 3-5. Relationship between IGF-II concentrations and the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells. Cells were incubated for 24 hours in the presence of various concentrations of IGF-II (5 - 100 ng/ml). The CAT activity of pOCAT (ANG N-1498/+18) in the absence of IGF-II is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). ANOVA was used for comparison.

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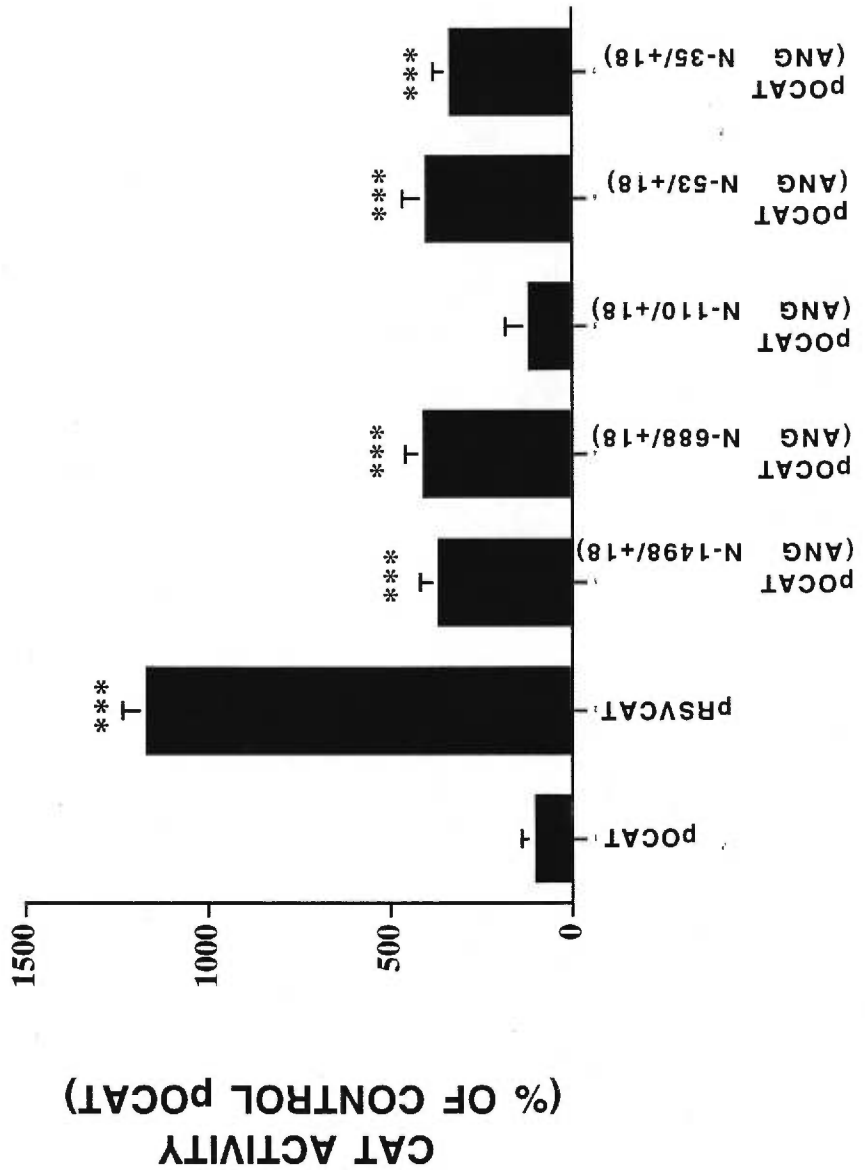

Figure 3-6. Expression of various ANG-CAT fusion genes in Hepa 1-6 cells. Results are expressed as the percentage of the CAT activity of the control, pOCAT, its CAT activity shown as 100%. Transfection efficiency has been normalized by the cotransfection of 2 μg pTKGH. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from Student's *t* test (***P<0.005).

Reproduced from Ming M., Sikstrom R., Lachance S., Delalandre A., Carriere S. and Chan J.S.D.: (1993) Hormonal regulation of expression of the angiotensinogen gene in mouse hepatoma cells. Am.J. Hypertension 6:141-148.

of these ANG-CAT fusion genes over the promoterless pOCAT was observed in Sertoli TM_4 cells (personal communication with Dr. M-L. Liu, University of Montreal). TM_4 cells are derived from mouse testis. As ANG mRNA is undetectable in mouse testis, TM_4 cells have been used as a negative control in the investigation of ANG gene expression. These results indicated that the Hepa 1-6 cell was useful for studing the regulation of ANG gene expression.

In summary, based on these results (Figures 3-1 to 3-6), we routinely cotransfected 2 μg of pTKGH with 20 μg of various ANG-CAT fusion genes into Hepa 1-6 cells. The cells were harvested after 48 hours of transfection, and the amount of RIA-hGH was used to normalize the transfection efficiency.

III.1.2. Effect of Steroid Hormones on the Expression of ANG-CAT Fusion Genes in Hepa 1-6 Cell Line

It was reported that dexamethasone, estradiol, and the thyroid hormone, L-T_3, enhanced the ANG mRNA in hepatocytes both *in vivo* and *in vitro* (Chang et al., 1987; Klett et al., 1988). Therefore, we sought to determine whether steroid hormones would increase the expression of ANG-CAT fusion genes in the Hepa 1-6 cell line. Figure 3-7 shows a relationship between dexamethasone concentrations and the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells. The addition of dexamethasone (10^{-4} M) stimulated the expression to 220±27% ($P<0.005$) compared with the control (without the addition of dexamethasone).

Figure 3-8 shows the effect of 8-Bromo-cAMP plus various steroid hormones and the thyroid hormone, L-T_3, on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells. There was a non-significant increase with 8-Bromo-cAMP (10^{-3} M) alone (20±26%), and a 118±9% increase with DEX alone. The sum of these two effects (138±35%) was significantly less than the increase obtained by 8-Bromo-cAMP and DEX together (241±12%). Thus, 8-Bromo-cAMP potentiated the effect of DEX. On the other hand, it appears that other steroid hormones (estradiol 10^{-6} M; testosterone 10^{-6} M and progesterone 10^{-6} M) and thyroid hormone

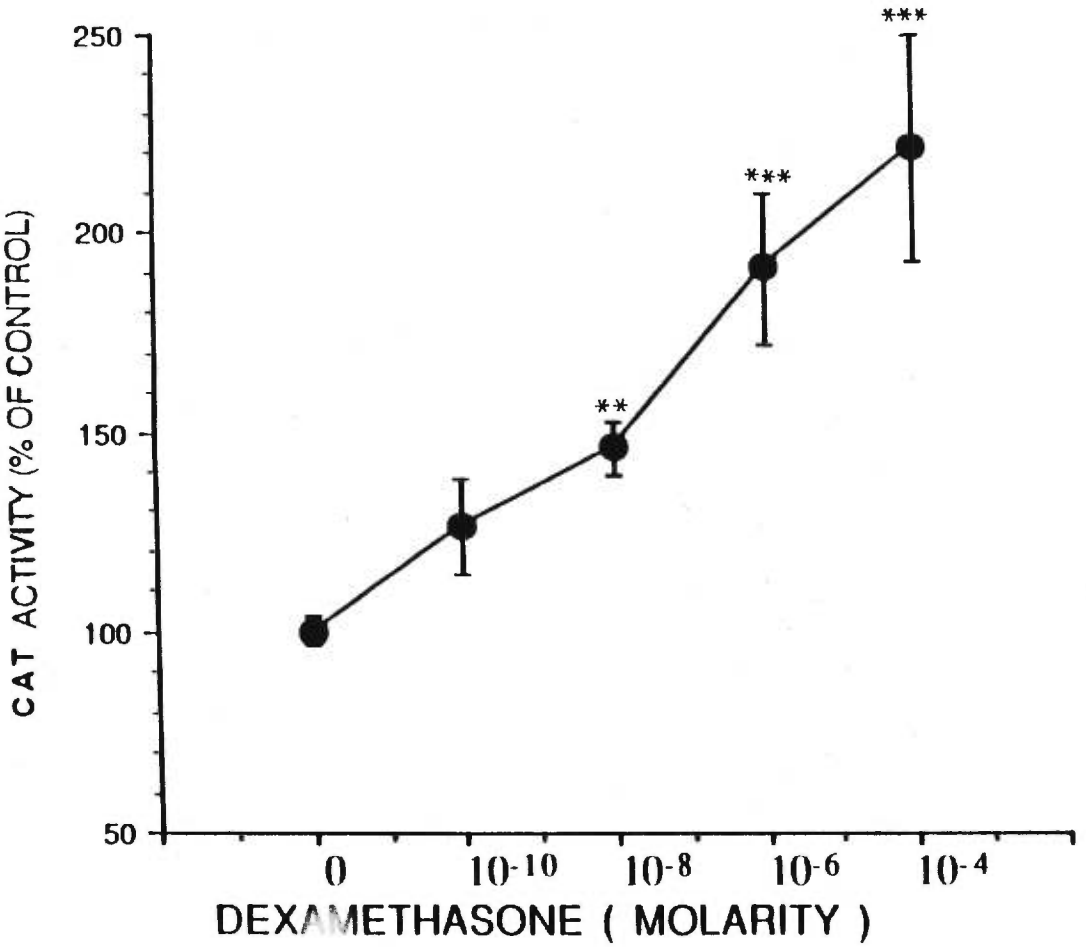

Figure 3-7. Relationship between dexamethasone concentrations and the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells. Cells were preincubated for 24 hours in 1% depleted fetal bovine serum (dFBS) after DNA transfection. Then the cells were incubated in medium without FBS for an additional 16 hours to arrest cell growth. Dexamethasone was then added to fresh medium without FBS and the system was incubated for an additional 24 hours. The CAT activity of pOCAT (ANG N-1498/+18) in the absence of dexamethasone is shown as 100%. Each point represents the mean$\pm$SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (**P<0.01, ***P<0.005).

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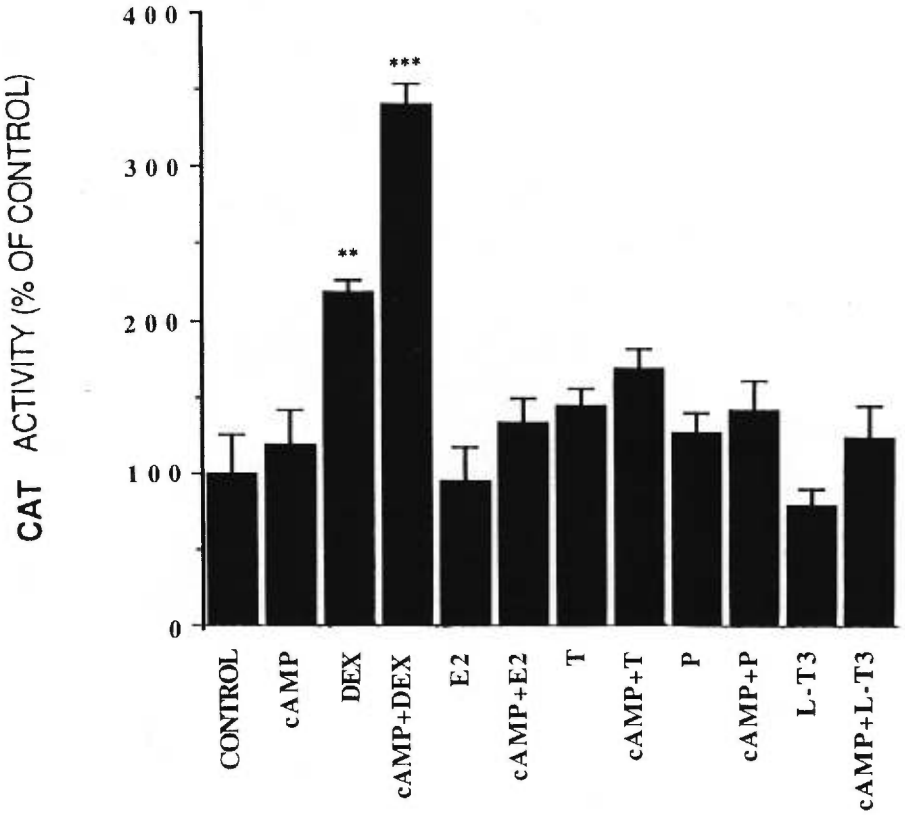

Figure 3-8. The effect of various hormones on the expression of pOCAT (ANG N-1498/+18) in the presence or absence of 8-Bromo-cAMP in Hepa 1-6 cells. Cells were incubated for 24 hours with various hormones in the presence or absence of 8-Bromo-cAMP (10^{-3}M). The CAT activity of pOCAT (ANG N-1498/+18), in the absence of the hormones and 8-Bromo-cAMP, is shown as 100%. Comparisons were made between the control and samples treated only with hormones; similarly, the effect of the combination of various hormones and 8-Bromo-cAMP is compared to that of cells treated with hormones alone. (DEX, dexamethasone 10^{-6} M; E_2, estradiol 10^{-6} M; T, testosterone 10^{-6} M; P, progesterone 10^{-6} M; L-T_3, thyroid hormone 10^{-6} M). Each point represents the mean$\pm$SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (**P<0.01, ***P<0.005).

Reproduced from Ming M., Sikstrom R., Lachance S., Delalandre A., Carriere S. and Chan J.S.D.: (1993) Hormonal regulation of expression of the angiotensinogen gene in mouse hepatoma cells. Am.J. Hypertension 6:141-148.

(L-T_3 10^{-6} M) had no significant effect on the expression of pOCAT (ANG N-1498/+18) either in the presence or absence of 8-Bromo-cAMP.

III.1.3. Effect of 8-Bromo-cAMP on the Expression of ANG-CAT Fusion Genes in Hepa 1-6 Cells

III.1.3.1. Effect of 8-Bromo-cAMP plus Dexamethasone on the Expression of ANG-CAT Fusion Genes in Hepa 1-6 Cells

Figure 3-9 shows the effect of dexamethasone on the expression of other ANG-CAT fusion gene constructs in the presence or absence of 8-Bromo-cAMP. As with the pOCAT (ANG N-1498/+18), dexamethasone (10^{-6} M) increased the expression of pOCAT (ANG N-688/+18) by 116±18%, P<0.01. There was a non-significant increase with 8-Bromo-cAMP alone (10^{-3} M) (13±17%). The sum of these two effects (129± 35%) was significantly less than the increase obtained by 8-Bromo-cAMP and DEX together (264±10%). Other ANG-CAT fusion genes, such as pOCAT (ANG N-110/+18), pOCAT (ANG N-53/+18), and pOCAT (ANG N-35/+18) were not responsive to the addition of dexamethasone alone or in combination with 8-Bromo-cAMP.

To localize the region that responds to DEX plus 8-Bromo-cAMP, the ANG-CAT fusion genes which contained different lengths of the 5'-flanking region of the ANG gene fused with the CAT reporter gene were also assessed as shown in Figure 3-10. DEX (10^{-6} M) also significantly increased the expression of pOCAT (ANG N-1138/+18), pOCAT (ANG N-960/+18), and pOCAT (ANG N-814/+18) compared with the controls. The addition of 8-Bromo-cAMP (10^{-3} M) alone had no effect on the expression of these fusion genes. However, it potentiated the effect of DEX. The combination of both reagents significantly increased the expression of these fusion genes compared with DEX alone (368±13% versus 252±16%, P<0.01; 203±21% versus 142±63%, P<0.05; 263±29% versus 166±13%, P<0.05; respectively). Other ANG-CAT fusion genes, such as pOCAT (ANG N-280/+18) and pOCAT (ANG N-196/+18) showed no significant response to the addition of DEX alone or in combination with 8-Bromo-cAMP.

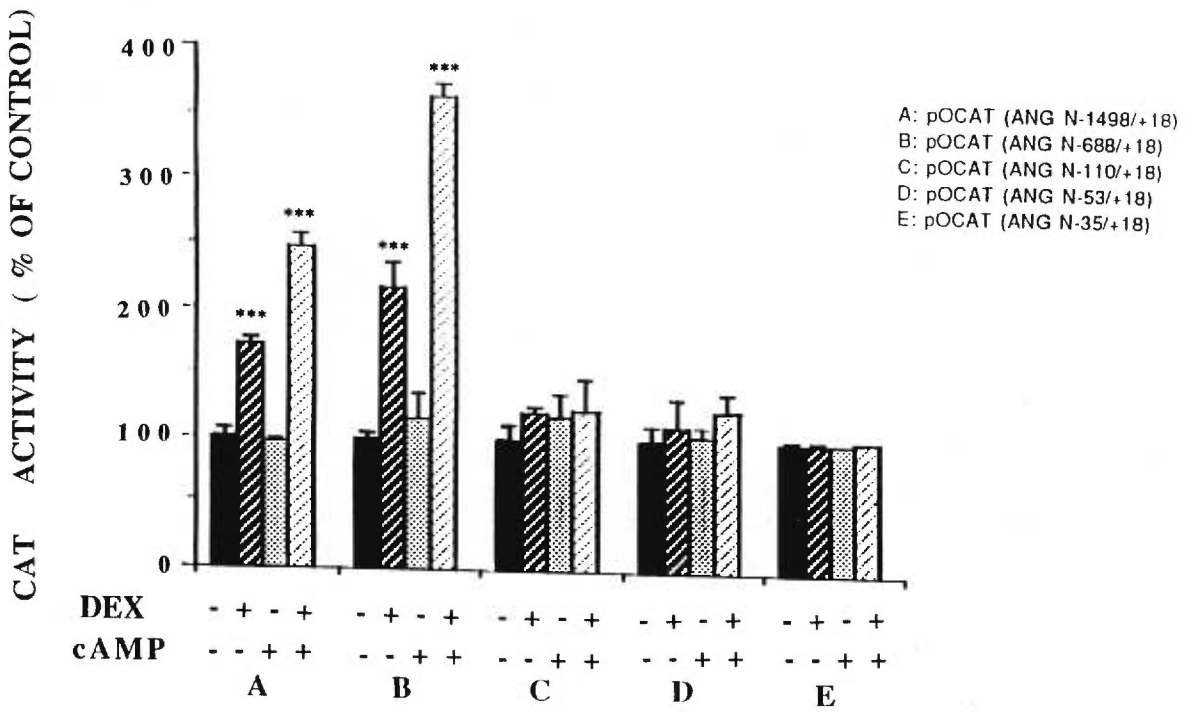

Figure 3-9. Effect of dexamethasone (10^{-6} M) on the expression of various ANG-CAT fusion genes in Hepa 1-6 cells in the presence or absence of 8-Bromo-cAMP (10^{-3} M). Cells were incubated for 24 hours in the presence of 8-Bromo-cAMP (10^{-3} M) or dexamethasone (10^{-6} M) or both. Results are expressed as a percentage of the CAT activity of the control (in the absence of both dexamethasone and 8-Bromo-cAMP; its CAT activity is shown as 100%). Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (***$P<0.005$). The solid black bar is without hormone treated, the solid hatched bar is from dexamethasone treated cells, the stippled bar is from cAMP treated cells and the empty hatched bar on the extreme right is from dexamethasone/cAMP treated cells.

Reproduced from Ming M., Sikstrom R., Lachance S., Delalandre A., Carriere S. and Chan J.S.D.: (1993) Hormonal regulation of expression of the angiotensinogen gene in mouse hepatoma cells. Am.J. Hypertension 6:141-148.

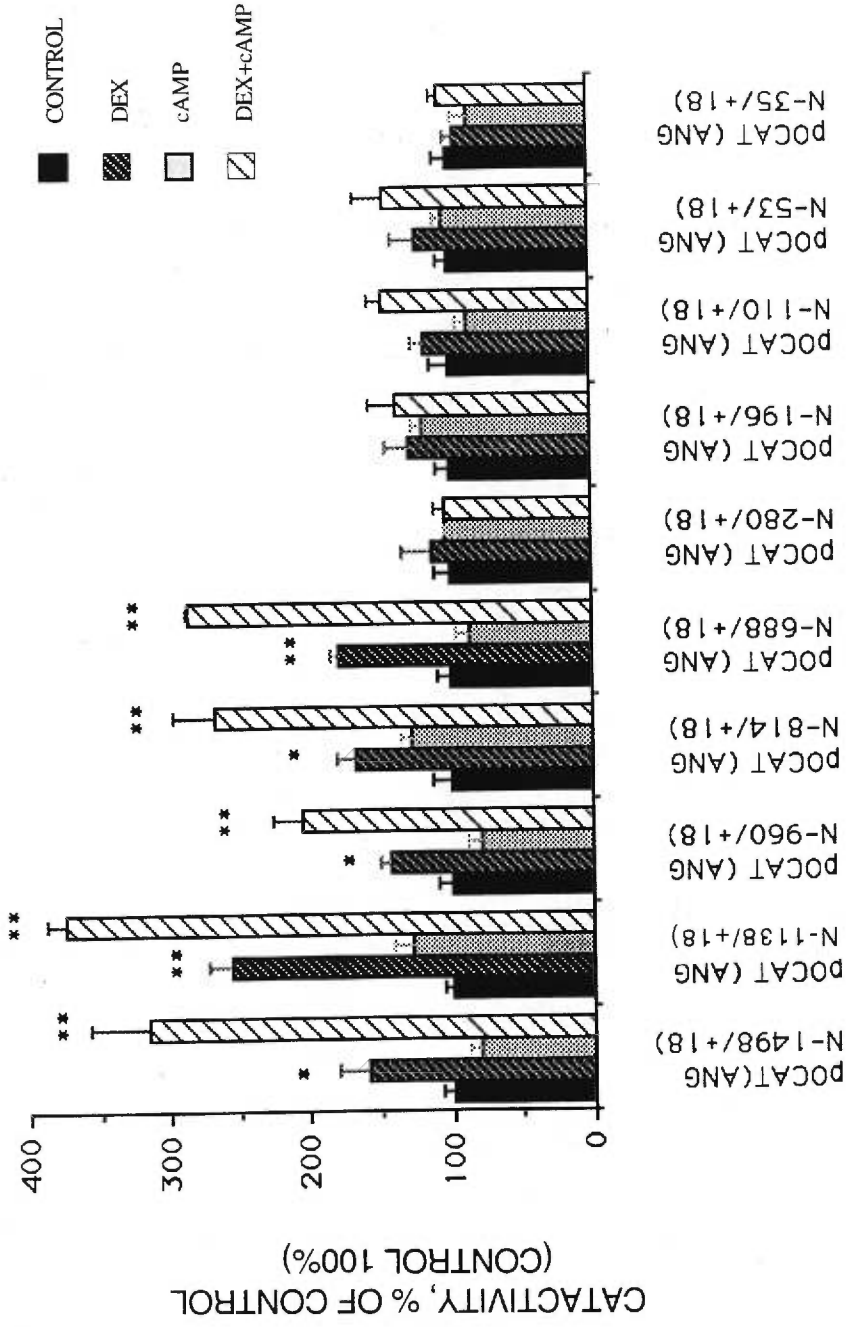

Figure 3-10. Effect of dexamethasone (10^{-6} M) on the expression of various ANG-CAT fusion genes in Hepa 1-6 cells in the presence or absence of 8-Bromo-cAMP (10^{-3} M). Cells were incubated for 24 hours in the presence of 8-Bromo-cAMP (10^{-3} M) or dexamethasone (10^{-6} M) or both. Results are expressed as percentage of the CAT activity of the control (in the absence of both 8-Bromo-cAMP and DEX; its CAT activity is shown as 100%). Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (*P<0.05, **P<0.01).

The above studies (Figures 3-6 to 3-10) demonstrated that: (a) The region of ANG N-688/-280 is essential for the effect of DEX to stimulate ANG gene expression; and (b) 8-Bromo-cAMP alone does not significantly stimulate ANG-CAT fusion gene expression in the Hepa 1-6 cell line, but it potentiates the effect of DEX.

To further confirm the possible effect of 8-Bromo-cAMP, the effects of forskolin (a post-receptor activator of adenylate cyclase) and glucagon (a plasma membrane receptor mediated activator of adenylate cyclase) on the expression of the ANG-CAT fusion gene in Hepa 1-6 cells were also examined.

Figure 3-11 shows a relationship between the addition of various concentrations of forskolin and the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells. Forskolin (10^{-9} to 10^{-5} M) alone did not significantly stimulate the expression of pOCAT (ANG N-1498/+18), but forskolin (10^{-5} M) potentiated the effect of DEX (10^{-6} M) on the expression of this fusion gene (450$\pm$23% increase (both) compared with 34$\pm$27% increase (forskolin alone) and 112$\pm$4% increase (DEX alone); $P<0.01$). Similar results were obtained by using different concentrations of glucagon. Figure 3-12 shows that different concentrations (10^{-10} to 10^{-6} M) of glucagon alone did not stimulate the expression of pOCAT (ANG N-1498/+18), but glucagon (10^{-6} M) potentiated the effect of DEX (10^{-6} M) on the expression of this fusion gene (163$\pm$30% increase (both) compared with a non-significant increase, 0$\pm$20% (glucagon alone) and 60$\pm$6% increase (DEX alone); $P<0.01$). The results obtained with forskolin and glucagon supported that obtained with 8-Bromo-cAMP. Thus, activators of the PKA pathway (8-Bromo-cAMP, forskolin, or glucagon) alone did not significantly stimulate the expression of pOCAT (ANG N-1498/+18), but they potentiated the effect of DEX on the expression of this fusion gene in Hepa 1-6 cells.

III.1.3.2. Identification of A Putative cAMP Response Element (CRE) in Rat ANG Gene

Studies in our laboratory have shown that there is a putative cAMP response element (CRE)

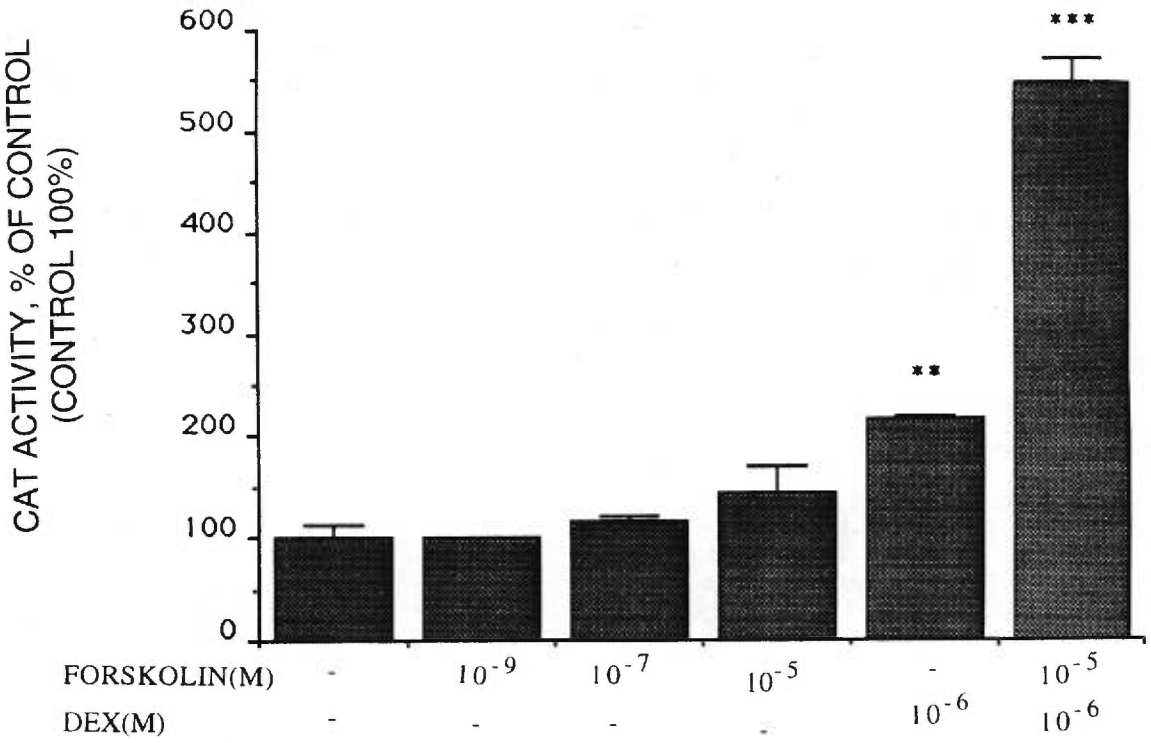

Figure 3-11. Effect of forskolin on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells in the presence or absence of dexamethasone (10^{-6} M). The CAT activity of pOCAT (ANG N-1498/+18) in the absence of both forskolin and dexamethasone is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (*P<0.05, **P<0.01, ***P<0.005).

Reproduced from Chan, J.S.D., Ming, M., Sikstrom, R., Lachance, S., Delalandre, A. and Carrière, S.: 8-Bromo-cyclic AMP (cAMP) enhances the effect of dexamethasone on the expression of the angiotensinogen (ANG) gene in cultured mouse hepatoma cells. The 8th Scientific Meeting of Amer. Soc. Hypertens., May 19-22, 1993, New York, NY

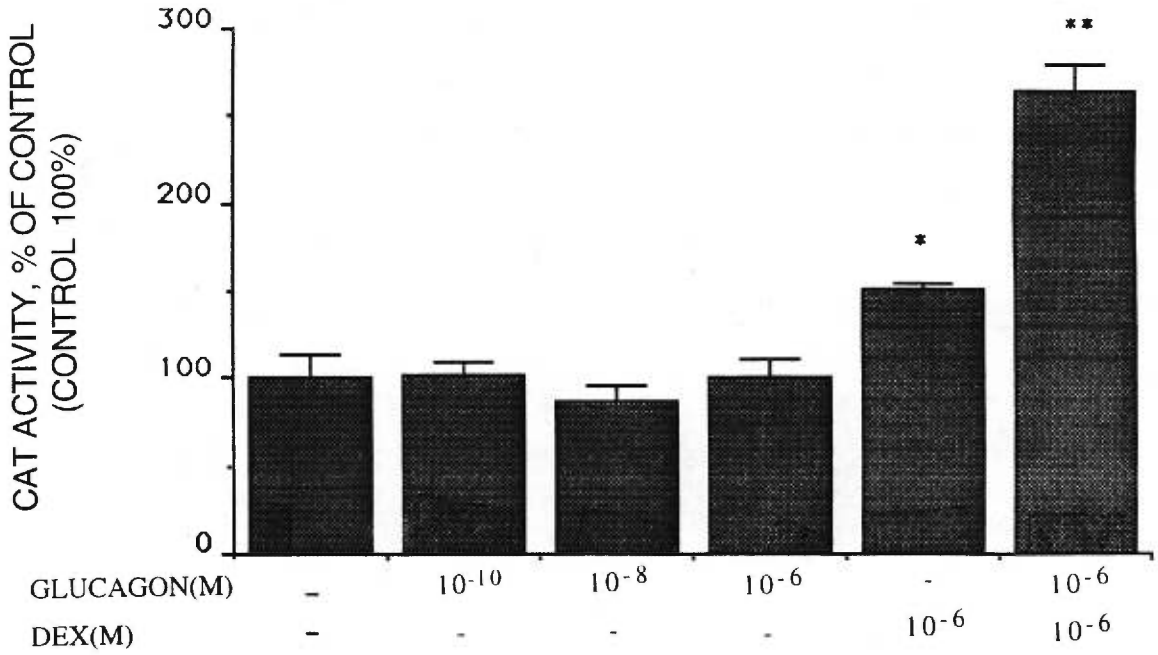

Figure 3-12. Effect of glucagon on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells. The CAT activity of pOCAT (ANG N-1498/+18) in the absence of both glucagon and DEX is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (*P<0.05, **P<0.01).

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in the rat ANG gene (ANG N-795/-788) (Chan et al., 1990). When this sequence (5'-TGACGTAC-3') was compared to the consensus CRE (5'-TGACGTCA-3') (Montminy et al., 1986), only the last two nucleotides were reversed. To investigate whether this DNA sequence could mediate the effect of cAMP on the expression of ANG-CAT fusion genes, various fusion genes with or without the putative ANG-CRE fused to the TK promoter were transfected into Hepa 1-6 cells. Figure 3-13 shows the results of these transfections. The addition of 8-Bromo-cAMP (10^{-3} M) stimulated the expression of pTKCAT (ANG N-814/-689), pTKCAT (ANG N-814/-761), and pTKCAT (ANG N-760/-689) to 130±11% (NS), 214±39% ($P<0.05$) and 105+5%, (NS) of the control (in the absence of both 8-Bromo-cAMP and forskolin, its CAT activity is shown as 100%). Similarly, the addition of forskolin (10^{-5} M) stimulated the expression of pTKCAT (ANG N-814/-689), pTKCAT (ANG N-814/-761), and pTKCAT (ANG N-760/-689) to 141±12% (NS), 288±26% ($P<0.01$) and 115±3% (NS) of the control value. Cells transfected with pTKCAT or pOCAT (ANG N-1498/+18) were not responsive to either cAMP or forskolin.

Figure 3-14 shows the relationship between various concentrations of 8-Bromo-cAMP (10^{-9} -10^{-3} M) and the expression of pTKCAT (ANG N-806/-779), the minimal DNA fragment containing the putative ANG CRE. The addition of 8-Bromo-cAMP (10^{-3}M) increased the expression by 120% ($P<0.05$).

The results provided evidence that the putative CRE of the rat ANG gene could mediate the effect of 8-Bromo-cAMP on the expression of the ANG gene in Hepa 1-6 cells.

In the absence of DEX, the addition of 8-Bromo-cAMP or forskolin alone stimulated the expression of pTKCAT (ANG N-814/-761) and 8-Bromo-cAMP alone also stimulated the expression of pTKCAT (ANG N-806/-779). Both ANG N-814/-761 and ANG N-806/-779 contained the DNA sequences of the putative CRE and no other response element has been identified in these regions. The pTKCAT (ANG N-760/-689) without the putative CRE was not responsive to the addition of either 8-Bromo-cAMP or forskolin. This fragment also may be responsible for the lowered responsiveness of pTKCAT (ANG N-814/-689) to the stimulation of

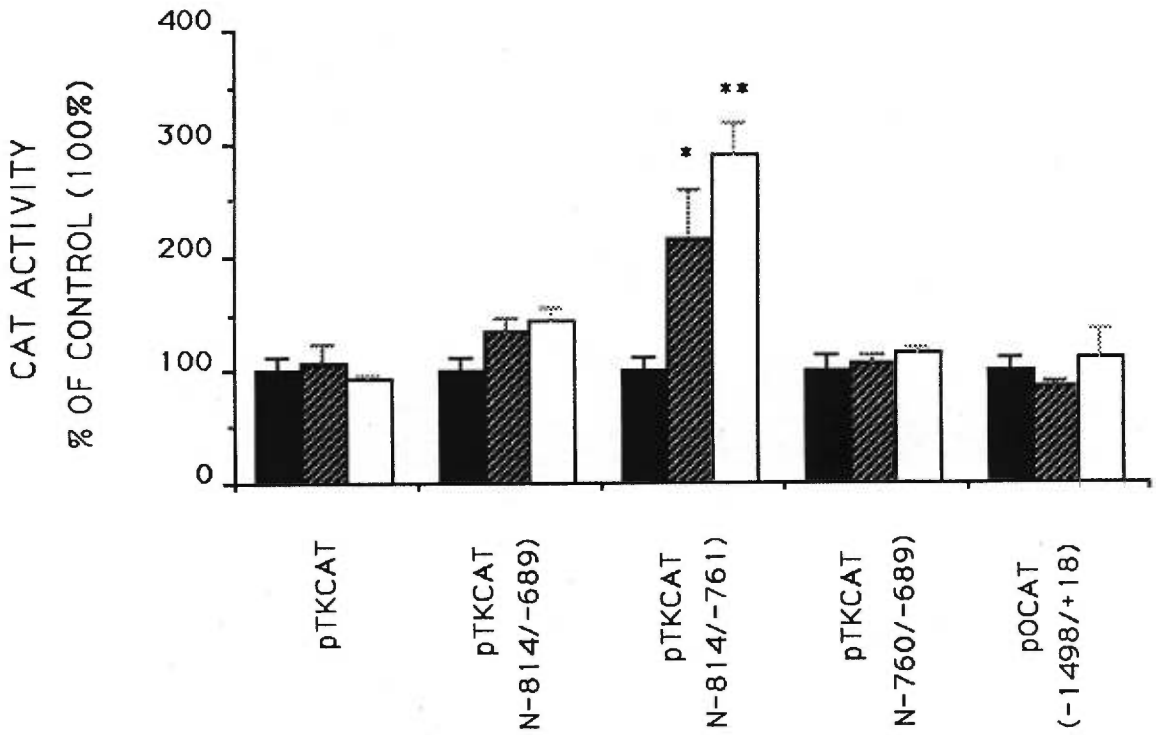


Figure 3-13. Effect of 8-Bromo-cAMP (10^{-3} M) and forskolin (10^{-5} M) on the expression of various ANG-CAT fusion genes with or without the putative cAMP response element fused with the thymidine kinase (TK) promoter in Hepa 1-6 cells. Results are expressed as a percentage of the CAT activity of the control (in the absence of both 8-Bromo-cAMP and forskolin; its CAT activity is shown as 100%). Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from Student's *t* test (*P<0.05, **P<0.01). Solid bar, control cells; hatched bar, 8-Bromo-cAMP treated cells; open bar, forskolin treated cells.

Reproduced from Chan, J.S.D., Wu, J., Ming, M., Sikstrom, R., Lachance, S., Delalandre, A. et Carrière, S.: Caracterization of a novel-cAMP responsive element (CRE) in the rat angiotensinogen gene. 15th Scientific Meeting of the International Society of Hypertension, Melbourne, Australia, March 20-24, 1994.

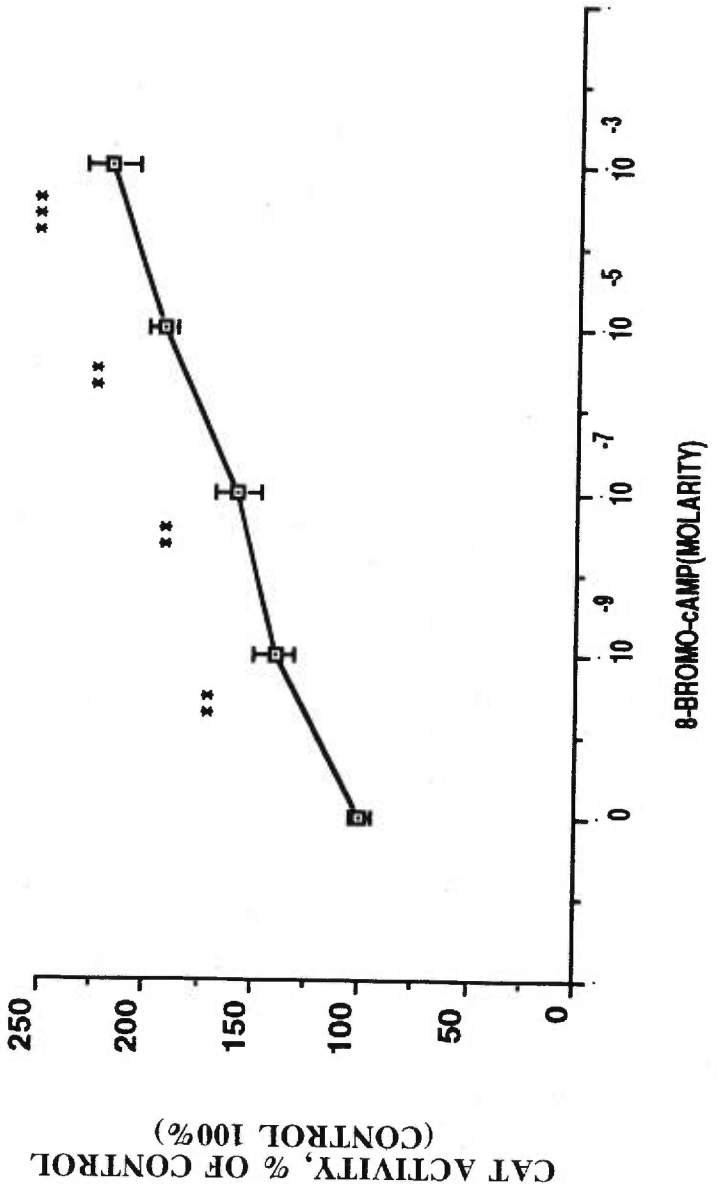


Figure 3-14. Relationship between the concentrations of 8-Bromo-cAMP and the expression of pTKCAT (ANG N-806/-779) in Hepa 1-6 cells. Cells were incubated for 24 hours in the presence of various concentrations of 8-Bromo-cAMP (10^{-9} to 10^{-3} M). The CAT activity of pTKCAT (ANG N-806/-779) in the absence of 8-Bromo-cAMP is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (**P<0.01, ***P<0.005).

8-Bromo-cAMP or forskolin, because pTKCAT (ANG N-814/-689) displayed a minimal response compared with pTKCAT (ANG N-814/-761) (130±11% versus 214±39%; 141±12 % versus 288±26%) (Figure 3-13). These results suggested that the fragment ANG (N-760/-689) could inhibit the putative CRE response to 8-Bromo-cAMP.

In summary, the results (Figures 3-8 to 3-14) showed that: (a) DEX stimulated the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells; (b) 8-Bromo-cAMP or forskolin alone did not stimulate the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells, but either of them had a potentiating effect on DEX; (c) 8-Bromo-cAMP or forskolin alone stimulated the expression of ANG-TK-CAT fusion genes containing the putative ANG CRE, but not the ANG-TK-CAT fusion genes without the putative ANG CRE; and (d) The fragment ANG (N-760/-689) could inhibit the putative CRE response to 8-Bromo-cAMP.

III.1.4. Effect of Isoproterenol on the Expression of ANG-CAT Fusion Genes in Hepa 1-6 Cells

III.1.4.1. Effect of Isoproterenol plus Dexamethasone on the Expression of ANG-CAT Fusion Genes in Hepa 1-6 Cells

Upon finding the potentiating effect of 8-Bromo-cAMP on dexamethasone for the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells, we studied the effect of isoproterenol on the expression of pOCAT (ANG N-1498/+18) .

Figure 3-15 shows that while isoproterenol alone (10^{-9} to 10^{-5} M) had no significant effect on the expression of pOCAT (ANG N-1498/+18), it enhanced the stimulatory effect of dexamethasone (10^{-6} M) in a concentration-dependent manner. The combination of either 10^{-7} M or 10^{-5} M isoproterenol with dexamethasone significantly increased the expression of pOCAT (ANG N-1498/+18) compared with dexamethasone alone (207±5% versus 174±5%, $P<0.05$; and 274±18% versus 174±5%, $P<0.01$, respectively).

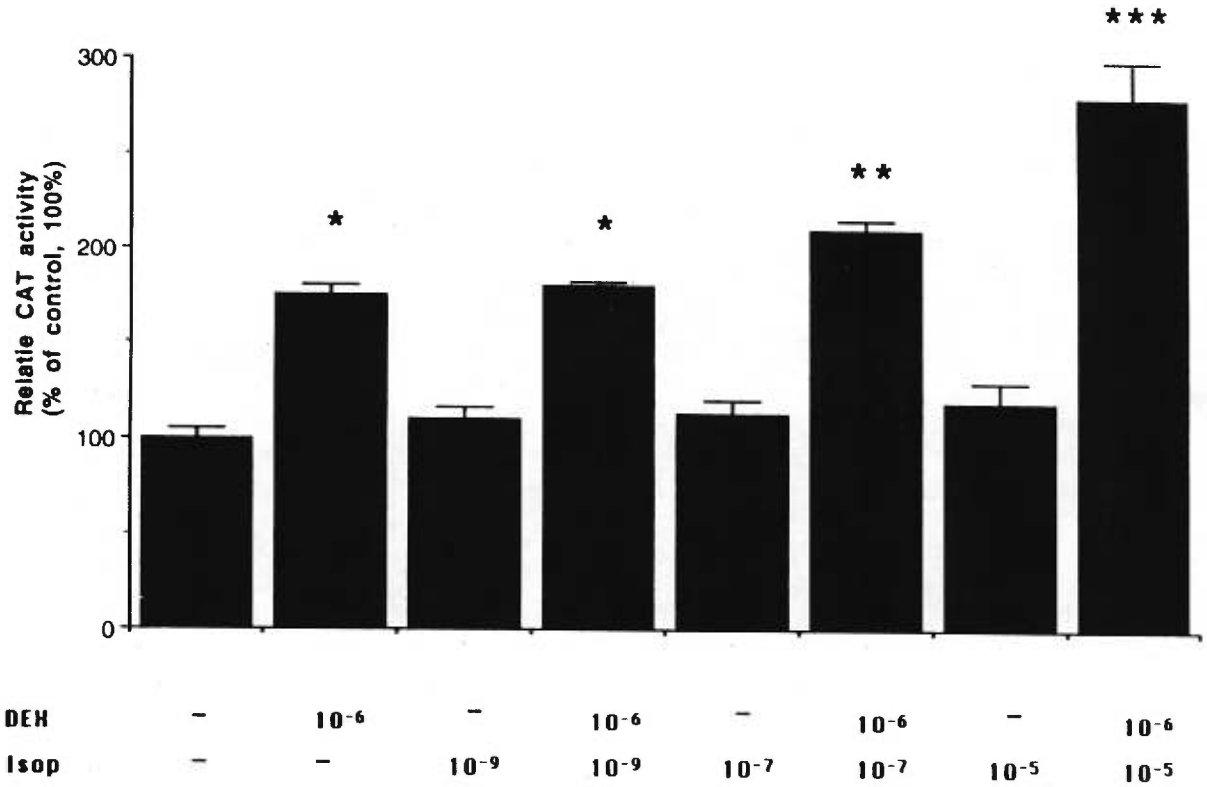


Figure 3-15. The effect of isoproterenol (Isop; 10^{-9} to 10^{-5} M) alone or combined with dexamethasone (DEX; 10^{-6} M) on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells. The chloramphenicol acetyltransferase (CAT) activity of pOCAT (ANG N-1498/+18) in the absence of both dexamethasone and isoproterenol is shown as 100% (control). Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). . The probability values are derived from ANOVA (*P<0.05, **P<0.01, ***P<0.005).

Reproduced from Ming M., Wu J., Lachance S., Delalandre A., Bouvier M., Carriere S. and Chan J.S.D.: (1995) β-adrenergic receptors and angiotensinogen gene expression in mouse hepatoma cells in vitro. Hypertension 25: (No 1) 105-109.

Figure 3-16 shows the effect of isoproterenol (10^{-5} M) on the expression of various ANG-CAT fusion genes with or without the putative ANG CRE fused to the TK promoter in Hepa 1-6 cells. The addition of isoproterenol (10^{-5} M) stimulated the expression of pTKCAT (ANG N-814/-689), pTKCAT (ANG N-814/-761), and pTKCAT (ANG N-760/-689) to 110±23% (NS), 253±18% ($P<0.01$), and 127±7% (NS) compared with the control (in the absence of isoproterenol, where CAT activity was shown as 100%). The pTKCAT alone was not responsive to isoproterenol. Like 8-Bromo-cAMP (Figure 3-13), isoproterenol alone stimulated the expression of pTKCAT (ANG N-814/-761), which contained the putative CRE fused to the TK promoter. Again, similarly to 8-Bromo-cAMP (Figure 3-9), isoproterenol alone did not stimulate the expression of pOCAT (ANG N-1498/+18), but it potentiated the effect of DEX (Figure 3-15). These results indicate that the effect of isoproterenol on the expression of the ANG gene in Hepa 1-6 cells is mediated by a signal transduction pathway involving cAMP.

III.1.4.2. Effect of Rp-cAMP on the Expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 Cells

Figure 3-17 shows that addition of Rp-cAMP (an inhibitor of cAMP-dependent protein kinase A I and II) inhibited the enhancing effect of isoproterenol (10^{-5} M) on the expression of pOCAT (ANG N-1498/+18) in a concentration-dependent manner in Hepa 1-6 cells in the presence of dexamethasone (10^{-6} M). Rp-cAMP (10^{-6} M) and Rp-cAMP (10^{-5} M) produced a significantly inhibitory effect. The expression of pOCAT (ANG N-1498/+18) was decreased from 252±10% to 215±6% ($P<0.05$) and 187±7% ($P<0.01$), respectively. These results suggested that the effect of isoproterenol on ANG gene expression was mediated by the cAMP-dependent protein kinase pathway.

III.1.4.3. Effect of β-Adrenergic Receptor Antagonists on the Expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 Cells

Figure 3-18 shows that propranolol (a non-selective β_1- and β_2-adrenergic receptor

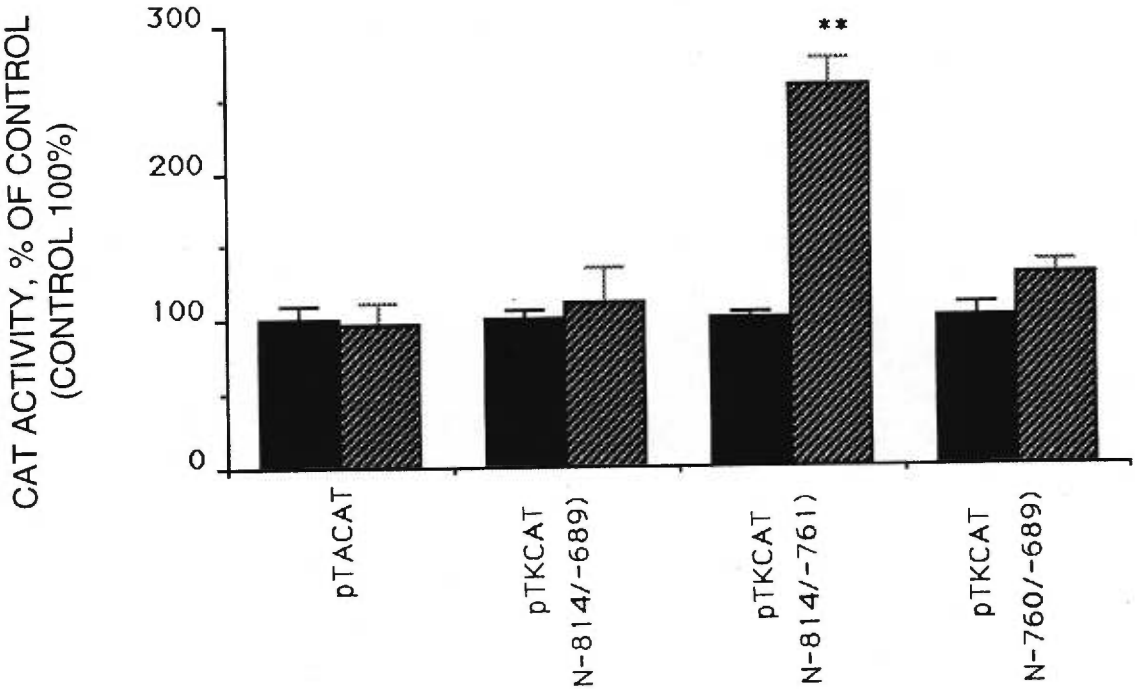


Figure 3-16. Effect of isoproterenol (10^{-5} M) on the expression of various ANG-CAT fusion genes with or without the putative cAMP response element fused to the thymidine kinase (TK) promoter in Hepa 1-6 cells. Results are expressed as a percentage of the CAT activity of the control (in the absence of isoproterenol, its CAT activity is shown as 100%). Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from Student's *t* test (**P<0.01). Solid bar, control cells; hatched bar, isoproterenol treated cells.

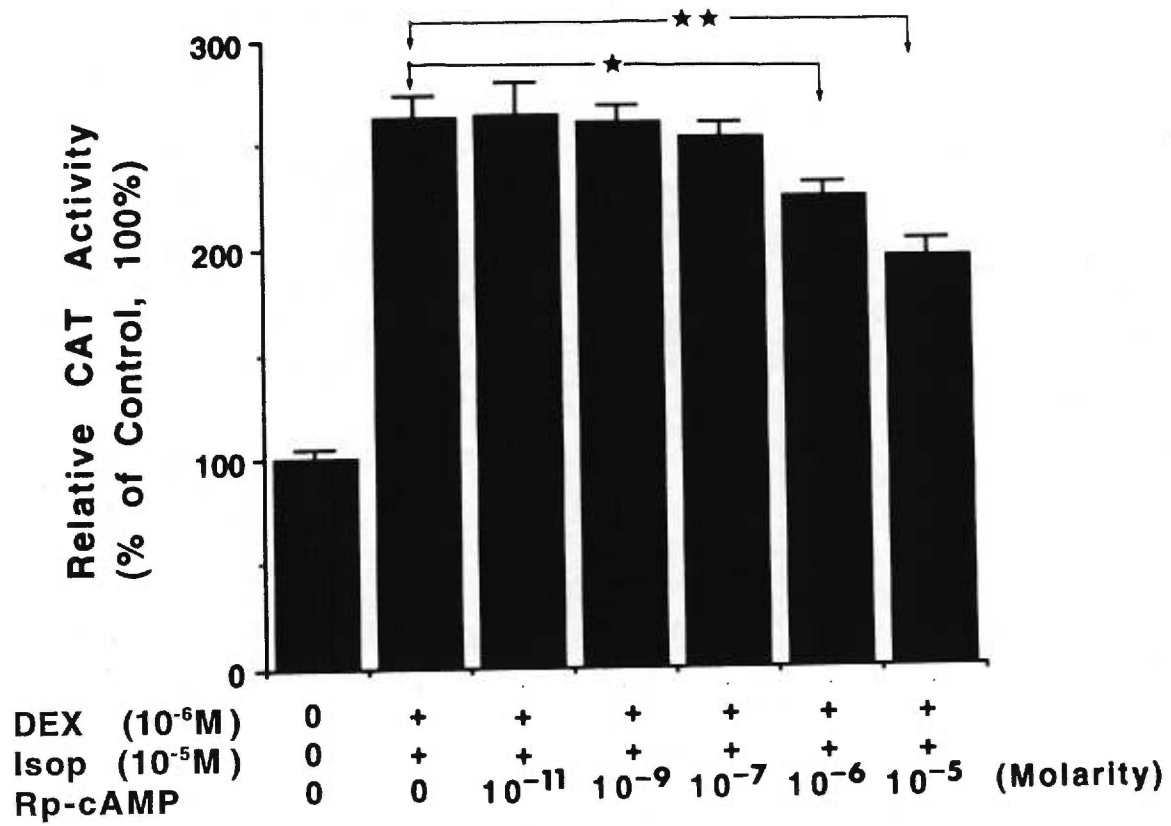


Figure 3-17. Inhibitory effect of Rp-cAMP on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells stimulated by isoproterenol (Isop) and dexamethasone (DEX). Cells were incubated for 24 hours in the presence of dexamethasone (10^{-6} M), isoproterenol (10^{-5} M), and various concentrations of Rp-cAMP (10^{-11} to 10^{-5} M). The chloramphenicol acetyltransferase (CAT) activity of pOCAT (ANG N-1498/+18) in the absence of dexamethasone, isoproterenol, and Rp-cAMP is shown as 100% (control). Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (*P<0.05, **P<0.01).

Reproduced from Ming M., Wu J., Lachance S., Delalandre A., Bouvier M., Carriere S. and Chan J.S.D.: (1995) β-adrenergic receptors and angiotensinogen gene expression in mouse hepatoma cells in vitro. Hypertension 25: (No 1) 105-109.

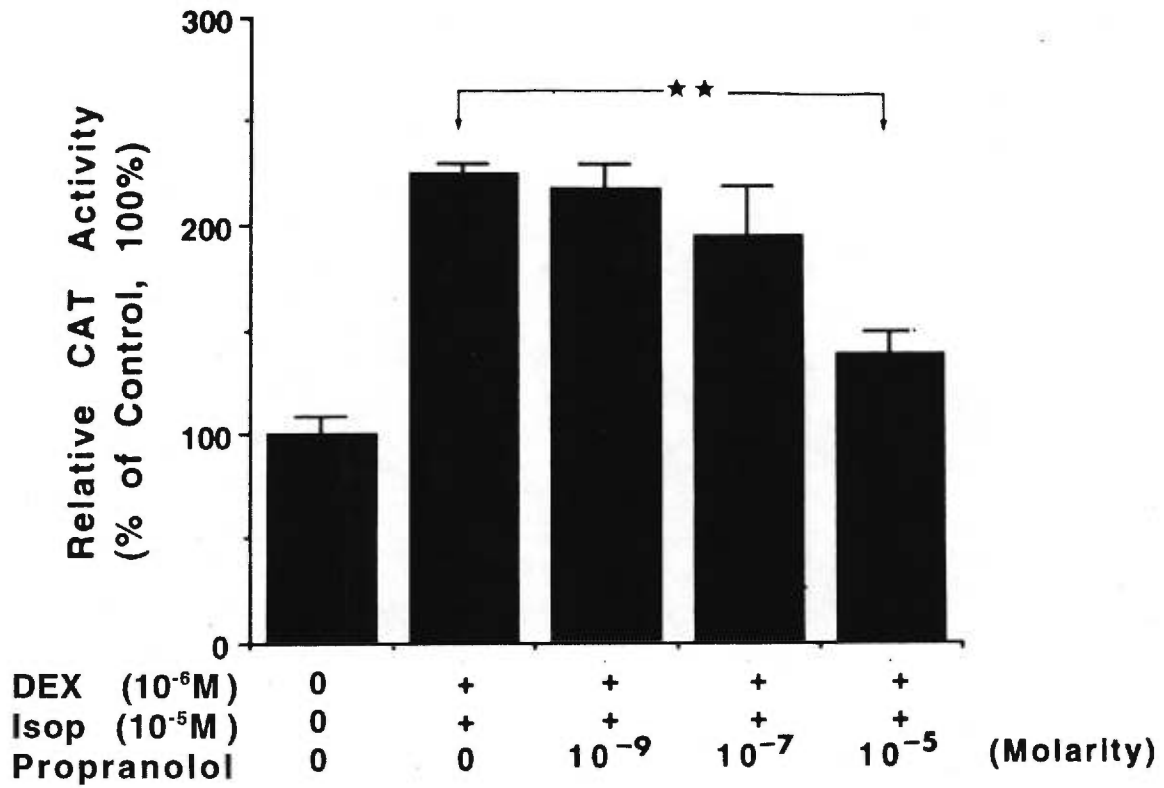


Figure 3-18. Inhibitory effect of propranolol on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells stimulated by dexamethasone (DEX) and isoproterenol (Isop). Cells were incubated for 24 hours in the presence of dexamethasone (10^{-6} M), isoproterenol (10^{-5} M), and various concentrations of propranolol (10^{-9} to 10^{-5} M). The CAT activity of pOCAT (ANG N-1498/+18) in the absence of dexamethasone, isoproterenol and propranolol is shown as 100% (control). Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (**P<0.01).

Reproduced from Ming M., Wu J., Lachance S., Delalandre A., Bouvier M., Carriere S. and Chan J.S.D.: (1995) β-adrenergic receptors and angiotensinogen gene expression in mouse hepatoma cells in vitro. Hypertension 25: (No 1) 105-109.

antagonist) blocked the enhancing effect of isoproterenol on the expression of pOCAT (ANG N-1498/+18) in the presence of dexamethasone (10^{-6} M). The addition of propranolol (10^{-5} M) decreased the expression from 223±4% to 137±10% ($P<0.01$). It appears that lower concentrations of propranolol, less than 10^{-5} M, did not exhibit an inhibitory effect.

Similarly, Figure 3-19 shows that addition of a β_2-adrenergic receptor antagonist (ICI 118,551) blocked the enhancing effect of isoproterenol in a concentration-dependent manner. ICI 118,551 (10^{-7} M) and ICI 118,551 (10^{-5} M) produced a significantly inhibitory effect. The expressions were decreased from 242±5% to 190±2% ($P<0.01$) and 160±8% ($P<0.01$), 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) even at a concentration as high as 10^{-5} M (Figure 3-20).

These results (Figure 3-18, 3-19, 3-20) suggest that the enhancing effect of isoproterenol was mediated predominantly by β_2-adrenergic receptors in Hepa 1-6 cells.

In summary, in the mouse hepatoma cell line, Hepa 1-6:

(a) Dexamethasone (10^{-6}M) stimulated the expression of pOCAT (ANG N-1498/+18), pOCAT (ANG N-1138/+18), pOCAT (ANG N-960/+18), pOCAT (ANG N-814/+18), and pOCAT (ANG N-688/+18), but not pOCAT (ANG N-280/+18), pOCAT (ANG N-196/+18), pOCAT (ANG N-110/+18), pOCAT (ANG N-53/+18), and pOCAT (ANG N-35/+18). These results indicated that the DNA sequence(s) that mediated the effect of dexamethasone on ANG gene expression, was located within ANG N-688/-280.

(b) 8-Bromo-cAMP or isoproterenol alone did not stimulate the expression of pOCAT (ANG N-1498/+18), but either of them potentiated the effect of DEX on the expression of this ANG-CAT fusion gene. In contrast, 8-Bromo-cAMP or isoproterenol alone did stimulate the expression of pOCAT (ANG N-814/-761), which contained the putative CRE (ANG N-795/-788). These results indicated that ANG gene expression in this cell line is controlled by multiple factors, and the effects

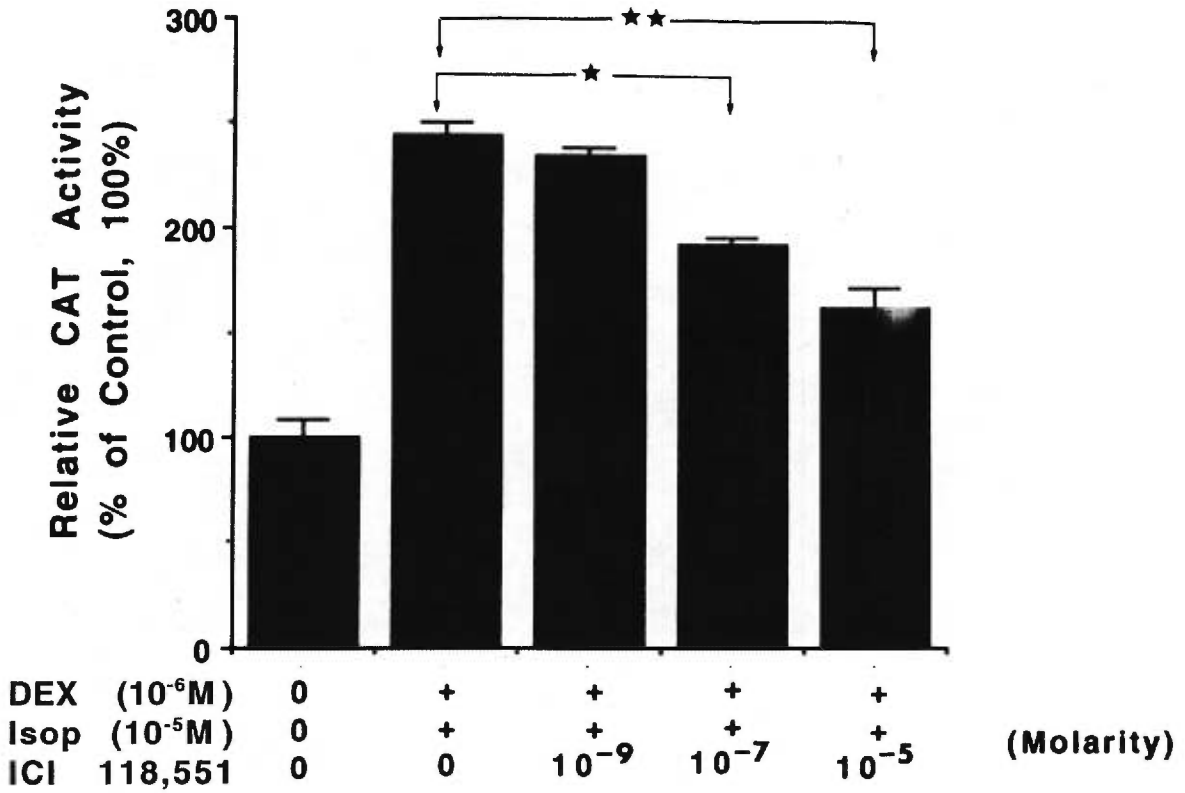


Figure 3-19. Inhibitory effect of ICI 118,551 on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells stimulated by dexamethasone (DEX) and isoproterenol (Isop). Cells were incubated for 24 hours in the presence of dexamethasone (10^{-6} M), isoproterenol (10^{-5} M), and various concentrations of ICI 118,551 (10^{-9} to 10^{-5} M). The chloramphenicol acetyltransferase (CAT) activity of pOCAT (ANG N-1498/+18) in the absence of dexamethasone, isoproterenol, and ICI 118,551 is shown as 100% (control). Each point represents the mean$\pm$SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (*P<0.05, **P<0.01).

Reproduced from Ming M., Wu J., Lachance S., Delalandre A., Bouvier M., Carriere S. and Chan J.S.D.: (1995) β-adrenergic receptors and angiotensinogen gene expression in mouse hepatoma cells in vitro. Hypertension 25: (No 1) 105-109.

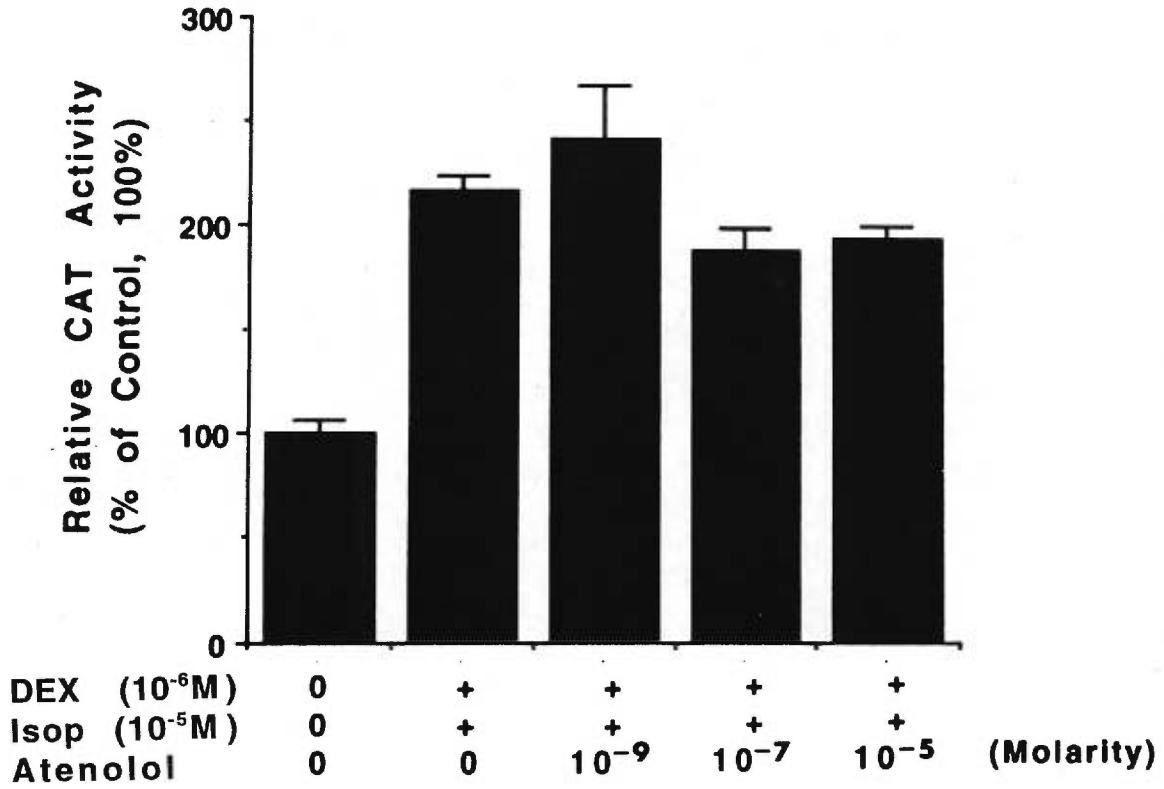


Figure 3-20. Effect of atenolol on the expression of pOCAT (ANG N-1498/+18) in Hepa1-6 cells stimulated by dexamethasone (DEX) and isoproterenol (Isop). Cells were incubated for 24 hours in the presence of dexamethasone (10^{-6} M), isoproterenol (10^{-5} M), and various concentrations of atenolol (10^{-9} - 10^{-5} M). The chloramphenicol acetyltransferase (CAT) activity of pOCAT (ANG N-1498/+18) in the absence of dexamethasone, isoproterenol, and atenolol is shown as 100% (control). Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). ANOVA was used for comparison.

Reproduced from Ming M., Wu J., Lachance S., Delalandre A., Bouvier M., Carriere S. and Chan J.S.D.: (1995) β-adrenergic receptors and angiotensinogen gene expression in mouse hepatoma cells in vitro. Hypertension 25: (No 1) 105-109.

of 8-Bromo-cAMP or isoproterenol on ANG gene expression were mediated by the putative CRE.

(c) The enhancing effect of isoproterenol on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells stimulated by dexamethasone was attenuated by Rp-cAMP (an inhibitor of protein kinase AI and II), propranolol (a non selective β_1- and β_2-adrenergic receptor antagonist), and ICI 118,551 (a β_2-adrenergic receptor antagonist), but was not inhibited by atenolol (a β_1-adrenergic receptor antagonist). These results indicated that the enhancing effect of isoproterenol on dexamethasone stimulation of the expression of pOCAT (ANG N-1498/+18) was mediated by the protein kinase A pathway and β_2-adrenergic receptors in Hepa 1-6 cells.

III.2. EXPRESSION OF THE ANG-CAT FUSION GENES IN OK CELLS

III.2.1. Preliminary Experiments

Figure 3-21 shows the efficiency of the transfection of pOCAT, pRSVCAT, and pOCAT (ANG N-1498/+18) fusion genes into OK cells. A plasmid containing the Rous sarcoma virus enhancer/promoter sequence fused to the CAT reporter gene (pRSVCAT) was used as a positive control, whereas the plasmid containing no promoter (pOCAT) was used as a negative control. Figure 3-21A shows a typical autoradiogram of duplicate transfections of these three plasmids, whereas Figure 3-21B shows the results expressed as the ratio of the relative amount of CAT activity by pRSVCAT (10 μg), pOCAT (10 μg), and pOCAT (ANG N-1498/+18) (10 μg) fusion genes in percentages after normalizing for the efficiency of transfection of 50 μg of intracellular protein. The full-length ANG-CAT fusion gene, pOCAT (ANG N-1498/+18), had 22-fold ($26\pm4\%$) higher enzymatic CAT activity than pOCAT ($1.2\pm0.2\%$), but had only one-half that of pRSVCAT ($65\pm12\%$).

III.2.1.1. Effect of hGH and IGF-I on the Expression of the ANG-CAT Fusion Genes in OK Cells

In order to use the human growth hormone as an internal control to normalize the transfection

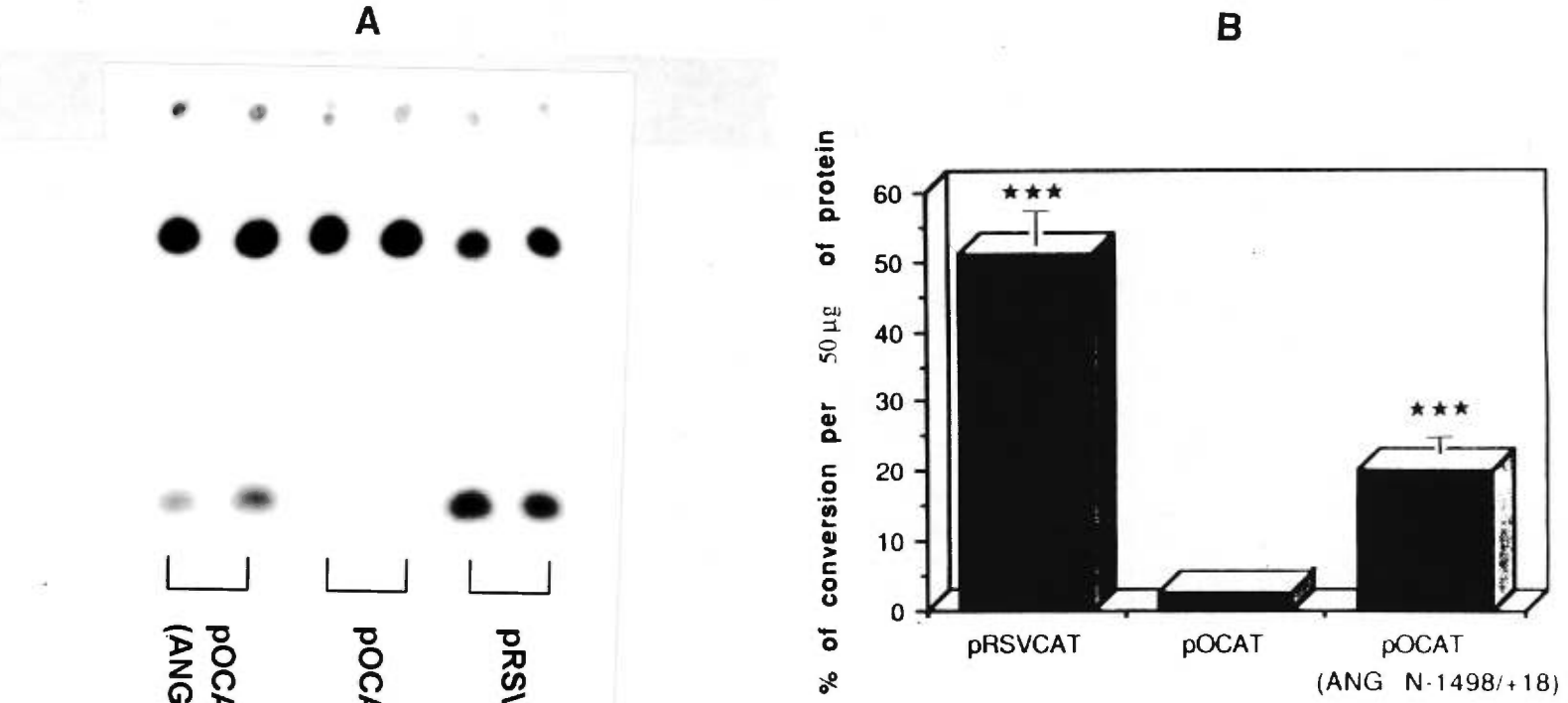


Figure 3-21. (A) A typical transient expression (autoradiogram) of the plasmids pRSVCAT, pOCAT and the ANG-CAT fusion gene, pOCAT (ANG N-1498/+18), in OK cells. (B) Results of transient gene expression expressed as a percentage of conversion of the [^{14}C] chloramphenicol per 50 µg of cellular protein. Each point represents the mean±SD of five independent transfections in duplicate plates (n=5). The probability values are derived from Student's *t* test (***P<0.005).

Reproduced from Chan J.S.D., Ming M., Nie Z.R., sikstrom R., Lachance S. and Carriere S.: (1992) Hormonal regulation of expression of the angiotensinogen gene in cultured opossum kidney (OK) proximal tubular cells. J. Amer Soc. Nephrol. 2: 1516-1522.

efficiency of different ANG-CAT fusion genes in OK cells, we examined the effect of hGH and IGF-I on the expression of the ANG-CAT fusion gene in OK cells. Figure 3-22 shows the effect of human growth hormone (hGH) on the expression of pOCAT (ANG N-1498/+18). The addition of various concentrations of hGH (0.1-100 ng/ml) did not stimulate the expression of pOCAT (ANG N-1498/+18) in OK cells.

Similarly, Figure 3-23 shows the effect of IGF-I on the expression of pOCAT (ANG N-1498/+18). The addition of various concentrations of IGF-I (5-100 ng/ml) did not stimulate the expression of the ANG-CAT fusion gene in OK cells.

These studies demonstrated that neither hGH nor IGF-I had a stimulatory effect on the expression of the ANG gene in OK cells. These observations were similar to those found in Hepa 1-6 cells (cf., Figure 3-3, 3-4). Hence, we co-transfected the OK cells with various ANG-CAT fusion genes (10 μg) and pTKGH (1 μg). Subsequently, CAT activity in the cell extract and the amount of IRA-hGH secreted into the medium were measured. The levels of IRA-hGH were used to normalize the transfection efficiency of various ANG-CAT fusion genes.

III.2.2. Effect of Steroid Hormones on the Expression of ANG-CAT Fusion Genes in OK Cells

Figure 3-24 shows a relationship between the addition of various concentrations of dexamethasone and the expression of pOCAT (ANG N-1498/+18). Dexamethasone (10^{-6} M) stimulated the expression of pOCAT (ANG N-1498/+18) as compared to the control ($357\pm49\%$ versus $100\pm23\%$, $P<0.01$). However, at 10^{-4} M DEX, the expression of pOCAT (ANG N-1498/+18) was profoundly decreased ($357\pm49\%$ versus $133\pm4\%$, $P<0.01$). This decrease appeared to be due primarily to a high number of dead cells in the culture (approximately 30 to 50% of cells were dead as analyzed by hemocytometer), while there was no noticeable cell death when 10^{-6} M DEX was used.

Figure 3-25 shows the effect of different hormones on the expression of the ANG-CAT

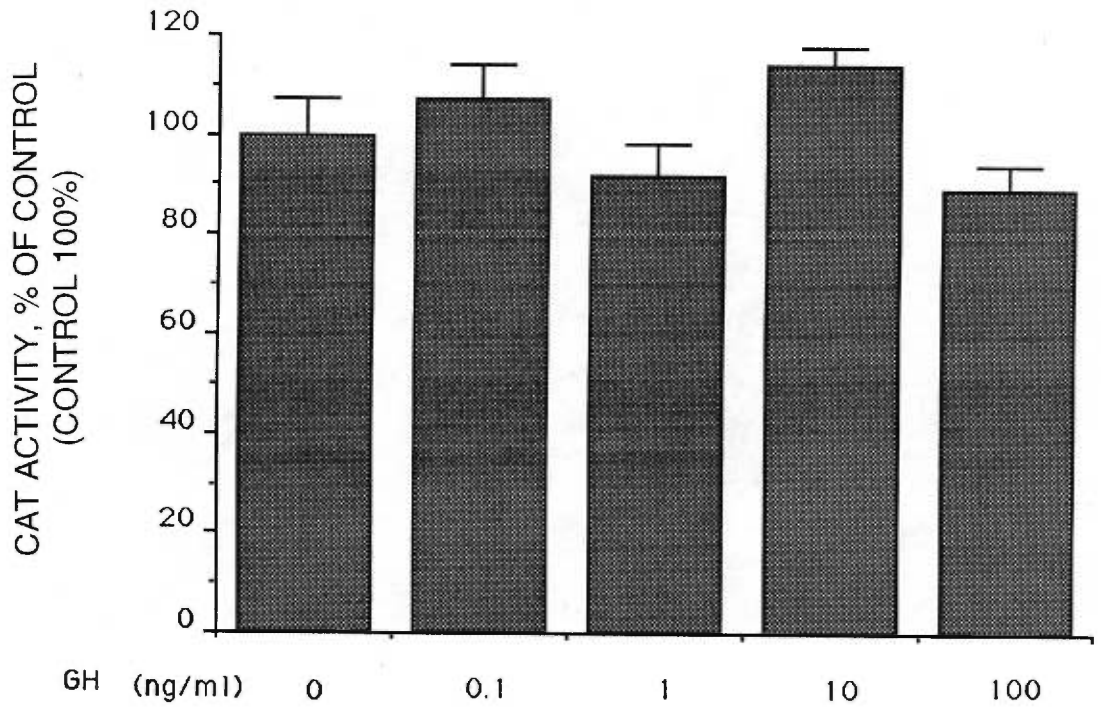


Figure 3-22. Relationship between hGH concentrations and the expression of pOCAT (ANG N-1498/+18) in OK cells. Cells were incubated for 24 hours in the presence of various concentrations of hGH (0.1 - 100 ng/ml). The CAT activity of pOCAT (ANG N-1498/+18) in the absence of hGH is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). ANOVA was used for comparisons.

Reproduced from Ming, M., Nie, Z-R., Sikstrom, R., Lachance, S. and Carrière, S.: Effect of human growth hormone on the expression of the angiotensinogen gene in opossum kidney (OK) cells. 60th Annual Meeting, Can. Soc. Clin. Invest., Quebec city, Canada, September 19-23, 1991.

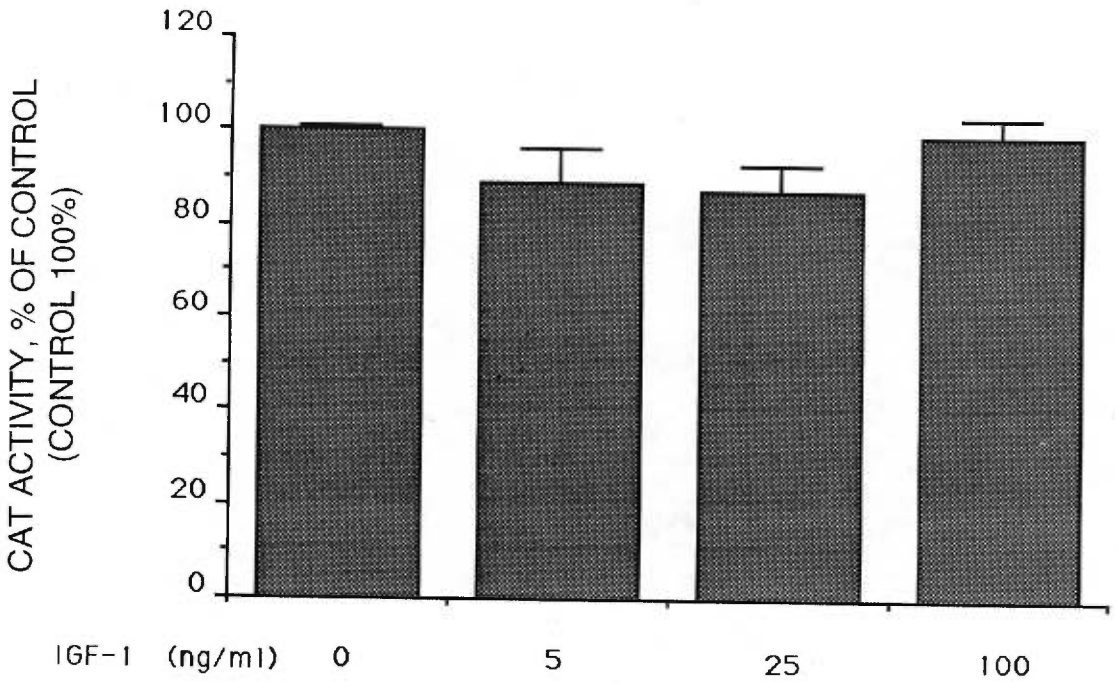


Figure 3-23. Relationship between IGF-I concentrations and the expression of pOCAT (ANG N-1498/+18) in OK cells. Cells were incubated for 24 hours in the presence of various concentrations of IGF-I (5-100 ng/ml). The CAT activity of pOCAT (ANG N-1498/+18) in the absence of IGF-I is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). ANOVA was used for comparisons.

Reproduced from Ming, M., Nie, Z-R., Sikstrom, R., Lachance, S. and Carrière, S.: Effect of human growth hormone on the expression of the angiotensinogen gene in opossum kidney (OK) cells. 60th Annual Meeting, Can. Soc. Clin. Invest., Quebec city, Canada, September 19-23, 1991.

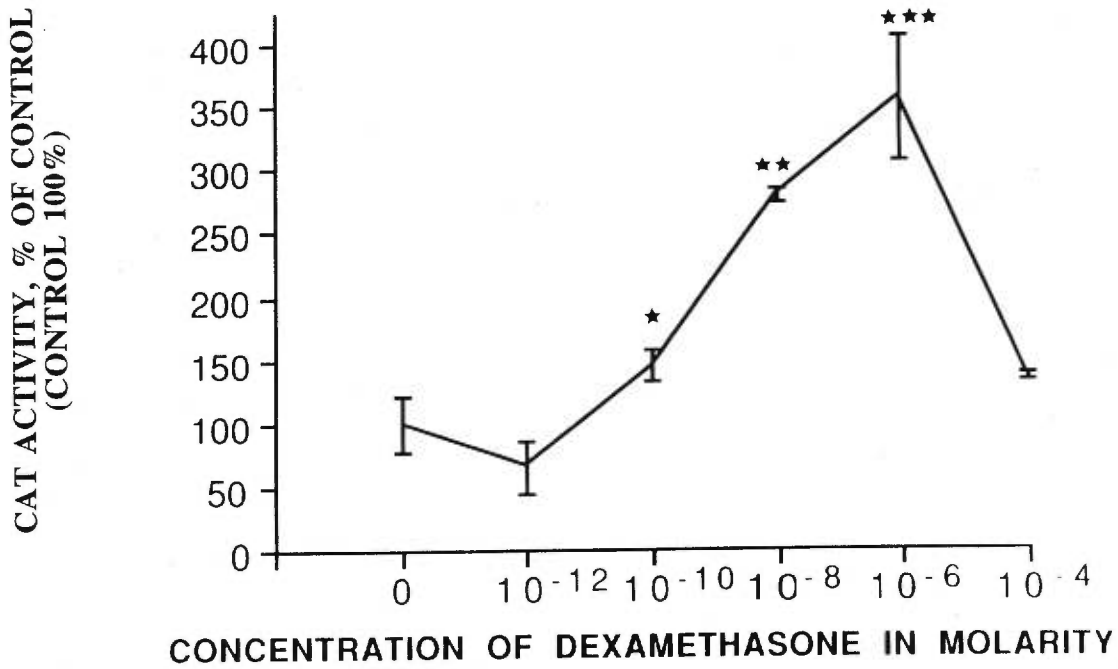


Figure 3-24. Relationship between dexamethasone concentrations and the expression of pOCAT (ANG N-1498/+18) in OK cells. Cells were incubated for 24 hours in the presence of various concentrations of dexamethasone (10^{-12} -10^{-4} M). The CAT activity of pOCAT (ANG N-1498/+18) in the absence of dexamethasone is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (*P<0.05, **P<0.01, ***P<0.005).

Reproduced from Chan J.S.D., Ming M., Nie Z.R., sikstrom R., Lachance S. and Carriere S.: (1992) Hormonal regulation of expression of the angiotensinogen gene in cultured opossum kidney (OK) proximal tubular cells. J. Amer Soc. Nephrol. 2: 1516-1522.

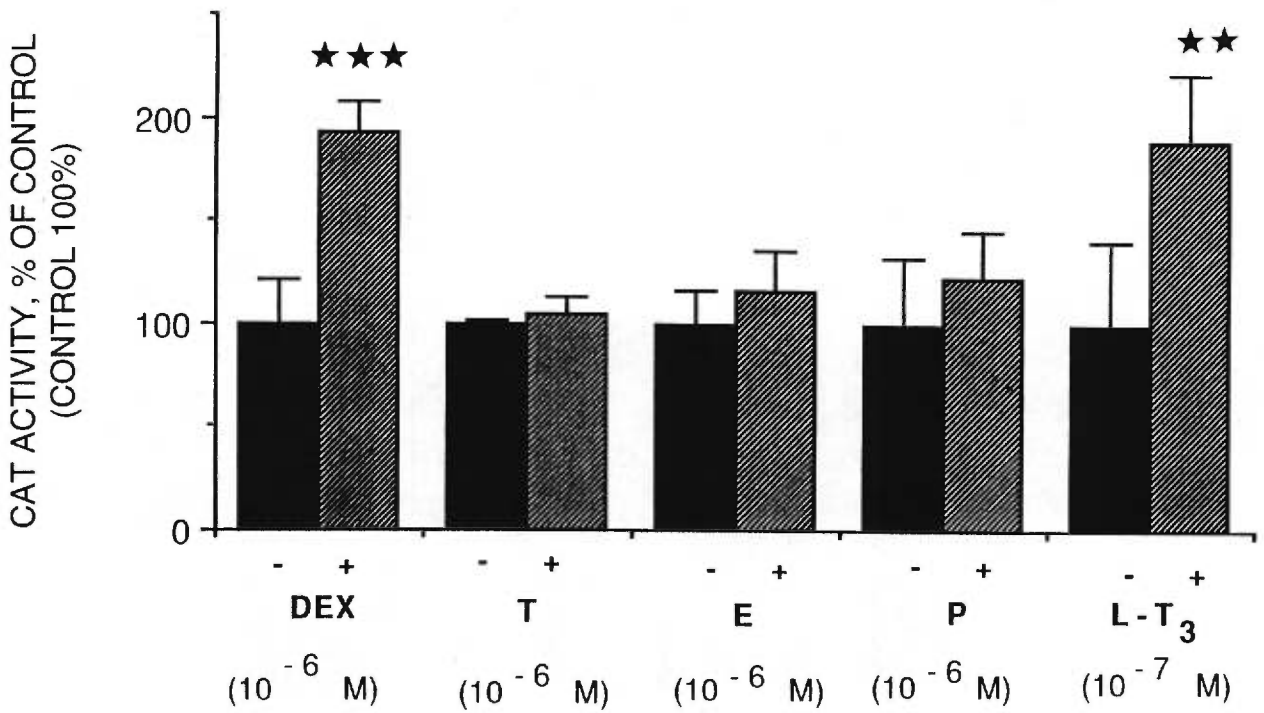


Figure 3-25. The effect of the addition (with, +; without, -) of different hormones (DEX, dexamethasone; E_2, estradiol; T, testosterone; P, progesterone; and L-T_3, thyroid hormone) on the expression of pOCAT (ANG N-1498/+18) in OK cells. Cells were preincubated for 24 hours in 1% depleted fetal bovine serum (dFBS) after DNA transfection. Hormones were then added to fresh medium containing 1% dFBS and cells were incubated for an additional 24 hours. The CAT activity of pOCAT (ANG N-1498/+18) in the absence of hormones is shown as 100%. Each point represents the mean$\pm$SD of three independent transfections in triplicate plates (n=3). The probability values are derived from Student's *t* test (*P<0.05).

Reproduced from Chan J.S.D., Ming M., Nie Z.R., sikstrom R., Lachance S. and Carriere S.: (1992) Hormonal regulation of expression of the angiotensinogen gene in cultured opossum kidney (OK) proximal tubular cells. J. Amer Soc. Nephrol. 2: 1516-1522.

fusion gene in OK cells. The addition of dexamethasone (10^{-6} M) stimulated the expression of pOCAT (ANG N-1498/+18) to 194±10% ($P<0.05$) compared with the control (without the addition of hormones). Other steroid hormones (estradiol, testosterone, and progesterone) had no effect on the expression of pOCAT (ANG N-1498/+18) even at a concentration as high as 10^{-6} M. Thyroid hormone, L-T_3 (10^{-7} M), increased the expression of pOCAT (ANG N-1498/+18) by 90% ($P<0.05$).

The effect of dexamethasone on the expression of the other ANG-CAT fusion genes in OK cells is shown in Figure 3-26. Dexamethasone (10^{-6} M) increased the expression of pOCAT (ANG N-688/+18) 50% ($P<0.05$). The ANG-CAT fusion genes, pOCAT (ANG N-110/+18), pOCAT (ANG N-53/+18), and pOCAT (ANG N-35/+18), were not responsive to the addition of dexamethasone.

In summary, in the opossum kidney cell line, OK: (a) DEX stimulated the expression of pOCAT (ANG N-1498/+18) in a concentration-dependent manner; (b) Thyroid hormone, L-T_3, (10^{-7}-10^{-6} M), stimulated the expression of pOCAT (ANG N-1498/+18); and (c) Other steroid hormones (estradiol, testosterone, and progesterone) had no effect on the expression of pOCAT (ANG N-1498/+18).

III.2.3. Effect of 8-Bromo-cAMP on the Expression of ANG-CAT Fusion Genes in OK Cells

III.2.3.1. Effect of 8-Bromo-cAMP or Forskolin on the Expression of pOCAT (ANG N-1498/+18) in OK Cells in the Presence or Absence of DEX

Figure 3-27 shows that the addition of 8-Bromo-cAMP (10^{-7}-10^{-3} M) stimulated the expression of pOCAT (ANG N-1498/+18) in a concentration-dependent manner. 8-Bromo-cAMP (10^{-3} M) alone stimulated the expression of pOCAT (ANG N-1498/+18) compared with the control (152±9% versus 100±7%, $P<0.01$). Dexamethasone alone (10^{-6} M) stimulated the

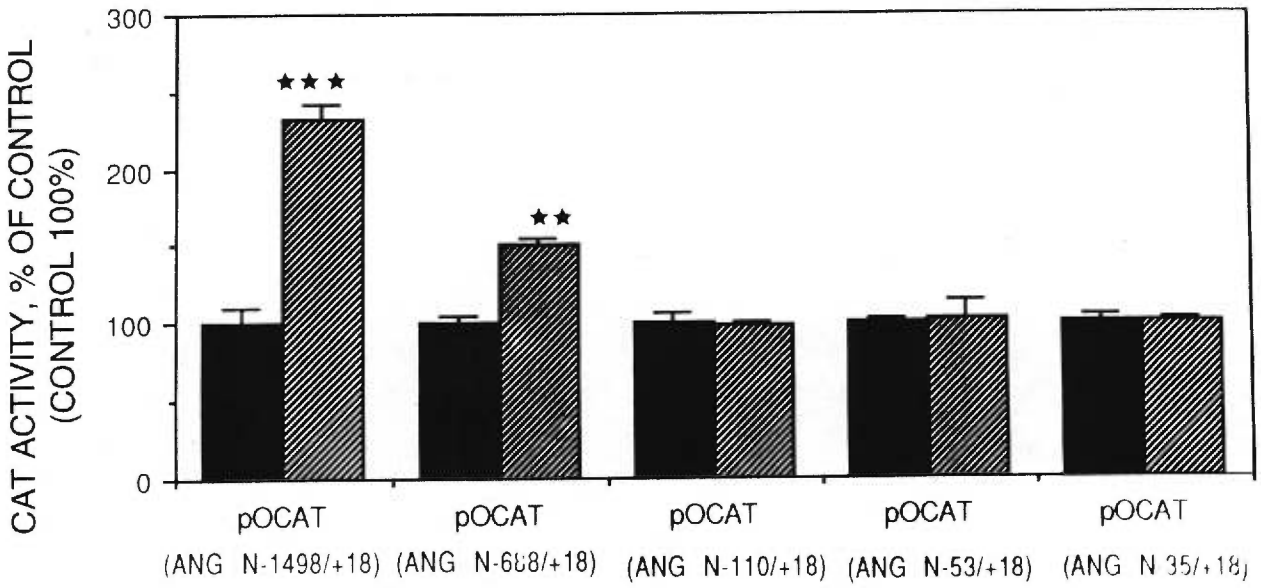


Figure 3-26. Effect of dexamethasone on the expression of various ANG-CAT fusion genes in OK cells. Cells were incubated for 24 hours with or without the presence of dexamethasone (10^{-6} M). Results are expressed as a percentage of the CAT activity of the control (in the absence of dexamethasone; its CAT activity is shown as 100%). Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from Student's *t* test (**P<0.01, ***P<0.005). The solid bar on the left is from cells without hormone added, and the hatched bar on the right is from dexamethasone treated cells.

Reproduced from Chan J.S.D., Ming M., Nie Z.R., sikstrom R., Lachance S. and Carriere S.: (1992) Hormonal regulation of expression of the angiotensinogen gene in cultured opossum kidney (OK) proximal tubular cells. J. Amer Soc. Nephrol. 2: 1516-1522.

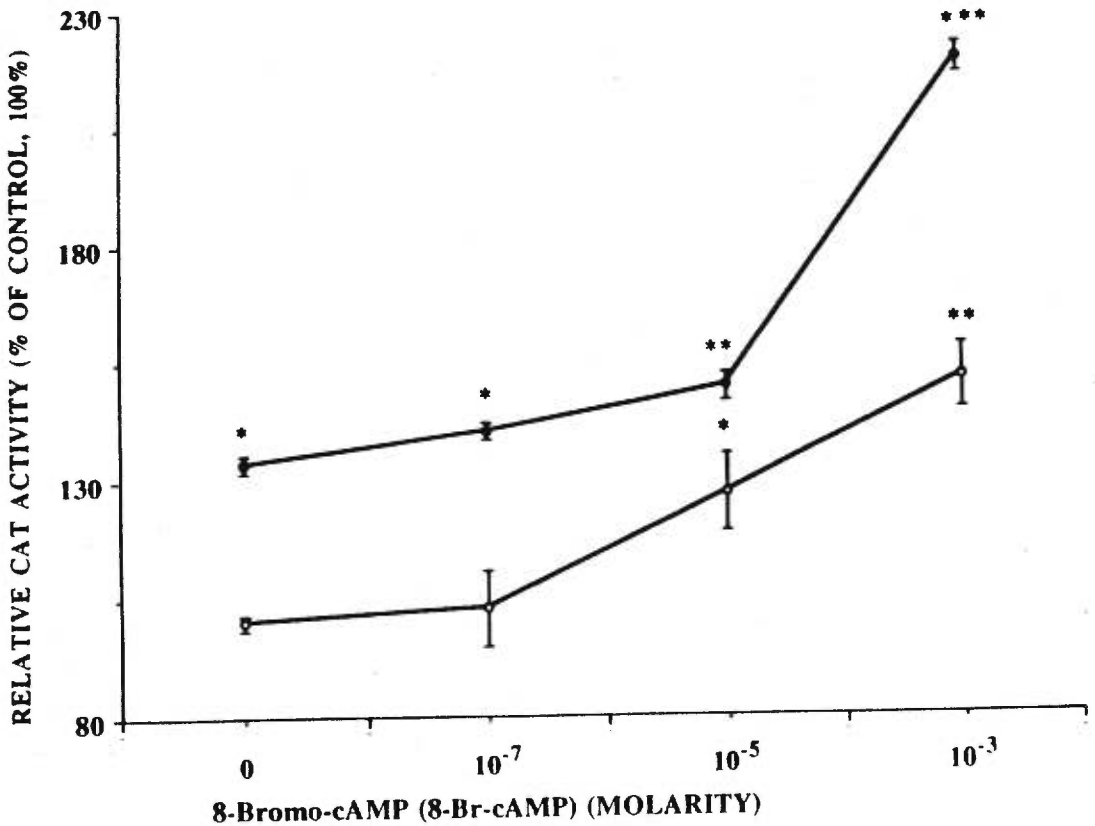


Figure 3-27. Effect of 8-Bromo-cAMP on the expression of pOCAT (ANG N-1498/+18) in OK cells in the presence or absence of DEX (10^{-6} M). Cells were incubated for 24 hours in the presence of various concentrations of 8-Bromo-cAMP (10^{-7} to 10^{-3} M) with or without DEX (10^{-6} M). The levels of transcriptional activity were quantified by chloramphenicol acetyltransferase activity. The effect of 8-Bromo-cAMP is compared to the control cells (in the absence of both dexamethasone and 8-Bromo-cAMP; its CAT activity is shown as 100%). The effect of the combination of 8-Bromo-cAMP and dexamethasone is compared to that of the cells in the presence of 8-Bromo-cAMP alone. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (**P<0.01, ***P<0.005;) ○, without DEX; ●, with DEX (10^{-6} M).

Reproduced from Ming M., Wang T.T., Lachance S., Delalandre A., Carriere S. and Chan J.S.D.: (1995) Expression of the angiotensinogen gene is synergistically stimulated by 8-Br cAMP and Dex in opossum kidney cells. Am. J. Physiol. 268: R105-R111.

expression of pOCAT (ANG N-1498/+18) to 137±2%. Moreover, 8-Bromo-cAMP (10^{-3} M) and dexamethasone (10^{-6} M) acted additively to stimulate the expression of this fusion gene (120±4% increase (both) compared to 52±9% increase (8-Bromo-cAMP alone) and 37±2% increase (DEX alone)).

The studies demonstrated that 8-Bromo-cAMP alone stimulated the expression of the ANG-CAT fusion gene pOCAT (ANG N-1498/+18) in OK cells in the absence of dexamethasone, whereas the addition of 8-Bromo-cAMP alone did not significantly stimulate the expression of the same ANG-CAT fusion gene in Hepa 1-6 cells (Figure 3-9, 3-10). To further confirm these observations, the effect of forskolin on the expression of pOCAT(ANG N-1498/+18) was also studied.

Figure 3-28 shows that forskolin (10^{-5} M) or dexamethasone (10^{-6} M) alone increased the expression of pOCAT (ANG N-1498/+18) in OK cells by 125±14% ($P<0.01$) and 65±7% ($P<0.05$), respectively, compared with the control. Forskolin plus dexamethasone acted synergistically to stimulate its expression (347±42% increase (both) compared with 125±14% increase (forskolin alone) ($P<0.01$) and 65±7% increase (DEX alone) ($P<0.01$), respectively).

These results suggest that 8-Bromo-cAMP or forskolin alone could stimulate the expression of pOCAT (ANG N-1498/+18), in the absence of dexamethasone in OK cells. This is in contrast to Hepa 1-6 cells, where neither 8-Bromo-cAMP nor forskolin alone could significantly stimulate the expression of pOCAT (ANG N-1498/+18), althrough they were able to enhance the stimulatory effect of DEX. These data suggest that the expression of the ANG gene in these two cell lines is probably under somewhat different controls.

III.2.3.2. Effect of the cAMP-Dependent Protein Kinase AI and II Inhibitor, (Rp)-cAMP, on the Expression of pOCAT (ANG N-1498/+18) in OK Cells

Figure 3-29 shows that the addition of (Rp)-cAMP (10^{-10}-10^{-4} M) blocked the stimulatory

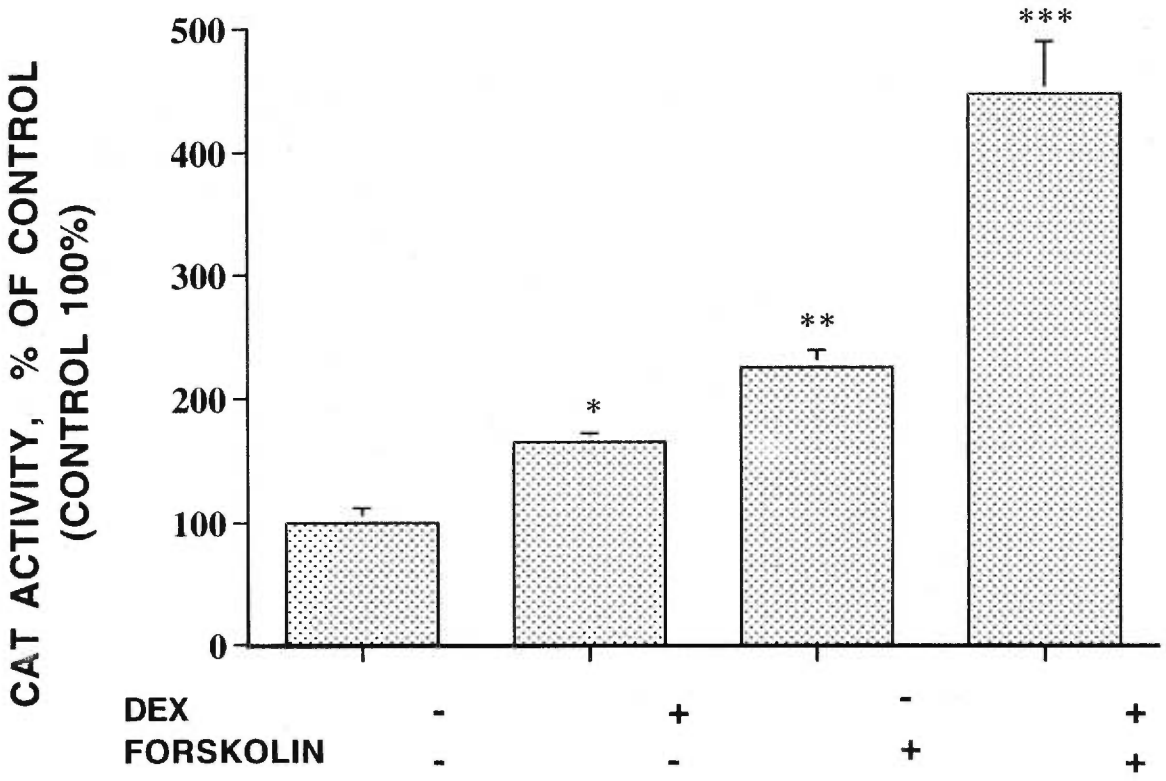


Figure 3-28. Effect of forskolin (10^{-5} M), DEX (10^{-6} M), or both on the expression of pOCAT (ANG N-1498/+18) in OK cells. Cells were incubated for 24 hours in the presence of forskolin, DEX, or both. The CAT activity of the control (in the absence of both forskolin and DEX) is shown as 100%. Each point represents the mean$\pm$SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.005$).

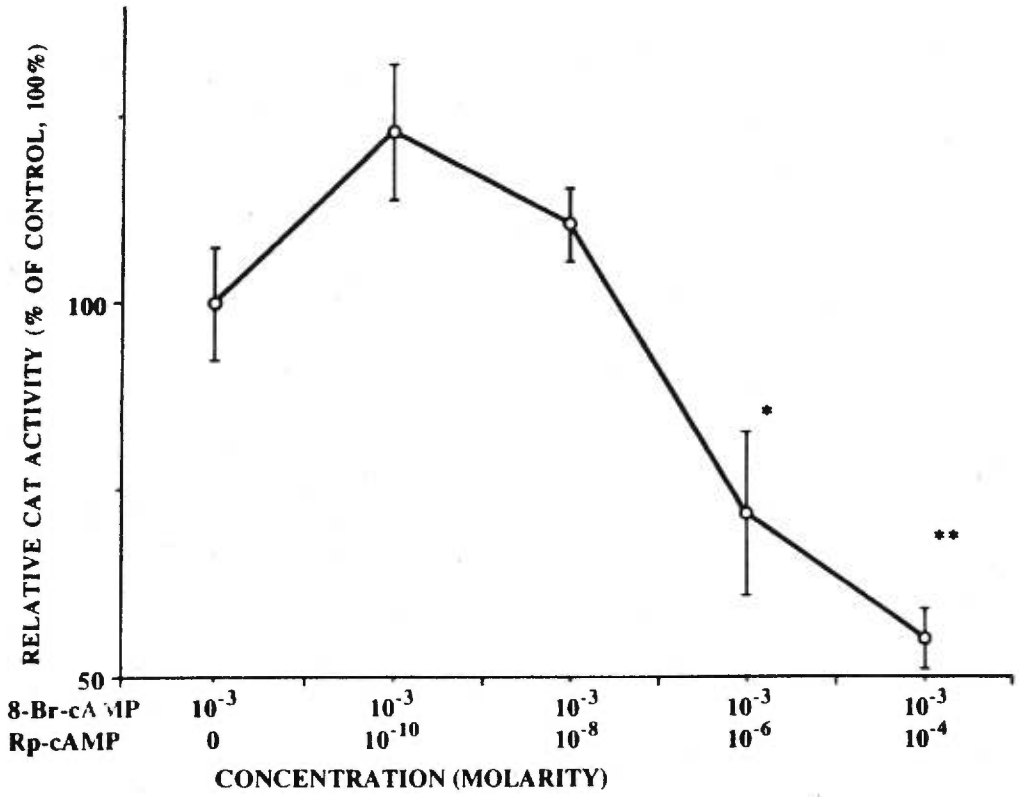


Figure 3-29. Inhibitory effect of Rp-cAMP on the expression of pOCAT (ANG N-1498/+18) in OK cells stimulated by 8-Bromo-cAMP. Cells were incubated for 24 hours in the presence of 8-Bromo-cAMP (10^{-3} M), with or without various concentrations of Rp-cAMP (10^{-10} to 10^{-4} M). The CAT activity of the control cells (in the absence of Rp-cAMP) is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (*P<0.05, **P<0.01).

Reproduced from Ming M., Wang T.T., Lachance S., Delalandre A., Carriere S. and Chan J.S.D.: (1995) Expression of the angiotensinogen gene is synergistically stimulated by 8-Br cAMP and Dex in opossum kidney cells. Am. J. Physiol. 268: R105-R111.

effect of 8-Bromo-cAMP (10^{-3} M) on the expression of pOCAT (ANG N-1498/+18 in OK cells in a concentration-dependent manner. At 10^{-6} and 10^{-4} M, (Rp)-cAMP significantly inhibited the stimulatory effect of 8-Bromo-cAMP (10^{-3} M); the expression levels were decreased to 72% ($P<0.05$) and 55% ($P<0.01$), respectively.

III.2.3.3. Identification of Putative CRE in Rat Angiotensinogen Gene

Figure 3-30 shows the effect of 8-Bromo-cAMP (10^{-3} M), DEX (10^{-6} M) or both on the expression of various ANG-CAT fusion genes in OK cells. The addition of 8-Bromo-cAMP or DEX alone significantly stimulated the expression of pOCAT (ANG N-1498/+18), pOCAT (ANG N-1138/+18), pOCAT (ANG N-960/+18), pOCAT (ANG N-814/+18), and pOCAT (ANG N-688/+18), as compared to the control (without the addition of 8-Bromo-cAMP or DEX) ($P<0.05$). 8-Bromo-cAMP plus DEX acted additively or synergistically to stimulate the expression of these fusion genes. For instance, there was a $102\pm12\%$ increase (both) compared with $66\pm14\%$ increase (DEX alone) and $30\pm3\%$ increase (cAMP alone) for the expression of pOCAT (ANG N-1498/+18). Similar results were found on the expression of pOCAT (ANG N-1138/+18), pOCAT (ANG N-960/+18), pOCAT (ANG N-814/+18), and pOCAT (ANG N-688/+18). However, other ANG-CAT fusion genes, such as pOCAT (ANG N-280/+18), pOCAT (ANG N-196/+18), pOCAT (ANG N-110/+18), pOCAT (ANG N-53/+18), and pOCAT (ANG N-35/+18), did not significantly respond to 8-Bromo-cAMP, DEX, or both.

Figure 3-31 shows the effect of 8-Bromo-cAMP or forskolin on the expression of various ANG-CAT fusion genes with or without the putative CRE fused to the TK promoter in OK cells. The addition of 8-Bromo-cAMP (10^{-3} M) stimulated the expression of pTKCAT (ANG N-814/-689), pTKCAT (ANG N-814/-761), and pTKCAT (ANG N-760/-689) to $139\pm11\%$ ($P<0.05$), $295\pm36\%$ ($P<0.01$) and $124\pm8\%$ (NS) as compared to the control (in the absence of 8-Bromo-cAMP or forskolin). Similarly, the addition of forskolin (10^{-5} M) stimulated the expression of pTKCAT (ANG N-814/-689), pTKCAT (ANG N-814/-761), and pTKCAT (ANG N-760/-689)

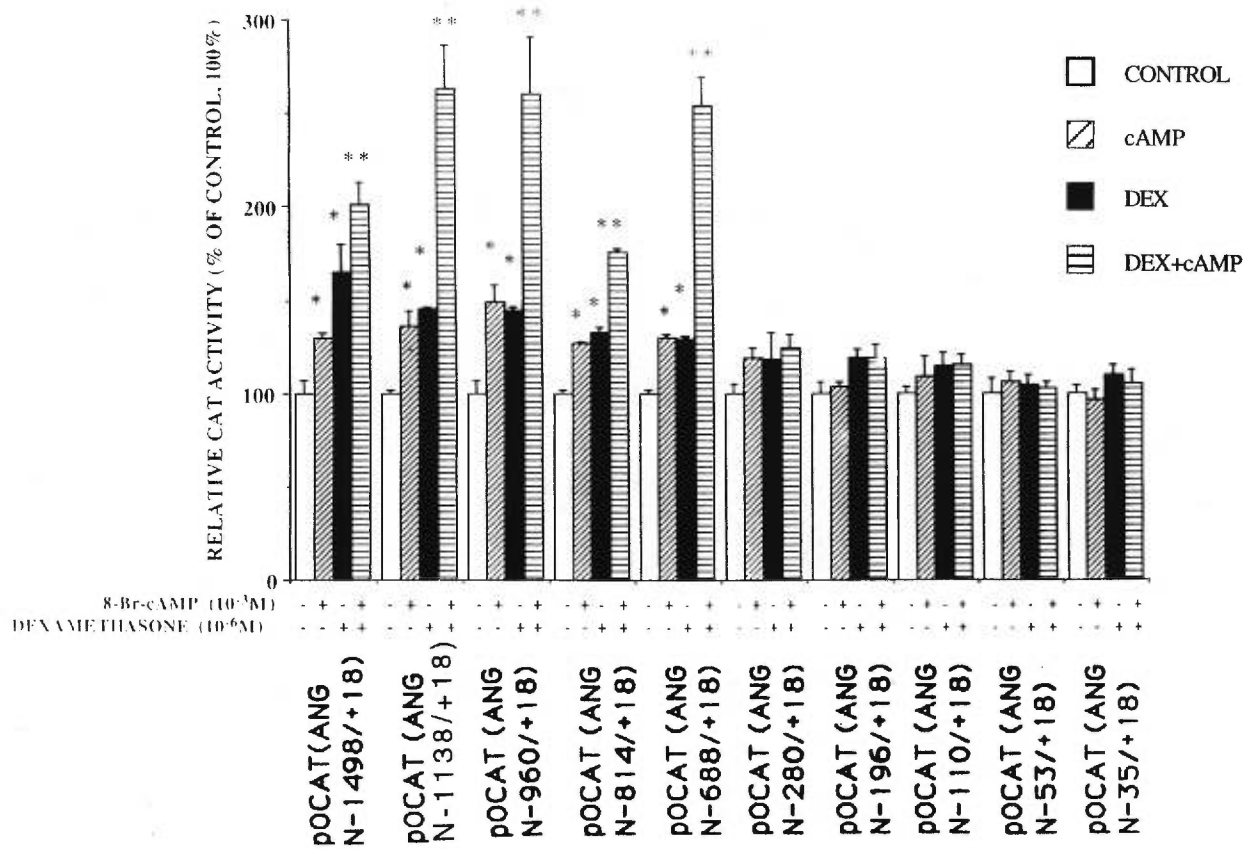


Figure 3-30. Effect of 8-Bromo-cAMP (10^{-3} M) or DEX (10^{-6} M) or both on the expression of various ANG-CAT fusion genes in OK cells. Cells were incubated for 24 hours in the presence of 8-Bromo-cAMP (10^{-3} M), DEX (10^{-6} M) or both. Results are expressed as a percentage of the CAT activity of the control (in the absence of both 8-Bromo-cAMP and DEX; its CAT activity is shown as 100%). Each point represents the mean$\pm$SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (*P<0.05, **P<0.01). Open bar, without the addition of hormones; hatched bar, 8-Bromo-cAMP treated cells; solid bar, DEX treated cells; striped bar, 8-Bromo-cAMP and DEX treated cells.

Reproduced from Ming M., Wang T.T., Lachance S., Delalandre A., Carriere S. and Chan J.S.D.: (1995) Expression of the angiotensinogen gene is synergistically stimulated by 8-Br cAMP and Dex in opossum kidney cells. Am. J. Physiol. 268: R105-R111.

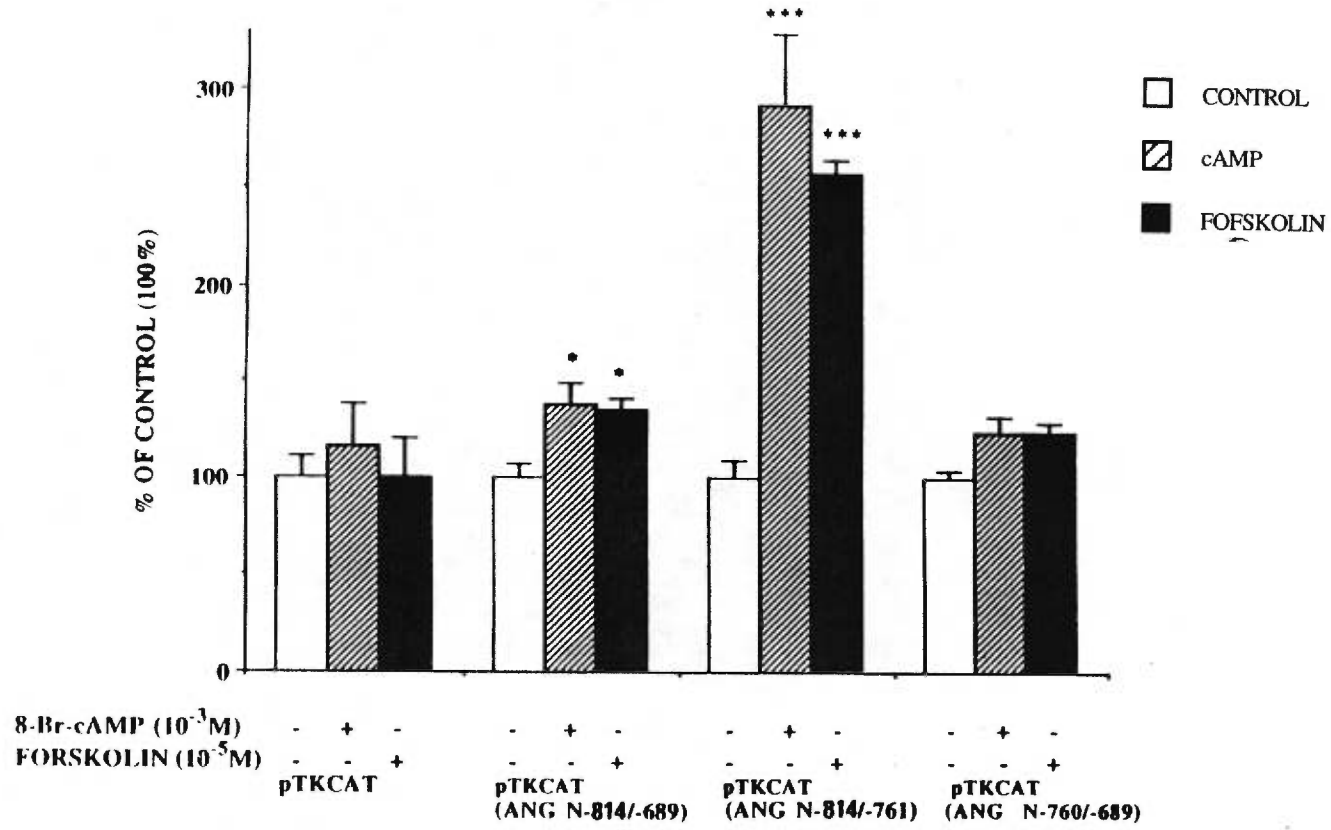


Figure 3-31. Effect of 8-Bromo-cAMP (10^{-3} M) and forskolin (10^{-5} M) on the expression of various ANG-CAT fusion genes with or without the putative cAMP response element fused to the thymidine kinase (TK) promoter in OK cells. Cells were incubated for 24 h in the presence of 8-Bromo-cAMP (10^{-3} M) or forskolin (10^{-5} M). Results are expressed as a percentage of the CAT activity of the control (in the absence of both 8-Bromo-cAMP and forskolin; its CAT activity is shown as 100%). Each point represents the mean±SD of five independent transfections in triplicate plates (n=5). The probability values are derived from Student's *t* test (*P<0.05, ***P<0.005). Open bar, control cells (without the addition of 8-Bromo-cAMP or forskolin); hatched bar, 8-Bromo-cAMP treated cells; solid bar, forskolin treated cells.

Reproduced from Ming M., Wang T.T., Lachance S., Delalandre A., Carriere S. and Chan J.S.D.: (1995) Expression of the angiotensinogen gene is synergistically stimulated by 8-Br cAMP and Dex in opossum kidney cells. Am. J. Physiol. 268: R105-R111.

to 136±5% (P<0.05), 259±7% (P<0.01) and 123±5% (NS) of the control value. pTKCAT was not responsive to the addition of either 8-Bromo-cAMP or forskolin.

Figure 3-32 shows the effect of 8-Bromo-cAMP and forskolin on the expression of pTKCAT (ANG N-806/-779), the minimum gene construct that contains the putative CRE, in OK cells. Both cAMP (10^{-3} M) and forskolin (10^{-5} M) stimulated the expression of pTKCAT (ANG N-806/-779) as compared to the control (206±2% versus 100±21% (P<0.01) and 192±9% versus 100±21 (P<0.01), respectively).

These studies (Figure 3-31, 3-32) demonstrated that the putative CRE of the ANG gene was located within the region of ANG N-806/-779.

In summary, in the opossum kidney (OK) cell line: (a) Either 8-Bromo-cAMP or forskolin alone stimulated the expression of pOCAT (ANG N-1498/+18). 8-Bromo-cAMP plus DEX acted additively to stimulate the expression of this fusion gene. However, forskolin plus DEX acted synergistically to stimulate the expression of pOCAT (ANG N-1498/+18); and (b) 8-Bromo-cAMP and forskolin also stimulated the expression of pTKCAT (ANG N-814/-761) and pTKCAT (ANG N-806/-779), which contain the sequence of a putative CRE (TGACGTAC; ANG N-795/-788). By contrast, the fragment ANG N-760/-689, which was immediately downstream of the fragment ANG N-814/-761, did not respond to either 8-Bromo-cAMP or forskolin.

The stimulatory effects of 8-Bromo-cAMP or forskolin on the expression of ANG-TK- CAT fusion genes with or without the putative CRE in Hepa 1-6 and OK cells are shown in Table 3-1.

III.2.4. Effect of Isoproterenol on the Expression of ANG-CAT Fusion Genes in OK Cells

III.2.4.1. Effect of Isoproterenol and Dexamethasone on the Expression of ANG-CAT Fusion Genes in OK Cells

Figure 3-33 shows the effect of isoproterenol on the expression of pOCAT (ANG N

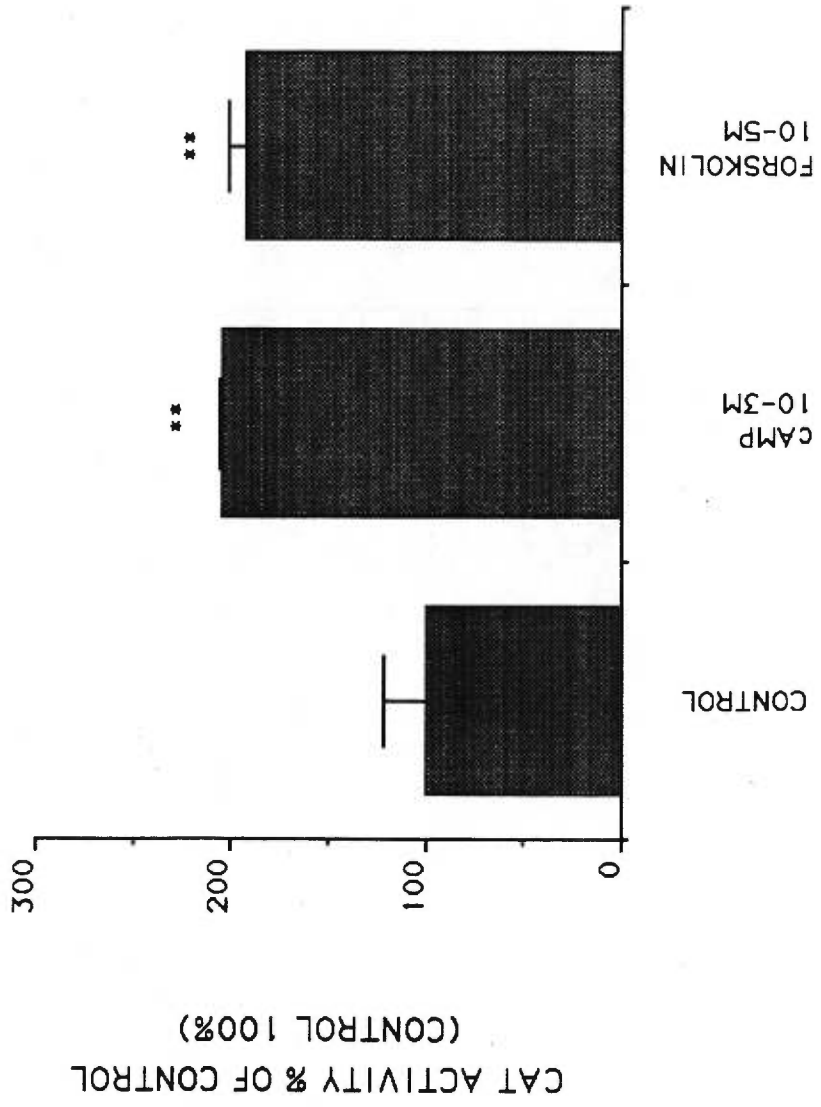


Figure 3-32. Effect of 8-Bromo-cAMP (10^{-3} M) and forskolin (10^{-5} M) on the expression of a fusion gene containing the putative cAMP response element fused with the thymidine kinase (TK) promoter, pTKCAT (ANG N-806/-779), in OK cells. Cells were incubated for 24 hours in the presence of 8-Bromo-cAMP or forskolin. Results are expressed as a percentage of the CAT activity of the control (in the absence of 8-Bromo-cAMP or forskolin; its CAT activity is shown as 100%). Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from Student's *t* test (**P<0.01).

Table 3-1. Effects of 8-Bromo-cAMP or Forskolin on the Putative CRE Mediated Expression of ANG-TK-CAT Fusion Genes in Hepa 1-6 and OK Cells

	cAMP				Forskolin			
	Hepa 1-6		OK		Hepa 1-6		OK	
	mean±SD	P	mean±SD	P	mean±SD	P	mean±SD	P
	%		%		%		%	
N-814/-689	130±12	N.S.	139±11	<0.05	141±12	ND	136±5	N.S.
N-814/-761	2.14±39	<0.01	295±36	<0.01	288±26	<0.01	259±7	<0.01
N-806/-779	220±12	<0.01	210±14	<0.01	N.D.		190±14	<0.01
N-760/-689	105±5	N.S.	124±8	N.S.	115±3	N.S.	125±5	N.S.

N.S. = no significant difference. N.D. = no determination.

The effect of 8-Bromo-cAMP or forskolin is compared to the control (in the absence of 8-Bromo-cAMP or forskolin), and the CAT activity of the control is 100%.

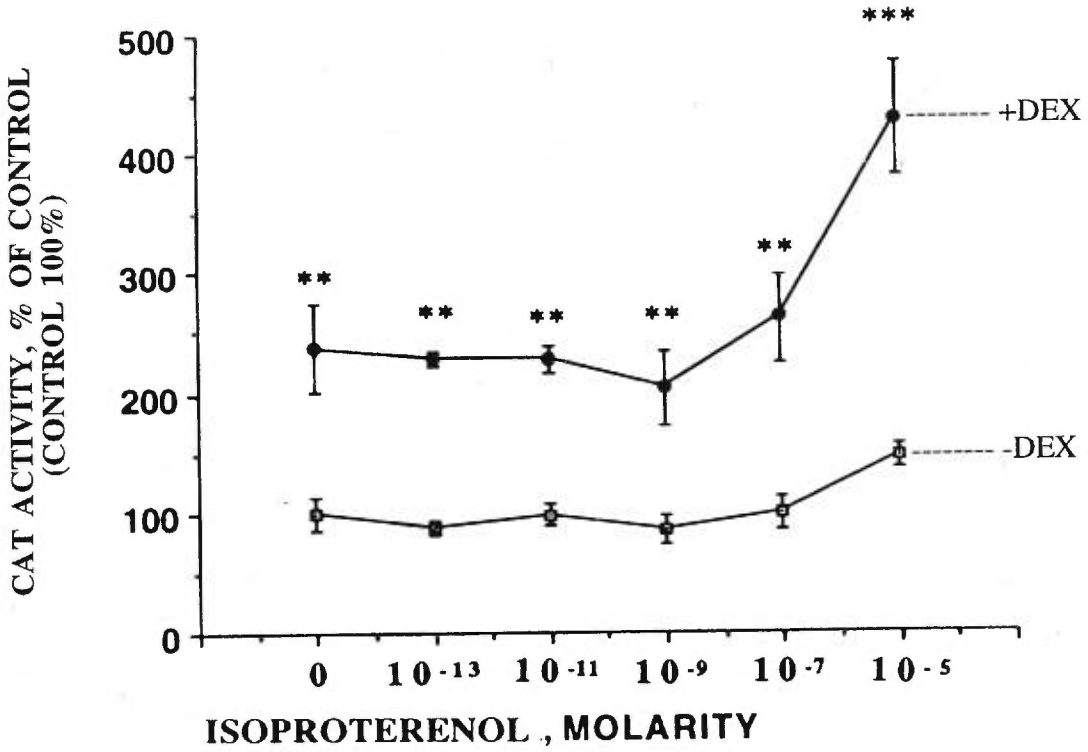


Figure 3-33. Relationship between isoproterenol concentrations and the expression of pOCAT (ANG N-1498/+18) in OK cells in the presence or absence of dexamethasone (10^{-6} M). The effect of isoproterenol is compared to that of the control cells (in the absence of both dexamethasone and isoproterenol; its CAT activity is shown as 100%). The effect of both isoproterenol and dexamethasone is compared to that of isoproterenol alone. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (**P<0.01, ***P<0.005). ❐, absence of dexamethasone; ●, presence of dexamethasone (10^{-6} M).

Reproduced from Ming M., Chan W., Wang T.T., Roberts K.D., Bouvier M., Lachance S., Carriere S. and Chan J.S.D.: (1996) β-adrenoceptors and dexamethasone synergistically stimulate the expression of the angiotensinogen gene in opossum kidney cells. Kid ney Intern. 50:94-101.

-1498/+18). Isoproterenol (10^{-11} to 10^{-5} M) did not significantly stimulate the expression of pOCAT (ANG N-1498/+18) as compared to the control (without isoproterenol), even at the high concentration, 10^{-5} M (141±9% versus 100±13%, NS). Dexamethasone (10^{-6} M) alone stimulated the expression of pOCAT (ANG N-1498/+18). Isoproterenol (10^{-5} M) plus DEX acted synergistically on the expression of pOCAT (ANG N-1498/+18) compared with DEX alone (427±47% versus 238±36%, $P<0.05$).

We have demonstrated that either 8-Bromo-cAMP or forskolin alone stimulated the expression of pOCAT (ANG N-1498/+18) in the absence of dexamethasone in OK cells (Figure 3-27, 3-30, 3-31). The present results showed that isoproterenol alone did not stimulate the expression of pOCAT (ANG N-1498/+18) in these cells. Furthermore, neither pTKCAT (ANG N-814/-761) nor pTKCAT (ANG N-806/-779), which contain the putative CRE of the rat ANG gene, responded to isoproterenol in OK cells (data not shown). These results raised the possibility that OK cells might have lost some β-adrenoceptors, and the amount of remaining receptors was too low to mediate the effect of isoproterenol. In order to explore this possibility, β_1-adrenoceptor cDNA (pBC-β_1AR) or β_2-adrenoceptor cDNA (pBC-β_2AR) and ANG-CAT fusion genes were cotransfected into OK cells. In this way the effect of isoproterenol on the expression of ANG-CAT fusion genes in OK cells could be ascertained.

III.2.4.2. Effect of Co-transfection of pBC-β_1AR or pBC-β_2AR on the Expression of pOCAT (ANG N-1498/+18) in OK Cells

Figure 3-34 shows that co-transfection of pBC-β_1AR or pBC-β_2AR (1 to 5 μg of DNA) with pOCAT (ANG N-1498/+18) stimulated the expression of pOCAT (ANG N-1498/+18) in OK cells. A 60% to 80% increase was found with 2 μg of pBC-β_1AR ($P<0.01$). The highest concentration of pBC-β_1AR (that is, 5 μg) did not significantly stimulate the expression of pOCAT (ANG N-1498/+18). Similarly, 2 μg of pBC-β_2AR increased the expression of pOCAT (ANG N-1498/+18) 60% to 80%. At 5 μg of pBC-β_2AR, the stimulatory effect was diminished but it was

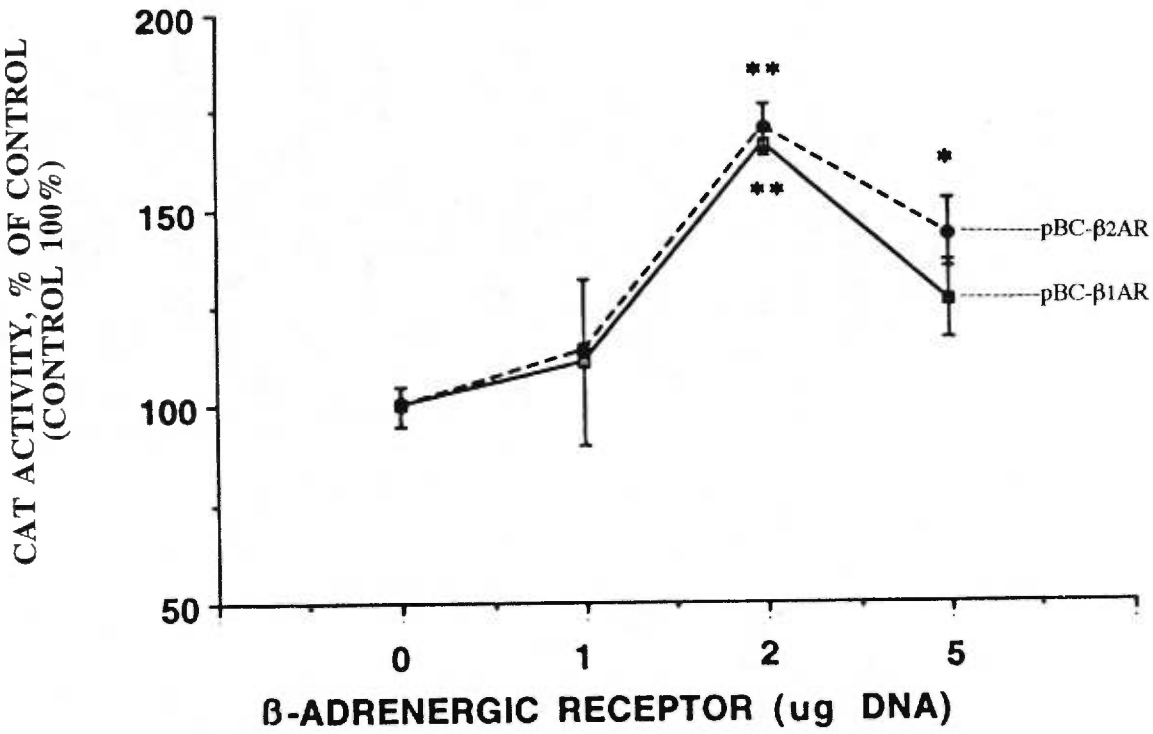


Figure 3-34. Expression of pOCAT (ANG N-1498/+18) co-transfected with different concentrations of plasmid containing the cDNA for the β1-adrenoceptor (pBC-β1AR) or β2-adrenoceptor (pBC-β2AR) in OK cells. Ten μg of pOCAT (ANG N-1498/+18), 2 μg of pTKGH and different concentrations (1 to 5 μg) of pBC-β1AR or pBC-β2AR per well ($5x10^5$) cells were used in the transfection. The levels of transcriptional activity were quantified by CAT activity. The efficiency of transfection was normalized by the amount of IR-hGH in the medium. The CAT activity of the control cells (without co-transfection of pBC-β1AR or pBC-β2AR) is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (*P<0.05, **P<0.01). ❐, pBC-β1AR; ●, pBC-β2AR.

Reproduced from Ming M., Chan W., Wang T.T., Roberts K.D., Bouvier M., Lachance S., Carriere S. and Chan J.S.D.: (1996) β-adrenoceptors and dexamethasone synergistically stimulate the expression of the angiotensinogen gene in opossum kidney cells. Kid ney Intern. 50:94-101.

still significantly higher than the control (143±9% versus 100±4 (P<0.05)). These studies indicated that the basal expression of pOCAT (ANG N-1498/+18) was stimulated by cotransfection of either β_1- or β_2-adrenoceptors in OK cells.

Figure 3-35 shows that the addition of a high concentration of isoproterenol (10^{-5} M) stimulated the expression of pOCAT (ANG N-1498/+18) in OK cells co-transfected with 2 μg of pBC-β_1AR compared with cells only transfected with pOCAT (ANG N-1498/+18) (186±16% versus 125±7, P<0.05), whereas lower concentrations (10^{-13} M to 10^{-7} M) had no significantly stimulatory effect on its expression. In cells transfected with pOCAT (ANG N-1498/+18) alone or co-transfected with either pBC-β_2AR (2μg) or pBC-BC12 (2μg), the plasmid vector used to carry the β-adrenoceptor cDNA in these experiments, the addition of isoproterenol (10^{-13} M to 10^{-5} M) had no significantly stimulatory effect. These results demonstrated that the vector carrying the β-adrenoceptor cDNA (pBC-BC12) did not mediate the isoproterenol effect on the expression of pOCAT (ANG N-1498/+18) in OK cells. Hence, this vector was appropriate for the study of β-adrenoceptor effects on ANG gene expression.

III.2.4.3. Effect of Isoproterenol and Dexamethasone on the Expression of pOCAT (ANG N-1498/+18) Co-transfected with pBC-β_1AR or pBC-β_2AR in OK Cells

Figure 3-36 shows the synergistic effect of isoproterenol (10^{-5} M) plus dexamethasone (10^{-6} M) on the expression of pOCAT (ANG N-1498/+18) co-transfected with different concentrations of pBC-β_1AR. It appears that the maximal effect of dexamethasone or isoproterenol was found at 2 μg of pBC-β_1AR. Isoproterenol plus DEX acted synergistically to stimulate the expression of this fusion gene (694±20% increase (both) compared with 140±2% increase (isoproterenol alone) and 218±8% increase (DEX alone)). On the other hand, the stimulatory effect was not significantly different between isoproterenol plus DEX and DEX alone on the expression of pOCAT (ANG N-1498/+18) when co-transfected with pBC-β_2AR (1 to 5 μg DNA) (Figure 3-37).

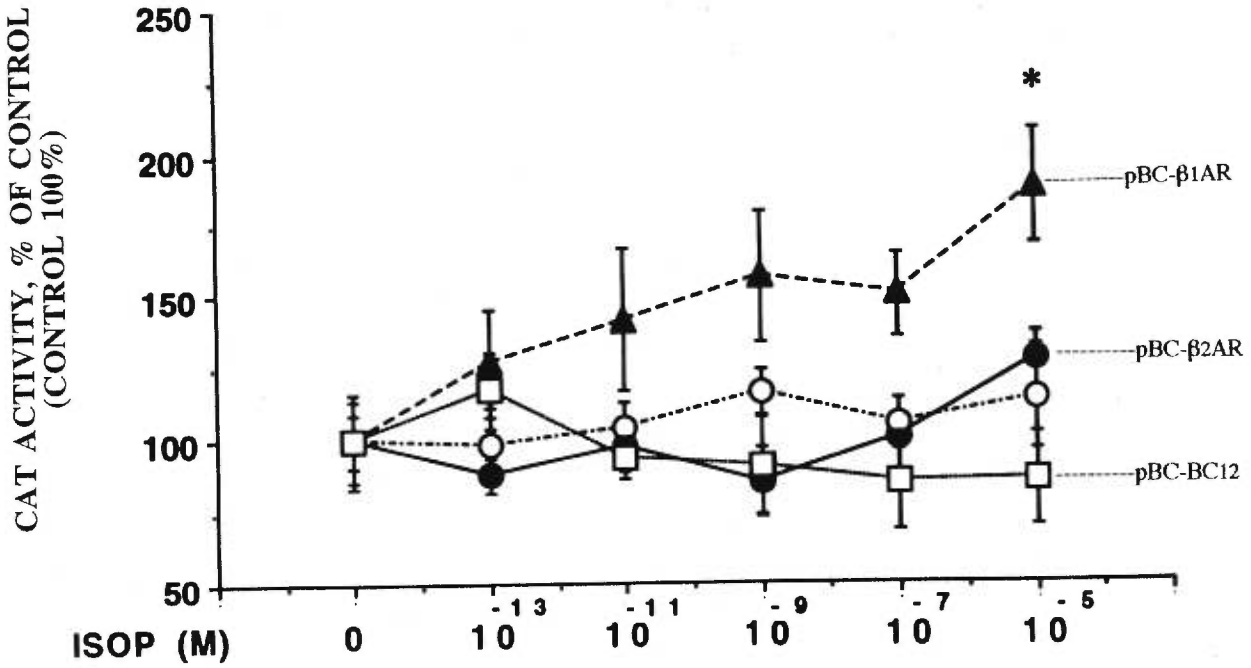


Figure 3-35. Relationship between the concentrations of isoproterenol and the expression of pOCAT (ANG N-1498/+18) co-transfected with pBC-β1AR or pBC-β2AR or the empty vector pBC-BC12 in OK cells. Ten μg of pOCAT (ANG N-1498/+18) and 2 μg of pBC-β1AR or pBC-β2AR or pBC-BC12 per well ($5x10^5$cells) were used in the transfection. The effect of isoproterenol is compared to that of the control cells (in the absence of isoproterenol; its CAT activity is shown as 100%). Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (*P<0.05). ○, without co-transfection with pBC-β1AR or pBC-β2AR or the empty vector pBC-BC12; □, pBC-BC12; ▲, pBC-β1AR; ●, pBC-β2AR.

Reproduced from Ming M., Chan W., Wang T.T., Roberts K.D., Bouvier M., Lachance S., Carriere S. and Chan J.S.D.: (1996) β-adrenoceptors and dexamethasone synergistically stimulate the expression of the angiotensinogen gene in opossum kidney cells. Kid ney Intern. 50:94-101.

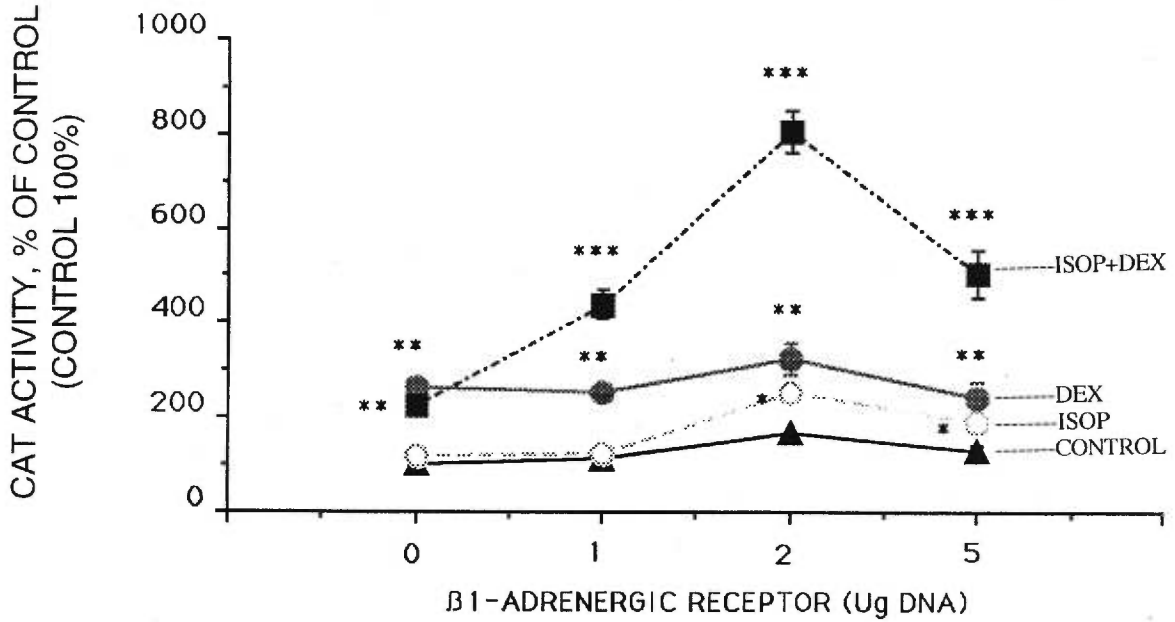


Figure 3-36. Effect of isoproterenol (10^{-5} M) or dexamethasone (10^{-6} M) or both on the expression of pOCAT (ANG N-1498/+18) co-transfected with different concentrations of pBC-β1AR in OK cells. The effect of isoproterenol or dexamethasone alone is compared to that of the control cells (in the absence of both isoproterenol and dexamethasone; its CAT activity is shown as 100%). The effect of the combination of isoproterenol and dexamethasone is compared to that of the cells in the presence of isoproterenol or dexamethasone alone. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (*P<0.05, **P<0.01, ***P<0.005). ▲, control; ○, isoproterenol; ●, dexamethasone; ■, isoproterenol and dexamethasone.

Reproduced from Ming M., Chan W., Wang T.T., Roberts K.D., Bouvier M., Lachance S., Carriere S. and Chan J.S.D.: (1996) β-adrenoceptors and dexamethasone synergistically stimulate the expression of the angiotensinogen gene in opossum kidney cells. Kid ney Intern. 50:94-101.

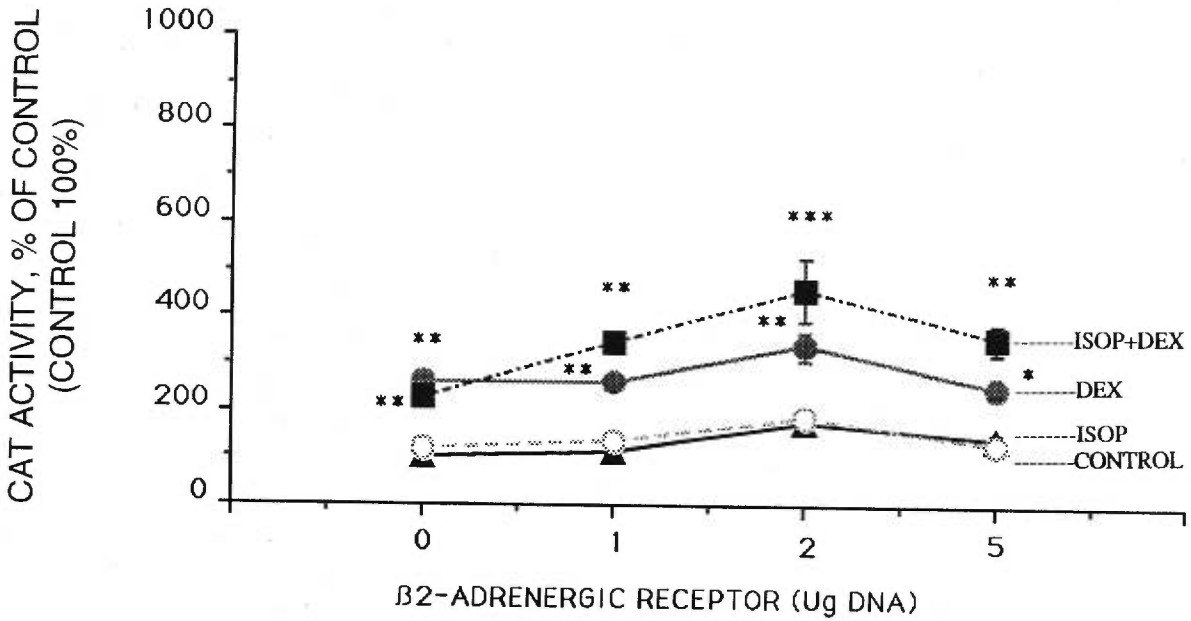


Figure 3-37. Effect of isoproterenol (10^{-5} M), dexamethasone (10^{-6} M) or both on the expression of pOCAT (ANG N-1498/+18) co-transfected with different concentrations of pBC-β2AR in OK cells. The effect of isoproterenol or dexamethasone alone is compared to that of the control cells (in the absence of both dexamethasone and isoproterenol; its CAT activity is shown as 100%). The effect of both isoproterenol and dexamethasone is compared to that of the cells in the presence of isoproterenol or dexamethasone alone. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA. (* $P< 0.05$; ** $P< 0.01$ and *** $P< 0.005$). ▲, control; ○, isoproterenol; ●, dexamethasone; ■, isoproterenol and dexamethasone.

Reproduced from Ming M., Chan W., Wang T.T., Roberts K.D., Bouvier M., Lachance S., Carriere S. and Chan J.S.D.: (1996) β-adrenoceptors and dexamethasone synergistically stimulate the expression of the angiotensinogen gene in opossum kidney cells. Kid ney Intern. 50:94-101.

III.2.4.4. Effects of Dexamethasone, RU486, Isoproterenol and Rp-cAMP on the Expression of pOCAT (ANG N-1498/+18) Co-transfected with pBC-β1AR in OK Cells

Figure 3-38 shows that DEX alone (10^{-12} to 10^{-4} M) stimulated the expression of pOCAT (ANG N-1498/+18) when co-transfected with 2 μg of pBC-β1AR in a concentration-dependent manner. DEX (10^{-6} M) stimulated the expression by 348±5% ($P<0.01$) and isoproterenol (10^{-5} M) stimulated the expression by 86±5% ($P<0.05$) compared with the control. DEX (10^{-6} M) and isoproterenol (10^{-5} M) thus acted synergistically to stimulate the expression of pOCAT (ANG N-1498/+18) by 548±22%.

Figure 3-39 shows the inhibitory effect of RU486 (a glucocorticoid antagonist) on the expression of pOCAT (ANG N-1498/+18) co-transfected with pBC-β1AR in OK cells stimulated by DEX. The addition of RU486 (10^{-11} to 10^{-5} M) blocked the stimulatory effect of DEX (10^{-6} M) in a concentration-dependent manner. RU486 (10^{-5} M) completely inhibited the stimulatory effect of DEX (123±13% versus 263±33% of control (100%), $P<0.01$).

Figure 3-40 shows that the addition of a high concentration of isoproterenol (10^{-5} M) alone stimulated the expression of pOCAT (ANG N-1498/+18) when co-transfected with 2 μg of pBC-β1AR as compared to the control (lacking isoproterenol or DEX) in OK cells (168±17% versus 100±6%, $P<0.05$). Lower concentrations (less than 10^{-5} M) had no significant effect on the expression of pOCAT (ANG N-1498/+18). DEX (10^{-6} M) plus isoproterenol (10^{-5} M) acted synergistically to stimulate the expression of this fusion gene (701±113% increase (both) compared with 68±17% increase (isoproterenol alone) and 200±39% increase (DEX alone)).

The addition of Rp-cAMP (an inhibitor of cAMP-dependent protein kinases AI and II) (10^{-10} to 10^{-4} M) blocked the stimulatory effect of isoproterenol (10^{-5} M) on the expression of pOCAT (ANG N-1498/+18) when co-transfected with pBC-β1AR in OK cells (Figure 3-41). Rp-cAMP (10^{-4} M) completely blocked the effect of isoproterenol (96±14% versus 193±11% of control

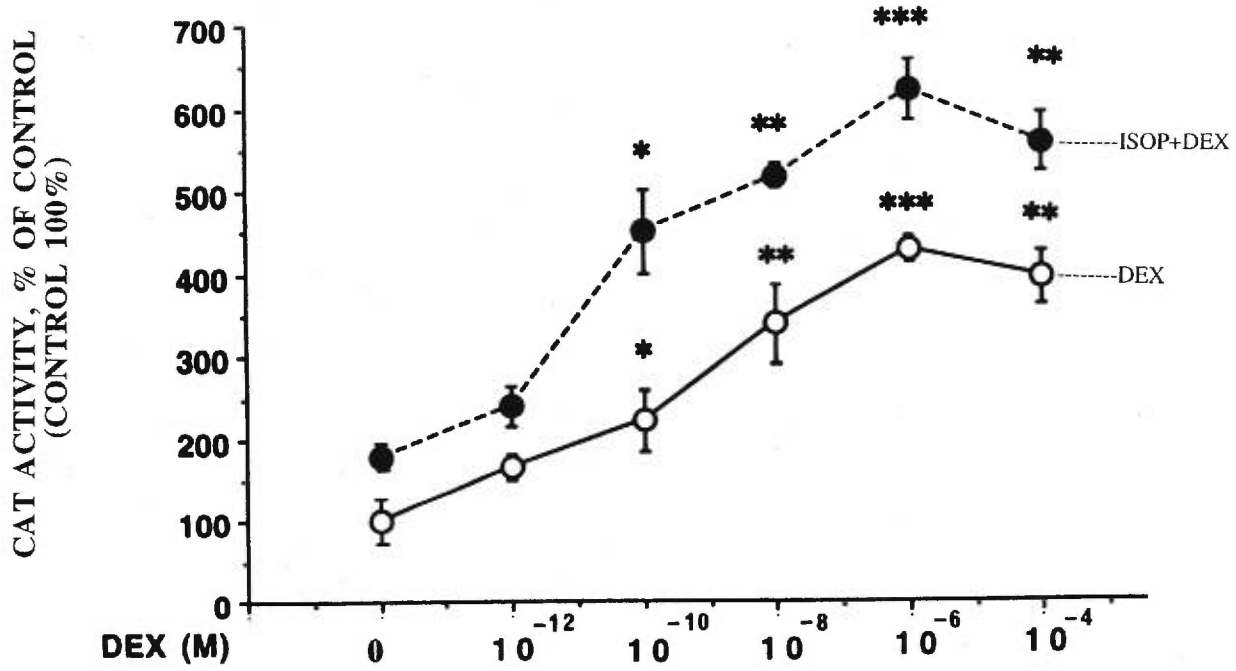


Figure 3-38. Relationship between the dexamethasone concentrations and the expression of pOCAT (ANG N-1498/+18) co-transfected with pBC-β1AR in OK cells in the presence or absence of isoproterenol (10^{-5} M). Ten μg of pOCAT (ANG N-1498/+18) and 2 μg of pBC-β1AR per well ($5x10^5$ cells) were used in the transfection. The effect of dexamethasone is compared to that of the control cells (in the absence of both dexamethasone and isoproterenol; its CAT activity is shown as 100%). The effect of both dexamethasone and isoproterenol is compared to that of the cells in the presence of DEX alone. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (* $P< 0.05$; ** $P< 0.01$ and *** $P< 0.005$). ○, various concentrations of dexmethasone without isoproterenol; ●, presence of isoproterenol.

Reproduced from Ming M., Chan W., Wang T.T., Roberts K.D., Bouvier M., Lachance S., Carriere S. and Chan J.S.D.: (1996) β-adrenoceptors and dexamethasone synergistically stimulate the expression of the angiotensinogen gene in opossum kidney cells. Kid ney Intern. 50:94-101.

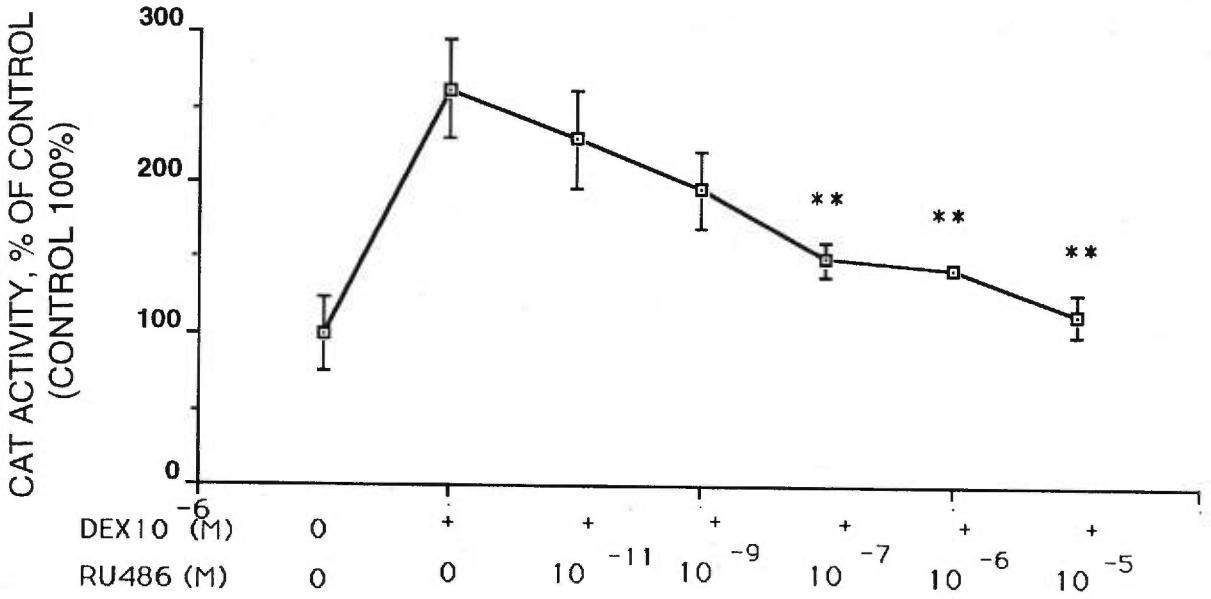


Figure 3-39. Inhibitory effect of RU486 on the expression of pOCAT (ANG N-1498/+18) co-transfected with pBC-β1AR in OK cells stimulated by dexamethasone. After DNA transfection cells were pre-incubated with various concentrations of RU486 for 16 hours and then further incubated for 24 hours in the presence of dexamethasone (10^{-6} M). The CAT activity of the control cells (in the absence of RU486 and dexamethasone) is shown as 100%. The inhibitory effect of RU486 was compared to the cells which were incubated with dexamethasone but in the absence of RU486. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (** $P < 0.01$).

Reproduced from Ming M., Chan W., Wang T.T., Roberts K.D., Bouvier M., Lachance S., Carriere S. and Chan J.S.D.: (1996) β-adrenoceptors and dexamethasone synergistically stimulate the expression of the angiotensinogen gene in opossum kidney cells. Kid ney Intern. 50:94-101.

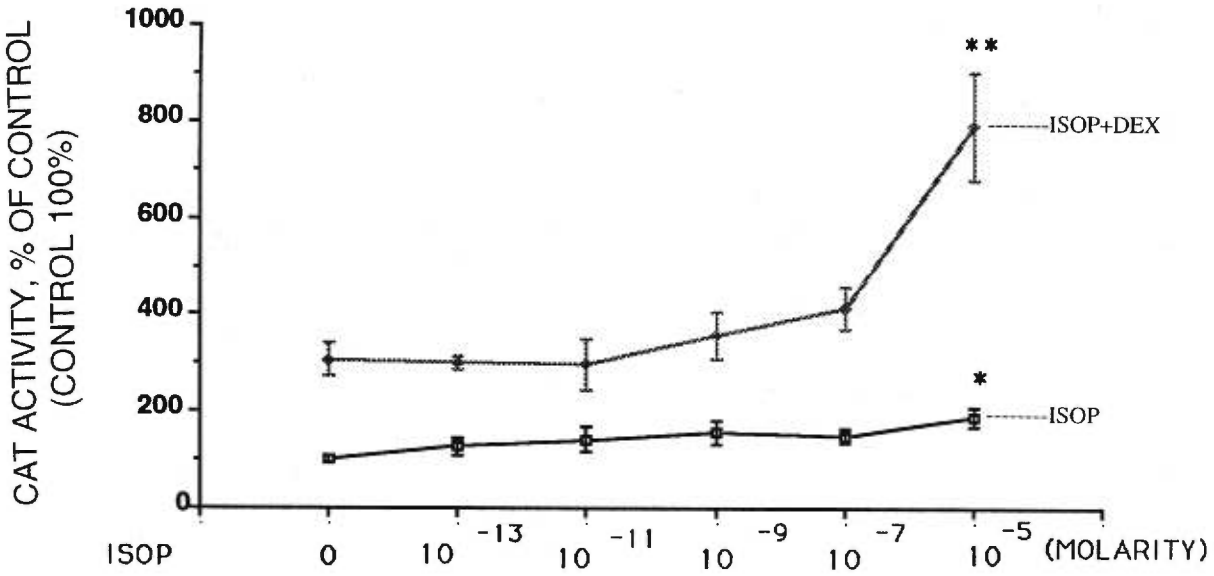


Figure 3-40. Relationship between isoproterenol concentrations and the expression of pOCAT (ANG N-1498/+18) in OK cells co-transfected with pBC-β1AR in the presence or absence of dexamethasone (10^{-6} M). Ten μg of pOCAT (ANG N-1498/+18) and 2 μg of pBC-β1AR per well ($5x10^5$ cells) were used in the experiment. The effect of isoproterenol is compared to that of the control cells (in the absence of dexamethasone and isoproterenol; its CAT activity is shown as 100%). The effect of both isoproterenol and dexamethasone is compared to that of the cells in the presence of isoproterenol alone. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (*P< 0.05; **P< 0.01). ❐, absence of dexamethasone; ●, presence of dexamethasone.

Reproduced from Ming M., Chan W., Wang T.T., Roberts K.D., Bouvier M., Lachance S., Carriere S. and Chan J.S.D.: (1996) β-adrenoceptors and dexamethasone synergistically stimulate the expression of the angiotensinogen gene in opossum kidney cells. Kid ney Intern. 50:94-101.

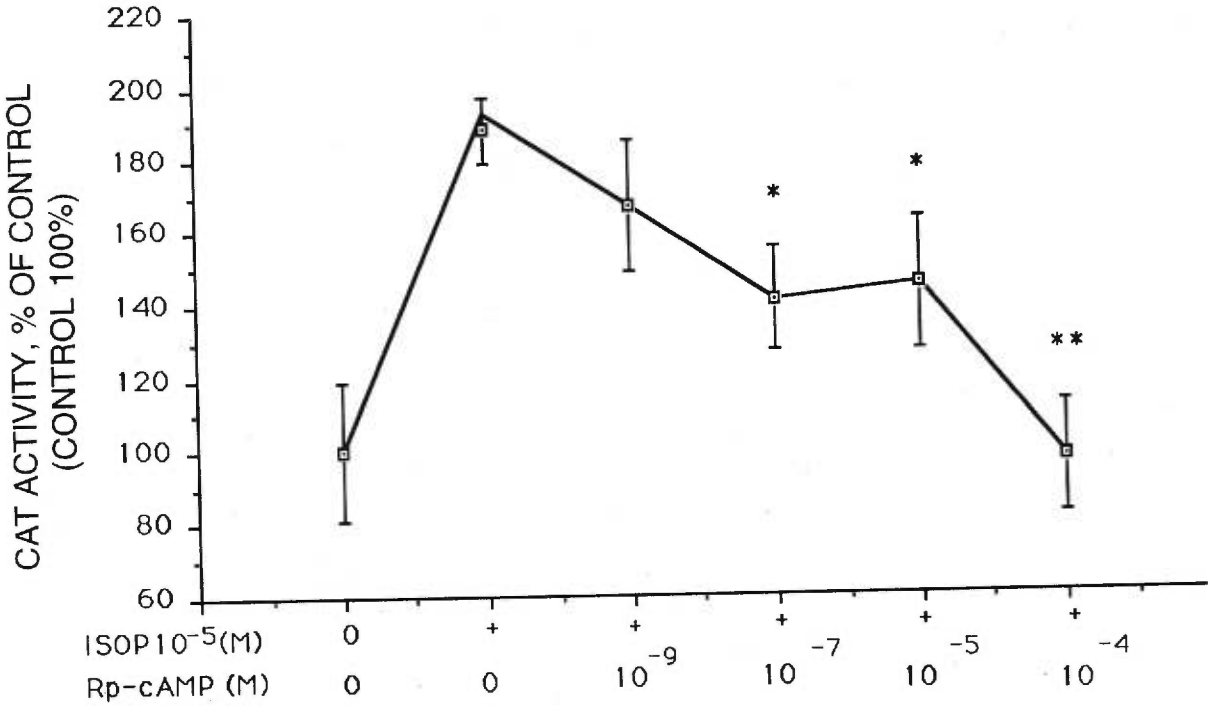


Figure 3-41. Inhibitory effect of Rp-cAMP on the expression of pOCAT (ANG N-1498/+18) co-transfected with pBC-β1AR in OK cells stimulated by isoproterenol. Cells were incubated for 24 hours in the presence of isoproterenol (10^{-5}M) with or without various concentrations of Rp-cAMP. The CAT activity of the control cells (in the absence of isoproterenol or Rp-cAMP) is shown as 100%. The inhibitory effect of Rp-cAMP was compared to that of the cells which were incubated with isoproterenol alone. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (* $P< 0.05$; ** $P< 0.01$).

Reproduced from Ming M., Chan W., Wang T.T., Roberts K.D., Bouvier M., Lachance S., Carriere S. and Chan J.S.D.: (1996) β-adrenoceptors and dexamethasone synergistically stimulate the expression of the angiotensinogen gene in opossum kidney cells. Kid ney Intern. 50:94-101.

(100%) P<0.01).

Figure 3-42 shows the effect of RU486 (10^{-5} M), Rp-cAMP (10^{-4} M) or a combination of both, on the expression of pOCAT (ANG N-1498/+18) when co-transfected with pBC-β_1AR in OK cells stimulated by isoproterenol (10^{-5} M) plus DEX (10^{-6} M). The addition of Rp-cAMP (10^{-4} M) alone partially but significantly inhibited the effect of isoproterenol plus DEX on the expression of pOCAT (ANG N-1498/+18) (313±6% versus 419±35% (P<0.05)). The addition of RU486 (10^{-5} M) alone or in combination with Rp-cAMP (10^{-4} M) completely blocked the effect of isoproterenol plus DEX on the expression of pOCAT (ANG N-1498/+18) in OK cells (102±11% versus 419±35% (P<0.005) and 101±12% versus 419±35% (P<0.005), respectively).

In summary, in the opossum kidney cell line (OK):

(a) Dexamethasone (10^{-6}M) and thyroid hormone L-T_3 (10^{-7}M) stimulated the expression of pOCAT (ANG N-1498/+18). Other steroid hormones, testosterone (10^{-6}M), estrogen (10^{-6}M) and progesterone (10^{-6}M) had no significant effect on the expression of pOCAT (ANG N-1498/+18).

(b) Dexamethasone (10^{-6}M) also stimulated the expression of pOCAT (ANG N-1138/+18), pOCAT (ANG N-960/+18), pOCAT (ANG N-814/+18), and pOCAT (ANG N-688/+18) but not the expression of pOCAT (ANG N-280/+18), pOCAT (ANG N-196/+18), pOCAT (ANG N-110/+18), pOCAT (ANG N-53/+18), or pOCAT (ANG N-35/+18).

(c) 8-Bromo-cAMP (10^{-3}M) alone stimulated the expression of pOCAT (ANG N-1498/+18), pOCAT (ANG N-1138/+18), pOCAT (ANG N-960/+18), pOCAT (ANG N-814/+18), and pOCAT (ANG N-688/+18) but not the expression of pOCAT (ANG N-280/+18), pOCAT (ANG N-196/+18), pOCAT (ANG N-110/+18), pOCAT (ANG N-53/+18), or pOCAT (ANG N-35/+18).

(d) Dexamethasone (10^{-6}M) plus 8-Bromo-cAMP (10^{-3}M) acted additively on the expression

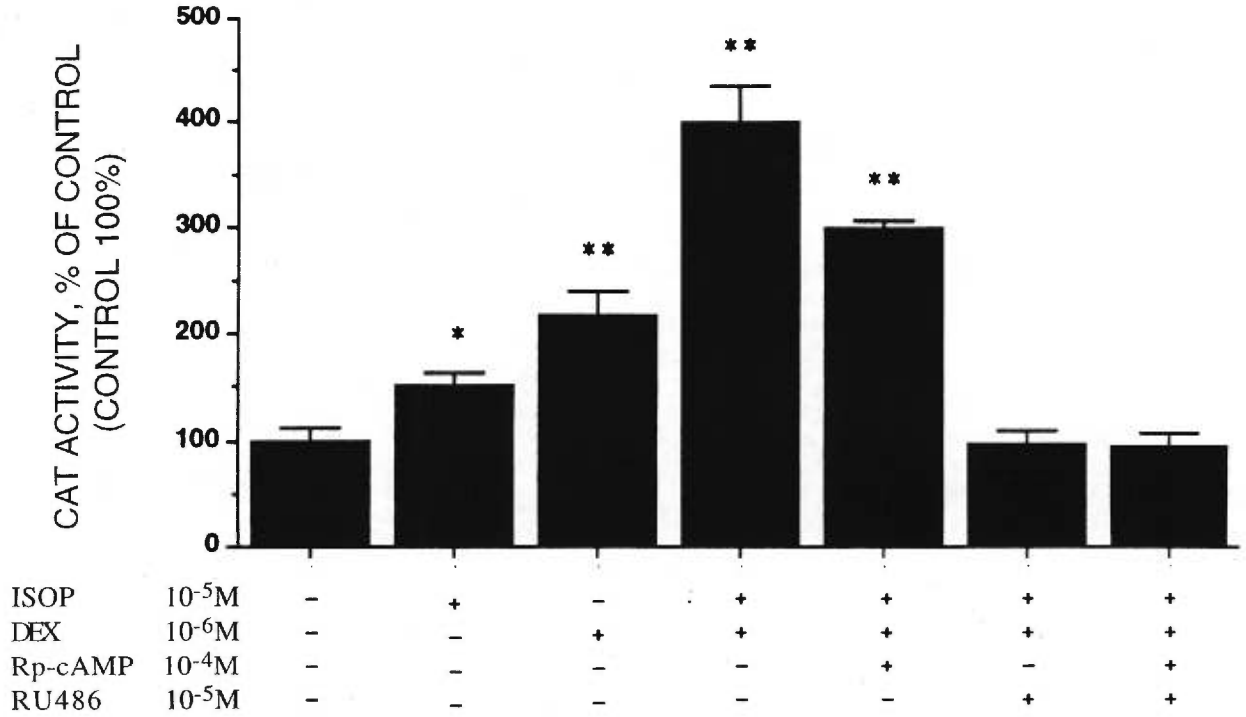


Figure 3-42. Effect of RU486 (10^{-5} M), Rp-cAMP(10^{-4} M) or both on the expression of pOCAT (ANG N-1498/+18) co-transfected with pBC-β1AR in OK cells stimulated by isoproterenol (10^{-5} M) and dexamethasone (10^{-6} M). After DNA transfection, cells were pre-incubated with RU486 for 16 hours and then further incubated for 24 hours in the presence of other drugs. The CAT activity of the control cells (in the absence of any drugs is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (* $P<0.05$; ** $P<0.01$).

Reproduced from Ming M., Chan W., Wang T.T., Roberts K.D., Bouvier M., Lachance S., Carriere S. and Chan J.S.D.: (1996) β-adrenoceptors and dexamethasone synergistically stimulate the expression of the angiotensinogen gene in opossum kidney cells. Kid ney Intern. 50:94-101.

of pOCAT (ANG N-1498/+18).

(e) Isoproterenol (10^{-5}M) alone did not stimulate the expression of pOCAT (ANG N-1498/+18) in OK cells. However, it did stimulate the expression of this fusion gene when it was co-transfected with β_1-adrenergic receptor cDNA (pBC-β_1AR), but not with β_2-adrenergic receptor cDNA (pBC-β_2AR). Isoproterenol (10^{-5}M) plus dexamethasone (10^{-6}M) acted synergistically to stimulate the expression of pOCAT (ANG N-1498/+18). The effect of isoproterenol was inhibited by Rp-cAMP (an inhibitor of protein kinase AI and II). These results indicated that the stimulatory effect of isoproterenol in OK cells was mediated through the β_1-adrenergic receptor and the protein kinase A pathway.

III.3. MOLECULAR MECHANISM OF DEXAMETHASONE PLUS 8-BROMO-cAMP ON THE EXPRESSION OF ANG GENE IN OK CELLS

To investigate the molecular mechanism of the interaction between glucocorticoids (DEX) and the protein kinase A pathway (isoproterenol, cAMP) on the expression of the ANG gene, we used ANG fusion genes containing either the putative cAMP response element (CRE) or glucocorticoid response elements (GREs) or both CRE and GREs fused to the 5'-end of the thymidine kinase promoter/enhancer in our gene transfection experiments. A summary of which response elements are present in each construct is included in Table 3-2.

Table 3-2. Constructs of the ANG-TK-CAT Fusion Genes

pTKCAT (ANG N-806/-465)	ANG CRE/GREI/GREII
pTKCAT (ANG N-806/-563)	ANG CRE/GREI
pTKCAT (ANG N-806/-779)	ANG CRE
pTKCAT (ANG N-591/-465)	ANG GREI/GREII
pTKCAT (ANG N-591/-563)	ANG GREI
pTKCAT (ANG N-485/-465)	ANG GREII

III.3.1. Effect of RU486, Rp-cAMP, or The Combination of both on the Expression of pTKCAT in OK Cells Stimulated by DEX plus 8-Bromo-cAMP

Figure 3-43 shows the effect of RU486 (10^{-5} M), Rp-cAMP (10^{-4} M), or both on the expression of plasmid vector pTKCAT in OK cells stimulated by DEX (10^{-6} M) plus 8-Bromo-cAMP (10^{-3} M). DEX, 8-Bromo-cAMP alone, or the combination of both did not stimulate the expression of pTKCAT as compared to the control (without DEX or 8-Bromo-cAMP). The addition of RU486, Rp-cAMP alone, or the combination of both had no effect on the expression of pTKCAT in the presence of DEX plus 8-Bromo-cAMP in OK cells. These studies demonstrated that the promoter/enhancer sequence of the thymidine kinase gene did not respond to DEX, 8-Bromo-cAMP, or the two together. Hence, the plasmid pTKCAT is an appropriate vector for the study of CRE and GRE of the rat ANG gene.

III.3.2. Effect of RU486, Rp-cAMP, or The Combination of Both on the Expression of pTKCAT (ANG N-806/-465) in OK Cells Stimulated by DEX plus 8-Bromo-cAMP

Figure 3-44 shows the effect of RU486 (10^{-5} M), Rp-cAMP (10^{-4} M) or both on the expression of pTKCAT (ANG N-806/-465, containing CRE/GREI/GREII) in OK cells stimulated by DEX (10^{-6} M) plus 8-Bromo-cAMP (10^{-3} M). The addition of DEX, 8-Bromo-cAMP or the combination of both stimulated the expression of pTKCAT (ANG N-806/-465) to 185%, 212% and 294%, respectively, as compared to the control, lacking DEX or 8-Bromo-cAMP. DEX plus 8-Bromo-cAMP acted additively to stimulate the expression of this fusion gene ($194\pm13\%$ increase (both) compared with $85\pm9\%$ increase (DEX alone), and $112\pm18\%$ increase (8-Bromo-cAMP alone)). RU486 or Rp-cAMP alone partially but significantly inhibited the effect of DEX plus 8-Bromo-cAMP ($234\pm13\%$ versus $294\pm13\%$ ($P<0.05$) and $186\pm9\%$ versus $294\pm13\%$ ($P<0.01$), respectively). The combination of RU486 and Rp-cAMP completely blocked the effect of DEX plus 8-Bromo-cAMP on the expression of pTKCAT (ANG N-806/-465) in OK cells ($132\pm8\%$ versus $294\pm13\%$ ($P<0.01$)).

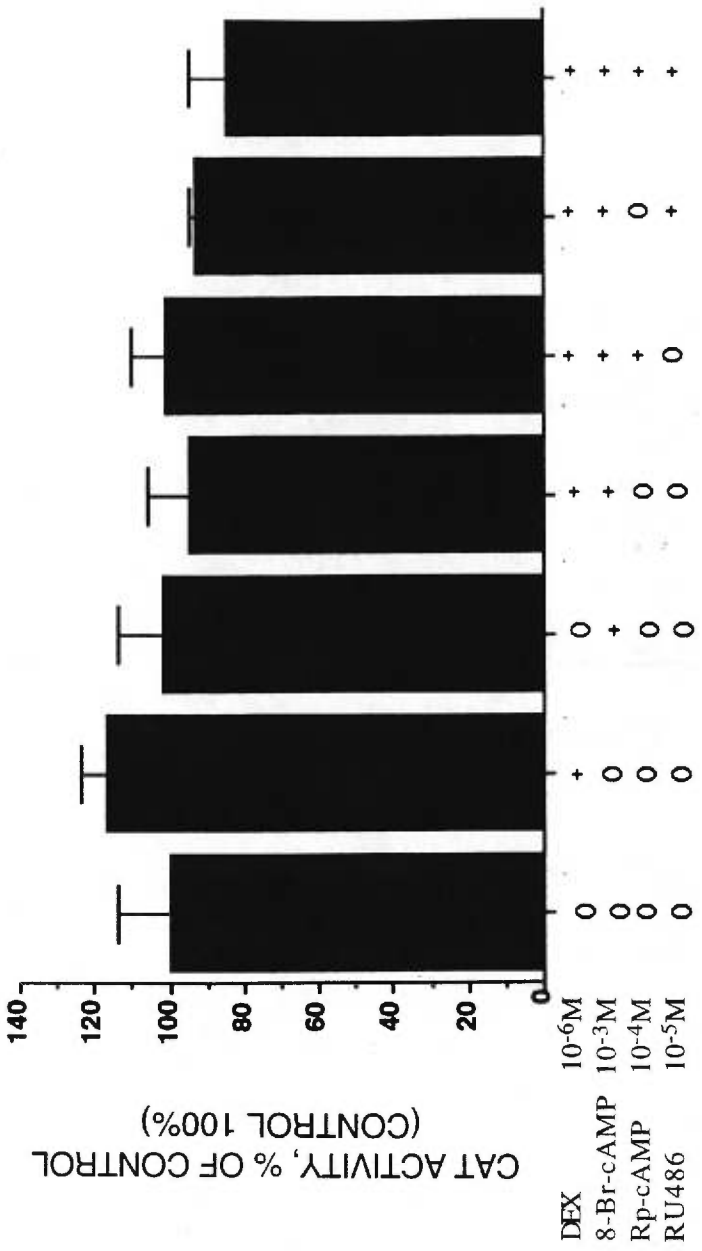


Figure 3-43. Effect of RU486 (10^{-5} M), Rp-cAMP(10^{-4} M) or both on the expression of pTKCAT in OK cells stimulated by 8-Bromo-cAMP (10^{-3} M) and dexamethasone (10^{-6} M). Cells were incubated for 24 hours in the presence of drugs. The CAT activity of the control cells (in the absence of any drugs) is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). ANOVA was used for comparisons.

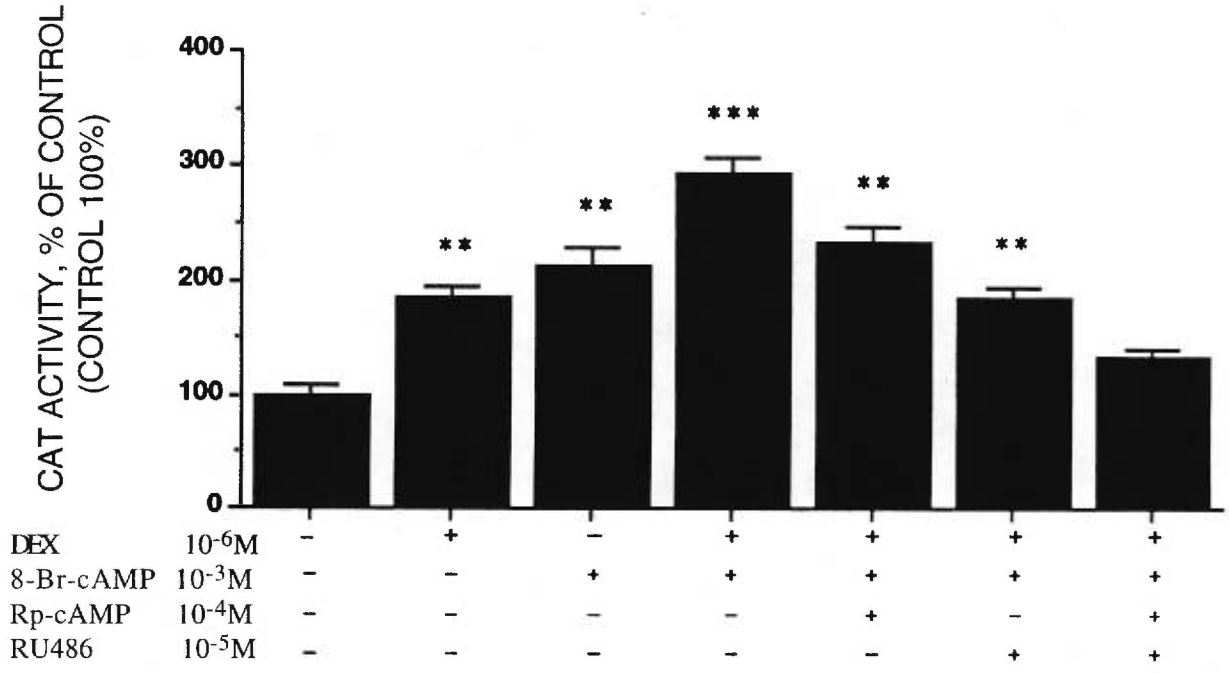


Figure 3-44. Effect of RU486 (10^{-5} M), Rp-cAMP (10^{-4} M) or both on the expression of pTKCAT (ANG N-806/-465, containing CRE/GREI/GREII) in OK cells stimulated by 8-Bromo-cAMP (10^{-3} M) and dexamethasone (10^{-6} M). All drugs were added simultaneously and cells were incubated for 24 hours in the presence of drugs. The CAT activity of control cells (in the absence of any drugs) is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA. (** $P< 0.01$ and *** $P< 0.005$).

III.3.3. Effect of RU486, Rp-cAMP, or both on the Expression of pTKCAT (ANG N-806/-465) Co-transfected with pBC-β1AR in OK Cells Stimulated by DEX plus Isoproterenol

Figure 3-45 shows the effect of RU486 (10^{-5} M), Rp-cAMP (10^{-4} M) or both on the expression of pTKCAT (ANG N-806/-465, containing CRE/GREI/GREII) co-transfected with 2 μg of pBC-β1AR in OK cells stimulated by DEX (10^{-6} M) plus isoproterenol (10^{-5} M). The addition of DEX, isoproterenol or both stimulated the expression of pTKCAT (ANG N-806/-465) to 180%, 212% and 308% as compared to the control, lacking DEX or isoproterenol. DEX plus isoproterenol acted additively on the expression of this fusion gene. There was a 80±19% increase with DEX alone, and a 112±19% increase with isoproterenol alone. The expected sum of these two effects, 192±38%, was almost the same as the increase obtained by isoproterenol and DEX together, 208±25%. Rp-cAMP alone partially but significantly inhibited the additive effect (212±13% versus 308±25% ($P<0.05$)). RU486 alone or combined with Rp-cAMP completely blocked the effect of DEX plus isoproterenol on the expression of pTKCAT (ANG N-806/-465) in OK cells (114±11% versus 308±25%, ($P<0.005$) and 94±10% versus 308±25% ($P<0.005$), respectively).

III.3.4. Effect of RU486, Rp-cAMP, or both on the Expression of pTKCAT (ANG N-806/-563) in OK Cells Stimulated by DEX plus 8-Bromo-cAMP

Figure 3-46 shows the effect of RU486 (10^{-5} M), Rp-cAMP (10^{-4} M) or both on the expression of pTKCAT (ANG N-806/-563, containing CRE/GRE I) in OK cells stimulated by DEX (10^{-6} M) plus 8-Bromo-cAMP (10^{-3} M). The addition of DEX, 8-Bromo-cAMP or both stimulated the expression of pTKCAT (ANG N-806/-563) to 160%, 158% and 254% as compared to the control (without the addition of DEX or 8-Bromo-cAMP), respectively. DEX plus 8-Bromo-cAMP acted additively to stimulate the expression of this fusion gene (154±18% increase (both) compared with 60±17% increase (DEX alone), and 58±8% increase (8-Bromo-cAMP

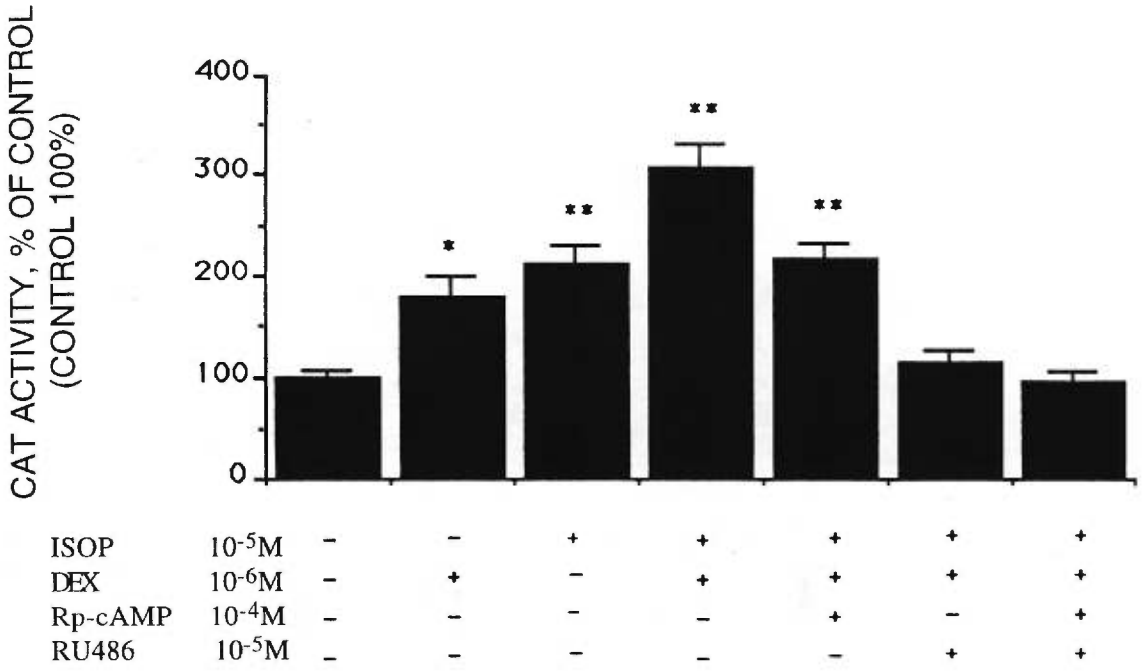


Figure 3-45. Effect of RU486 (10^{-5} M), Rp-cAMP (10^{-4} M) or both on the expression of pTKCAT (ANG N-806/-465, containing CRE/GRE-I/GRE-II) cotransfected with pBC-β1AR in OK cells stimulated by isoproterenol (10^{-5} M) and dexamethasone (10^{-6} M). After DNA transfection, cells were pre-incubated with RU486 for 16 hours and then were further incubated for 24 hours in the presence of other drugs. The CAT activity of control cells (in the absence of any drugs) is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (* $P< 0.05$; ** $P< 0.01$).

Reproduced from Ming M., Chan W., Wang T.T., Roberts K.D., Bouvier M., Lachance S., Carriere S. and Chan J.S.D.: (1996) β-adrenoceptors and dexamethasone synergistically stimulate the expression of the angiotensinogen gene in opossum kidney cells. Kid ney Intern. 50:94-101.

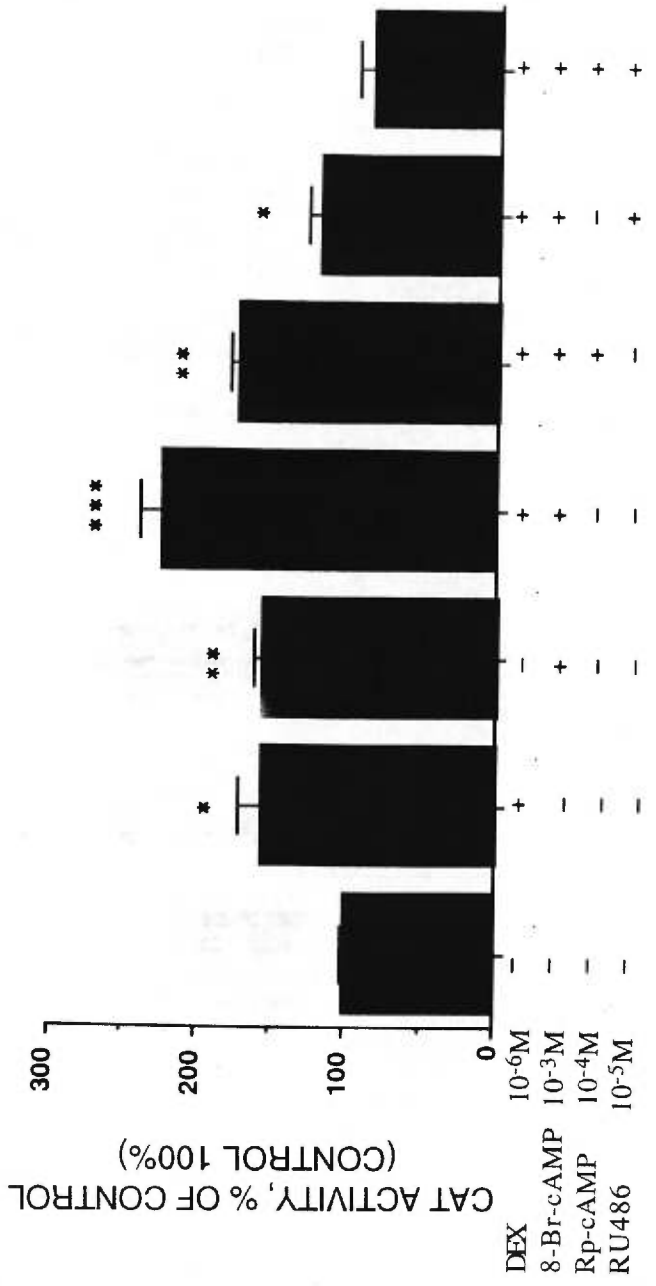


Figure 3-46. Effect of RU486 (10^{-5} M) or Rp-cAMP(10^{-4} M) or both on the expression of pTKCAT (ANG N-806/-563, containing CRE/GREI) in OK cells stimulated by 8-Bromo-cAMP (10^{-3}M) and dexamethasone (10^{-6} M). All drugs were added simultaneously and cells were incubated for 24 hours. The CAT activity of the control cells (in the absence of any drugs) is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (* $P< 0.05$; ** $P< 0.01$ and *** $P< 0.005$).

alone)). Either Rp-cAMP or RU486 alone partially, but significantly inhibited the additive effect (174±4% versus 254±18 (P<0.01) and 120±7% versus 254±18 (P<0.005), respectively). The combination of Rp-cAMP and RU486 completely blocked the effect of DEX plus 8-Bromo-cAMP on the expression of pTKCAT (ANG N-806/-563) in OK cells (85+9% versus 254+18, P<0.005).

The results (Figure 3-44, 3-45, and 3-46) indicated that: (a) Both ANG N-806/-465 and ANG N-806/-563 are involved in the response to the stimulation of DEX, 8-Bromo-cAMP, and both on the expression of the rat ANG gene; and (b) DEX and 8-Bromo-cAMP act additively on the expression of ANG N-806/-465 and ANG N-806/-563.

III.3.5. Effect of RU486, Rp-cAMP, or both on the Expression of pTKCAT (ANG N-806/-779) in OK Cells Stimulated by DEX plus 8-Bromo-cAMP

Figure 3-47 shows the effect of RU486 (10^{-5} M), Rp-cAMP (10^{-4} M) or both on the expression of pTKCAT (ANG N-806/-779, containing CRE) in OK cells stimulated by DEX (10^{-6} M) plus 8-Bromo-cAMP (10^{-3} M). Either 8-Bromo-cAMP alone or combined with DEX stimulated the expression of pTKCAT (ANG N-806/-779) to 154% and 151%, respectively, as compared to the control, lacking DEX or 8-Bromo-cAMP. There was no significant difference between 8-Bromo-cAMP alone and 8-Bromo-cAMP plus DEX (54±11% increase versus 51±11% increase, NS). Furthermore, DEX alone did not stimulate the expression of pTKCAT (ANG N-806/-779) compared with the control (104±14% versus 100±12, NS). Rp-cAMP alone or combined with RU-486 completely blocked the effect of 8-Bromo-cAMP plus DEX on the expression of pTKCAT (ANG N-806/-779) (101±7% versus 151±11% (P<0.05) and 102±12% versus 151±11% (P<0.05), respectively). However, RU486 alone did not block the effect of DEX plus 8-Bromo-cAMP on the expression of pTKCAT (ANG N-806/-779) in OK cells (137±4% versus 151±11%, NS).

These results showed that ANG N-806/-779 mediated the effect of 8-Bromo-cAMP but not

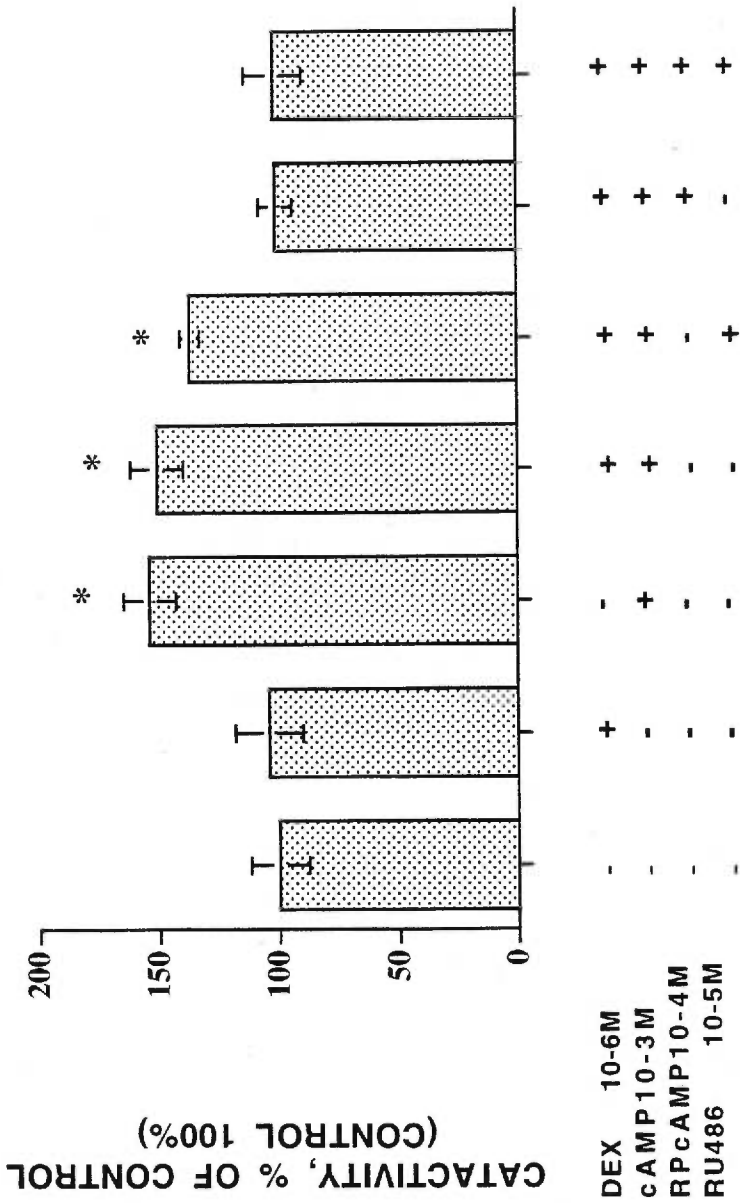


Figure 3-47. Effect of RU486 (10^{-5} M) or Rp-cAMP(10^{-4} M) or both on the expression of pTKCAT (ANG N-806/-779, CRE) in OK cells stimulated by 8-Bromo-cAMP (10^{-3} M) and dexamethasone (10^{-6} M). All drugs were added simultaneously and cells were incubated for 24 hours. The CAT activity of the control cells (in the absence of any drugs) is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (* P< 0.05).

DEX on the expression of the ANG gene. It provided evidence that the DNA fragment ANG N-806/-779 contains a cAMP responsive element (CRE) of the rat ANG gene.

III.3.6. Effect of RU486, Rp-cAMP, or Both on the Expression of pTKCAT (ANG N-591/-465) in OK Cells Stimulated by DEX plus 8-Bromo-cAMP

Figure 3-48 shows the effect of RU486 (10^{-5} M), Rp-cAMP (10^{-4} M) or both on the expression of pTKCAT (ANG N-591/-465, containing GREI/GREII) in OK cells stimulated by DEX (10^{-6} M) plus 8-Bromo-cAMP (10^{-3} M). Either DEX alone or in combination with 8-Bromo-cAMP stimulated the expression of pTKCAT (ANG N-591/-465) to 191±4%, and 221±15%, respectively, as compared to the control, lacking DEX or 8-Bromo-cAMP. However, 8-Bromo-cAMP alone did not stimulate this fusion gene expression (108±4% versus 100±13% (NS)). There was no significant difference between DEX alone and DEX plus 8-Bromo-cAMP (91±3% increase versus 121±15% increase (NS)). RU486 alone or combined with Rp-cAMP completely blocked the effect of DEX plus 8-Bromo-cAMP on the expression of pTKCAT (ANG N-591/-465) (130±14% versus 221±15% ($P<0.05$) and 90±6% versus 221±15% ($P<0.01$)) whereas, Rp-cAMP alone did not block the effect of DEX plus 8-Bromo-cAMP (180±16% versus 221±15% (NS)). The results provided the evidence that the DNA fragment ANG N-591 to N-465 contains the glucocorticoid response elements (GREs) of the rat ANG gene.

III.3.7. Effect of RU486, Rp-cAMP, or Both on the Expression of pTKCAT (ANG N-591/-563) in OK Cells Stimulated by DEX plus 8-Bromo-cAMP

Figure 3-49 shows the effect of RU486 (10^{-5} M), Rp-cAMP (10^{-4} M) or both on the expression of pTKCAT (ANG N-591/-563, containing GREI) in OK cells stimulated by DEX (10^{-6} M) plus 8-Bromo-cAMP (10^{-3} M). Either DEX alone or in combination with 8-Bromo-cAMP stimulated the expression of pTKCAT (ANG N-591/-563) to 144% and 141%, respectively, as compared to the control, lacking DEX and 8-Bromo-cAMP ($P<0.05$), whereas 8-Bromo-cAMP alone did not stimulate the expression of this fusion gene. There was no significant difference

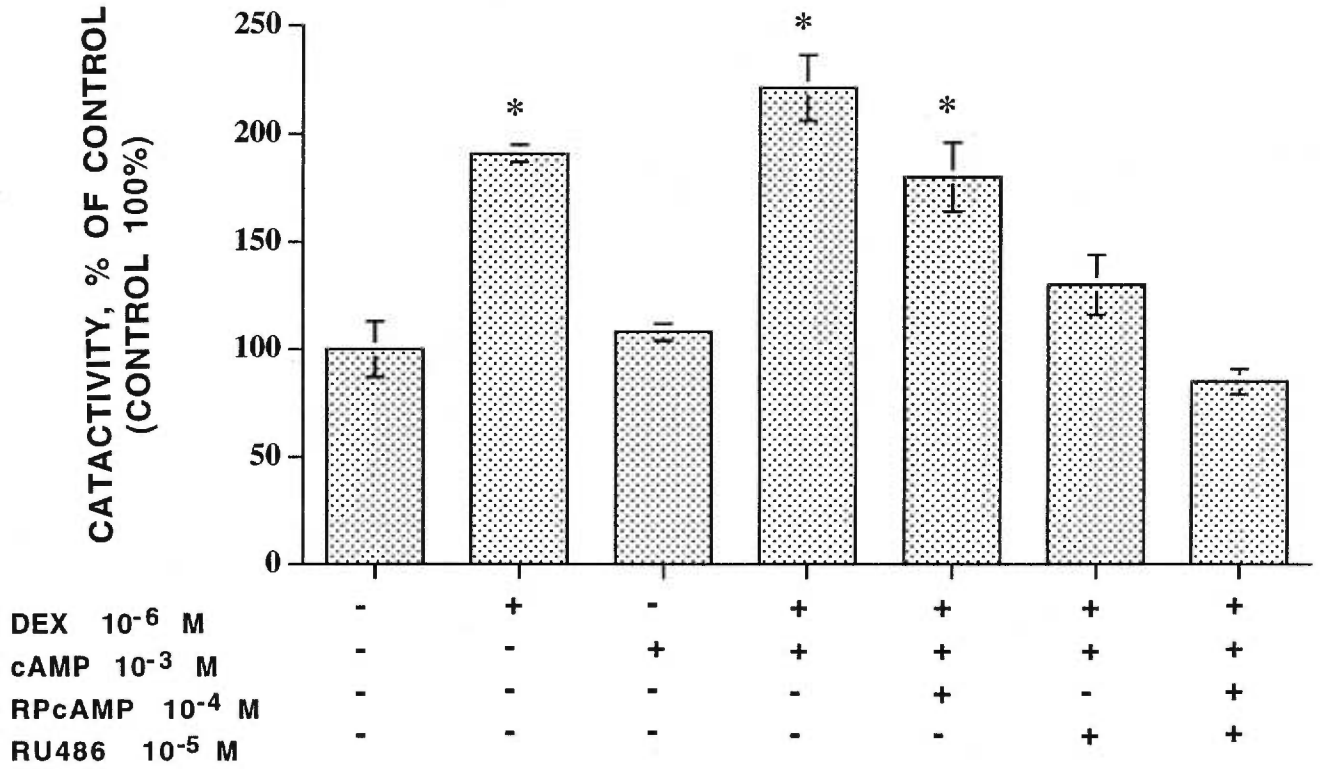


Figure 3-48. Effect of RU486 (10^{-5} M) or Rp-cAMP(10^{-4} M) or both on the expression of pTKCAT (ANG N-591/-465, GREI/GREII) in OK cells stimulated by 8-Bromo-cAMP (10^{-3} M) and DEX (10^{-6} M). All drugs were added simultaneously and cells were incubated for 24 hours. The CAT activity of the control cells (in the absence of any drugs) is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (* $P < 0.05$).

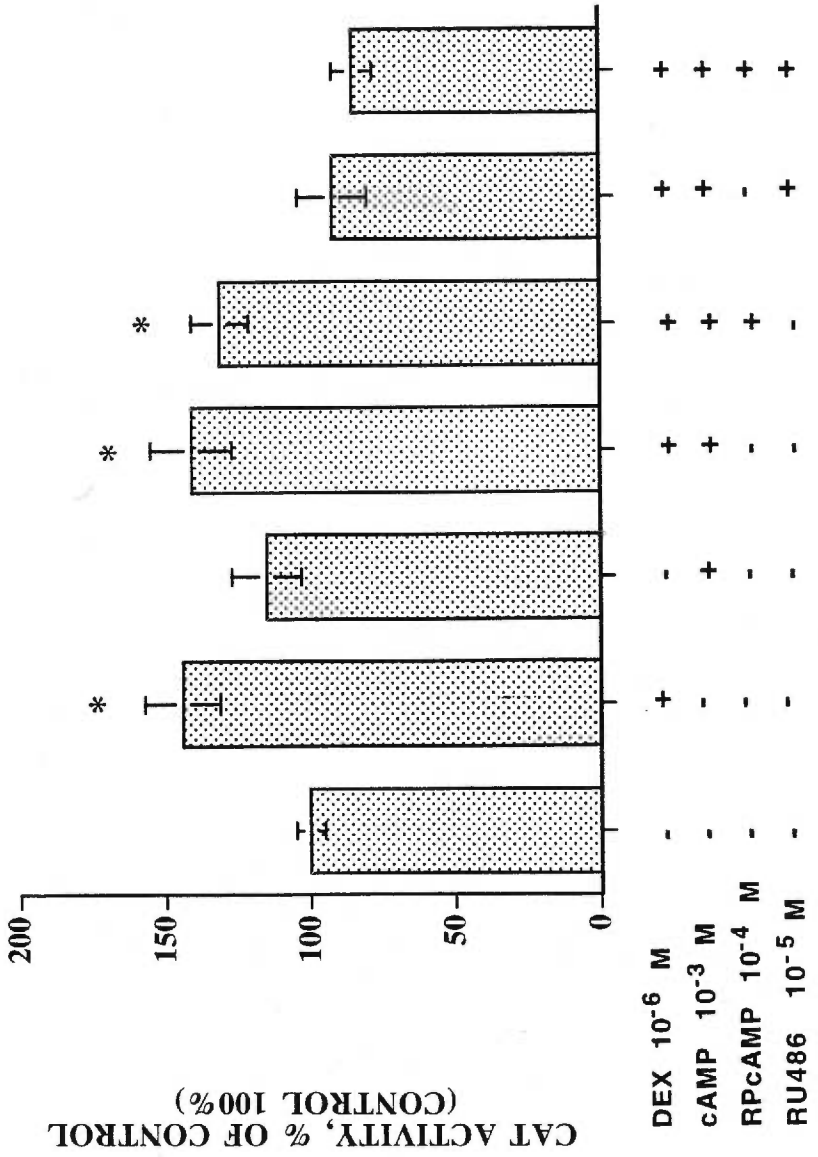


Figure 3-49. Effect of RU486 (10^{-5} M) or Rp-cAMP(10^{-4} M) or both on the expression of pTKCAT (ANG N-591/-563, GREI) in OK cells stimulated by 8-Bromo-cAMP (10^{-3} M) and dexamethasone (10^{-6} M). All drugs were added simultaneously and cells were incubated for 24 hours. The CAT activity of the control cells (in the absence of any drugs) is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (* $P< 0.05$).

between the effects of DEX alone and DEX plus 8-Bromo-cAMP (44±13% increase versus 41±13% increase (NS)). RU-486 alone or combined with Rp-cAMP completely blocked the effect of DEX plus 8-Bromo-cAMP on the expression of pTKCAT (ANG N-591/-563) (92±11% versus 141±13% ($P<0.05$) and 85±7% versus 141±13% ($P<0.05$), respectively). However, Rp-cAMP alone did not block the effect of DEX plus 8-Bromo-cAMP. The results indicated that the DNA fragment ANG N-591/-563 contained a glucocorticoid response element (GRE) of the rat ANG gene.

III.3.8. Effect of RU486, Rp-cAMP, or Both on the Expression of pTKCAT (ANG N-485/-465) in OK Cells Stimulated by DEX plus 8-Bromo-cAMP

Figure 3-50 shows the effect of RU486 (10^{-5} M), Rp-cAMP (10^{-4} M) or both on the expression of pTKCAT (ANG N-485/-465, containing GREII) in OK cells stimulated by DEX (10^{-6} M) plus 8-Bromo-cAMP (10^{-3} M). DEX, 8-Bromo-cAMP, or both did not stimulate the expression of pTKCAT (ANG N-485/-465). The addition of RU486, Rp-cAMP, or both had no effect on the expression of pTKCAT (ANG N-485/-465) in the presence of DEX plus 8-Bromo-cAMP in OK cells. The results indicated that the DNA fragment ANG N-485/-465 is not responsive to the addition of DEX, 8-Bromo-cAMP, or both.

These results (Figure 3-48, 3-49, and 3-50) shows that:

(a) Dexamethasone stimulates the expression of pTKCAT (ANG N-591/-465, containing GREI/GREII) and pTKCAT (ANG N-591/-563, containing GREI), but not the expression of pTKCAT (ANG N-485/-465, containing GREII).

(b) The fragments ANG N-591/-465, ANG N-591/-563, and ANG N-485/-465 do not mediate the effect of 8-Bromo-cAMP on the expression of the ANG gene.

(c) 8-Bromo-cAMP does not enhance the stimulatory effect of DEX on the expression of pTKCAT (ANG N-591/-465), pTKCAT (ANG N-591/-563) or pTKCAT (ANG N-485/-465).

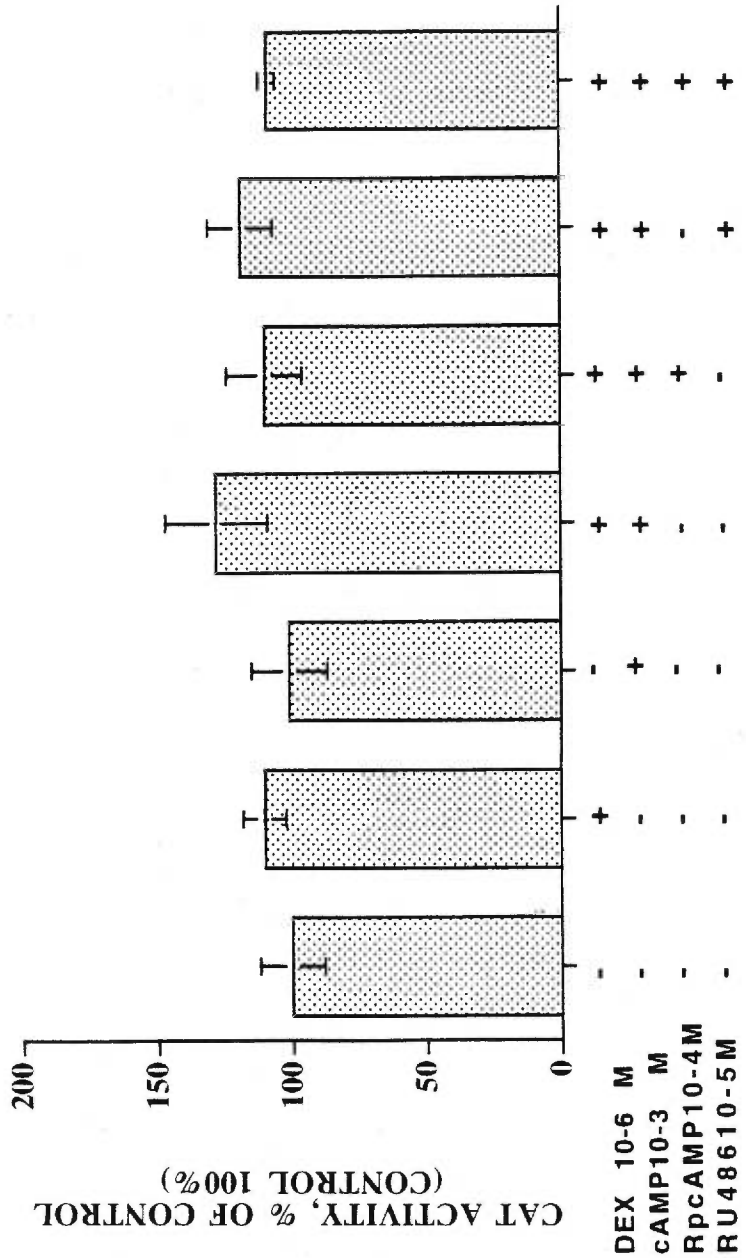


Figure 3-50. Effect of RU486 (10^{-5} M) or Rp-cAMP(10^{-4} M) or both on the expression of pTKCAT (ANG N-485/-465, GREII) in OK cells stimulated by 8-Bromo-cAMP (10^{-3} M) and dexamethasone (10^{-6} M). All drugs were added simultaneously and cells were incubated for 24 hours. The CAT activity of the control cells (in the absence of any drugs) is shown as 100%. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). ANOVA was used for comparisons.

(d) The fragment ANG N-485/-465 does not respond to stimulation by DEX, 8-Bromo-cAMP or the combination ofboth.

III.3.9. Effect of DEX, 8-Bromo-cAMP or Both on the Expression of pTKCAT (ANG N-806/-465), pTKCAT (ANG N-806/-563), pTKCAT (ANG N-806/779), pTKCAT (ANG N-591/-465), pTKCAT (ANG N-591/-563), and pTKCAT (ANG N-485/-465) in OK Cells

Finally, Figure 3-51 shows the effect of DEX, 8-Bromo-cAMP, or both on the expression of pTKCAT (ANG N-806/-465, CRE/GREI/GREII), pTKCAT (ANG N-806/-563, CRE/GREI), pTKCAT (ANG N-806/-779, CRE), pTKCAT (ANG N-591/-465, GREI/GREII), pTKCAT (ANG N-591/-563, GREI), and pTKCAT (ANG N-485/-465, GREII) in OK cells. DEX alone significantly stimulated the expression of pTKCAT (ANG N-806/-465), pTKCAT (ANG N-806/-563), pTKCAT (ANG N-591/-465), and pTKCAT (ANG N-591/-563) as compared to the control (without the addition of DEX or 8-Bromo-cAMP) (168$\pm$16% versus 100$\pm$17% ($P<0.05$), 151$\pm$5% versus 100$\pm$14% ($P<0.05$), 184$\pm$29% versus 100$\pm$11% ($P<0.05$), 153$\pm$17% versus 100$\pm$7% ($P<0.05$)), but DEX had no effect on pTKCAT (ANG N-806/-779) and pTKCAT (ANG N-485/-465). The addition of 8-Bromo-cAMP alone stimulated the expression of pTKCAT (ANG N-806/-465), pTKCAT (ANG N-806/-563), and pTKCAT (ANG N-806/-779) as compared to the control (190$\pm$19% versus 100$\pm$17% ($P<0.05$), 152$\pm$10% versus 100$\pm$14% ($P<0.05$), 169$\pm$20% versus 100$\pm$13% ($P<0.05$)), but 8-Bromo-cAMP had no effect on the expression of pTKCAT (ANG N-591/-465), pTKCAT (ANG N-591/-563) and pTKCAT (ANG N-485/-465). Moreover, DEX plus 8-Bromo-cAMP additively stimulated the expression of pTKCAT (ANG N-806/-465) and pTKCAT (ANG N-806/-563) as compared to the effect of DEX and 8-Bromo-cAMP (167$\pm$21% increase (both) compared with 68$\pm$16% increase (DEX alone) and 90$\pm$19% increase (cAMP alone) for the expression of pTKCAT (ANG N-806/-465), and 93$\pm$10% increase (both) compared with 51$\pm$5% increase (DEX alone) and 52$\pm$10% increase (cAMP alone) for the expression of pTKCAT (ANG N-806/-563).

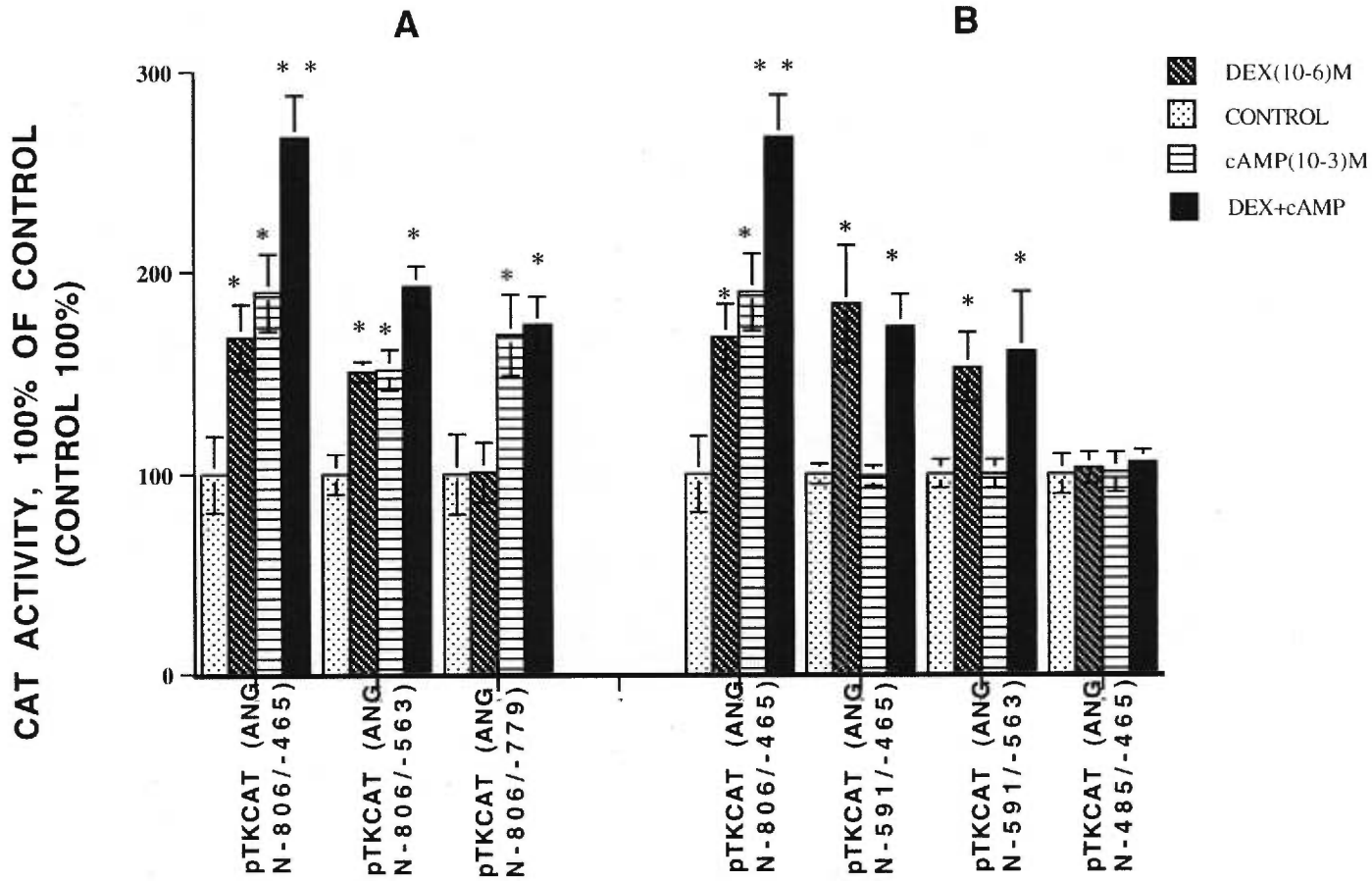


Figure 3-51. Effect of 8-Bromo-cAMP (10^{-3} M) or dexamethasone (10^{-6} M) or both on the expression of the various ANG-TK-CAT fusion genes in OK cells. The effect of 8-Bromo-cAMP or dexamethasone alone is compared to that of the control cells (in the absence of both dexamethasone and 8-Bromo-cAMP, its CAT activity is shown as 100%). The effect of both 8-Bromo-cAMP and dexamethasone is compared to that of the cells in the presence of 8-Bromo-cAMP or dexamethasone alone. Each point represents the mean±SD of three independent transfections in triplicate plates (n=3). The probability values are derived from ANOVA (* $P < 0.05$; ** $P < 0.01$).

The results (Figures 3-44 to 3-51) provide evidence that: (a) The fragment N-806/-563 of the rat ANG gene contains the essential elements to mediate the effect of DEX plus 8-Bromo-cAMP; (b) The fragment N-806/-779 of the rat ANG gene contains the cAMP response element, which mediates the effect of 8-Bromo-cAMP and isoproterenol on the expression of the ANG gene; and (c) The fragment N-591/-563 of the rat ANG gene contains the glucocorticoid response element, which mediates the effect of DEX on the expression of the ANG gene.

CHAPTER IV DISCUSSION

IV.1. JUSTIFICATION OF CELL LINES AND REPORTER SYSTEMS IN THE PRESENT STUDY

IV.1.1. Cell Lines

The renin-angiotensin axis is a phylogenetically ancient system for control of systemic blood pressure and fluid and electrolyte balance. The first reaction in the renin-angiotensin cascade is the cleavage of angiotensinogen by renin. The liver is the major source of plama ANG (Nasjletti and Masson, 1972). Some diseases, such as cirrhosis and congestive heart failure, can decrease the capacity of the liver to synthesize ANG and reduce the circulating ANG level. Several physiologic and pathologic conditions, such as stress, pregnancy, Cushing's syndrome, and glucocorticoid therapy can enhance the liver synthesis of ANG and increase the circulating ANG levels and systemic blood pressure. It has been reported that during the terminal stages of malignant hypertension, the plasma ANG levels may increased 2-4 fold (Haynes et al., 1953; Gould et al., 1966). Althrough ANG mRNAs have been identified in other organs (brain, kidney, adrenal gland, ovary, lung, and heart) (Ohkubo et al., 1986; Dzau et al., 1987), they do not contribute in a major way to the circulating ANG level. Therefore, to study the regulation of ANG gene expression, the cultured hepatocyte is one of the best model systems, and one in which the hormonal milieu can be carefully controlled (Chang and Perlman, 1987). A detailed explanation of the choice of the Hepa 1-6 cell line over other hepatoma cell lines has been provided in the Materals and Methods section (II.2.2.).

The kidney plays an essential role in regulating the RAS by secreting renin in response to a variety of stimuli, and it also serves as an important target tissue of AII. The discovery of the existence of all the components of the RAS in the kidney indicates the presence of an intrarenal

renin-angiotensin system (Burns et al., 1993), which has led to the hypothesis that in addition to being a plasma hormone, AII can be formed locally and therefore possess aditional autocrine and paracrine actions (Navar et al., 1997). AII plays an important role in tubular electrolyte, and subsequently, fluid reabsorption. It has been determined that proximal tubule cells contain all the components of the RAS necessary for the synthesis and secretion of AII (Navar et al., 1997). Experimental evidence has shown that the intratubular AII concentrations are regulated independently of the circulating ANG concentrations (Navar et al., 1997). The OK cell line, derived from the opossum kidney, expresses low levels of ANG mRNA and the angiotensin-converting enzyme (Chan et al., 1991; Ingelfinger et al., 1991), making it an excellent model to investigate the mechanism of ANG gene regulation in proximal tubule cells.

Both Hepa 1-6 and OK cells have low levels of ANG mRNA, indicating that the trans-acting factors regulating ANG gene expression are likely to be present in these cells. Hence, they become very useful cell models for transfection of ANG-CAT fusion genes and study of their regulation. Indeed, following co-transfection with β-adrenergic receptor cDNA into OK cells, but not into Hepa 1-6 cells which already show a response to β-adrenergic receptor stimulation in the presence of DEX, both cell lines could be used to study ANG gene expression in response to β-adrenergic stimulation in the presence/absence of β-adrenergic inhibitors. As these cell lines respond to the stimulation of DEX and 8-Bromo-cAMP, an analog of the second messenger of isoproterenol, they should also be good models for characterizing glucocorticoid-dependent and PKA-dependent regulation of ANG gene expression. In short, their high efficiency of transfection, and their responsiveness to DEX and β-adrenergic agonists, are major advantages for investigating the interaction between DEX and isoproterenol on the expression of the ANG gene.

IV.1.2. Reporter Systems

A detailed justification of the use of the CAT reporter system in the present study has been given in the Materials and Methods section (II.2.3.1.1.). A concern in using human growth hormone (hGH) as an internal control for monitoring transfection efficiency, along with any other

transient expression system, is the possibility that any hGH secreted into the medium may be biologically active, leading to the expression of other genes (Selden et al., 1986). To eliminate this possibility, we assessed the effect of hGH on the expression of the ANG gene in preliminary experiments as shown in Figures 3-3 and 3-22. Different concentrations of hGH (0.1-100 ng/ml) had no significant effect on the expression of the ANG gene in either Hepa 1-6 cells or OK cells.

The effects of growth hormone on growth and protein metabolism, including proliferation and protein synthesis in both skeletal and nonskeletal tissues, are mediated by somatomedins (Van and Underwood, 1975), i.e. the relative roles of hGH and IGF-I in stimulating bone growth may be most accurately described by a "dual-effect" mode of hGH action, in which hGH stimulates cartilage precursor cells first to differentiate and subsequently to produce, and become responsive to, the autocrine and paracrine mitogenic effects of IGF-I (Isaksson et al., 1987). The somatomedins are polypeptide growth factors secreted by the liver and other tissues in response to the stimulation by GH (D' Ercole et al., 1984). The principal circulating somatomedins are insulin-like growth factor I (IGF-I, somatomedin C) and insulin-like growth factor II (IGF-II).

Both IGF-I and IGF-II have multiple physiological effects on target tissues (Guler et al., 1988). IGF-I has pronounced growth-stimulating activity and is much affected by GH. IGF-II plays a role in the growth of the fetus and is much less affected by GH. As the mouse IGF-I and IGF-II are unavailable, the IGF-I and IGF-II used in our experiments were recombinant human IGF I and IGF II. Both of them are effective in rodents (Jonet et al., 1993; Kunner et al., 1993; Miyagishima et al., 1993), so they were used to examine the effect of IGF-I and IGF-II on the expression of the ANG gene in Hepa 1-6 and OK cells. Addition of IGF-I or IGF-II had no significant effects on the expression of the ANG-CAT fusion gene, pOCAT (ANG N-1498/+18), in Hepa 1-6 or OK cells (Figures 3-4, 3-5, 3-23).

In conclusion, as the preliminary studies indicated that the CAT activity encoded by the ANG-CAT fusion gene was predominantly intracellular, the cell extracts were used for measurement of CAT activity (Figure 3-1). hGH, IGF-I, and IGF-II have no significant effects on

ANG gene expression (Figures 3-3, 3-4, 3-5, 3-22,3-23). Hence, the level of RIA-hGH in the medium was used as the internal control to monitor the transfection efficiency in all the present studies using the CAT transient expression system.

IV.2. CELL LINE SPECIFICITY OF ANG GENE EXPRESSION

IV.2.1. Cell Line Specificity

Cell-specific expression of genes is regulated to a large extent at the transcriptional level by the interactions of DNA binding proteins (transacting factors) with specific DNA control elements (response elements) organized in modular arrays within the 5'-flanking regions of the transcribed genes (Maniatis et al., 1987). DNA control elements resulting in specific gene transcription include more proximal core promoter elements, and upstream promoter and distal enhancer or silencer elements; these locations are defined relative to the site of initiation of transcription. Core promoter elements are defined as spatially constrained DNA sequences which accurately determine the site of transcriptional initiation (e.g., TATAA box). Upstream promoter and enhancer or silencer elements of transcription are located at variant distances from the core promoter. Both promoter and enhancer or silencer elements of genes may determine cellular specificity of gene expression by uniquely directing transcription within discrete cellular phenotypes. In addition, the transcription of many genes expressed in specific tissues may be induced by specific effectors, such as cAMP (Nagamine and Reich, 1985; Deutsch et al., 1987), phorbol esters (Chiu et al., 1987), and steroid hormones (Hollenberg et al., 1985; Toohey et al., 1986; Brasier et al., 1986; Chang and Perlman, 1987; Evans, 1988). All of these exert their effects via interaction with upstream promoter and distal enhancer or silencer DNA elements.

We have previously shown different levels of expression of ANG-reporter fusion genes in different cell lines (Chan et al., 1992a; Ming et al., 1993). In Hepa 1-6 cells, the basal level of expression for the full-length ANG-CAT fusion gene pOCAT (ANG N-1498/+18) was 3.7-fold that of the promoterless pOCAT (Figure 3-6). Deletion of the DNA sequence in the 5'-flanking

region located between N-1498 and N-689, pOCAT (ANG N-688/+18) yielded a similar activity as compared to pOCAT (ANG N-1498/+18). However, with the further removal of the sequence between N-688 to N-111, pOCAT (ANG N-110/+18) yielded an activity comparable to the promoterless pOCAT (1.1-fold). These results suggested that the sequence of the ANG gene from positions N-1498 to N-111 might contain multiple cell line-specific enhancer and silencer *cis*-regulatory elements. Further deletion of N-110 to N-54, pOCAT (ANG N-53/+18), and N-53 to N-36, pOCAT (ANG N-35/+18), resulted in a 3- and 2-fold increase, respectively, above that of pOCAT alone, suggesting that nucleotide N-110 to N-54 contains a dominant silencing regulatory element and N-53 to N+18 contains multiple enhancer/promoter *cis*-regulatory elements. These results were in agreement with the results reported by others (Brasier et al., 1989; Feldmer et al., 1992; Zhao et al., 1992). The experiments of the former group showed that the plasmid containing ANG N-1600 to +39 was as active as the plasmid containing ANG N-688 to +39 in HEP G2 cells (Brasier et al., 1989). They proposed that the DNA sequence that determined the cell line specificity of the ANG gene was located in N-688 to N+39.

Previous studies in our laboratory have demonstrated that OK cells can also express the full-length ANG-hGH fusion gene, pOGH (ANG N-1498/+18), and the RIA-hGH levels observed were 226-fold that of the promoterless pOGH (Chan et al., 1991). The truncated ANG-hGH fusion genes, pOGH (ANG N-688/+18), pOGH (ANG N-110/+18), pOGH (ANG N-53/+18) and pOGH (ANG N-35/+18), yielded, respectively, 4.5-, 1.0-, 12.0-, and 2.5-fold hGH products compared to pOGH alone. The full-length ANG-hGH fusion gene, pOGH (ANG N-1498/+18), was not expressed either in a rat pancreatic islet tumor cell line (RIN 1056A) or a human placental choriocarcinoma cell line (JEG-3) (Chan et al., 1990). In contrast, the truncated ANG-hGH fusion genes, pOGH (ANG N-688/+18), pOGH (ANG N-110/+18), pOGH (ANG N-53/+18), and pOGH (ANG N-35/+18) were 1.8-, 1.5, 12, and 3.0-fold higher than pOGH alone on the expression of hGH activity in RIN 1056A cells, while neither the full-length nor the truncated fusion genes were expressed in JEG-3 cells (Chan et al., 1990). Studies have shown both the OK

cell and the RIN 1056A cell express ANG mRNA (Chan et al., 1991), whereas no ANG mRNA is expressed in JEG-3 cells. These data demonstrate that the DNA fragments which determine the cell line specificity of ANG gene expression are located in ANG N-1498 to N+18.

The above results obtained with OK cells, RIN 1056A cells, and JEG-3 cells differ from the results obtained with hepatoma cells. In both HEP G2 and Hepa 1-6 cells, the plasmids containing ANG N-1600 to N+39, or ANG N-1498 to N+18, are as active as the plasmids containing ANG N-688 to N+39, or ANG -688 to N+18. In contrast, in OK cells, the deletion of DNA sequence between N-1498 and N-689 yielded a dramatically decreased activity (from 226-fold to 4.5-fold). However, in RIA 1056A cells, the deletion of the same DNA sequence yielded an increased activity (from 1-fold to 1.8-fold). The reason for the difference might be explained by cell-line specificity. Hepatocytes are the major cells that synthesize the plasma ANG. HEP G2 and Hepa 1-6 cells were derived from hepatoma cells, but both of them still retain some characteristics of hepatocytes and express ANG mRNA. The local renal RAS plays an important role in the paracrine regulation of renal hemodynamics and tubular transport (Navar et al., 1997). AII can be produced in the kidney by using the ANG that is synthesized in proximal tubular cells. The urine AII levels were 10-fold higher than circulating AII levels, and the intratubular AII concentrations are regulated independently of the circulating ANG concentrations (Navar et al., 1997). ANG mRNA has been localized to the proximal tubule (Dzau et al., 1987). OK cells are derived from renal tissue and Northern blot analysis has demonstrated that ANG mRNA is expressed in this cell line (Chan et al., 1992a). RIN 1056A cells are derived from a pancreatic islet tumor. JEG-3 cells do not express the endogenous ANG genes and Northern blot analysis did not detect the expression of ANG mRNA in these cells (Brasier et al., 1989). Many genes have been identified containing cell-specific enhancers, such as the immunoglobulin heavy chain gene (Banerji et al., 1983; Gilles et al., 1983), the α-fetal protein gene (Godbout et al., 1986), the rat elastase I gene (Ornitz et al., 1987) and the growth hormone gene (Bodner and Karin, 1987). This suggests that gene expression can be directed to unique cell types by combining appropriate enhancer elements. On the other hand, transacting factors also can be tissue-specific, because some of them are

synthesized only in a particular cell. Or availability of a factor could vary--for example, NFκB is present in many cell types, but it is sequestered in the cytoplasm by the inhibitory protein I-κB. In B lymphocytes, NFκB is released from I-κB and moves to the nucleus, where it activates transcription (Beg and Balwin, 1993; Reya and Grosschedl, 1998). Which combinations of the multiple sequences restrict ANG gene activity to different cell types and how the different transacting factors affect ANG gene expression in different cell types require further study.

There was a noticeable difference between the basal levels of expression of pOGH (ANG N-1498/+18) and pOCAT (ANG N-1498/+18) in OK cells. The expression of pOGH (ANG N-1498/+18) and pOCAT (ANG N-1498/+18) was 200-fold and 20-fold higher than the promoterless pOGH and pOCAT, respectively. One possible explanation for such a difference is that the hGH reporter system is extremely sensitive, and the accumulation of hGH protein in the medium is 10-fold greater than the accumulation of CAT protein in cells. Another possible explanation is that the secretory pathway for ANG is constitutive in OK cells. Thus, the amount of hGH in the medium as measured by RIA-hGH represents the accumulated amount of the protein secreted by OK cells in a given time period. In contrast, the amount of cellular CAT activity as measured by CAT assays represents a lesser amount of protein retained by OK cells. The basal expression of various ANG-reporter fusion genes in different cell lines is shown in Table 4-1.

IV.2.2. The Promoter and Proximal Enhancer/Silencer of The Rat ANG Gene

At least six transcriptional response elements have been identified within 688 bp of the 5'-flanking region of the rat ANG gene using a DNase protection assay (footprinting) (Brasier et al., 1989). The TATAA box and CAAT box are located 30 bp and 50 bp upstream from the transcription start site (Tanaka et al., 1984). The CAT activities of pOCAT (ANG N-53/+18) and pOCAT (ANG N-35/+18) were 4- and 3-fold greater than that of pOCAT (Figure 3-6). One element located at position N-108 to N-60 in the rat ANG gene can serve as either a silencer or activator (SOAP box) of transcription depending upon its location with respect to the basal

TABLE 4-1. The Basal Expression of Various ANG-Reporter Fusion Genes in Different Cell Lines

	0	-1498/+18	-688/+18	-110/+18	-53/+18	-35/+18	
ANG-CAT [1]							
mouse hepatoma cell (Hepa1-6)	1.0	3.7	4.0	1.1	4.0	3.0	(1)
ANG-hGH [2]							
opposum kidney cell (OK)	1.0	200.0	4.5	1.1	11.0	2.5	(2)
rat pancreatic islet tumor cell (RIN1056A)	1.0	1.0	1.8	1.5	12.0	3.0	(2)
human placental choriocarcinoma cell (JEG-3)	1.0	1.0	1.0	1.0	1.0	1.0	(2)
ANG-Luc [3]							
human hepatoma cell (HAP G2)	1.0	4.0	5.0	1.0	3.5	-	(3)

[1] ANG-CAT fusion gene
[2] ANG-hGH fusion gene
[3] ANG-luciferase fusion gene

(1). Ming et al., 1993
(2). Chan et al., 1991
(3). Brasier et al., 1989

promoter. Deletion of the bases located between N-108 to N-60 resulted in an increase in the transcription of the ANG-luciferase fusion gene (Brasier et al., 1989). Analagous results were obtained in the present study (Figure 3-6). Either pOCAT (ANG N-110/+18) or pOGH (ANG N-110/+18) (Chan et al., 1991; Chan et al., 1992a) yielded an activity comparable to pOCAT or pOGH, respectively. Deletion of N-110 to N-54 resulted in increased transcription in both OK and Hepa 1-6 as well as in RIN 1056A cells (Table 4-1). Such suppression of transcription is not restricted to the angiotensinogen gene, because this element also down-regulates the transcription driven by the 72 bp repeat enhancer of the SV40 gene (Kadesch and Berg, 1986). This DNA element of the ANG gene, N-110 to N-54, acts independently of orientation but has narrowly defined spatial constraints in its capacity to suppress transcription optimally (Brasier et al., 1989). However, it can also activate transcription when located at a distance as great as 150 bp upstream from the ANG gene core promoter. There is a perfect 12 bp palindrome (5'-CTCTGTACAGAG-3') from N-77 to N-66 in this region. A substitution mutation in the palindrome abolished transcriptional activity, which suggests that the palindromic sequence may be a critical site for the binding of a trans-acting factor.

In summary, the expression of the rat ANG gene is cell line-specific. Multiple *cis*-regulatory elements control the expression of the ANG gene. The basal expression levels of various lengths of ANG-CAT fusion genes are different. Since the basal expression of pOCAT (ANG N-110/+18) is comparable to the CAT activity of pOCAT, and deletion of N-110 to N-54 results in increased transcription in both Hepa 1-6 and OK cells, it is highly likely that a silencer is located within N-110/-53.

IV.3. EFFECT OF STEROID HORMONES AND THYROID HORMONE ON ANG GENE EXPRESSION IN HEPA 1-6 AND OK CELL LINES

Studies *in vivo* and *in vitro* have shown that exposure of isolated hepatocytes or hepatoma cells to glucocorticoids increase the expression of ANG mRNA and ANG secretion (Cheng and Perlman, 1987; Ohtani et al., 1992; Feldmer et al., 1992; Hong-Brown and Deschepper, 1992).

Such studies indicate that expression of the ANG gene in the liver is under the control of glucocorticoids (Brasier et al., 1989; Ohtani et al., 1992). Whether other steroid hormones (estrogen, testosterone, and progesterone) and thyroid hormone can directly stimulate the expression of the ANG gene is less well characterized. Furthermore, it appears that the stimulatory effect of various hormones depends upon the condition of the isolated hepatocytes, e.g., Ben-Ari and Garrison (1988) reported that ethenyl estradiol did not cause a significant increase of ANG mRNA in isolated hepatocytes of Wistar rats, whereas Klett et al. (1992) obtained contradictory results in isolated hepatocytes of SD rats; or the types of hepatoma cell lines used, e.g., rat hepatoma cell lines Reuber H35 and FTO-2B did not respond to estrogen, but Fe33, a subclone of FTO-2B did respond to estrogen stimulation (Chang and Perlman, 1987; Ohtani et al., 1992; Feldmer et al., 1992).

IV.3.1. Effect of Dexamethasone on ANG Gene Expression

In the present study, we have shown that dexamethasone stimulates the expression of pOCAT (ANG N-1498/+18) in a concentration-dependent manner (10^{-12} to 10^{-4} M) in Hepa 1-6 cells (Figure 3-7). These results are in close agreement with the results of Brasier et al (1989) and Feldmer et al (1992), whose studies have shown that glucocorticoids directly stimulate the expression of ANG-luciferase fusion genes or ANG-CAT fusion genes in human and rat hepatoma cells, respectively. These studies demonstrate that glucocorticoids directly control the transcription of the ANG gene in liver cells.

With evidence for a localized renal renin-angiotensin system (Richou et al., 1983; Champbell et al., 1986; Seikaly et al., 1990), it is speculated that steroid hormones and thyroid hormone might also influence the expression of the ANG gene in the kidney. However, the few studies on hormonal regulation of the expression of the ANG gene in the kidney reported conflicting results. For example, Campbell and Habener (1986) reported that treatment with a combination of DEX, ethinyl estradiol, and thyroid hormone *in vivo* increased the abundance of ANG mRNA in rat liver, kidney, and several other organs. By contrast, studies by Kalinyak and Perlman (1987) in

rats showed that treatment with DEX increased ANG mRNA in both the liver and brain, but not in the kidney. Thus, it is not clear whether steroid hormones and thyroid hormone have a direct effect on the expression of the ANG gene in the kidney. In the present study, we therefore investigated the effect of steroid hormones on the expression of the ANG gene not only in Hepa 1-6 cells but also in the opossum kidney (OK) proximal tubular cells to determine the specific regions of the ANG gene that regulate its expression in both cell lines, and to ascertain the specific hormonal effects in renal cells as opposed to that in liver cells.

DEX stimulated the expression of pOCAT (ANG N-1498/+18) in a concentration-dependent manner (10^{-12} to 10^{-4} M) in OK cells (Figure 3-24). The addition of RU486, a glucocorticoid antagonist that competes for binding to glucocorticoid receptors (Yang et al., 1994), blocked the stimulatory effect of DEX (Figure 3-39). These results demonstrated that the effect of DEX on ANG gene expression is mediated via the glucocorticoid receptor complex, and confirmed reports of the presence of endogenous glucocorticoid receptors in OK cells (Vrtovsnick et al.; 1994).

Our results did not support the report of Kalinyak and Perlman (1987) that treatment with DEX alone in rats did not increase the level of ANG mRNA in the kidney. One possible explanation is that the results of these investigators were obtained *in vivo*, and our results were obtained *in vitro*. Thus, it could be argued that *in vivo*, other regulatory elements, involved in the transportation, distribution, metabolism and excretion of DEX, as well as the influence of other hormones, are involved (Ganon, 1990 f). All of these can modulate the effect of DEX on ANG gene expression in renal cells in the intact animal. A second possible explanation is a species difference between the rat and opossum. The observation that the stimulatory effect of DEX can be completely blocked by RU-486, which competes with glucocorticoids in their receptor binding (Figure 3-39), indicates that the stimulation is mediated via the glucocorticoid receptor in OK cells. These results are similar to that obtained by Ben-Ari and Garrion (1988) in rat hepatocytes showing that DEX stimulated ANG mRNA accumulation up to 3.5-fold and this effect was inhibited competitively by RU 486.

The expression of pOCAT (ANG N-1498/+18) was proportional to the concentration of DEX over the range of 10^{-12} - 10^{-6} M (0.5- to 3.5-fold compared with the control), but it was profoundly decreased at 10^{-4} M (from 3.5-fold to 1.5-fold) in OK cells (Figure 3-24). The decrease appeared to be due primarily to a high number of dead cells in the culture (approximately 30 to 50% of cell death as ascertained with a hemocytometer), whereas at lower concentration (10^{-6} M) no dead cells were observed. In contrast to OK cells, DEX (10^{-4} M) stimulated the expression of pOCAT (ANG N-1498/+18) to 2.2-fold ($p < 0.005$) over control in Hepa 1-6 cells (Figure 3-7). At such high concentration of DEX, cells grew well and no obviously dead cells were observed. The different response to high concentrations of DEX in OK and Hepa 1-6 cells may be explained by cell line specificity. The liver is the principal site of glucocorticoid catabolism. Enzymes in hepatocytes can convert glucocorticoids. Hepa 1-6 cells are derived from hepatomas, but several hepatocyte enzymes are still expressed in these cells (Darlington et al., 1980; Darlington, 1987). If the glucocorticoid catabolic pathway is retained, this could explain the tolerance of Hepa 1-6 cells to high concentration of DEX.

The present studies also showed that pOCAT (ANG N-1138/+18), pOCAT (ANG N-960/+18), pOCAT (ANG N-814/+18), and pOCAT (ANG N-688/+18) were responsive to the addition of DEX, but that pOCAT (ANG N-280/+18), pOCAT (ANG N-196/+18), pOCAT (ANG N-110/+18), pOCAT (ANG N-53/+18), and pOCAT (ANG N-35/+18) were not, and that this was true for both Hepa 1-6 (Figure 3-10) and OK cells (Figure 3-30). Thus, our results are in agreement with the report by Brasier et al. (1989), that the ANG sequence N-688 to N-110 contains functional glucocorticoid response elements. Indeed, on the basis of the DNA sequence reported by us (Chan et al., 1990) as well as by others (Ohkubo et al., 1983; Gailard et al., 1989; Brasier et al., 1990), two putative glucocorticoid-response elements have been identified in the rat ANG gene. One, located at N-585 to N-570 (GRE I), is composed of the sequence 5'-AGAACATTTTGTTTC-3'. It is a near-palindromic GRE. The other, at N-477 to N-472 (GRE II), is composed of the sequence 5'-AGAACA-3'. It is a hexameric "half-site" sequence (Brasier et al., 1990).

IV.3.2. Effect of Estrogen on ANG Gene Expression

It is generally accepted that the systemic renin-angiotensin system might play an important role in the pathogenesis of hypertension in pregnancy. Hypertension is present in 10 to 20% of pregnant women (Skinner et al., 1969; Lindheimer and Katz, 1986). Angiotensinogen increases gradually throughout pregnancy, and reaches about five times pre-pregnancy levels by the end of the third trimester in response to increasing estrogen levels (Skinner et al., 1975; Weir et al., 1975; Wilson et al., 1980). Animal experiments showed that after estradiol treatment of rats, the plasma ANG concentration (Hiwada et al., 1976; Krakoff and Eisenfeld, 1977; Shionoiri et al., 1983; Hassager et al., 1987) and ANG mRNA levels in the liver are increased (Ohkubo et al., 1986; Kunapuli et al., 1987). Estradiol also caused a significant increase in ANG mRNA in isolated hepatocytes (Klett et al., 1992). There was a two hour lag between exposure to estradiol and the rise in ANG mRNA. The rise in the ANG protein secretion rate was further delayed by about 2 hours with respect to the increase in mRNA, which corresponded to the transit time from translation to secretion of ANG. Therefore, it is surprising that cells transfected with pOCAT (ANG N-1498/+18) were not responsive to the addition of estradiol in both Hepa 1-6 and OK cells (Figures 3-8 ; 3-25). We attempted to use different concentrations of estradiol (10^{-12} to 10^{-6} M), but none of these concentrations appeared to stimulate the expression of pOCAT (ANG N-1498/+18) (data not shown) in either Hepa 1-6 cells or OK cells.

A putative estrogen response element (ERE) has been identified in human, mouse and rat ANG genes. In the human ANG gene, the ERE occurs at -337 to -324 (Fukamizu et al., 1986). In the mouse ANG gene, the ERE has been located closely associated with the GRE in the promoter region of the ANG gene (Clouston et al., 1988). Analysis of the 5'-flanking sequence of the rat ANG gene revealed that several putative estrogen response elements (CTATTCCAC, CCATCCAC, and CTTTACCAC) were located at N-655/-647, N-598/-591, and N-562/-554 of the ANG gene (Campbell and Habener, 1986). The observed sequences have approximately 80% homology with the consensus estrogen response element in chicken vitellogenin II genes (Jost et

al., 1984). Using a deletion mutation, Feldmer et al. (1992) found the estrogen effect was mediated by the region from -92 to -60 in the rat ANG gene, and there was a sequence (5'-GGTCC-3') between nucleotide -91 and -87, identical to one which had been reported to confer estrogen inducibility in the chicken ovalbumin gene promoter. Thus, we were puzzled by the lack of response of pOCAT (ANG N-1498/+18) to estrogen. A possible explanation for this observation may be that Hepa 1-6 cells lack functional estrogen receptors. As we did not measure the estrogen receptor, we are not sure if this is the case, but this possibility is supported by the results of other investigators. The hepatoma cell lines FTO-2B and H4IIE did not respond to estrogen stimulation. In contrast, the hepatoma cell line Fe 33, derived from FTO-2B, which had been stably transfected with the human estrogen receptor, did respond to estrogen stimulation with regard to ANG gene expression (Klett et al., 1992).

Using reverse transcription and the polymerase chain reaction, it was found that estrogen receptor mRNA was present in OK cells, and at physiologic concentrations (10^{-11} M), estrogen inhibited intracellular cAMP accumulation induced by PTH stimulation (Stock et al., 1992). A possible explanation for the reason that estrogen failed to stimulate the expression of the ANG-CAT fusion gene in OK cells may be the different incubation times. Stock et al. (1992) preincubated OK cells with estrogen for 7 to 10 days, whereas we carried out incubations for only 24 hours. The length of incubation affected estrogen effect on ANG gene expression has been indicated by other investigators (Ben-Ari and Garrison, 1988). In rat, estrogen is a potent stimulus of ANG synthesis, but the estrogen level must be elevated for several days before the effect is observed (Helmer et al., 1952; Clauser et al., 1983).

IV.3.3. Effect of Androgen on ANG Gene Expression

In the rat ANG gene, no androgen-response elements (AREs) have been identified to date. Wang et al. (1994) reported that testosterone treatment of both female and castrated male rats did not change the plasma ANG level or the hepatic ANG mRNA. However, other investigators reported that dihydrotestosterone may influence ANG mRNA levels in both hepatic and non-

hepatic cells (Ellison et al., 1989; Klett et al., 1992). In the present experiments, different concentrations of dihydrotestosterone (10^{-12} to 10^{-6} M) failed to increase ANG gene expression in Hepa 1-6 cells (data not shown). This result is in agreement with those of Wang et al. (1994) and Klett et al. (1992) showing that dihydrotestosterone injections did not affect plasma ANG concentration. Although Klett et al. (1992) found that dihydrotestosterone increased only marginally, but nonsignificantly, the amount of hepatic ANG mRNA compared to total RNA, the total RNA was significantly increased (about 1.6-fold), and consequently ANG mRNA was increased relative to tissue wet weight. A similar observation was reported for isolated hepatocytes exposed to dihydrotestosterone. Both *in vivo* and *in vitro* effects peaked at 2 hours. However, no increased ANG secretion was observed. The investigators proposed that dihydrotestosterone may induce transient sequestration of ANG mRNA into a nondegradable, and at the same time, nontranslatable form or compartment. This phenomenon has been observed in cells exposed to stress or in developmental systems (Jackson and Standart, 1990). In the present study, as the ANG mRNA was not detected after the addition of dihydrotestosterone, it is possible that ANG mRNA was also sequestered in Hepa 1-6 cells. Another possibility is that the Hepa 1-6 cells, as a cell line derived from hepatoma, lack the functional androgen receptor, and this is supported by the observation that the expression of some enzymes is diminished or absent in this cell line (Darlington and Bernhard, 1980; Darlington, 1987). As the androgen receptor was not measured in this cell line, this possibility can not be excluded.

Renal ANG mRNA levels in the male WKY rat increased significantly during puberty (Ellison et al., 1989). The renal ANG mRNA level in the adult female rat was similar to pre-pubertal levels and was considerably lower than that in the adult male rat. Castration lowered ANG mRNA levels in the male kidney by $\geq$60% (Ellison et al., 1989). Male rats castrated as weanlings and normal adult female rats implanted with testosterone displayed significant increases in renal ANG mRNA levels. These results suggested that androgen might be involved in the regulation of the ANG gene in the kidney.

Various investigators have demonstrated that androgens may affect the renal metabolic process (Maunsbach, 1966; Swank et al., 1973; Schiebler and Danner, 1978; Watson et al., 1981; Mackovic et al., 1986), stimulate the synthesis of many renal enzymes, and induce the hypertrophy of renal proximal tubular cells. The renotrophic effect of androgens is of particular interest with regard to the Na^+/H^+ exchanger, which secretes H^+ and resorbs HCO_3^- in the proximal tubule. Sex differences in the rate of Na^+/H^+ exchange in mouse kidney have been reported (Mackovic et al., 1986). These authors found testosterone could increase the rate of exchange by more than 100%, while castration of male mice decreased Na^+/H^+ exchange. Since androgens may stimulate the expression of renal ANG mRNA, and AII also increases Na^+/H^+ exchange in the proximal tubular cells, it is possible that the Na^+/H^+ exchanger may be stimulated by androgen regulated increases in AII production or the interaction of these two hormones.

Since the ANG mRNA level in OK cells is very low, it is very difficult to study the hormonal effect at the messenger RNA level; on the other hand, as the ANG mRNA induced by testosterone can be sequestered in hepatocytes (Klett et al., 1992), studies on mRNA level only are insufficient to determine whether the induced mRNA can be translated to ANG protein. Using gene transfection, our results did not show that dihydrotestosterone had any effect on ANG gene expression. Some possible explanations are: (a) mRNA sequestration: The induced ANG mRNA in OK cells was sequestered, as is known to occur in hepatocytes; (b) Sexual differences: OK cells were derived from kidney cells of an adult female American opossum. It has been reported that the renal ANG mRNA level in adult female rats is considerably lower than that in adult male rats (Ellison et al., 1989); (c) OK cells have lost the androgen receptor, or as a cell line derived from the female opossum kidney, never had the androgen receptor. As the androgen receptor was not measured in the present experiments, this possibility can not be excluded; (d) The incubation time with dihydrotestosterone was insufficient. Testosterone treatment in Ellison's experiments was performed over 20 days in both female and castrated male WKY rats. In the present experiment, OK cells were treated with dihydrotestosterone for only 24 hours.

IV.3.4. Effect of Progesterone on ANG Gene Expression

Progesterone may play an important role in the regulation of blood pressure and thus may be related to the pathogenesis of hypertension during pregnancy. However, no progesterone response elements (PREs) have been identified in the rat ANG gene and no studies have been reported on the effect of progesterone on ANG gene expression. We have found that addition of different concentrations of progesterone (10^{-12} to 10^{-6} M) failed to induce ANG gene expression in either Hepa 1-6 cells or OK cells. Possible explanations might be: (a) Progesterone influences the function of the renin-angiotensin system independently of increasing ANG gene expression. Studies have demonstrated that progesterone may increase plasma renin activity and the increased renin activity may produce more angiotensin II from its precursor, ANG (Fukamizu et al., 1986); (b) The progesterone receptor has been lost in both Hepa 1-6 cells and OK cells. Thus, the two cell lines did not respond to progesterone. To explore this possibility, the progesterone receptor levels should be measured in both cell lines; (c) There were no progesterone response elements (PREs) in the rat ANG gene. Gene expression is regulated by the interaction between the DNA binding proteins (transacting factors) and the specific DNA control elements in the 5'-flanking region of the transcribed genes (Maniatis et al., 1987). Lack of progesterone response elements could explain why progesterone fails to induce rat ANG gene expression in both Hepa 1-6 and OK cells.

IV.3.5. Effect of Thyroid Hormone on ANG Gene Expression

It is well recognized that thyroid hormones have profound effects on the cardiovascular system and blood pressure regulation. Hyperthyroid patients have an increased blood volume, and the reverse occurs in hypothyroidism (Anthonism et al., 1960). In perfused rat liver slices, ANG production was increased significantly in the presence of thyroid hormone (Ruiz et al., 1987; Semia et al., 1989). The release of ANG was decreased by 60% in hypothyroid animals and this decrease was reversed by thyroxine treatment (Murakami et al., 1981; Dzau and Herrmann, 1982;

Clauser et al., 1983). These studies indicate that thyroid hormone plays an important role in the development of hypertension in hyperthyroid patients via the renin-angiotensin system.

Thyroid hormone L-T_3 had no effect on the expression of the ANG-CAT fusion genes in Hepa 1-6 cells. We attempted to use different concentrations of L-T_3 (10^{-12} to 10^{-6} M) to stimulate the expression of pOCAT (ANG N-1498/+18), but none of these concentrations appeared to stimulate the expression of this fusion gene (data not shown). These results agree with the work of Ruiz et al. (1987) who showed that L-T_3 failed to stimulate ANG gene expression in hepatoma cell lines. However, these results are in contrast to the work of other investigators (Chang and Perlman, 1987; Darby et al., 1991; Hong-Brown and Deschepper, 1992). Their results have shown that L-T_3 stimulation increases ANG mRNA and ANG protein production in the rat hepatoma cell lines H35 and H4IIEC-3. The possible explanations for this discrepancy are: (a) Species and cell line specificity: Hepa 1-6 cells were derived form a mouse hepatoma, whereas H35 and H4IIEC-3 cells were derived from a rat hepatoma. However, even though both H35 and H4IIEC-3 cells (an H35 cell subclone) were derived from the same rat hepatoma, H35 were consistently more responsive to L-T_3 than H4IIEC-3 cells. Thus, this subline could have lost additional thyroid hormone receptors (Hong-Brown and Deschepper, 1992); (b) deficient thyroid hormone receptor: as a cell line derived from a mouse hepatoma, Hepa 1-6 cells have lost the thyroid hormone receptor. This possibility is supported by absence of various enzyme activities in this cell line as already alluded to. In order to further study this phenomenon, thyroid hormone receptor levels in Hepa 1-6 cells should be measured; (c) Growth status: subconfluent H35 cells did not respond to L-T_3, and this was not due to the number of thyroid hormone receptors (Hong-Brown and Deschepper, 1992). An ongoing synthesis of short lived proteins was a general requirement for the action of L-T_3 in liver cells (Seeling et al., 1982; Hamblin et al., 1987). It is therefore possible that induction of such proteins was elevated when the cells were in a confluent state. In the present study, in order to obtain a high transfection efficiency, the cells were transfected at 70-75% confluence, but not treated with hormones until they reached 90-95% confluence. Hence, the growth status is not likely to be a major factor in the present study.

The thyroid hormone, L-T_3, stimulates the Na^+/H^+ exchanger activity in cultured OK cells (Yonemura et al., 1990), suggesting that OK cells contain functional thyroid hormone receptors, and therefore thyroid hormones might have multiple functions in proximal tubular cells. Our studies have shown that, when induced by L-T3 (10^{-7} M), OK cells transfected with pOCAT (ANG N-1498/+18) displayed 1.9-fold CAT activity of the controls, lacking L-T_3 (Figure 3-25). These results demonstrated that L-T_3 could directly stimulate the transcription of the ANG gene in kidney cells. Furthermore, our results support the existence of a putative thyroid hormone response element within nucleotides N-1498 to N+18 of the rat ANG gene. Indeed, the structural analysis of the 5'-flanking region of the ANG gene revealed that a DNA sequence 5'-CCTGGCATGTCCT-3' was located at positions N-379 to N-367 (Chan et al., 1990) and this DNA sequence has 70% homology with a thyroid hormone response element in the rat growth hormone gene (Glass et al., 1987).

In summary, DEX stimulated the expression of ANG-CAT fusion genes in both Hepa 1-6 and OK cells via the glucocorticoid receptor. RU 486 completely blocked this effect. Other steroid hormones (estrogen, testosterone and progesterone) did not stimulate the expression of ANG-CAT fusion genes in either cell line. The possible explanations include: (a) Hepa 1-6 and OK cells lack the functional receptors. (b) These hormones influence the function of the RAS independently of ANG gene expression, for example progesterone could induce the expression of the renin gene but not the ANG gene. (c) To observe the effects of estrogen and androgen, longer incubation times may be necessary. (d) Finally, thyroid hormone (L-T3) stimulated the expression of ANG-CAT fusion genes in OK cells, but not in Hepa 1-6 cells.

IV.4. ROLE OF CYCLIC AMP ON THE EXPRESSION OF THE ANG GENE IN HEPA 1-6 AND OK CELLS

Many polypeptide hormones affect the functions of their target cells through the second messenger cAMP. These signals often cause profound changes in cellular morphology,

intermediary metabolism, and enzyme (protein) biosynthesis. The biological effects of cAMP are mediated not only through the action of cAMP-dependent protein kinase leading to phosphorylation of specific substrate proteins, thereby altering the activities or functions of these proteins (Taylor, 1989; Ganong, 1991e), but also through the synthesis of many proteins at the gene transcription level.

In eukaryotic cells, the effects of cAMP on gene expression have been shown to occur at one or several levels: (a) Cyclic AMP has been demonstrated to modulate the transcriptional activity of genes (Dynan, 1989; Ptashne and Gann 1990; Hunter and Karin, 1992). The cAMP response element (CRE) has been identified in many genes, and cAMP response element binding protein (CREB) has also been found in many cell types; (b) Cyclic AMP not only regulates the rate of transcription but also stabilizes the mRNA. It has been shown that cAMP affects the rate of degradation of specific mRNAs, including the phosphoenolpyruvate carboxykinase (PEPCK) and lactate dehydrogenase genes (Nachaliel et al., 1993; Chung et al., 1995).

The cAMP effect is often mediated by a short palindromic core motif, 5'-TGACGTCA-3' (designed as a cAMP-response 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 response element displays properties of a classical enhancer sequence, which stimulates transcription at a distance, and is functionally independent of orientation. This CRE core motif is also observed in promoters of certain viruses including HTLV-I and -II, cytomegalovirus and adenovirus.

Activation of the transcription of a cAMP-responsive gene is initiated by the binding of a CRE by the cAMP response element binding protein (CREB). There are three CREs in the regulated region of the CREB gene. Several potential phosphorylation sites for protein kinase A (PKA), protein kinase C (PKC), and casein kinase II have been found in the CREB molecule. Experiments have shown that the phosphorylation of CREB plays an important role in its biological function. For instance, phosphorylation of CREB at a single residue, Ser 133, within

the kinase A motif, is critical for transcriptional activation (Gonzales and Montminy, 1989). Thus, cAMP may not only stimulate CREB gene expression to increase the CREB level but also activate CREB to bind to a CRE.

The effect of cAMP on gene transcription is rapid. In the transcription of the tyrosine hydroxylase gene induced by cAMP in PC12 cells, the peak occurred within 30 minutes of forskolin treatment and declined gradually over 24 hours (Bowyer et al., 1992).

A putative CRE, 5'-TGCGTCA-3', was 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 DNA structure of the 5'-flanking sequence of the rat ANG gene showed that the DNA sequence of nucleotides N-795 to N-788 (5'-TGACGTAC-3') was almost identical to the consensus CRE of the somatostatin gene (Montminy et al., 1986), except that the last two nucleotides were in the reverse order. It was termed a 'CRE-like' response element. This observation raised the possibility that cAMP may stimulate the expression of the rat ANG gene via this putative CRE.

IV.4.1. cAMP Effect on ANG Gene Expression in Hepa 1-6 Cells

8-Bromo-cAMP alone did not stimulate the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells, whereas 8-Bromo-cAMP enhances the effect of DEX on the expression of pOCAT (ANG N-1498/+18) (Figures 3-8, 3-9, 3-10). Our results are in agreement with the studies of Ohtani et al. (1992), who reported that whereas the basal secretion of ANG decreased during the period of culture in primary cultures of rat hepatocytes, the addition of DEX and $(Bu)_2$-cAMP completely prevented this decrease. ANG secretion by freshly plated hepatocytes was slightly increased in response to DEX, but after 24 hours in culture, hepatocytes no longer responded to DEX alone. However, after treatement with $(Bu)_2$-cAMP, glucagon, or forskolin, ANG secretion increased in response to DEX in a concentration-dependent manner. DEX stimulated ANG mRNA accumulation 3.5-fold after 2.5 to 3 hours of incubation in isolated rat liver cells (Ben-Ari and

Garrison, 1988). The stimulatory diastereomer of adenosine 3', 5'-cyclic phosphorothioate, an active adenosine 3', 5'-cyclic monophosphate analogue, alone caused a 1.8-fold increase in ANG mRNA accumulation, and this effect was additive with that of DEX. Hence, DEX and cAMP effects depend on cell type and culture conditions.

Indeed, 8-Bromo-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 (Figure 3-8), whereas 8-Bromo-cAMP alone stimulated the expression of the deleted mutants pTKCAT (ANG N-814/-761) and pTKCAT (ANG N-806/-779), which contain the putative CRE, in these cells (Figures 3-13, 3-14). The molecular mechanism responsible for the absence of the effect of 8-Bromo-cAMP alone on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells might be that there are some inhibitory factors in Hepa 1-6 cells which may counteract the stimulatory effect of 8-Bromo-cAMP on the expression of the full length ANG gene. The addition of DEX may decrease the effect of these inhibitory factors. Thus, the effect of cAMP on ANG gene expression appeared only in the presence of DEX. This possibility is supported by studies showing that following deletions that removed the presumed binding element(s) for such inhibitory factors but in which the putative CRE was retained (pTKCAT (ANG N-814/-761) and pTKCAT (ANG N-806/-779)), 8-Bromo-cAMP alone was able to induce the expression of the fusion genes in Hepa 1-6 cells.

IV.4.2. The Role of cAMP on ANG Gene Expression in OK Cells

In contrast to the situation for Hepa 1-6 cells, our studies showed that addition of cAMP directly stimulated the expression of pOCAT (ANG N-1498/+18) in a concentration-dependent manner in OK cells (Figure 3-27). At present, the mechanism is unclear with regard to the differential response to 8-Bromo-cAMP on the expression of the full length ANG-CAT fusion gene in OK and Hepa 1-6 cells. One possible explanation is cell line specificity. We have shown different levels of expression of ANG-CAT fusion genes in Hepa 1-6 and OK cells (section IV.2.1.). The expression of pOCAT (ANG N-1498/+18) in Hepa1-6 cells is only 3.7-fold that of the promoterless pOCAT, while the expression of the same fusion gene in OK cells is 20-fold that

of the pOCAT. It has been reported that the expression of the ANG gene in response to hormones differs markedly in liver compared to other tissues (Kalinyak and Perlman, 1987, Kunapuli et al., 1987). Many genes have been identified containing cell-specific enhancers, and the transacting factors vary in cell lines from different species and tissue sources. Many of them are functional in some cell lines, but inactive in others (Leven, 1990).

We also found in OK cells, that pOCAT (ANG N-1136/+18), pOCAT (ANG N-960/+18), pOCAT (ANG N-814/+18), and pOCAT (ANG N-688/+18) were responsive to the addition of cAMP, but that pOCAT (ANG N-280/+18), pOCAT (ANG N-196/+18), pOCAT (ANG N-110/+18), pOCAT (ANG N-53/+18) and pOCAT (ANG N-35/+18) were not (Figure 3-30). These results indicated that a putative CRE was located in the 5'-flanking region (ANG N-1498 to N-280) of the rat ANG gene.

To assess whether the putative CRE (ANG N-795/-788) mediated the stimulatory effect of cAMP on ANG gene expression, mutants of different lengths, either with the putative CRE [pTKCAT (ANG N-814/-689), pTKCAT (ANG N-814/-761), and pTKCAT (ANG N-806/-779)] or without the putative CRE [pTKCAT (ANG N-760/-689)] were used for gene transfection experiments. Addition of 8-Bromo-cAMP or forskolin stimulated the expression of pTKCAT (ANG N-814/-761) to 2.6- to 3-fold compared with controls, lacking cAMP and forskolin (Figure 3-31). Deletion of the DNA sequence in both 5'- and 3'-flanking regions located between N-814 and N-761, as in the pTKCAT (ANG N -806/-779) construct did not alter the response to either 8-Bromo-cAMP (2.1-fold) or forskolin (1.9-fold) (Figure 3-32), whereas the stimulation of the expression of pTKCAT (ANG N-814/-689) and pTKCAT (ANG N-760/-689) were only 1.4- and 1.2-fold, respectively.

Although 8-Bromo-cAMP or forskolin increased the expression of pTKCAT (ANG N-814/-689) about 0.4-fold, we do not know the reason for such a low stimulation with cAMP and forskolin compared to pTKCAT (ANG N-814/-761) (1.6- to 2-fold increase). The similar results was also obtained in Hepa 1-6 cells. One possible explanation is that the DNA sequence ANG N-

760/-689 may interact with a CREB-associated protein(s), which may modulate or inhibit the effect of cAMP. Following a deletion removing the presumed binding elements for the CREB-associated proteins, either 8-Bromo-cAMP or forskolin can induce the expression of the remainder, pTKCAT (ANG N-814/-761), more efficiently. Indeed, this possibility is supported by the report of Miller et al. (1993) that CREB-associated proteins bind to the DNA sequence adjacent to the CRE motif of the glucagon gene and inhibit the response of the gene to cAMP stimulation.

In OK cells, 8-Bromo-cAMP alone stimulated the expression of pOCAT (ANG N-688/+18), but not the expression of pOCAT (ANG N-280/+18) (Figure 3-30). In Hepa 1-6 cells, 8-Bromo-cAMP enhanced the effect of DEX on the expression of pOCAT (ANG N-688/+18), but not the expression of pOCAT (ANG N-280/+18) (Figure 3-10). These results were puzzling as no CRE motif has been described in the segment ANG N-688/-280 to date, hence a response to cAMP stimulation was not expected. One possible explanation is that there is another CRE between the nucleotides N-688 to N-280 of the ANG gene, and its sequence differs from the concensus CRE, because we have not found any consensus CRE motif within this region. Another possibility relates to glucocorticoid recepter phosphorylation. Both GREI and GREII are located in ANG N-688/-280. The addition of 8-Bromo-cAMP may activate the PKA pathway which may then phosphorylate the glucocorticoid receptor, sequentially enhancing the binding affinity of the glucocorticoid receptor for GRE and thereby increasing ANG gene transcription. This possibility has been described in model II (section I.6). However, the results of our study do not support it. Indeed, the addition of 8-Bromo-cAMP did not enhance the effect of DEX on the expression of other ANG GRE(s) constructs, pTKCAT (ANG N-591/-465), pTKCAT (ANG N-591/-563), and pTKCAT (ANG N-485/-465) compared with DEX alone (Figures 3-48, 3-49, 3-50, and 3-51). Hence, these data indicate that phosphorylation of the glucocorticoid receptor does not play an important role on the expression of pOCAT (ANG N-688/+18) induced by 8-Bromo-cAMP. This raises the possibility, therefore, that other sequences within ANG N-688/-592 or ANG N-464/-280 are involved in a cAMP-dependent modulation of ANG expression.

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. DEX plus 8-Bromo-cAMP acted additively to stimulate the transcription of the ANG gene (Figure 3-27), whereas DEX plus forskolin acted synergistically to stimulate ANG gene expression in OK cells (Figure 3-28). Moreover, our studies also showed that the stimulatory effect of 8-Bromo-cAMP plus DEX on the expression of pOCAT (ANG N-1498/+18) was no more than 2- to 3-fold compared with controls (in the absence of both DEX and cAMP) (Figures 3-8, 3-9, 3-10, 3-27, 3-30). Such observations are in agreement with the studies of Ohtani et al. (1992), who showed the maximal effect of DEX plus dibutyryl cAMP [(db)-cAMP] on ANG secretion by primary cultured hepatocytes was about 2- to 3-fold over the control.

In summary, 8-Bromo-cAMP alone did not stimulate the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells, but it enhanced the effect of DEX. Hence, 8-Bromo-cAMP potentiated the effect of DEX. In contrast, 8-Bromo-cAMP alone stimulated the expression of pOCAT (ANG N-1498/+18) in OK cells, and 8-Bromo-cAMP plus DEX acted additively on the expression of this fusion gene. Explanations for the different responses to 8-Bromo-cAMP in Hepa 1-6 and OK cells may be explained by the presence of cell-specific enhancers in the rat ANG gene, or by the effect of different transacting factors in these two cell lines (see IV. 5.1.). In contrast, in both Hepa 1-6 and OK cells, 8-Bromo-cAMP alone stimulated the expression of pTKCAT (ANG N-814/-761) and pTKCAT (ANG N-806/-779), both deleted mutants containing the putative CRE. These data provide evidence that the putative CRE may mediate the effect of 8-Bromo-cAMP on ANG gene expression.

IV.5. ROLE OF ISOPROTERENOL ON THE EXPRESSION OF THE ANG GENE IN HEPA 1-6 AND OK CELLS

Isoproterenol is generally viewed as a pure β-adrenoceptor agonist. The binding of isoproterenol with its membrane receptors activates G-proteins. The activated G-proteins stimulate adenylate cyclase to form cAMP, the important second messenger, which activates protein kinase

A (PKA). The activated PKA can phosphorylate various substrates, including enzymes and transcription factors. Phosphorylation and dephosphorylation are important mechanisms that can alter protein activity and therefore the physiological functions of the cell. Isoproterenol is known to stimulate the synthesis of hepatic intracellular cAMP via the β-adrenoceptor and we have demonstrated that 8-Bromo-cAMP stimulated the expression of the ANG-CAT fusion gene pOCAT (ANG N-1498/+18) in Hepa 1-6 cells and OK cells. We thus wished to investigate whether isoproterenol had any effect on the expression of the ANG gene in these cell lines.

IV.5.1. β-adrenoceptors and ANG Gene Expression in Hepa 1-6 Cells

An increased activation of the sympathetic nervous system (Sesoki et al., 1987; Anderson et al., 1989) and of the renin-angiotensin axis (Laragh et al., 1960; Ito et al., 1980) is believed to be involved in the pathogenesis of hypertension. Numerous *in vitro* and *in vivo* studies have shown that AII enhances the release of norepinephrine by postganglionic sympathetic nerve termini (Hughes and Roth, 1971; Zimmerman, 1981; Matsukawa et al., 1991). Administration of captopril (an ACE inhibitor) significantly decreased smooth muscle sympathetic nerve activity in accelerated hypertensive patients as compared to normotensive patients, suggesting that levels of plasma norepinephrine, which may reflect sympathetic nerve activity in the hypertensive patients, could depend on the concentration of AII (ie, activation of the renin-angiotensin system) (Matsukawa et al., 1993). On the other hand, there are only a few studies demonstrating that catecholamines have an effect on the release of AII (Nakamaru et al., 1986b; Richards et al., 1989a; Tang et al., 1990). Isoproterenol (10^{-6} to 10^{-4} M) caused an increase in the release of AII from isolated perfused mesenteric arteries (Nakamaru et al., 1986a). The increases in AII release were blocked by propranolol and captopril ($2x10^{-6}$ M). A high dosage of isoproterenol (100 mM) significantly stimulated the release of AII from neuronal cultures (Richards et al., 1989b). Isoproterenol (10^{-7} to 10^{-5} M) increased the secretion of angiotensin from cultured bovine aortic endothelial cells in a concentration-dependent manner (Tang et al., 1990). The addition of ICI 118,551 (10^{-6} M), but not atenolol (10^{-6} M) blocked the effect of isoproterenol. The infusion of

isoproterenol into the brachial artery of hypertensive subjects stimulated a local outflow of AII (Taddei et al., 1991; Taddei and Salvetti, 1992).

These studies showed that release of locally generated AII by isoproterenol was mediated by β-adrenoceptors. However, there are no reports demonstrating that isoproterenol has an effect on the expression of the ANG gene in hepatic and/or extrahepatic cells.

Our studies have shown that isoproterenol (10^{-5} M) alone did not significantly stimulate the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells (Figure 3-15). We attempted to use different concentrations of isoproterenol (10^{-13} - 10^{-5} M) to stimulate the expression of this ANG-CAT fusion gene, but none of them had a significant effect (data not shown). However, the addition of isoproterenol enhanced the stimulatory effect of DEX on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells (Figure 3-15), indicating that isoproterenol potentiated the effect of DEX on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells. That isoproterenol alone did not stimulate the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells could not be explained by the lack of β-adrenoceptors in this cell line, because isoproterenol alone stimulated the expression of another ANG-CAT fusion gene, pTKCAT (ANG N-814/-761), which was a deleted mutant containing the putative CRE (Figure 3-16), and isoproterenol enhanced the effect of DEX on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells. Both these effects of isoproterenol had to be mediated by the membrane receptor. It has been demonstrated that β_1-, β_2-, and β_3-adrenoceptor subtypes are present in the liver (Schmelck and Hanoune, 1980; Sulakle, 1987; Lefkowitz and Caron, 1988) and isoproterenol increases liver cAMP levels (Hagino and Shigei, 1976). The present study shows that the addition of propranolol (a non-selective β-adrenoceptor antagonist) or ICI 118,551 (a selective β_2-adrenoceptor antagonist) inhibited the potentiating effect of isoproterenol on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells stimulated by DEX, but the addition of atenolol (a selective β_1-adrenoceptor antagonist) had no inhibitory effect (Figures 3-18, 3-19, 3-20). These studies demonstrate that the enhancing effect of isoproterenol in Hepa 1-6 cells is mediated by the β_2-adrenoceptor, not the β_1-

adrenoceptor. This is in agreement with the studies of other investigators (Graziano et al., 1985; Schmelck and Hanoune, 1980) who demonstrated that β_2-adrenoceptors predominated in the liver.

Our results on isoproterenol are in close agreement with our previous results with 8-Bromo-cAMP (Ming et al., 1993). 8-Bromo-cAMP alone did not significantly stimulate the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells, but it enhanced the stimulatory effect of DEX on the expression of this fusion gene (Figures 3-8, 3-9, 3-10). The current investigations indicate that the effect of isoproterenol may be mediated by the cAMP-dependent protein kinase pathway. Indeed the addition of Rp-cAMP (an inhibitor of cAMP-dependent protein kinase AI and II) (Landsberg and Jastorff, 1985) inhibited the enhancing effect of isoproterenol (Figure 3-17). It has been reported that β_2-adrenoceptor couples with various signaling pathways, such as, PKC, ras, and mitogen-activated protein kinase; it is possible that the ANG gene expression induced by isoproterenol in Hepa 1-6 cells is mediated by one of these other transduction pathways. However, our results do not support this possibility. We have shown that the effect of isoproterenol on ANG gene expression is very similar to the effect of 8-Bromo-cAMP, forskolin, and glucagon (Figure 3-8, 3-11, 3-12 and 3-15). All three are activators of the PKA pathway. The effect of isoproterenol on ANG gene expression can be blocked by Rp-cAMP (an inhibitor of cAMP dependent protein kinases AI and II). Isoproterenol alone can stimulate the expression of pTKCAT (ANG N-814/1761), which contains the putative CRE and no other response element has been demonstrated to locate in this region.

IV.5.2. β-adrenoceptors and ANG Gene Expression in OK Cells

Low levels of renal nerve stimulation decrease sodium excretion and increase the level of ANG mRNA in the rat kidney *in vivo*. The administration of the β_1-adrenoceptor antagonist, atenolol, blocked the effect of renal nerve stimulation (Nakamura and Johns, 1994). This observation was supported by our previous studies (Wang et al., 1994) that isoproterenol directly stimulated the expression of the ANG gene in a concentration-dependent manner in OK 27 cells, an

OK cell line into which a fusion gene had been stably integrated. This fusion gene, pOGH (ANG N-1498/+18), contains the 5'-flanking sequence of the rat ANG gene fused with the coding sequence of the human growth hormone (hGH) gene as a reporter. The stimulatory effect of isoproterenol in OK 27 cells was blocked by the presence of Rp-cAMP, propranolol, and atenolol. The presence of ICI 118,551, however, was 1,000-fold less effective in inhibiting the effect of isoproterenol compared to atenolol, which suggested that the effect of isoproterenol was probably mediated via the β_1-adrenoceptor, not the β_2-adrenoceptor, in OK 27 cells.

It was surprising that isoproterenol alone did not stimulate the expression of either pOCAT (ANG N-1498/+18) (Figures 3-33, 3-35), pTKCAT (ANG N-814/-761), or pTKCAT (ANG N-806/-779) (data not shown) in OK cells, whereas 8-Bromo-cAMP or forskolin, a direct activator of adenylyl cyclase, both appeared to stimulate the expression of the three ANG-CAT fusion genes in OK cells (Figures 3-27, 3-28). These data conformed to the presence of a functional adenylate cyclase in OK cells.

The different response to isoproterenol in OK and OK 27 cells might be explained by the fact that OK 27 is a more homogeneous cell line. It is a monoclonal cell line derived from OK cells which had stably integrated a fusion gene, pOGH (ANG N-1498/+18), and had been clonally selected in our laboratory. The OK cells that were obtained from ATCC are somewhat heterogeneous. It is also conceivable that several passages after the OK cells were obtained from ATCC, some cells expressed β-adrenoceptors whereas others did not. Thus, the amount of β-adrenoceptors in OK cells may be too low to mediate the effect of isoproterenol on the expression of the ANG gene. This possibility was supported by the results of Cheng et al. (1990), showing in OK cells, dopamine stimulated cAMP production via the DA_1 receptor, whereas isoproterenol had no effect on cAMP production. A high concentration of isoproterenol (10^{-5} M) did not stimulate the expression of pOCAT (ANG N-1498/+18) in OK cells, but it potentiated the effect of DEX (Figure 3-33). A possible explanation for this phenomenon is that DEX increased the sensitivity of the remaining β-adrenoceptors to the stimulation of catecholamines (Stiles et al.,

1984). This is supported by the studies of other investigators (Allolio et al., 1997, Chong et al., 1997) showing that DEX increases the β-adrenoceptor density and reduces receptor desensitization. Several consensus sequences for glucocorticoid response elements exist in the human β-adrenoceptor genes, and exposure to glucocorticoids leads to an enhanced rate of transcription of β-adrenoceptor mRNA (Collins et al., 1988). Glucocorticoids also reverse the down-regulation of β-adrenoceptors exposed to β-agonists. Pretreatment of human lung mast cells with DEX protected against the functional desensitization of β-adrenoceptors induced by incubation with isoproterenol (Chong et al., 1997). In order to further study this phenomenon, β-adrenoceptors should be measured before and after the transfection of β-adrenoceptor cDNA in the presence versus the absence of DEX in this cell line.

To investigate the isoproterenol effect on ANG gene expression, we cotransfected β-adrenoceptors and ANG-CAT fusion genes into OK cells. Our studies showed that co-transfection of the plasmid containing β_1-adrenoceptor cDNA (pBC-β_1AR) or β_2-adrenoceptor cDNA (pBC-β_2AR) stimulated the basal expression of pOCAT (ANG N-1498/+18) (Figure 3-34), suggesting that these receptors may directly couple to the adenylyl cyclase system in OK cells. Indeed, studies by Suzuki et al. (1992) have shown these β_1- and β_2- adrenoceptor expression vectors are positively coupled to adenylyl cyclase when transfected into Chinese hamster fibroblasts.

The addition of isoproterenol (10^{-5} M) stimulated the expression of pOCAT (ANG N-1498/+18) when cotransfected with pBC-β_1AR but not with pBC-β_2AR (Figure 3-35). These studies confirmed our previous studies (Wang et al., 1994) that isoproterenol stimulated the expression of pOGH (ANG N-1498/+18) in OK 27 cells via the β_1-adrenoceptor. At present, we do not known why isoproterenol fails to stimulate ANG gene expression via the β_2-adrenoceptor in OK cells. One likely explanation is that there is a different degree (or rate) of desensitization (or sequestration) for the β_1-and β_2-adrenoceptors when exposed to high levels of agonists. This possibility is supported by studies demonstrating that the β_2-adrenoceptor is subjected to sequestration by its own agonist much faster than the β_1-adrenoceptor (Suzuki and Bouvier,

1992). Both the β_1-adrenoceptor cDNA and β_2-adrenoceptor cDNA were gifts from Dr. Bouvier (University of Montreal) who has reported that in Chinese hamster fibroblasts which were transfected with either β_1-adrenoceptor cDNA or β_2-adrenoceptor cDNA, treatment of the cells expressing the β_2-adrenoceptor with isoproterenol leads to a rapid sequestration of >30% of receptors away from the cell surface; in contrast, virtually no agonist-induced sequestration is observed in the cells expressing the β_1-adrenoceptor. Longer exposure of the cells to isoproterenol leads to a time-dependent reduction in the total number of β-adrenoceptors in both β_1-adrenoceptor and β_2-adrenoceptor expressing cell lines. However, this down-regulation is significantly slower in the cells expressing the β_1-adrenoceptor. In fact, no appreciable down-regulation of the β_1-adrenoceptor is detected in the first hour of isoproterenol treatment, compared with >50% down-regulation of β_2-adrenoceptors over the same time period. After 24 hours of treatment with isoproterenol, 20% of the original number of β_2-adrenoceptors remain, whereas 60% of the β_1-adrenoceptors are still present after the same treatment (Suzuki et al., 1992).

The addition of DEX directly stimulated the expression of pOCAT (ANG N-1498/+18) in a concentration-dependent manner in OK cells and the combination of both DEX and isoproterenol acted synergistically on the expression of this fusion gene when cotransfected with pBC-β_1AR (Figure 3-36) but not with pBC-β_2AR (Figure 3-37). These data confirmed our studies that DEX directly stimulated the expression of pOCAT (ANG N-1498/+18) (Figure 3-24), and that DEX plus 8-Bromo-cAMP acted additively on ANG gene expression (Figures 3-27, 3-30) in OK cells. It is unclear why 8-Bromo-cAMP plus DEX acted additively on the pOCAT (ANG N-1498/+18) expression in OK cells (Figure 3-28), whereas isoproterenol plus DEX acted synergistically on expression of the same ANG gene construct in OK cells cotransfected with the β1-adrenoceptor. It is possible that the permeability of 8-Bromo-cAMP is low, and the intracellular 8-Bromo-cAMP level is too low to mediate a synergistic effect with DEX. This possibility is supported by the fact that only high concentrations of 8-Bromo-cAMP (10^{-3} M) can stimulate the ANG-CAT fusion gene expression in OK cells, while forskolin at 10^{-5} M can act as efficiently as 8-Bromo-cAMP (10^{-3}

M). Forskolin plus DEX acted synergistically in inducing pOCAT (ANG N-1498/+18) expression (Figure 3-28) as did isoproterenol plus DEX. To further study this possibility, intracellular cAMP levels should be measured in OK cells after 8-Bromo-cAMP and forskolin treatment. However, after cotransfection with β_1-adrenoceptor cDNA, the β_1-adrenoceptor will be expressed at the plasma membrane, which can be demonstrated by the different responses to isoproterenol stimulation between pBC-β_1AR cotransfected cells and non-cotransfected cells. The binding of isoproterenol with the receptor will activate Gs proteins and adenylate cyclase, leading to increased intracellular cAMP and activation of PKA. This is more likely a physiological stimulation rate and the increased intracellular cAMP level is not dependent on the permeability of 8-Bromo-cAMP. This possibility is supported by the result that the addition of isoproterenol increases intracellular cAMP in OK 27 cells (Wang et al., 1994).

The addition of Rp-cAMP blocked, in a concentration-dependent manner, the stimulatory effect of isoproterenol on the expression of pOCAT (ANG N-1498/+18) in OK cells co-transfected with β_1-adrenoceptor cDNA (Figure 3-41). The data indicate that the effect of β_1- adrenoceptors on the expression of the ANG gene is mediated via the cAMP-dependent protein kinase A pathway. The addition of Rp-cAMP alone only partially blocked the stimulatory effect of DEX plus isoproterenol on the expression of pOCAT (ANG N-1498/+18) when cotransfected with pBC-β_1AR (Figure 3-42), whereas the pre-incubation (overnight) with RU486 completely blocked the effect of DEX plus isoproterenol. The combined addition of RU486 and Rp-cAMP also completely blocked the stimulation by DEX plus isoproterenol on the expression of pOCAT (ANG N-1498/+18) cotransfected with pBC-β_1AR. The reason that pre-incubation with RU486 can completely inhibit the synergistic effect of DEX plus isoproterenol might be explained as follows (a) GREs have been identified in β-adrenoceptor genes and exposure to DEX enhances the rate of transcription of β-adrenoceptor mRNA (Collins et al., 1988) and increases the expression of β-adrenoceptors (Davies et al., 1981) as well as increases the efficiency of the coupling mechanism of the receptors to adenylyl cyclase (Stiles et al., 1984); (b) DEX enhances the sensitivity of β-adrenoceptors to catecholamines via reversing the down-regulation of β-adrenoceptors exposed to

β-agonists (Stiles et al., 1984); (c) DEX positively affects the PKA pathway, as supported by the studies of Chang and Bourne (1987) showing that glucocorticoids activate adenylyl cyclase in GH3 cells.

In summary, the mechanisms for the absence of effect of isoproterenol alone on the expression of pOCAT (ANG N-1498/+18) in both Hepa 1-6 and OK cells are different. In Hepa 1-6 cells, the effect of isoproterenol on the expression of pOCAT (ANG N-1498/+18), as well as the effect of 8-Bromo-cAMP, depended on the presence of DEX. But isoproterenol alone stimulated the expression of pTKCAT (ANG N-814/-761) and pTKCAT (ANG N-806/-779), both deleted mutants containing the putative CRE. These results provided evidence that the lack of stimulation of the expression of pOCAT (ANG N-1498/+18) by isoproterenol in Hepa 1-6 cells was not due to the lack of functional β-adrenoceptors.

In OK cells, isoproterenol alone did not stimulate the expression of pOCAT (ANG N -1498/+18), pTKCAT (ANG N-814/-761) or pTKCAT (ANG N-806/-779). These results were different from those elicited with 8-Bromo-cAMP and forskolin. As 8-Bromo-cAMP is an analog of cAMP, the second messenger of isoproterenol, and forskolin is a post-receptor activator of the adenylate cyclase, their effects on ANG gene expression are plasma membrane independent and their effectiveness indicated that adenylate cyclase in OK cells is functional. These data raise the possibility that isoproterenol did not stimulate the expression of the ANG-CAT fusion gene in OK cells due to the lack of functional β-adrenoceptors. This is supported by the results of Cheng et al. (1990) showing that in OK cells, dopamine stimulated cAMP production via DA_1 receptors, whereas isoproterenol had no effect on cAMP production, indicating a deficiency of β-adrenoceptors in OK cells.

In Hepa 1-6 cells, isoproterenol potentiated the effect of DEX on the expression of pOCAT (ANG N-1498/+18), whereas in OK cells, isoproterenol plus DEX acted synergistically on the expression of the same fusion gene when cotransfected with β_1 adrenoceptor cDNA. Both the

potentiating and synergistic effect could be blocked by either Rp-cAMP or RU 486. These data demonstrated that both glucocorticoid receptors and the PKA pathway were involved. ICI 118,551 blocked the potentiating effect of isoproterenol in Hepa 1-6 cells. However, the synergistic effect was observed in OK cells only when they were cotransfected with β_1-adrenoceptor cDNA. These results indicate that the effect of isoproterenol was mediated by β_2-adrenoceptors in Hepa 1-6 cells, whereas in OK cells the effect of isoproterenol was mediated by β_1-adrenoceptors.

IV.6. THE MOLECULAR MECHANISM OF THE SYNERGISTIC EFFECT OF GLUCOCORTICOID PLUS ISOPROTERENOL ON THE EXPRESSION OF THE ANG GENE IN HEPA 1-6 AND OK CELL LINES

Transcriptional control by steroid hormones is mediated by a family of nuclear receptors which are activated by their ligands, and bind to *cis* regulatory elements in specific target genes (Beato, 1989; Tsai and O'Malley, 1994). Some studies have provided evidence of functional interactions between the nuclear receptors and other transcription factors (Drouin et al., 1987; Strahle et al., 1988; Akerblom et al., 1988; Schule et al., 1988a; Schule et al., 1990; Yang et al., 1990). In particular, this has been shown in the case of glucocorticoid-inducible genes which frequently show GREs contiguous with DNA regulatory sequences for other transcription factors. These include binding sites for SP1, NF1, CACCC and CCAAT box binding proteins (Strahle et al., 1988; Schule et al., 1988b), CREB (Drouin et al., 1987; Akerblom et al., 1988) and AP-1 factors (Schule et al., 1990; Yang et al., 1990), or a second GRE (Strahle et al., 1988). Interaction between the glucocorticoid receptor and these nuclear DNA binding proteins can lead to either enhancement or repression of gene transcription.

Synergism between the glucocorticoid receptor and other transcription factors was first reported for NF1, SP1, and CACCC binding proteins in genes encoding tyrosine aminotransferase and tryptophan oxygenase (Strahle et al., 1988; Schule et al., 1988b). Functional cooperation in these instances was shown to be critically dependent on the spacing between the regulatory

elements but not on their orientation, and it is mediated by protein-protein interaction rather than cooperative DNA binding (Strahle et al., 1988; Schule et al., 1988a, 1988b). These results further revealed that a single copy consensus GRE sequence isolated at a distance from the transcription start site was insufficient for gene induction, but became active when a second regulatory element including a second GRE was positioned nearby (Strahle et al., 1988). This suggests a basic model of glucocorticoid-induced transcriptional activation requiring multiple GREs or a combination of a GRE with other *cis* elements to constitute a hormone-inducible enhancer.

Our results showed that both pTKCAT (ANG GRE I/GRE II) (ANG N-591/-465); and pTKCAT GRE I (ANG N-591/-563) responded to the addition of DEX (Figures 3-48, 3-49, 3-51), while pTKCAT GRE II alone (ANG N-485/-465) did not respond to DEX (Figures 3-50, 3-51). The reason that ANG GRE II did not respond to the stimulation of DEX might be explained if it is not a glucocorticoid response element in the rat ANG gene, despite the fact that it has a high degree of homology with the consensus GRE sequence. Another possibility is GRE II by itself is insufficient to respond to the stimulation by DEX. Several observations have indicated that the presence of one GRE is not sufficient for hormone inducibility (Jantzen et al., 1987; Brasier and Li, 1996), but another GRE or other regulatory elements are required. In the first 600 base pairs of the 5'-flanking region of the rat ANG gene, there is a near-palindromic GRE I containing the sequence 5'-AGAACATTTTGTTTC-3' (-585 to -570). The present studies have shown that GRE I is essential for glucocorticoid stimulation (Figures 3-49, 3-51). In contrast, GRE II contains a hexameric "half-site" sequence, 5'-AGAACA-3', located between -477 and -472, which is insufficient for glucocorticoid stimulation in the absence of a functional GRE I (Figures 3-50, 3-51). These data are in agreement with those of Brasier and Li (Brasier and Li, 1996). They showed that a site-directed mutation of GRE I within a sequence that did not bind with the recombinant glucocorticoid recepter (GR) abolished glucocorticoid stimulation of the reporter gene. The GRE II mutation attenuated, but did not abolish glucocorticoid stimulation in the presence of wild type GRE I. Both GRE I and GRE II bound to recombinant GR in *in vitro* DNA binding assays. On the basis of these data, they proposed a model in which GREs in the ANG promoter

were hierarchical (GRE I displayed the dominant activity) and synergistic. The involvement of both GREs resulted in more stimulation than either GRE alone. This mechanism of regulation is supported by other examples, such as the tyrosine aminotransferase gene, in which multiple GREs function to activate tyrosine aminotransferase transcription synergistically (Jantzen et al., 1987). The present result is in contrast to that obtained by Feldmer et al. (1992) showing that either GRE I or GRE II alone could partially increase the CAT activity, and the two GREs combined synergistically increased CAT activity. They explained that their results could not be considered as a physiological stimulation rate, because, in their constructs, the center-to-center distance between the two GREs was only 44 bp, whereas the two GREs were separated by 92 bp in the natural genomic localization. In our experiments, the fragment which contained all the sequence from GRE I to GRE II of the rat ANG gene was used. Thus our result may have represented a physiological stimulation rate, in contrast to that obtained by Feldmer et al. Our data is further supported by the studies of Schule et al. (1988a), showing that the synergism elicited by GREs depended on the distance between the two elements.

The present studies provided evidence of direct transcriptional stimulation of the rat ANG gene by 8-Bromo-cAMP and isoproterenol. The effect of 8-Bromo-cAMP on the expression of pOCAT (ANG N-1498/+18) was concentration-dependent and cell line-specific. The responses to 8-Bromo-cAMP were different in Hepa 1-6 and OK cells.

In Hepa 1-6 cells, the addition of 8-Bromo-cAMP alone was ineffective (Figure 3-8) in the expression of pOCAT (ANG N-1498/+18). The action of 8-Bromo-cAMP was dependent on the presence of DEX and the combination of both led to a synergistic effect as compared to DEX or cAMP alone. These results were confirmed by the effects of forskolin and glucagon. Either of them alone could not stimulate the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells; their action was dependent on the presence of DEX (Figures 3-11, 3-12). The reason that the effect of 8-Bromo-cAMP on the expression of pOCAT (ANG N-1498/+18) in Hepa 1-6 cells is dependent on the presence of DEX is not clear at present. One possibility might be that there was

an inhibitory factor(s) in Hepa 1-6 cells, that could abrogate the induction of the ANG gene by cAMP. The addition of DEX might negatively regulate the expression of this inhibitory factor(s). A cAMP effect on ANG gene expression would then appear in the presence of DEX. Glucocorticoids have been shown to repress gene expression by various mechanisms including the following: (a) Negative glucocorticoid response element (nGRE). The DNA sequence 5'-CAGATCTCAGCATCAT-3' can be recognized by GR as the determinant of negative regulation activators (Drouin et al., 1987; Akerblom et al., 1988; Schule et al., 1990; Yang et al., 1990). The inhibitory effect of nGRE on gene transcription has been demonstrated in both the bovine prolactin gene (Diamond et al., 1990) and the pro-opiomelanocortin (POMC) gene (Drouin et al., 1989); (b) Competitive GREs. The negative regulation by glucocorticoids could be due to the GR causing steric hindrance to the binding of a positive factor to the other response element which is close or adjacent to or overlapping the GRE (Akerblom et al., 1988; Diamond et al., 1990); (c) No DNA binding. GR and c-Jun protein can directly interact and form a complex which is unable to transactivate. Once the complex is formed, neither GR nor c-Jun protein can bind to the DNA (Jonet et al., 1990).

Since glucocorticoids act on numerous target genes, their effects can also be produced through the induction of one or more components of the cAMP signaling pathway. For instance, glucocorticoids have been reported to activate adenylyl cyclase in GH3 cells (Chang and Bourne, 1987) and enhance cAMP dependent gene transcription via this mechanism. It is not known whether glucocorticoids regulate the expression of PKA, although glucocorticoids stimulate CREB expression in rat C6 cells (Jungmann et al., 1992). Activated PKA or CREB could represent an indirect mechanism for glucocorticoid stimulation of ANG gene transcription. Either PKA or CREB may facilitate the effect of cAMP on the expression of the ANG gene, but it can not account for the results in our experiments. pTKCAT CRE (ANG N-806/-779) responded to 8-Bromo-cAMP, but not DEX. 8-Bromo-cAMP plus DEX did not evoke a larger increase in the expression of pTKCAT (ANG N-806/-779) than 8-Bromo-cAMP alone (Figures 3-47, 3-51). If DEX had stimulated adenylate cyclase in OK cells, addition of DEX plus 8-Bromo-cAMP would have

significantly increased the pTKCAT CRE expression as compared with 8-Bromo-cAMP alone. These results suggest that the effect of glucocorticoid plus 8-Bromo-cAMP on ANG gene expression can not be attributed to glucocorticoid inducing one or more components of the cAMP signaling pathway.

None of pTKCAT (ANG GRE I/GRE II) (ANG N-591/-465), pTKCAT GRE I (ANG N-591/-563), or pTKCAT GRE II (ANG N-485/-465) responded to 8-Bromo-cAMP, and there was no significant difference between the effect of DEX plus 8-Bromo-cAMP and the effect of DEX alone on the expression of any of these three ANG-TK-CAT fusion genes (Figures 3-48, 3-49, 3-50, 3-51). The data provided evidence that phosphorylation of glucocorticoid receptors did not play an important role on the expression of the ANG gene stimulated by 8-Bromo-cAMP plus DEX. These results are supported by the studies of Tienrungro et al. (1987). Their experiments showed that phosphorylation did not affect the function of the glucocorticoid receptor. Thus, these results do not support model II proposed in the Introduction. According to that hypothesis, binding of isoproterenol to its membrane receptors activates G-proteins, then the G-proteins stimulate adenylate cyclase to catalyze the generation of cAMP from ATP. The increased intracellular cAMP would bind to protein kinase A, which is a tetramer containing two regulatory and two catalytic subunits. Binding of four cAMP molecules to two regulatory subunits activates PKA and releases the catalytic subunits. The liberated catalytic subunits phosphorylate glucocorticoid receptors which have been activated by the binding of ligand (DEX). Then, the phosphorylated glucocorticoid receptor might bind to GRE more stably and increase the rate of transcription. As there is no difference between the DEX alone and DEX plus 8-Bromo-cAMP on the expression of pTKCAT (ANG GRE I/GRE II), pTKCAT (ANG GRE I), or pTKCAT (ANG GRE II), the synergistic effect of DEX plus isoproterenol could not be explained by isoproterenol-induced glucocorticoid receptor phosphorylation increasing the rate of ANG gene transcription, the basic premise of model II (section I.6.).

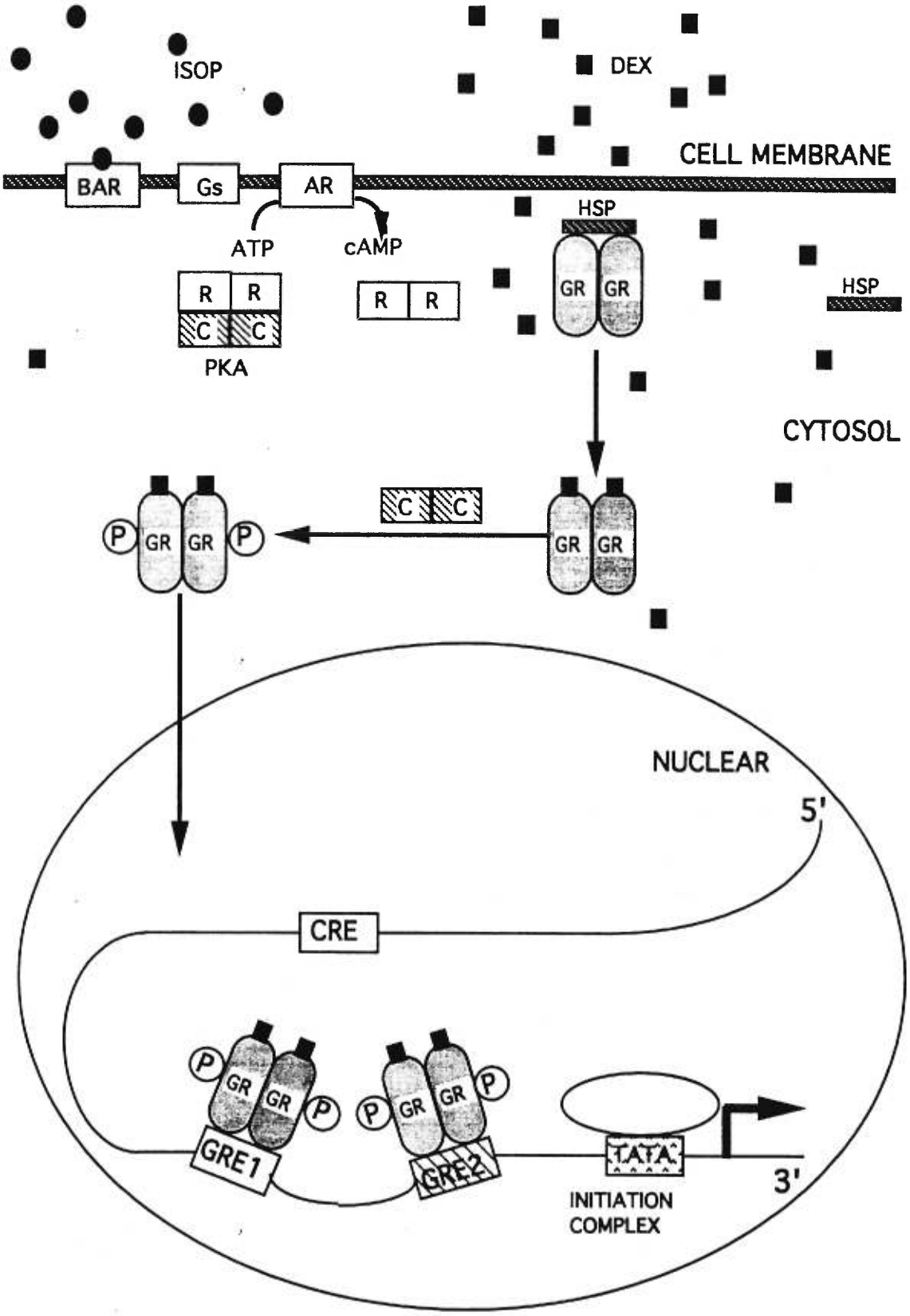


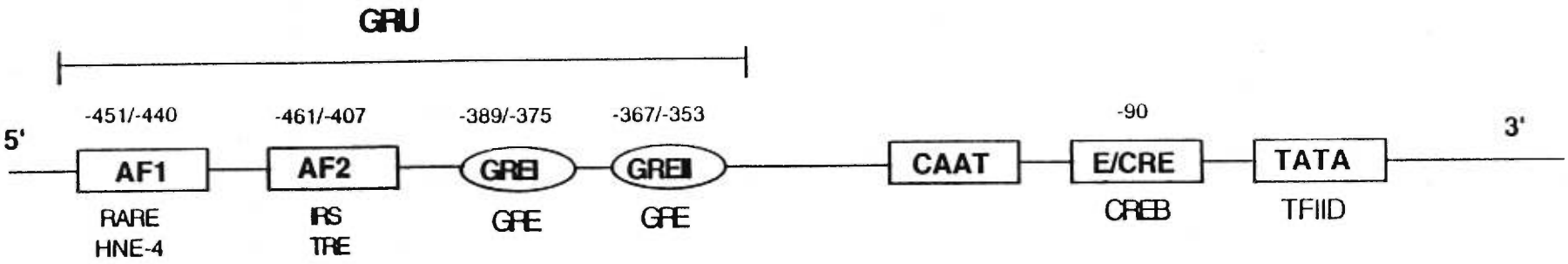
Figure 1-10 Model II

How do your data support model I?

Gene transcription induced by DEX is mediated by binding the activated glucocorticoid receptor (GR) to glucocorticoid response element(s) (GREs). Gene transcription induced by cAMP is mediated by binding the phosphorylated cAMP response element binding protein (CREB) to the cAMP response element (CRE). Our results (Figures 3-16, 3-36, 3-42) provide evidence that the synergistic effect of isoproterenol plus DEX was dependent on the presence of a functional interaction between the CREB/CRE and the GR/GRE on the expression of pOCAT (ANG N-1498/+18) in both cell lines.

There is an excellent example of functional cooperation between the glucocorticoid receptor and the cAMP signaling pathway, illustrated by the PEPCK gene (Granner et al., 1991; Lucas and Granner, 1992; Imai et al., 1993) (Figure 4-1). The proximal region of the PEPCK gene promoter (-100 to +1 bp) contains the TATAA and E/CRE elements. The glucocorticoid response element in this gene is a complex unit (hence termed glucocorticoid response unit or GRU) consisting of a tandem array (5' to 3') of a RARE (retinoic acid response element), an IRS (insulin response element), and two glucocorticoid receptor binding sites (GRE I, GRE II). The entire complex spans about 110 base pairs (from -452 to -353). The two glucocorticoid receptor binding sites are not homologous with the consensus GRE sequence which they match in only 7/12 and 6/12 positions, respectively. Each GRE functions independently accounting for half of the full response (Granner et al., 1991). Both glucocorticoids and cAMP alone induce PEPCK gene transcription, and in combination produce a synergistic effect. This is achieved through a protein-protein interaction between the glucocorticoid receptor and CREB occuring over a distance of 300 bp.

The DNA sequence of the rat ANG gene published by our and other laboratories has shown that the proximal region of the rat ANG gene (-100 to +1) contains the TATAA box (ANG N-30/-25) and CCAT box (ANG N-55/-44). The GRE(s) in this gene are also part of a complex unit consisting of a tandem array (5' to 3') of GRE I , APRE (acute phase response element) and GRE II (Figure 1-5). The entire complex spans about 113 bp (from -585 to -472). The GRE I is a near-palindrome containing the sequence 5'-AGAACATTTTGTTTC-3'. GRE II, a hexameric "half-



AF1: TGACCTTTGGCC

AF2: TGGTGTTTTG

GRE consensus	G/TG/TTACA/cNNNTGTT/cCT
GREI	cacACANNNTGTgCa
GREII	aGcAtANNNaGTCCa

Figure 4-1. PEPCK Gene Promoter Elements.

Modified from Imai et al., (1990) Chracterization of complex glucocorticoid response unit in the phosphoenolpyruvate carboxykinase gene. Mol Cell Biol 10: 4712-4719.

site" (5'-AGAACA-3') is located between -477 to-472. The present studies showed that GRE I is essential for DEX stimulation (Figures 3-49, 3-51), while GRE II alone is insufficient (Figures 3-50, 3-51). GRE II, however, is required for the maximal response to DEX and the combination of DEX and cAMP on ANG gene expression (Figure 3-45, 3-46, 3-48, 3-49). The CRE in the rat ANG gene (5'-TGACGTAC-3'), is located from -795 to -788, 250 bp upstream of the GRU. Its sequence is highly homologous with the sequence of the consensus CRE (5'-TGACGTCA-3'), with only the last two nucleotides being reversed. Our present studies showed that the fusion gene pOCAT (ANG N-806/-779), containing the CRE, only responded to 8-Bromo-cAMP, but not to DEX, since the addition of DEX did not enhance the effect of 8-Bromo-cAMP (Figure 3-47), whereas the fusion genes containing GRE(s) only respond to DEX, but not to 8-Bromo-cAMP. Likewise, the addition of 8-Bromo-cAMP did not enhance the effect of DEX (Figures 3-48, 3-49). Only the fusion genes containing both CRE and GRE responded to either 8-Bromo-cAMP or DEX, and the combination of both had an additive effect (Figures 3-45, 3-46, 3-51). These data indicate that the interaction between DEX and 8-Bromo-cAMP (or isoproterenol) on ANG gene expression involves both the CRE and GRE(s).

Serial deletion mutants of fragment ANG N-806/-465, containing CRE/GRE I/GRE II, were made from its 3'- or 5'- end. The responses of the different mutants to the stimulation of DEX, 8-Bromo-cAMP, or the combination of both were different (Figure 3-51). DEX, 8-Bromo-cAMP, as well as the combination of both stimulated the expression of pTKCAT (ANG N-806/-465) to 0.7-, 0.9- and 1.7-fold higher than that of the control. Deletion of the DNA sequence in the 3'-flanking region located between N-465 and N-562, pTKCAT (ANG N-806/-563, containing CRE/GREI) increased the response to DEX, 8-Bromo-cAMP, and the combination of both to 0.5-, 0.5-, and 0.9-fold compared with that of the control. However, with the further removal of the sequence between N-561 and N-778, pTKCAT (ANG N-806/-779, containing CRE) was only stimulated by 8-Bromo-cAMP. Neither DEX nor the combination of DEX and 8-Bromo-cAMP showed any effect compared to the control and 8-Bromo-cAMP alone. These data demonstrated

that the effect of 8-Bromo-cAMP on ANG gene expression is mediated by the cAMP response element.

In contrast, upon deletion of the DNA sequence in the 5'-flanking region located between N -806 and N-592, pTKCAT (ANG N-591/-465, containing GRE I/GRE II) only responded to stimulation by DEX. Neither 8-Bromo-cAMP nor the combination of DEX and 8-Bromo-cAMP showed any significant difference compared to the control and DEX alone. With the further removal of the sequence between N-591 and N-486, pTKCAT (ANG N-485/-465, containing ANG GRE II) did not respond to the stimulation by DEX, 8-Bromo-cAMP or the combination of both. This demonstrated that GRE II alone is insufficient to mediate the ANG gene expression induced by DEX. However, pTKCAT (ANG N-591/-563, containing GRE I) responded to the stimulation by DEX. These data agree with the model of Brasier and Li (1996) proposing that the two GREs in the ANG gene are hierarchical and GRE I is dominant.

These data further demonstrated that the effect of DEX plus 8-Bromo-cAMP on ANG gene expression is mediated by interaction between the glucocorticoid signal transduction pathway and the protein kinase A pathway, and involves both a CRE and GREs. Phosphorylation of the glucocorticoid receptor itself does not play an important role. It is more likely that the effect was produced by an interaction between the activated glucocorticoid receptor and a cAMP response element binding protein.

Our results are compatible with Model I proposed in the Introduction. According to that hypothesis, binding of isoproterenol to its membrane receptors activate G-proteins, then the activated G-proteins stimulate adenylate cyclase to catalyze the generation of cAMP from ATP. The increased intracellular cAMP binds to the regulatory subunits of PKA and releases the catalytic subunits. The liberated catalytic subunits are able to translocate into the nucleus and phosphorylate the cAMP response element binding protein (CREB). The phosphorylation of CREB increases the binding affinity of CREB for CRE and stimulates transcription. After its diffusion into the cytosol, DEX binds to glucocorticoid receptors and causes the release of heat shock proteins. The receptors

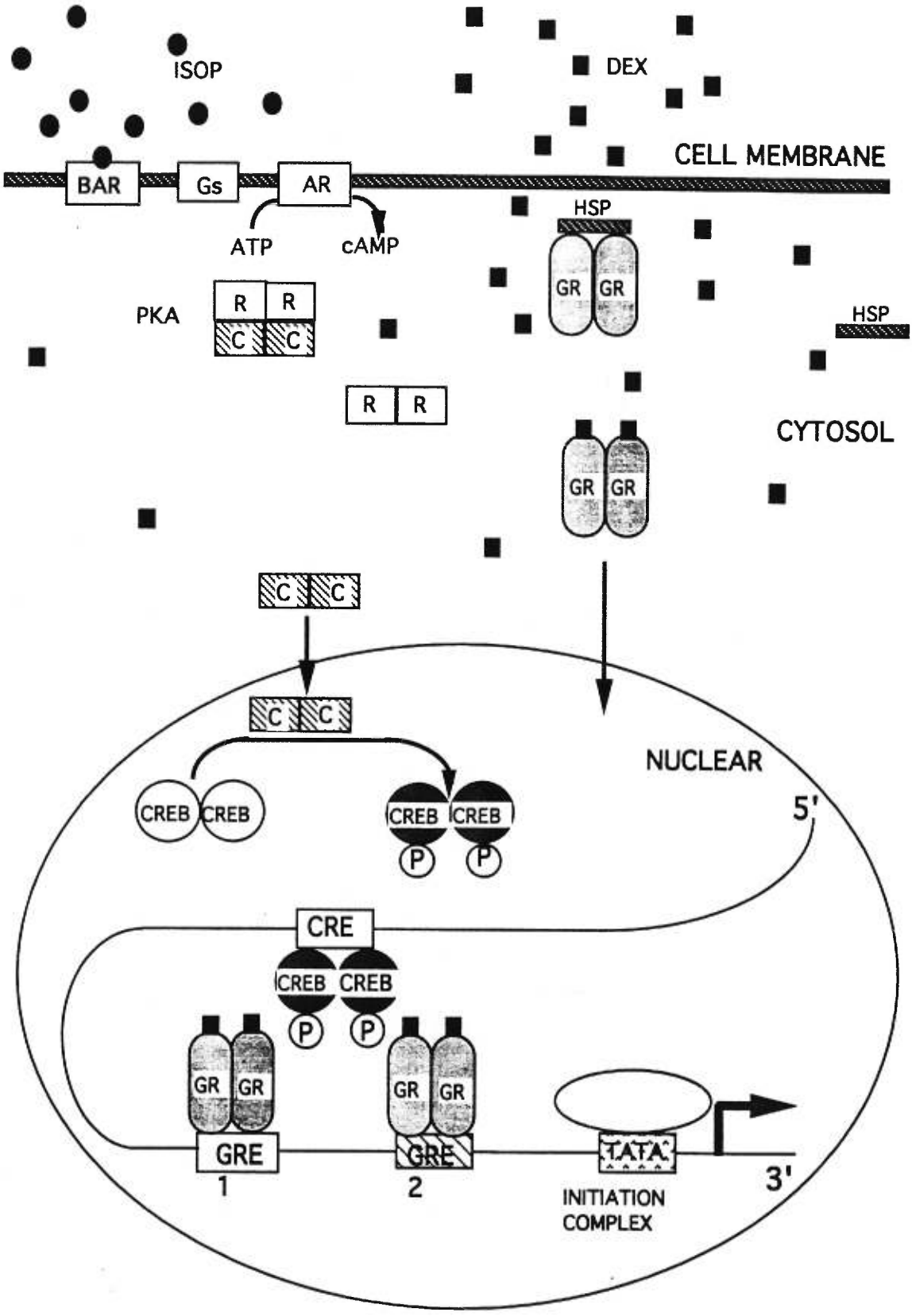


Figure 1-9 Model I

are activated and the DNA binding sites are exposed. The activated receptors are translocated into the nucleus and bind to the GRE, stimulating transcription. The combination of DEX and isoproterenol produces a synergy. It is achieved by the interaction between the phosphorylated CREB, which has bound to the CRE, and the activated glucocorticoid receptor, which has bound to the GRE. These interactions occur within 250 bp of each other, and may stimulate ANG gene transcription more effectively.

CONCLUSION AND FUTURE RESEARCH

Our experiments demonstrated that DEX stimulated the expression of the rat ANG gene in both the mouse hepatoma cell line (Hepa 1-6) and the opossum kidney cell line (OK). The stimulatory effects are concentration-dependent and can be inhibited by RU486, implicating the glucocorticoid receptor in the effect of DEX on ANG gene expression.

There is a likely cAMP response element in the rat ANG gene (ANG N-795/-788, 5'-TGACGTAC-3'); compared to the consensus CRE, the last two base pairs are reversed. It appears to mediate the effect of 8-Bromo-cAMP and isoproterenol on ANG gene expression in both Hepa 1-6 and OK cell lines.

Isoproterenol stimulated ANG gene expression in both cell lines. This response was inhibited by the β_2-adrenergic antagonist, ICI 118,551, in Hepa 1-6 cells, implicating β_2-adrenoceptors in the effect of isoproterenol on ANG gene expression in Hepa 1-6 cells. In contrast, only when OK cells were cotransfected with the β_1-adrenoceptor cDNA, was there a stimulation by isoproterenol, implicating β_1-adrenoceptors in the effect of isoproterenol in OK cells. As the effect of isoproterenol in both cell lines was inhibited by Rp-cAMP, the protein kinase A pathway was apparently involved in the effect of isoproterenol on ANG gene expression in both cell lines.

DEX and isoproterenol acted synergistically to stimulate the expression of the ANG gene in Hepa 1-6 and OK cell lines. Both RU486 and Rp-cAMP partially inhibited the synergistic effect, implicating both the glucocorticoid receptor signaling transduction pathway and the PKA pathway in this response.

8-Bromo-cAMP did not enhance the effect of DEX on the expression of fragments of the ANG gene containing only the GRE(s); thus, the synergistic effect can not be explained by phosphorylation of the glucocorticoid receptor. Rather, the synergy most likely occurs via a protein-protein interaction between the activated glucocorticoid receptor bound to GRE(s) and the

phosphorylated cAMP response element binding protein (CREB) bound to the CRE. The cross-talk between the two different signal transduction pathways stimulates ANG gene expression more efficiently than either one by itself.

These results provide evidence that increased sympathetic nervous system activity can increase blood pressure not only through its direct effect on the cardiovascular system, which may represent an acute regulatory process, but also through an indirect effect of stimulation of hepatic ANG gene expression, resulting in increased circulatory levels of ANG, which may represent a chronic regulatory process. The effect of catecholamine on ANG gene expression can be further enhanced by increased plasma glucocorticoid levels. This would explain the hypertension induced by stress, Cushing's syndrome and some forms of adrenal neoplasia, in which both increased sympathetic nervous system activity and increased plasma glucocorticoid levels are found.

As isoproterenol and DEX singly or in combination induce ANG gene expression in OK cells, a cell line possessing some characteristics of proximal tubular cells, it is probable that increased sympathetic activity, increased plasma glucocorticoid levels, or both not only induce the expression of the hepatic ANG gene to increase the systemic blood pressure, but also induce ANG gene expression in the proximal tubular cells themselves. The proximal tubular cells play an important role in the reabsorption of electrolytes, followed by water, and AII can be produced in the proximal tubule using the ANG that is synthesized in these cells. Thus, the increased local ANG synthesis may increase renal AII levels, altering renal vessel hemodynamics and the reabsorption of sodium and water. Our findings could account for the high percent of renal complications in hypertensive patients, especially those with Cushing's syndrome. It has been reported that 75% of Cushing's syndrome patients have hypertension and 6% have renal complications. Our experiments may further suggest that renal damage may be caused not only by hypertension, but also by the toxic effects of high plasma glucocorticoid levels.

Our results provide some evidence that in the treatment of hypertension, drugs that block the multiple pathways, such as the ACE inhibitor which decreases AII production, the catecholamine antagonist, and the glucocorticoid antagonist should be used together.

To provide direct evidence that the synergistic effect between DEX and isoproterenol on ANG gene expression is caused by a protein-protein interaction between the glucocorticoid receptor (GR) and the CREB as implied by our results and formulated in Model I, coprecipitation experiments can be carried out. In brief, following metabolic labeling of cells, one can use specific antibodies to either member of the putative complex, to detect proteins on non-denaturing or denaturing gels, along with labeled standards (CREB or GR) in adjacent lanes. Alternatively, cross-linking experiments, or the yeast two-hybrid system, can be used to detect specific protein-protein interactions.

To convincingly demonstrate the presence of a silencer in ANG N-760/-689, DNA footprint or methylation interference experiments can be performed to detect whether there is a protein-DNA interaction in this area. If a stretch of protected bases is detected, mutagenesis of this locus will be performed. Transfection of such mutants into Hepa 1-6 and OK cell lines, followed by treatment with 8-Bromo-cAMP or isoproterenol, should confirm that this sequence is a silencer element, if it no longer inhibits the stimulation by these drugs.

As the synergistic effect involves both the glucocorticoid receptor pathway and the PKA pathway, glucocorticoid receptor deficient animals or cells and PKA pathway member deficient animals or cells can be tested to find out whether the synergistic effect of DEX and isoproterenol on ANG gene expression is still present. For instance, cells lacking CREB but possessing all other elements of the β-adrenergic signal transduction pathway as well as the glucocorticoid receptor, may no longer demonstrate synergy when treated with DEX and isoproterenol. Various other "knockout" cells or animals lacking one or more members of the interacting pathways can be generated and analyzed in the same way. Finally, specific inhibitors of steps in each pathway can be used in cells possessing the full complement of pathway components. Using either of these

approaches, if synergy is lost, this will provide additional evidence in support of Model I.

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ORIGINAL CONTRIBUTION

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