The Transcriptional Regulation of 11ß-Hydroxysteroid Dehydrogenase Type 1

Louise J S Williams

A thesis submitted for the degree of Doctor of Philosophy

The University of Edinburgh

September 1996

Declaration

I declare that this thesis and the work presented here are entirely the result of my own independent investigation. Where I have received assistance this is indicated in the text or acknowledged on the following page.

Acknowledgements

I would like thank my supervisors Karen Chapman and Jonathan Seckl, for the continual guidance and encouragement that I have received over the past few years. In particular, a big thank you goes to Karen for always being there when I needed advice and for being such an excellent supervisor.

I acknowledge the assistance of Keith and Andy from the Biomedical Research Facility for help with animal work.

A big thank you to those in the Molecular Endocrinology Laboratory for all the help that I have received, in particular to Val Lyons for her good humour, helping me with various molecular techniques and for putting up with a less than tidy work environment. I'd like to thank everyone for making it such an enjoyable place to work especially Jill for being 'co-ordinated' and Joyce for being my 'best pal' also Caroline, Michael, Duncan, Heather, Amanda, June, Leonie and Karen French.

Abstract

The steroid metabolising enzyme 11ß-hydroxysteroid dehydrogenase (11ß-HSD) interconverts active and inactive glucocorticoids; in rats corticosterone and 11-dehydrocorticosterone, thus regulating access of glucocorticoids to intracellular receptors. Two 11ß-HSD isozymes exist; type 1 (11ß-HSD1) is NADPH-dependent, widely distributed and is highly expressed in liver, where it acts predominantly as a reductase in intact cells thereby generating active glucocorticoids from inactive substrates. Type 2 11ß-HSD (11ß-HSD2) is an NAD-dependent exclusive dehydrogenase, expressed in placenta where it protects the foetus from maternal glucocorticoids, and in aldosterone target tissues such as kidney, where it plays a well characterised role in conferring mineralocorticoid specificity upon the mineralocorticoid receptor.

The role of 11ß-HSD1 remains unclear, however, it is likely to play a central role in determining cellular sensitivity to glucocorticoids. Attenuation of hepatic 11ß-HSD1 expression is associated with increased insulin sensitivity, decreased blood glucose levels and reduced expression of glucocorticoid-inducible genes involved in gluconeogenesis in liver.

The aim of the work described in this thesis was to elucidate the molecular mechanisms which govern transcription of this important steroid metabolising enzyme, primarily in liver, the site of highest expression of 11ß-HSD1.

Work from this laboratory, investigating activity of the rat 11ß-HSD1 promoter in transfected HepG2 cells (a liver derived cell line) has shown the importance of the region between -812 and +47 in conferring C/EBPα inducibility upon a linked reporter gene, and has also demonstrated the presence of a repressor element between -812 and -599.

Within this region I have shown 13 sites to which proteins from rat liver nuclear extract bind; 11 of these sites were similarly bound by bacterially expressed C/EBPα. One of these sites was a high affinity C/EBPα binding site spanning the transcription start and was investigated in some detail. The majority of complexes formed by rat

liver nuclear extract on the transcription start contained C/EBPα and/or C/EBPß. Furthermore, a minimal promoter (-88 to +8), containing this site and 2 additional weak C/EBP binding sites at -54 to -36 and -75 to -62 was transcriptionally active *in vitro* in nuclear extract from rat liver but not from HeLa cells, which lack C/EBP.

A longer region of the promoter between, -599 and +8, is transcriptionally active *in vitro* in rat liver nuclear extract to a similar or slightly greater extent than the -88 to +8 promoter. This region contained 6 sites of liver nuclear protein binding between -599 and -88, all of which corresponded to recognition sites for C/EBP, suggesting that C/EBP-related transcription factors may be the major factors in liver which directly contact the 11ß-HSD1 promoter between -599 and -88.

Within the repressor region between -812 and -599 were 3 specific sites of protein binding by liver nuclear extract, one of these sites did not bind C/EBPα. One site, at -792 to -780, was similarly bound by liver nuclear extract and recombinant C/EBPα; the second site was protected by liver nuclear extract between -711 and -663, and within this site C/EBPα bound between -696 and -677; the third site was protected by liver nuclear extract only. The 2 sites of C/EBPα interaction with the promoter at -790 and -690 were further characterised. Protein-DNA complexes formed at -690 contained predominantly C/EBPα and C/EBPß; analysis of the -790 site on which DNase I footprints were formed by rat liver nuclear extract and recombinant C/EBPα failed to show distinct protein complexes, and did not bind recombinant C/EBPα protein.

Preliminary experiments to investigate the factors which may regulate 11ß-HSD1 transcription in other tissues (bovine adrenal cortex and undifferentiated/differentiated 3T3-F442A cells, a preadipocyte cell line) show a different spectrum of proteins binding to the 11ß-HSD1 promoter from that seen in liver.

11ß-HSD1 is expressed and regulated in a tissue-specific manner. The results described in this thesis suggest that this occurs through the tissue-specific transcription factors which bind to the 11ß-HSD1 promoter. In liver, the central role played by C/EBP in the expression of 11ß-HSD1 suggests that C/EBP may at least in part

modulate glucocorticoid effects indirectly by altering the transcription of 11ß-HSD1, and is consistent with the involvement of C/EBPα in regulating energy supply, and the role of C/EBPß in the acute phase response.

List of Figures

Chapter 1: Introduction

Chapter 2: Materials and Methods

Chapter 3: C/EBPα and C/EBPß bind to the proximal promoter of the rat 11ß-HSD1 gene

Chapter 4: Transcription factor interaction with the rat 11ß-HSD1 gene between -812 and +74

List of Tables

Chapter 1: Introduction

Chapter 2: Materials and Methods

Chapter 3: C/EBPα and C/EBPß bind to the proximal promoter of the rat 11ß-HSD1 gene

Chapter 6: Tissue specific transcriptional control of 11ß-HSD1 expression

Abbreviations

11ß-HSD1	11ß-hydroxysteroid dehydrogenase type 1
11ß-HSD2	11ß-hydroxysteroid dehydrogenase type 2
3ß-HSD	3ß-hydroxysteroid dehydrogenase
ACTH	adrenal corticotrophic hormone
ADH	alcohol dehydrogenase
AME	apparent mineralocorticoid excess
AdMLP	adeno virus major late promoter
AME	apparent mineralocorticoid excess
ATP	adenosine 5' triphosphate
AR	androgen receptor
bp	base pair
BSG	bovine serum globulin
cAMP	cyclic adenosine monophosphate
CBG	cortisol/corticosterone binding protein
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
COUP-TF	Chicken ovalbumin upstream promoter-transcription factor
CRH	corticotrophin relesing hormone
C-terminal	carboxy terminal
CTP	cytidine 5'-triphosphate
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
DEPC	diethyl pyrocarbonate
DHEA	dehydroepiandrosterone
dH_2O	distilled water
DMS	dimethyl sulphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol

EDTA	ethylenediaminetetracetic acid
EGTA	ethylene glycol-bis(ß-aminoethyl ethyr) N', N', N', N' -tetraacetic acid
ER	oestrogen receptor
ERE	oestrogen response element
G6Pase	glucose 6-phosphatase
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GTP	guanosine 5'-triphosphate
HEPES	N-2-hydroxyethylpiperazine N'-2-ethansulphonic acid
hsp	heat shock protein
IL	interleukin
IPTG	isopropylthiogalactoside
kb	kilobase
kDa	kilodalton
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NP40	nonidet P40
N-terminal	amino terminal
PBS	phosphate buffered saline
PEPCK	phosphoenolpyruvate carboxykinase
PMSF	phenylmethylsulphonyl fluoride
PR	progesterone receptor
rC/EBP	recombinant C/EBP
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate
TAF	TBP associated factor
TBE	tris-borate-EDTA
TBP	TATA binding protein
TE	tris - EDTA buffer

TEMED	N'N'N'N'-tetramethylethylenediamine
THE	tetrahydroxycortisone
THF	tetrahydroxycortisol
Tris	tris(hydroxymethyl)aminoethane
Triton X-100	octyl phenoxy polyethoxyethanol
tRNA	transfer ribonucleic acid
UTP	uridine 5'-triphosphate
UV	ultraviolet

Publications from this thesis

Paper submitted

Williams L.J.S., Edwards C.R.W., Seckl J.R., Chapman K.E. The proximal 88 base pairs of the rat 11ß-hydroxysteroid dehydrogenase 1 gene promoter is transcriptionally active *in vitro* and binds CCAAT/Enhancer binding protein

Abstracts

Williams L.J.S., Lyons V., Wallace R., Seckl J.R., Chapman K.E.(1996) C/EBP regulates the expression of the rat 11ß-hydroxysteroid dehydrogenase type 1 promoter in liver cells. *10th International Congress of Endocrinology*. P1-233

Williams L.J.S., Edwards C.R.W., Seckl J.R., Chapman K.E.(1995) The 11ß-hydroxysteroid dehydrogenase 1 promoter is transcriptionally active *in vitro*. *J. Endocrinol.* **147:** (suppl.) P46

Williams L.J.S., Edwards C.R.W., Seckl J.R., Chapman K.E.(1995) C/EBP binds to two distinct sites on the 11ß-hydroxysteroid dehydrogenase promoter. *77th Annual Meeting of the Endocrine Society*. P2-553

Williams L.J.S., Edwards C.R.W., Seckl J.R., Chapman K.E. (1995) C/EBP binds to the transcriptional start of the rat 11ß-HSD-1 gene. *J. Endocrinol.* **144:** (suppl.) P259

Williams L.J.S., Napolitano A., Rajan V., Edwards C.R.W., Seckl J.R., Chapman K.E. (1993) C/EBP - A putative transcriptional regulator of 11ß-hydroxysteroid dehydrogenase. *J. Endocrinol.* **139:** (suppl.) P6

Table of Contents

Chapter 7: Discussion

CHAPTER 1

Introduction

1.1 Steroid hormones

Steroid hormones are all derived from cholesterol and are synthesised by three embryologically related tissues; the testis, the ovary and the adrenal gland. The major classes of steroid hormone are androgens, oestrogens, progestins and corticosteroids. Androgens and oestrogens are synthesised and secreted by the gonads and play a role in reproduction and sexual behaviour. Progesterone is secreted by the corpus luteum and placenta, and plays a central role in female reproduction (Lydon et al., 1995). There are two types of corticosteroids; glucocorticoids and mineralocorticoids, both of which are synthesised in the adrenal cortex. Glucocorticoids play central roles in the maintenance of homeostasis, mood, behaviour and the stress response, while mineralocorticoids maintain salt and water balance. The action of steroid hormones can be modulated at a number of levels; synthesis, secretion, binding to plasma proteins e.g. corticosteroid binding globulin (CBG), sex steroid binding globulin (SHBG) (also called testosterone binding globulin) and albumin, uptake by tissues, binding and retention in tissues, metabolism within tissues, presence of receptors in cells, availability and activity of receptors inside cells and transcriptional activity of receptors in the nucleus.

This thesis is concerned with the tissue specific regulation of 11ß-HSD1, a crucial steroid metabolising enzyme which regulates intracellular access of glucocorticoids to corticosteroid receptors. In this chapter I shall review the processes which control the action of glucocorticoids, the biology and regulation of the 11ß-hydroxysteroid dehydrogenase enzymes and the process by which tissue-specific controls of gene transcription are achieved, with particular attention to hepatic gene transcription.

1.1.1 The adrenal cortex and biosynthesis of adrenocortical hormones

The adrenal cortex is divided into three morphologically distinct regions based on differences in vascular and connective tissue; the zona glomerulosa, the zona fasciculata, and the zona reticularis (Arnold, 1866). The narrow outer zona glomerulosa lies below the adrenal capsule and is the site of biosynthesis of the mineralocorticoid aldosterone. The bulk of the cortex is formed by the zona fasciculata/reticularis which lies below the glomerulosa and which secretes glucocorticoids, and in humans, dehydroepiandrosterone (DHEA) and other weak androgens (see Orth et al., 1992).

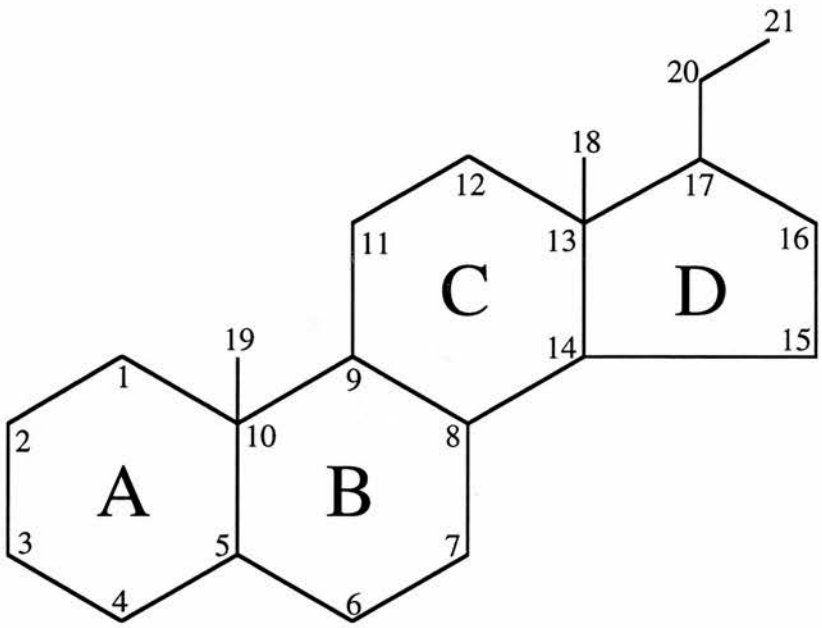


Figure 1.1 *Basic steroid ring structure*
The rings are named A-D and the numbers refer to the conventional designation of carbon atoms.

Cholesterol, the precursor to all steroid hormones, is either taken up from circulating blood by the adrenal gland or is synthesised *de novo*. The basic structure of steroid hormones is shown in Figure 1.1. The major steps in steroid biosynthesis are outlined in Figure 1.2 and involve 4 cytochrome P-450 enzymes and 3ß-hydroxysteroid dehydrogenase (3ß-HSD). The rate limiting step in the synthesis of all steroid hormones is the removal of the side chain from cholesterol to form the C21 steroid pregnenolone by cytochrome P-450_{SCC} (side chain cleavage enzyme). Subsequent reactions by 3ß-HSD, $P450_{C17}$ (17,20-lyase), $P450_{C21}$ (21-hydroxylase) and $P450_{C11}$ (11-hydroxylase) result in the production of predominantly corticosterone (in rats) or cortisol (in humans) (Figure 1.2). The unique expression in the zona glomerulosa of aldosterone synthase (related to $P450_{C11}$, but the product of a separate gene) is responsible for the conversion of corticosterone to aldosterone, and thus the production of mineralocorticoid by the glomerulosa (Domalik et al., 1991).

1.1.2 Control of corticosteroid synthesis and secretion

There is a natural diurnal rhythm of glucocorticoid synthesis and secretion, with peak levels before the period of activity as well as increased glucocorticoid synthesis and

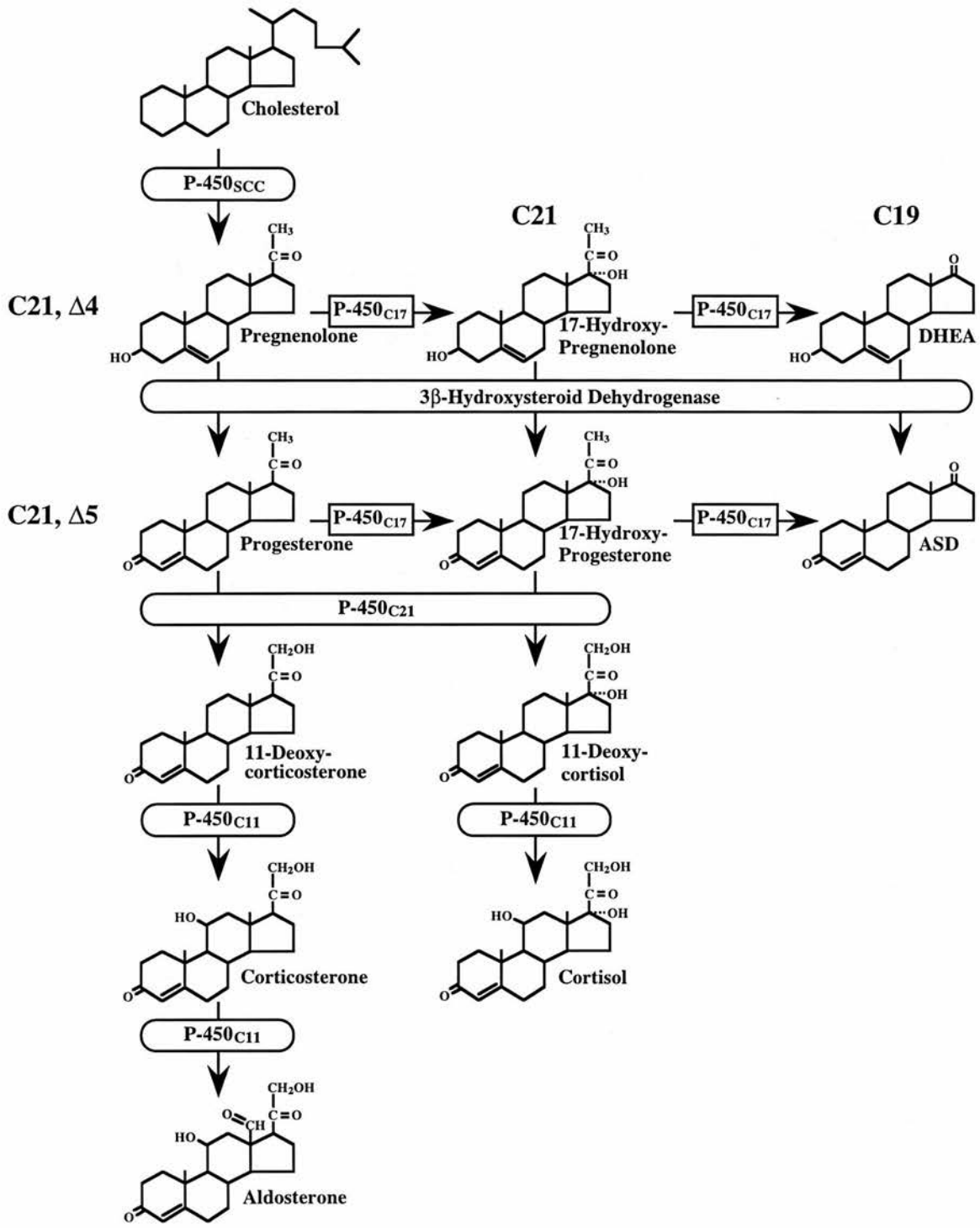


Figure 1.2 *Biosynthesis of adrenocortical steroids*

Adrenal steroid biosynthetic pathways in the cortex. Pathways for glucocorticoid, mineralocorticoid and androgen biosynthesis are shown. DHEA, Dehydroepiandrosterone; ASD, Androstenedione (Modified from Orth et al., 1992).

secretion during stress. Adrenal steroid hormones are not stored by the adrenal gland but are released into the circulation once synthesised.

Synthesis of the glucocorticoids cortisol and corticosterone, by the zona fasciculata/reticularis, is controlled by the interaction of hormonal and neuronal stimuli and their subsequent actions on the hypothalamus, anterior pituitary and adrenal cortex (HPA axis) (Figure 1.3). At the adrenal cortex, the main stimulus of glucocorticoid synthesis is adrenocorticotrophic hormone (ACTH) (Waterman & Simpson, 1989; White et al., 1994), secreted by the pituitary from the precursor polypeptide, proopiomelanocortin (POMC). ACTH binds to cell surface receptors on the adreno-cortical cell, subsequently resulting in increased intracellular cyclic AMP (cAMP). Within minutes, there is an increase in the activity of P-450$_{SCC}$, the rate limiting step in steroidogenesis. Additionally, over a period of hours/days, ACTH causes an increase in the synthesis of steroidogenic enzymes and proteins required for steroidogenesis e.g. adrenodoxin (reviewed in Simpson & Waterman, 1983).

ACTH synthesis and secretion from the pituitary is in turn regulated by multiple hormonal factors, the most important of which are corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) both of which are released from the hypothalamus. CRH and AVP are both synthesised in the parvicellular neurones of the paraventricular nucleus of the hypothalamus, and AVP is synthesised in the magnocellular neurones of the supraoptic and paraventricular nuclei, released into the hypophyseal portal blood which delivers CRH and AVP to the pituitary where they act to increase production of ACTH (Orth et al., 1992). In addition to direct simulation by these factors, AVP also potentates the action of CRH on the anterior pituitary (Antoni, 1993).

Activation of HPA axis activity is terminated by the actions of glucocorticoids, which negatively feedback at the levels of the hypothalamus (to decrease CRH and AVP secretion), the pituitary (to decrease POMC synthesis and secretion) (Lundbland & Roberts, 1988) and also the hippocampus (Bayer et al., 1988; Davis et al., 1986) (see Section 1.1.5.3).

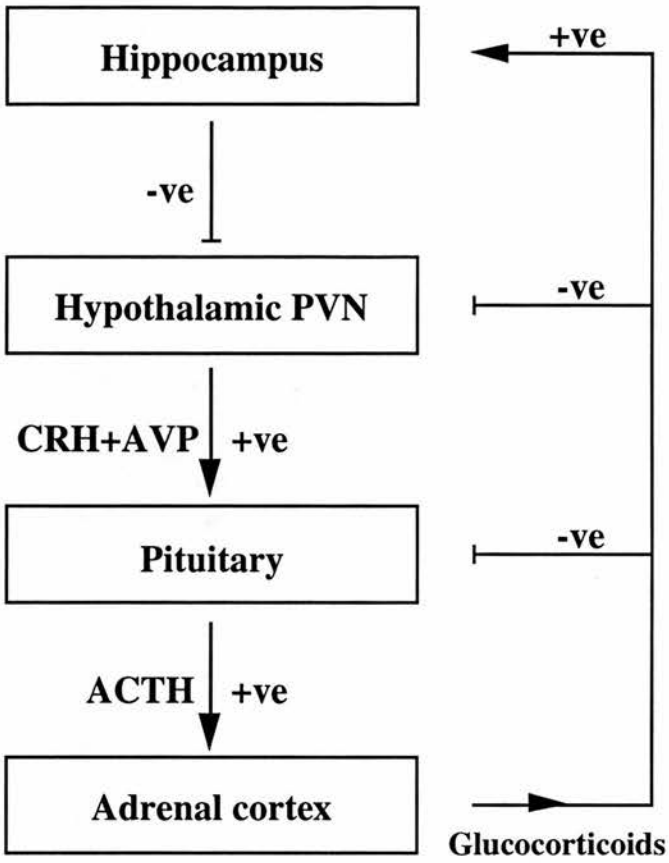


Figure 1.3 *The HPA axis*
Schematic representation of the HPA axis

1.1.3 Control of mineralocorticoid secretion

Secretion of aldosterone is controlled primarily by the renin-angiotensin system. When sodium concentrations in the distal tubule of the kidney nephron are low or if there is low renal arterial blood pressure, the juxta-glomerular cells release renin into the bloodstream (Orth et al., 1992). In the blood, renin acts to form angiotensin I from angiotensinogen (Goodfriend et al., 1980). Angiotensin I is converted to the active octapeptide angiotensin II by angiotensin converting enzyme (ACE) in the vasculature and lungs. Angiotensin II stimulates the release of aldosterone from the adrenal glomerulosa cells by binding to a G protein-linked receptor leading to activation of phospholipase C, and an increase in steroid production (Quinn & Williams, 1988).

1.1.4 Plasma steroid binding proteins

In the bloodstream, circulating steroid hormones are reversibly bound to plasma

proteins, namely CBG, SHBG, and albumin. 90% of cortisol and 80% of corticosterone are found associated with CBG, with remaining glucocorticoids normally associated with albumin (Dunn et al., 1981). Aldosterone in plasma is also associated with steroid binding proteins, but a higher percentage are 'free' with only 20% bound to CBG and 40% to albumin (Dunn et al., 1981; Pardridge, 1981).

1.1.5 Actions of glucocorticoids

Glucocorticoids affect many diverse physiological functions, for example, fuel metabolism, immunological and inflammatory function, electrolyte and fluid homeostasis, muscle and bone formation, mood, behaviour, and the stress response (Orth et al., 1992). The profound effects of glucocorticoids are apparent in patients with glucocorticoid deficiency (Addison's Disease) who typically have muscle weakness and weight loss. In contrast, patients with glucocorticoid excess (Cushing's syndrome) frequently have hypertension, wasting of muscles, osteoporosis, and fat redistribution. Excessive exposure to glucocorticoids during development may also be detrimental to the foetus resulting in a higher risk of hypertension in adult life (Seckl et al., 1995).

1.1.5.1 Effects on metabolism

Glucocorticoids are involved in the metabolism of carbohydrates, proteins and lipids and, together with insulin and glucagon, regulate blood glucose. After a high carbohydrate meal, blood glucose increases, resulting in insulin secretion from the pancreas (Cryer, 1992). Insulin inhibits gluconeogenesis and together with glucocorticoids causes the rapid uptake of glucose, its storage as glycogen and the use of glucose in liver (Cryer, 1992). Insulin in liver also promotes the conversion of excess glucose into fatty acids, which are transported to adipose tissue and deposited as fat (Cryer, 1992). In muscle, insulin decreases the release of amino acids thus decreasing the availability of precursors to gluconeogenesis (Cryer, 1992). During fasting, glucocorticoid have a permissive effect on the actions of glucagon (Exton, 1979), which is released from the pancreas and essentially has opposite effects to insulin, increasing blood glucose by promoting glycogenolysis and gluconeogenesis e.g. by activating phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase) (Exton, 1979). Glucocorticoids also directly inhibit glucose

transport into cells (Munck, 1962) and activate the breakdown of lipids in adipose tissue (Fain, 1979).

1.1.5.2 Immunological function and inflammatory processes

A normal physiological role of glucocorticoids is to suppress the immune response following initial activation (Graham & Tucker, 1984). Macrophages and T- and B-lymphocytes attack and destroy invading bacteria and viruses. During inflammation, glucocorticoids inhibit the movement of fluid and immune cells into the site of inflammation (Zweifach et al., 1953) and inhibit the action of mediators involved in the inflammatory response, for example, by inhibiting prostaglandin and cytokine synthesis (Hong & Levine, 1976). They also decrease thymus derived lymphocytes (T-cells) and bone marrow derived lymphocytes (B-cells) by inhibiting their generation (Cupps et al., 1985; Gillis et al., 1979). Glucocorticoids have the same effect on monocytes (the precursors of macrophages) and inhibit the phagocytotic and cytotoxic effects of macrophages (Reinehart et al., 1982).

1.1.5.3 Glucocorticoids and the stress response

Stresses such as trauma, illness and psychological stress activate the HPA axis, resulting in increased plasma ACTH and cortisol levels. The mechanisms by which stress induces these changes are not known, but result in CRH and/or AVP being released from the hypothalamus, consequently increasing ACTH secretion from the pituitary (Orth et al., 1992). Negative feedback action by glucocorticoids terminates the stress response by acting on the pituitary to decrease ACTH production and transcription of its precursor POMC, as well as exerting feedback effects on the hypothalamus, reducing CRH and AVP production (Orth et al., 1992).

1.1.5.4 Other effects of glucocorticoids

Glucocorticoids under normal physiological conditions have well described effects on development. Particularly well documented is the requirement of glucocorticoids for normal maturation of the lung, where they act on type II pneumocytes to increase surfactant production (Ballard, 1987), thereby allowing the lungs to function normally at birth. In the generation of the adrenal gland glucocorticoids are required for regulating formation of chromaffin cells from neural crest epithelial cells (Federoff et al., 1988). Mice homozygous for a targeted disruption of the

glucocorticoid receptor gene die of respiratory problems caused by a lack of surfactant production, and have defective chromaffin cell development (Cole et al., 1995). Glucocorticoids in excess cause retention of fluids, resulting in hypertension (Krakoff et al., 1975; Saruta et al., 1986).

Excess glucocorticoids cause muscular weakness which is frequently seen in patients with Cushing's Disease due to loss of proteins from muscle and their use as substrates for gluconeogenesis (Exton, 1979). Additionally, glucocorticoids promote loss of creatine from muscle, associated with oedema and fibrosis and inhibit bone formation by osteoblasts (Hahn et al., 1979). Excess glucocorticoids have many effects on mood and behaviour, and Cushings patients commonly have psychological disturbances; typically cognitive/memory dysfunction and depression (Jeffcoate et al., 1979; Plotz, 1952; Starkman et al., 1986). Glucocorticoids are also important in prenatal programming, if the foetus is exposed to high levels of glucocorticoids then there may be a retardation of foetal growth (Reinisch et al., 1978) and, in later adult life, a higher risk of hypertension (Benediktsson et al., 1993).

1.1.6 Actions of mineralocorticoids

As implied by the name, the actions of mineralocorticoids are concerned with maintaining salt and water homeostasis. The main site of mineralocorticoid action is at the kidney where aldosterone acts on the distal convoluted tubule and the collecting ducts; mineralocorticoids also affect the colon, sweat and salivary glands. In general, aldosterone causes sodium absorption and potassium excretion, and hence increased fluid retention, which increases blood pressure (see for example Orth et al., 1992). In addition, mineralocorticoids act on the central nervous system to regulate, for example, salt appetite (McEwen et al., 1986).

1.2 Steroid hormone receptors

It has long been assumed that steroid hormones enter cells by diffusion, however, recent evidence from yeast suggests that an active mechanism transports steroids into cells (Kralli et al., 1995). Inside target cells, steroid hormone action is mediated by specific receptor proteins. Steroid receptors (including the classical steroid receptors, glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR), oestrogen receptor (ER) and androgen receptor (AR)) all belong to the

nuclear receptor superfamily (summarised in Table 1.1). Members of this family also include receptors for other ligands including thyroid hormone (T_3), Vitamin D_3, retinoic acid, 9-cis retinoic acid, the insect hormone ecdysone and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (an endogenous ligand for PPARγ) (Evans, 1988; Forman et al., 1995; Isseman & Green, 1990; Koelle et al., 1991), and receptors for which no ligand exists, or has yet been identified (the 'orphan' receptors) (reviewed in Beato, 1989; Evans, 1988; O'Malley, 1990) (for classification of receptors see Section 1.2.5). These receptors all act in the nucleus, bind to DNA and share a common domain structure, conserved across species, from insects to mammals (Section 1.2.1, Figure 1.4), and play diverse roles, controlling homeostasis, development, differentiation and reproduction.

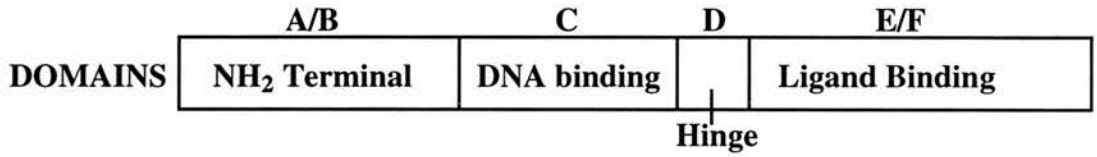
Receptors for which the ligand is known	Orphan Receptors	*Drosophila* Proteins
Glucocorticoid (GR)	COUP-TF I/COUP-TF II	Seven-up
Progesterone (PR)	Ear-2	
Mineralocorticoid (MR)	HNF-4	HNF-4(D)
Androgen (AR)	TR2	
Oestrogen (ER)	TR4	
	LXR	Ultraspiracle
Retinods (RARα, ß, γ) (RXRα, ß, γ)	PPARα/ß	Ecdysone Receptor
Thyroid hormone (T_3Rα,ß)	NGFI-B	DHR3
Vitamin D_3 (VDR)	NURR1	DHR39
PPARγ	SF-1	Ftz-TF
	RORα/ß	

Table 1.1 *The nuclear receptor superfamily*
Compilation of some members of the nuclear receptor family, taken from Amero et al., 1992; Forman et al., 1995; Laudet & Adelmant, 1995; Laudet et al., 1992.

The members of the steroid receptor superfamily probably number in the hundreds, and their properties are variable. All classical receptors and most other receptors are only active as dimers; a few orphan receptors, and possibly T_3 receptor (T_3R) probably also act as monomers (Section 1.2.5). Some (including GR), are complexed with heat shock proteins (Section 1.2.2), although most are not, and others repress transcription in the absence of ligand (e.g., T_3R) (Brent et al., 1989; Zhang et al., 1991). To review in detail the properties of all nuclear receptors is not practical for the purpose of this thesis, so I will provide a general review of the characteristics of this family of proteins concentrating largely on GR.

1.2.1 Steroid hormone receptor structure

The glucocorticoid receptor cDNA was the first steroid receptor cDNA to be cloned, and it showed homology to v-ERBA, subsequently found to be the oncogenic variant of $T_3R\alpha$ (Hollenberg et al., 1985). Comparison of cDNA sequences encoding members of the nuclear receptor family has shown that all share a conserved domain structure (Figure 1.4). Various functions have been ascribed to these domains and these will be discussed below.

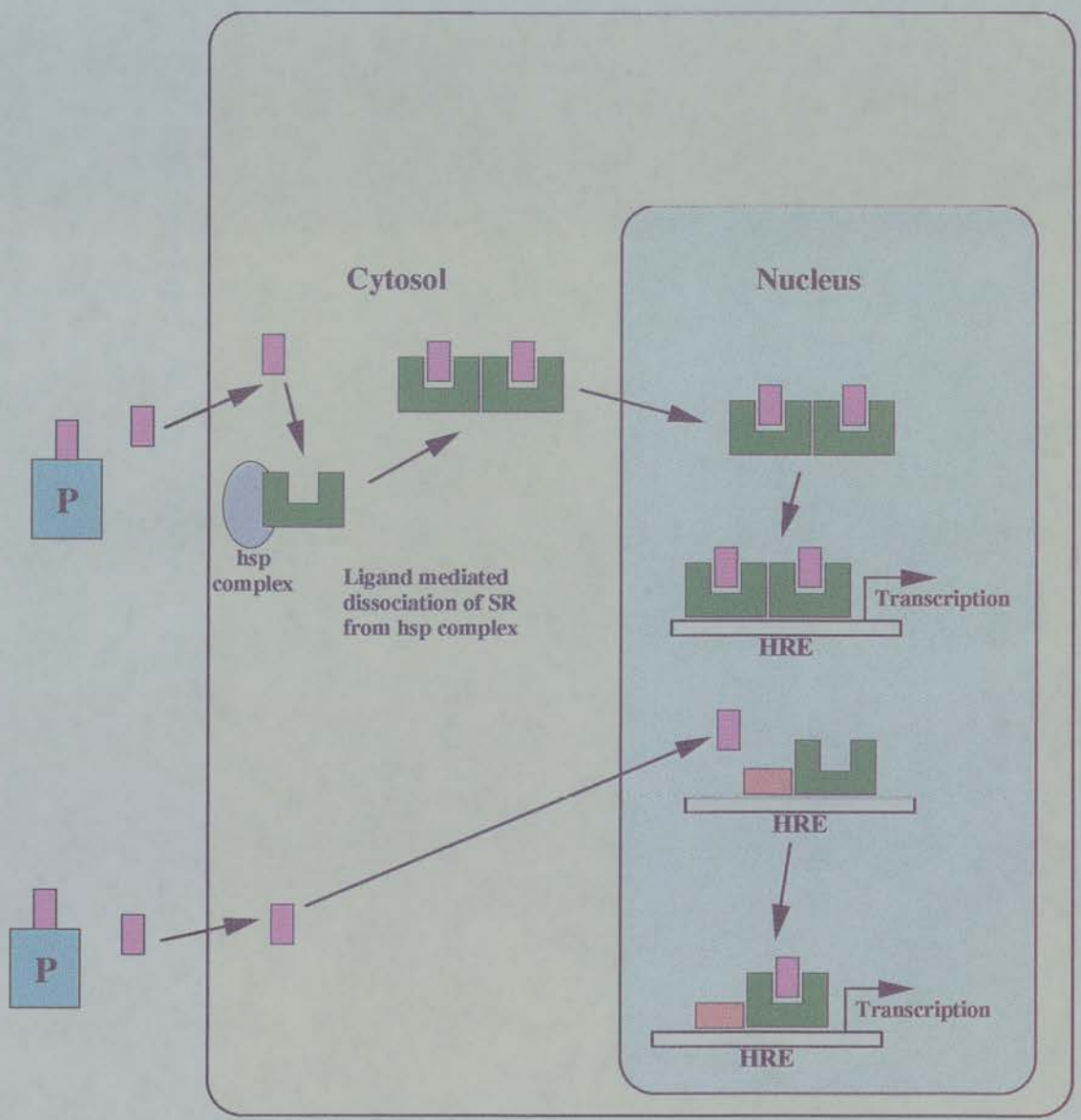


SEQUENCE IDENTITY	**Highly Variable**	**Highly Conserved**	**Moderately well conserved**
FUNCTION	**Transactivation**	**DNA binding** **Dimerisation**	**Ligand Binding** **Dimerisation** **Transactivation** **Nuclear Translocation** **hsp binding**

Figure 1.4 *Domain structure of steroid receptors*
The functions of domains are indicated. Domains A-F refer to terminology adopted for steroid receptor structure, originally assigned to the human ER (Krust et al., 1986).

Figure 1.5 *Activation of nuclear receptors*

The top part of the diagram represents the prevalent model for transcriptional activation by steroid receptors. Steroid hormones (purple boxes) are released from plasma proteins (P), they enter the cell and dissociate receptors (green boxes) from the hsp complex resulting in receptor dimerisation, translocation into the nucleus and gene activation. The bottom part of the diagram represents activation by receptors which are not associated with hsp in the cytoplasm. The ligand enters the nucleus and binds to nuclear receptors heterodimerised with other nuclear receptors and activate transcription.


Cytosol
Nucleus
P
hsp
complex
Ligand mediated
dissociation of SR
from hsp complex
Transcription
HRE
HRE
P
Transcription
HRE

The steroid receptors contain a highly conserved DNA binding domain (domain C) located roughly in the middle of the receptor (Section 1.2.4), connected by a less well conserved 'hinge' domain (domain D) to a C-terminal ligand binding domain (domain E/F), which confers specificity for ligand and which shows a moderate degree of conservation, particularly between receptors which bind the same ligand e.g. RARα, ß and γ (Krust et al., 1989).

The N-terminus (domain A/B) is generally variable in size and amino acid sequence, and is involved in cell type specific transcriptional regulation (Carlstedt-Duke et al., 1982). It is not required for hormone binding (Rusconi & Yamomoto, 1987), receptor activation (Gehring & Arndt, 1985), dimerisation (Eriksson & Wrange, 1990), DNA recognition (Freedman et al., 1988) or for transport into the nucleus (Jewell et al., 1995) and has been termed the modulatory domain. It is, however, required for full transcriptional activity of GR (Miesfeld et al., 1987), and loss of the N-terminal domain results in altered protein-protein contacts in the dimer which affects the interaction with DNA (Eriksson and Wrange, 1990).

1.2.2 Ligand binding domain

The original two step model for the activation of steroid hormone receptors proposed that in the absence of ligand steroid hormone receptors were retained in the cytoplasm (Jensen, 1990). Following binding of steroid to receptor, the steroid-receptor complex transformed into the active form of the receptor and translocated to the nucleus (Jensen et al., 1968). Data now suggests that the exact location of the steroid hormone receptor differs depending on the individual receptor type (reviewed by Jenson, 1990; Walters, 1985).

In the absence of ligand, heat shock proteins (hsp) (hsp90, hsp70 and hsp56) are found associated with certain steroid receptors, in a complex that is unable to bind to DNA (Rehberger et al., 1992; Rexin et al., 1992; Segnitz & Gehring, 1995), including GR (Dalman et al., 1990; Dalman et al., 1991). Binding of ligand to the ligand binding domain (domain E), leads to the dissociation of receptor from hsp's, followed by receptor dimerisation to form a complex capable of interacting with DNA and regulating gene transcription (Figure 1.5), (reviewed in Baniahmed & Tsai, 1993; Glass, 1994). This domain also contains a trans-activating function; deletions

in this domain of GR abolish ligand binding and activation of transcription (Godowski et al., 1987). Recent evidence from ER suggests that domain E interacts with coactivators (Cavailles et al., 1995; Le Dovarin et al., 1995; vom Baur, et al., 1996).

1.2.3 Nuclear translocation of steroid hormone receptors

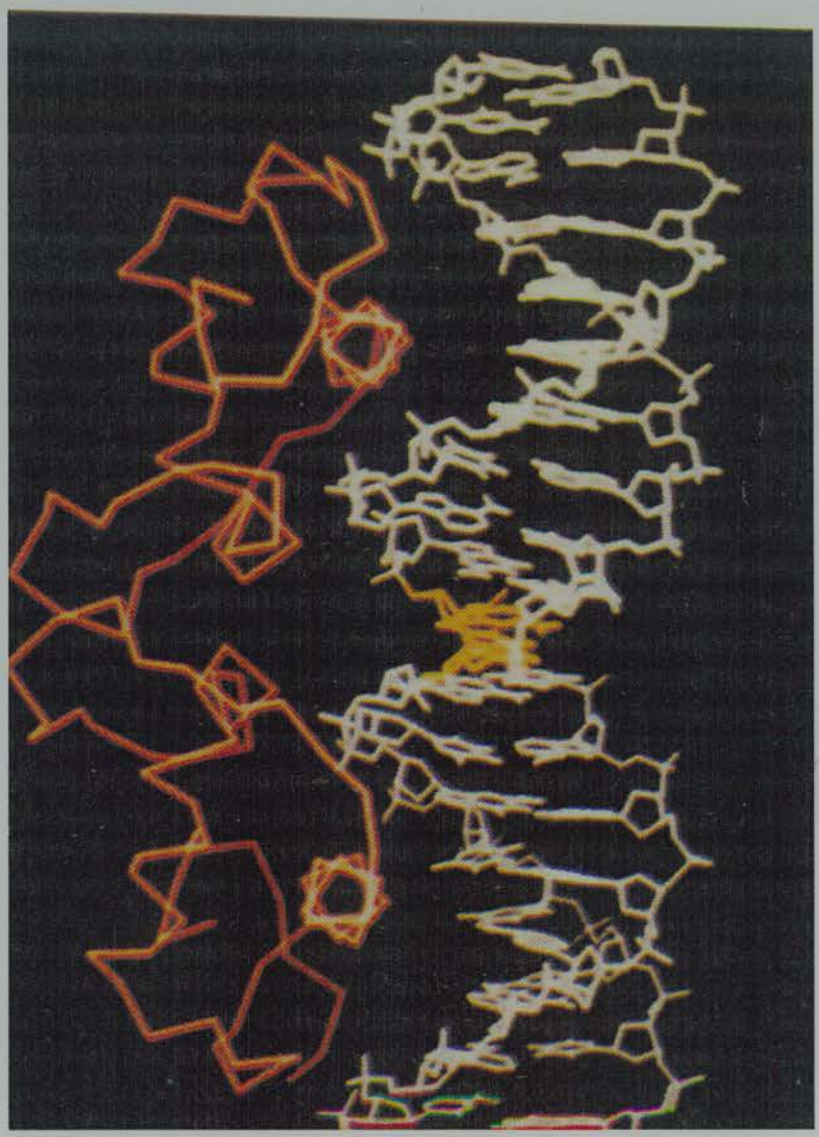
All proteins are synthesised in the cytoplasm, therefore, all nuclear receptors have a nuclear localisation signal to allow translocation of the receptor into the nucleus. The nuclear localisation signal is bipartate in character and has perhaps been best described for the progesterone and androgen receptors (Jenster et al., 1993; Ylikomi et al., 1993). It consists of two basic amino acids separated by 10 amino acid residues from another set of 4-5 basic amino acid residues. If not already situated in the nucleus, then following binding of ligand, nuclear receptors need to be transported into the nucleus to activate transcription. The precise details of nuclear translocation are unclear, but in yeast the glucocorticoid receptor is actively transported to the nucleus by a transporter (Kralli et al., 1995) and possibly nuclear transporters exist for other steroid receptors (Defranco et al, 1995).

1.2.4 DNA binding domain of steroid hormone receptors

The DNA binding domain (domain C) is the most highly conserved domain amongst members of the superfamily. Nuclear magnetic resonance spectroscopy has been used to characterise the structure of domain C for a number of receptors (Baumann et al., 1993; Knegtel et al., 1993; Lee et al., 1993; Schwabe et al., 1990). The structure of the GR DNA binding domain bound to DNA has been determined by x-ray crystallography (Luisi et al., 1991) (Figure 1.6). The DNA binding domain of GR has a high basic amino acid content, and contains 9 conserved cystines, 8 of which form two zinc fingers each containing one molecule of zinc (Luisi et al., 1991). The zinc is co-ordinated to the sulphur atom of the four cystines. The first zinc finger contains the amino acids that recognise specifically the steroid response element on DNA (section 1.2.5) and positions an α-helix to interact with DNA in the major groove (Green et al., 1988; Luisi et al., 1991). The second zinc finger is involved in receptor dimerisation and provides phosphate contacts with the DNA backbone (Luisi et al., 1991); mutations in the second zinc finger reduce efficiency of DNA binding (Hard et al., 1990; Luisi et al., 1991; Schwabe et al., 1990).

Figure 1.6 *The structure of the glucocorticoid receptor bound to a GRE*

Taken from Luisi, et al., 1991.



1.2.5 Classes of nuclear hormone receptors

Members of the nuclear hormone receptor family can be divided into 3 classes on the basis of their DNA binding activity (Laudet and Adelmant, 1995) (Figure 1.7).

Class I steroid receptors bind to DNA as dimers and recognise inverted repeats with consensus sequences GGTACAnnnTGTYCT for GR, PR, AR and MR, and AGGTCAnnnTGACCT for ER (Figure 1.7) (Beato, 1987; Freedman & Luisi, 1993). The DNA binding specificity primarily results from 3 amino acids in the first zinc finger which contact exposed edges of base pairs in the major groove, which are identical in AR, PR, GR and MR, but differ in ER (Green et al., 1988).

Class II nuclear receptors bind as dimers to sequences containing two copies of the consensus half site, AGGTCA, in various orientations (direct, inverted, everted repeats), separated by 0-5 nucleotides (depending on the receptor) (Figure 1.7) (Glass et al., 1991). This group of receptors includes the receptors T_3R, RARα, ß, γ, RXRα, ß, γ and VDR as well as many of the orphan receptors. Direct repeats with different spacings are selective for different receptors (Naar et al., 1991; Umesono et al., 1991), with VDR recognising a direct repeat with a 3 base pair spacing (DR+3), T_3R a 4 (DR+4), RAR a 5 or 1 nucleotide spacing (DR+5, DR+1), and RXR a 2 (DR+2) (Umesono et al., 1991). In addition, RXR forms dimers with, and enhances DNA binding of RAR, T_3R, VDR (Kliewer et al., 1992a; Leid et al., 1992; Marks et al., 1992; Yu et al., 1991; Zhang et al., 1992) and some orphan receptors including LXR, RLD-1 and MB67 (Apfel et al., 1994; Baes et al., 1994; Gearing et al., 1993; Kliewer et al., 1992b; Willy et al., 1995).

Class III nuclear receptors such as NGFI-B and SF-1 bind as monomers to an element containing the AGGTCA consensus sequence extended by 2-5 nucleotides at the 5' end (Wilson et al., 1993), with the nucleotides immediately 5' of the half site important in specifying receptor selectivity (Ueda et al., 1992; Wilson et al., 1993).

1.2.6 Steroid receptor phosphorylation

The glucocorticoid receptor is phosphorylated in its inactive state and rapidly undergoes hormone induced hyperphosphorylation after binding glucocorticoids (Orti et al., 1992). There are several phosphorylation sites on the mouse

	Binding Site		Receptor
Class I	AGRACAnnnTGTMCY → ←	GRE (IR+3)	GR, PR, MR, AR
	AGGTCAnnnTGACCT → ←	ERE (IR+3)	ER
Class II	RGGTCAn_xRGGTCA → →	DR+X	RXR, RAR, TR, VDR, COUP-TF, PPAR
	RGGTCAn_xTGACCY → ←	IR+X	
	TGACCYn_xRGGTCA ← →	ER+X	
Class III	AAAGGTCA →	NBRE	NGFI-B, NURR1
	YCAAGGTCA →	FRE	FTZ-F1, SF-1, ELP
	WAWNTAGGTCA →	RevRE	RZR/ROR, Rev-erb/RVR

Figure 1.7 *Classes of steroid hormone receptors*

Class I, II and III consensus binding sites are shown. (Laudet & Adelmant, 1995). The inverted repeat (IR), direct repeat (DR) or everted repeat (ER) are indicated by the arrows. N indicates any nucleotide and x the variable spacing between repeats. R indicates A/G, M indicates A/C, Y indicates C/T and W indicates A/T. Examples of the receptors that bind to the different classes of binding sites are listed.

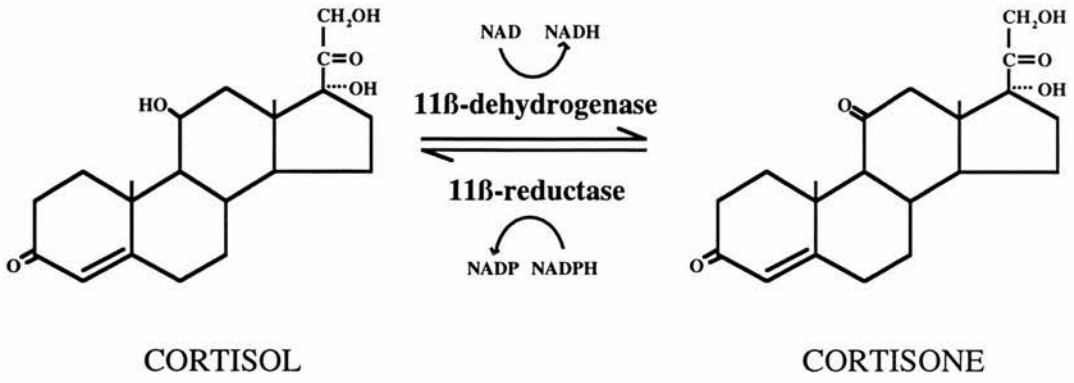
glucocorticoid receptor and most are found in the N-terminal domain (Dalman et al., 1988; Smith et al., 1989). Hormone-induced hyperphosphorylation increases the charge at the phosphorylated sites but also increases the overall negative charge around the region of the N-terminal domain that is involved in transactivation (Bodwell et al., 1995). Hyperphosphorylation may play a role in regulating transactivation of GR, or alternatively, may be of relevance to the cell cycle dependence of GR activity (Hu et al., 1994).

1.2.7 Modulation of ligand availability for nuclear receptors

The hormone ligands for nuclear receptors, including the receptors for glucocorticoids, oestrogens and T_3 are interconverted between active and inactive forms by enzymes which exhibit tissue-specific distributions. Different isoforms of an enzyme often exist, with one form activating and the other inactivating hormone, thereby controlling active intracellular ligand concentration. For example, during development, the differential expression of deiodinase enzymes (interconverting active and inactive thyroid hormones) in placenta and foetal tissue is essential for normal foetal development (Moreno et al., 1994; d'Escobar et al, 1993). Intracellular levels of active glucocorticoids are governed by the activity of the 11ß-hydroxysteroid dehydrogenase enzymes. The work presented in this thesis is on the transcriptional regulation of 11ß-hydroxysteroid dehydrogenase type 1 and in the next section (Section 1.3) I shall discuss the regulation and actions of the 11ß-hydroxysteroid dehydrogenase enzymes.

1.3 11ß-hydroxysteroid dehydrogenase

The enzyme 11ß-hydroxysteroid dehydrogenase (11ß-HSD) catalyses the interconversion of ß-hydroxy and keto groups at position C11 in glucocorticoids, thereby regulating the access of glucocorticoids to glucocorticoid receptors. The reaction catalysed by 11ß-HSD is shown in Figure 1.8.



CORTISOL CORTISONE

Figure 1.8 *Modulation of glucocorticoids by 11ß-hydroxysteroid dehydrogenase*
The figure shows the reaction carried out by 11ß-HSD in man. In rats, corticosterone and 11-dehydrocorticosterone are interconverted

In 1953, 11ß-HSD enzyme activity was first described in rat liver interconverting steroid 11-hydroxy and 11-keto groups (Amelung et al., 1953). Now, two forms of 11ß-HSD have been identified, designated type 1 and type 2 (in order of discovery). 11ß-HSD1 is a lower affinity, NADP(H) dependent enzyme which is widely expressed, with particularly high levels found in liver (Agarwal et al., 1989) where its expression *in vivo* is hormonally regulated (Lax et al., 1978; Low et al., 1993; Low et al., 1994c). In contrast, 11ß-HSD2 is a high affinity NAD^+ dependent enzyme expressed predominantly in aldosterone target tissues (e.g. kidney) and in placenta, which appears to function exclusively as a dehydrogenase, rapidly inactivating glucocorticoids (Albiston et al., 1994; Brown et al., 1993; Brown et al., 1996a; Rusvai & Naray-Fejes-Toth, 1993). The main properties of the two enzymes are summarised in Table 1.2. The biological role of 11ß-HSD1 is not clear, although in general its level of expression correlates with GR expression (Whorwood et al., 1992), and it may be important for regulating access of glucocorticoids to glucocorticoid receptors (reviewed in Section 1.3.2.1). 11ß-HSD2 is found predominantly in mineralocorticoid target tissues and placenta and its biological function in kidney has been well described (Section 1.3.2.2.1)

1.3.1 Properties, function and regulation of 11ß-HSD1

1.3.1.1 Properties of 11ß-HSD1

11ß-HSD1 was originally identified as a dehydrogenase and on that basis was purified from liver (Lakshmi & Monder, 1988). It is a 34kDa NADP-dependent

Properties	11ß-HSD1	11ß-HSD2
Molecular Weight (kDa)	34	44
Reaction direction (intact cells)	11ß-reductase	11ß-dehydrogenase
Reaction direction (cell homogenates)	reversible	11ß-dehydrogenase
pH optimum	10	7.5-8.5
Co-substrate	NADP	NAD
Tissue distribution	widespread, highest in liver	MR target tissues and placenta
Km (nM) (cortisol)	17000	54

Table 1.2 *Comparison of the properties of the type 1 and type 2 11ß-hydroxysteroid dehydrogenases*

See text for details (adapted from Seckl, 1993).

glycoprotein (Lakshmi and Monder, 1988) with a K_m for cortisol and corticosterone in the μM range (Monder & Lakshmi, 1989; Monder et al., 1991). Subsequently, the corresponding cDNA was isolated from a rat liver cDNA library (Agarwal et al., 1989), and shown to encode both 11ß-reductase and 11ß-dehydrogenase activity in transfected CHO cells (Agarwal et al., 1989). However, recent evidence suggests when assayed in intact cells, 11ß-HSD1 encoded predominantly reductase activity with little or no associated dehydrogenase activity, for example, in primary cultures of human fibroblasts (Hammami & Siiteri, 1991), primary cultures of rat Leydig cells (C. Leckie, personal communication), primary cultures of rat hepatocytes (Jamieson et al., 1995) and primary rat neuronal cultures (Rajan et al., 1996). Reductase activity was also found in cells transfected with 11ß-HSD1 cDNA; transiently transfected COS7 cells (Low et al., 1994a), stably transfected TBM cells (a toad bladder cell line) (Duperrex et al., 1993) and transiently transfected Y1 cells (P. Jamieson, personal communication). *In vivo*, indirect evidence also suggests that hepatic 11ß-HSD1 acts in the reductase direction. In humans given an oral dose of cortisone, plasma levels of cortisol increase suggesting that cortisol is produced from cortisone by 11ß-HSD1 in liver (Walker et al., 1992). 11ß-HSD1 cDNAs have now been isolated from human, monkey, sheep and mouse (Moore et al., 1993; Rajan et al., 1995; Tannin et al., 1991; Yang et al., 1992) and the corresponding human (Tannin et al., 1991) and rat (Moisan et al., 1992) genes isolated. There is widespread expression of a single mRNA species which exists in adult liver, testis, ovary, lung, vascular tissue and in the brain (Agarwal et al., 1989; Moisan et al., 1990a), but in kidney at least three mRNAs are present (Krozowski et al., 1990; Moisan et al., 1992). Transcription starts at a single site in both liver and hippocampus (Moisan et al., 1992), however, kidney uses two additional transcriptional start sites, together accounting for about 50% of 11ß-HSD1 transcripts in kidney (Moisan et al., 1992). One of the mRNAs has an extended 5' untranslated region, but does not encode an N-terminally extended protein (Moison et al., 1992); the other mRNA initiates within the first intron and is predicted to encode a truncated protein (Moison et al., 1992). There is however, no evidence to show the truncated RNA is translated *in vivo*. In liver, during foetal development, 11ß-HSD1 is expressed prior to birth in mouse and its expression corresponds with the onset of glucocorticoid receptor mediated functions such as glucose homeostasis (Section 1.1.5.1), 11ß-HSD1 may potentiate glucocorticoid action at this time.

1.3.1.2 Functions of 11ß-HSD1

11ß-HSD1 is widely expressed with highest levels found in liver and lower levels in other tissues including brain (hypothalamus, cerebellum, hippocampus, and cortex), kidney, adipose tissue, adrenal and gonads (Agarwal et al., 1989; Moison, et al., 1990a). The biological role of 11ß-HSD1 remains unclear. However, many lines of evidence point to a role for 11ß-HSD1 in modulating glucocorticoid access to glucocorticoid receptors. The reductase activity of 11ß-HSD1 predominates in intact cells and in perfused liver (Section 1.3.1.1), suggesting that *in vivo* 11ß-HSD1 is regenerating active glucocorticoid from inert substrates. Furthermore, the relative levels of expression of glucocorticoid receptor mRNA and 11ß-HSD1 mRNA in various tissues positively correlate, whereas no correlation is seen with mRNA encoding mineralocorticoid receptors (Whorwood et al., 1992), consistent with a role for 11ß-HSD1 in modulating access of glucocorticoids to glucocorticoid receptors. 11ß-HSD1 is not essential for viability or reproduction in mice as mice lacking an intact 11ß-HSD1 gene are healthy and fertile (Kotelevtsev et al., 1996b). Below I will review the possible biological functions in a limited number of tissues, for which evidence exists for a role for 11ß-HSD1.

1.3.1.2.1 Liver

Increased glucocorticoid levels result in decreased insulin sensitivity and increased hepatic glucose production (Cryer, 1992). In humans, administration of carbenoxolone (an inhibitor of 11ß-HSD) results in increased insulin sensitivity and decreased glucose production (Walker et al., 1995). Attenuation of hepatic 11ß-HSD1 expression in rat is associated with decreased levels of mRNA encoding the key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEPCK) (Jamieson et al., 1996). Mice homologous for a targeted disruption of the 11ß-HSD1 gene, also have reduced fasting glucose levels and decreased expression of G6Pase compared to wild type animals (Kotelevtsev et al., 1996a; Y. Kotelevtsev personal communication). All of this data is consistent with a role for 11ß-HSD1 in maintaining glucose homeostasis (Section 1.1.5.1).

1.3.1.2.2 Brain

The function of 11ß-HSD1 in the CNS is not clear, but may relate to mood, neuronal survival and glucocorticoid feedback. It is widely expressed in the brain, with highest

levels in cerebellum, hippocampus and cortex (Moisan et al., 1990a). It is likely that as in liver, neuronal 11ß-HSD1 also predominantly encodes reductase activity e.g. in primary cultures of rat hippocampal neurones (Rajan et al., 1996), suggesting that it is the reductase activity of 11ß-HSD1 and not dehydrogenase activity which is modulating glucocorticoid access to corticosteroid receptors. In the hippocampus, there is no 11ß-HSD2 to restrict access of glucocorticoid to hippocampal MR (Roland & Funder, 1996; Roland et al., 1995), which therefore functions as a high affinity glucocorticoid receptor, not as an aldosterone receptor. 11ß-HSD1 may be providing glucocorticoid to both receptors, although it is likely that MR is constitutively occupied at physiological concentrations of glucocorticoids, including during the diurnal nadir (Rajan et al., 1996).

1.3.1.2.3 Testis

Testosterone production by the rat testis is suppressed by glucocorticoids (Saez et al., 1977), through glucocorticoid receptor-mediated actions (Bambino & Hseuh, 1981; Welsh et al., 1982). 11ß-HSD1 immunoreactivity was absent from immature rat testis but became detectable in rats aged 33 days in the Leydig cells of the testis when oxidative activity appeared to predominate over reductase activity (Phillips et al., 1989). It was suggested that expression of 11ß-HSD1 at puberty led to the inactivation of glucocorticoids, allowing testosterone production (Gao et al., 1996; Phillips et al., 1989). In support of this, addition of carbenoxolone to Leydig cells in culture potentiated the inhibition of testosterone production by corticosterone (Monder, 1991). However, recent work in our laboratory has shown that primary cultures of rat Leydig cells contain 11ß-HSD1 mRNA and express reductase activity in intact cells, with little associated dehydrogenase activity (Leckie & Seckl, 1996). In contrast, 11ß-HSD2 mRNA was absent from rat testis. If indeed testicular 11ß-HSD1 is reductive rather than oxidative, then it cannot be responsible, by inactivating glucocorticoid, for the production of testosterone at puberty. The function of 11ß-HSD1 in the testis therefore remains controversial. It is noteworthy that mouse testis does not express 11ß-HSD1 mRNA (Rajan et al., 1995; C. Leckie, personal communication), suggesting that 11ß-HSD1 may not be involved in regulating testosterone production.

1.3.1.2.4 Ovary

Glucocorticoid receptors are found in the ovary (Schreiber et al., 1982) and 11ß-HSD1 protein and mRNA are located in the oocyte and luteal bodies (Benediktsson et al., 1992) indicating that 11ß-HSD1 may be modulating glucocorticoid access to GR and hence ovarian function. It has been suggested that high intrafollicular cortisol is necessary for the complete maturation of the oocyte (Michael et al., 1993b) and to inhibit the action of luteinising hormone on steroidogenesis in human granulosa-lutein cells (Michael et al., 1993a).

1.3.1.3 Regulation of 11ß-HSD1

Most studies investigating the regulation of 11ß-HSD have measured total activity and have been unable to distinguish between 11ß-HSD1 and 11ß-HSD2 activity, some do however measure 11ß-HSD1 mRNA levels which can be used to infer 11ß-HSD1 activity. Both 11ß-HSD1 and 11ß-HSD2 are regulated in a tissue specific and developmentally specific manner, and in adults are hormonally regulated.

There is very little expression of 11ß-HSD1 mRNA in the developing mouse embryo. 11ß-HSD1 mRNA first becomes detectable in mouse foetal liver, at E10.5 with expression in lung, kidney, stomach and liver of newborn mice, increasing to adult levels by approximately 3 weeks of age (V. Rajan, personal communication), with highest expression in the liver at all stages.

In the adult rat *in vivo* 11ß-HSD1 mRNA is regulated by glucocorticoids in all tissues examined including hippocampus, liver and kidney (Low et al., 1994c; Moisan et al., 1990b). Additionally, dexamethasone increases 11ß-HSD1 mRNA expression *in vitro* in fibroblasts (Hammami and Siiteri, 1991), primary hepatocytes (Jamieson et al., 1995) and 2S FAZA cells (Voice et al., 1996). In all three cell systems, expression is also regulated by insulin, with insulin decreasing 11ß-HSD1 activity (Hammami and Siiteri, 1991; Jamieson et al., 1995; Voice et al., 1996).

In rats, hepatic 11ß-HSD1 activity and mRNA is sexually dimorphic, with males having approximately 2-fold higher expression than females (Low et al., 1994b); this hepatic sexual dimorphism of 11ß-HSD1 expression is not, however, seen in mice (Rajan et al., 1995). The sexual dimorphism is due to the sex-specific patterns of

growth hormone secretion, which in turn is determined by sex steroids (Low et al., 1994b). In addition, administration of oestradiol to rats causes a dramatic decrease in hepatic 11ß-HSD1 activity and mRNA to very low or undetectable levels, whilst leaving hippocampal 11ß-HSD1 activity and mRNA unaffected (Low et al., 1994b). 11ß-HSD1 is also regulated by thyroid hormone which, attenuates 11ß-HSD1 activity and mRNA levels in liver and pituitary (Koerner & Hellman, 1964; Whorwood et al., 1993).

1.3.2 Properties, function and regulation of 11ß-HSD2

1.3.2.1 Properties of 11ß-HSD2

11ß-HSD2 is an NAD-dependant, exclusive dehydrogenase which has been best characterised in kidney (Rusvai and Naray-Fejes-Toth, 1993) and placenta (Brown et al., 1993). It has a molecular size of 44kDa, and high affinity for corticosterone and cortisol, with a K_m in the nM range (Albiston et al., 1994; Brown et al., 1993; Brown et al., 1996a; Rusvai and Naray-Fejes-Toth, 1993). 11ß-HSD2 cDNA clones have recently been isolated from human, sheep, rabbit and rat kidney (Albiston et al., 1994; Agarwal et al, 1994; Naray-Fejes-Toth & Feyes-Toth, 1995; Zhou et al., 1995) and human placental libraries (Brown et al., 1996a). These cDNAs are very similar to each other but show only 14% identity with 11ß-HSD1 cDNA (Albiston et al., 1994). 11ß-HSD2 mRNA is highly expressed in kidney, colon, pancreas and placenta. The human gene encoding 11ß-HSD2 has recently been cloned (Agarwal et al., 1995) as well as the mouse gene encoding 11ß-HSD2 (Cole, 1995). Patients with the syndrome of Apparent Mineralocorticoid Excess (AME) (Section 1.3.2.2.1) have mutations which disrupt the 11ß-HSD2 gene (Mune et al., 1995; Wilson et al., 1995a; Wilson et al., 1995b), suggesting that a lack of active 11ß-HSD2 is responsible for the disorder (Section 1.3.2.2.1).

1.3.2.2 Function of 11ß-HSD2

The function of 11ß-HSD2 appears to be protective. In adults it plays a crucial role in protecting MR from occupation by glucocorticoids (Section 1.3.2.2.1) and during development 11ß-HSD2 protects the foetus and foetal tissues from inappropriate exposure to glucocorticoids. The presence of 11ß-HSD2 by inactivating glucocorticoid allows selective access of aldosterone to MR in cells which co-express 11ß-HSD1 and MR.

1.3.2.2.1 Mineralocorticoid target tissues and the syndrome of AME

11ß-HSD2 in the distal nephron inactivates glucocorticoids, permitting only aldosterone present at approximately one hundredth the level of glucocorticoids to gain access to the otherwise non-selective MR (Figure 1.9). The function of 11ß-HSD2 has become apparent from studying patients who have defective 11ß-HSD2 activity and suffer from the syndrome of Apparent Mineralocorticoid Excess (AME) which results in glucocorticoid occupation of MR, thereby mimicking the effects of mineralocorticoids resulting in salt retention and hypokalaemia and hence high blood pressure (Stewart et al., 1988; Ulick et al., 1979). They have an increased ratio of cortisol:cortisone metabolites in urine, and 11α^3H-cortisol when administered exhibits a prolonged half life, confirming the impaired inactivation of cortisol (Stewart et al., 1988). This is now known as AME Type1, having a defect in the 11ß-dehydrogenase, but not 11-oxidase activities of 11ß-hydroxysteroid dehydrogenase. Type 2 AME has also been described. This is identical to Type-1 AME except that there is a normal cortisol:cortisone metabolite ratio. Proof of abnormal 11ß-dehydrogenation was obtained as 11α^3H-cortisol had a prolonged half life (Mantero et al., 1994).

1.3.2.2.2 Function of 11ß-HSD2 in placenta and during development

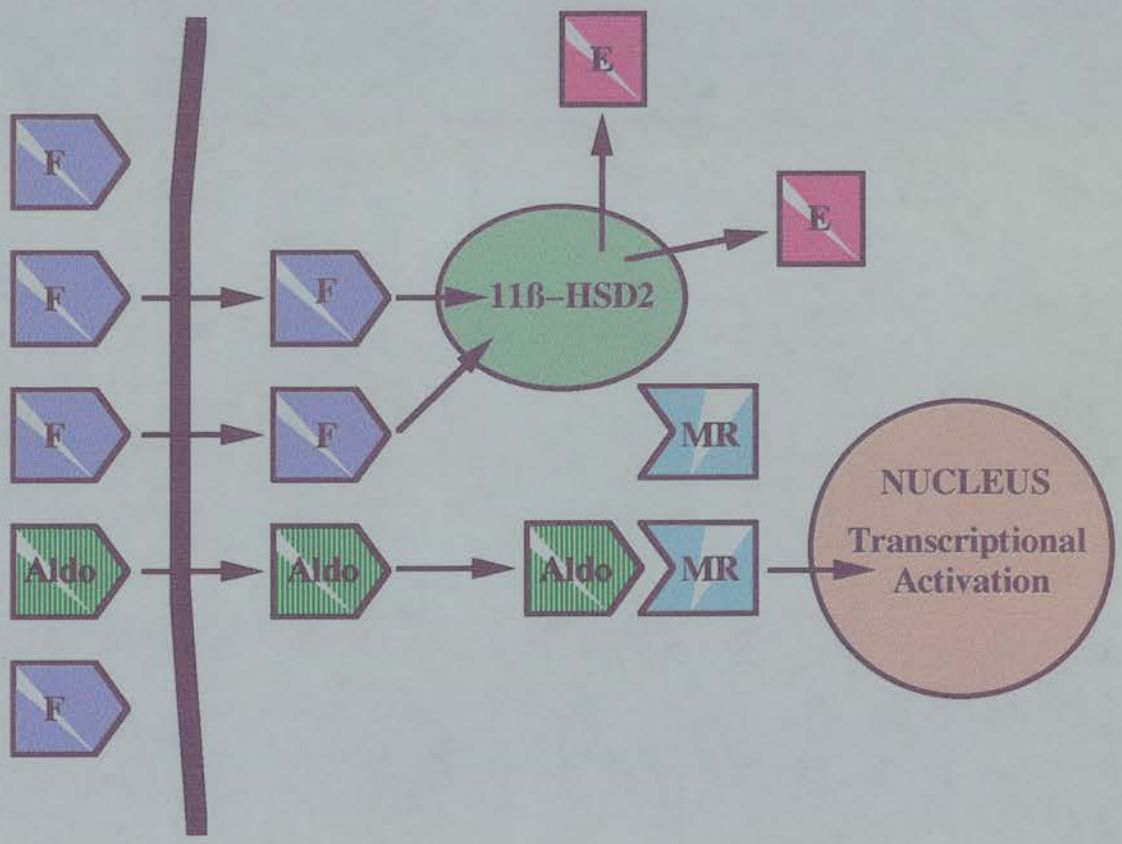
11ß-HSD2 is widely expressed in developing foetus which is consistent with a role in ensuring a glucocorticoid-free environment in early development (Brown et al., 1996b) which would otherwise result in detrimental effects on development (Brown et al., 1996b). 11ß-HSD2 mRNA disappears during mid-late gestation, permitting active glucocorticoid to rise in tissues. 11ß-HSD2 plays a similar role in placentas, restricting access of glucocorticoids across placenta during development of the foetus. It seems to be especially important during early foetal stages as expression in mouse placenta is high until E15.5, after which time it rapidly disappears. In addition exposure to high levels of maternal glucocorticoids results in low birth weight and an increased risk in adult life of hypertension (Benediktsson et al., 1993).

1.3.2.3 Regulation of 11ß-HSD2

Very few studies describe the regulation of 11ß-HSD2 in adults. Oestrogen probably increases 11ß-HSD2 activity in kidney (Low et al., 1993) and 11ß-HSD2 activity in the baboon placenta is increased by oestrogen treatment (Baggia et al., 1990). Rats

Figure 1.9 *The role of 11ß-HSD2 in the human distal nephron*

Both aldosterone and cortisol can enter the renal cortical collecting cells, however 11ß-HSD2 acts to protect mineralocorticoid receptor (MR). The active glucocorticoid cortisol (F) is inactivated to form cortisone (E), thus allowing aldosterone to bind and activate MR causing MR to enter the nucleus and activate transcription (adapted from Seckl, 1993).


E
F
E
F
F
11ß–HSD2
F
F
MR
NUCLEUS
Transcriptional
Activation
Aldo
Aldo
Aldo
MR
F

treated with dexamethasone, deoxycorticosterone and 9α-fluorocortisol show increased renal 11ß-HSD2 activity (Li et al., 1996) and activity in JEG3 cells, a human cytotrophoblastic cell line, was increased by treatment with forskolin or dibutyryl cAMP suggesting that hormones that activate protein kinase A may increase expression of 11ß-HSD2 (Pasquarette et al., 1996).

The bulk of this thesis is concerned with transcriptional regulation of hepatic 11ß-HSD1. In the next sections I shall briefly review what is known of the processes involved in initiation of transcription in eukaryotes and of the transcription factors involved in gene expression in mammalian liver.

1.4 Transcription in eukaryotes

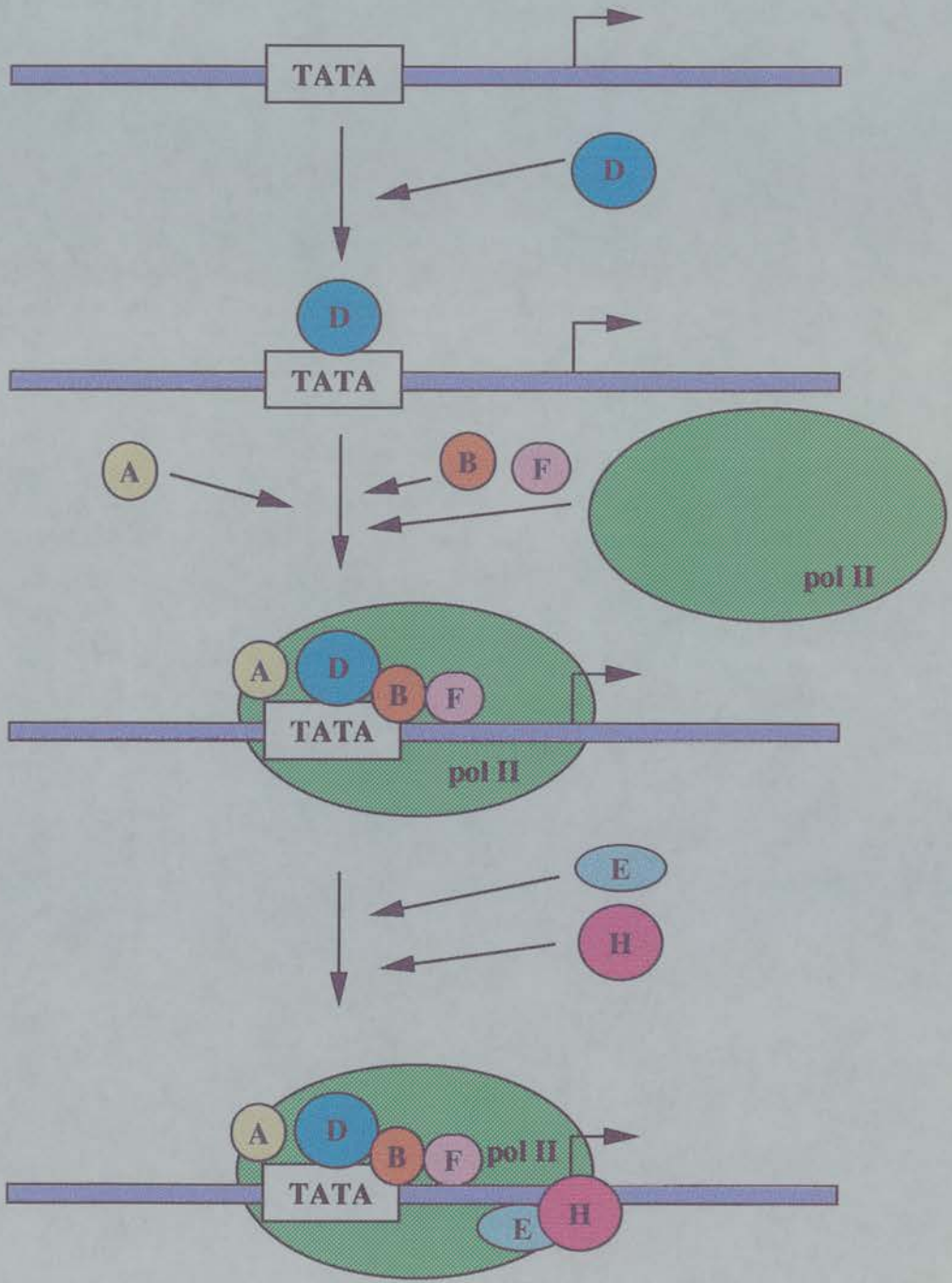
1.4.1 The assembly of the basal transcription initiation complex

In eukaryotes, three types of RNA polymerase exist which transcribe different types of RNA. Type I RNA polymerase transcribes the genes encoding 18S, 5.8S and 28S ribosomal RNA's; 5S ribosomal RNA and transfer RNA are transcribed by RNA polymerase III; RNA polymerase II transcribes mRNA, RNA that will be translated into proteins.

The basal promoter in most cases consists of the initiator element, a weakly conserved element encompassing the transcription start, first described in the TATA-less terminal deoxynucleotidyl transferase gene (Smale & Baltimore, 1989), and in many genes but by no means all, the TATA box, located 25 to 30 base pairs upstream of the transcription start (Conaway & Conaway, 1993). Initiation of eukaryotic mRNA synthesis is controlled by the action of multiple transcription factors that regulate the ordered assembly and activity of the transcription complex containing RNA polymerase II and the general transcription factors (some of which include TFIIA, IIB, IID, IIE, IIF and IIH) on the basal promoter (Hernandez, 1993). Transcription factors, chromatin structure, and the methylation state of promoter DNA all provide additional levels of control on the activity of the RNA polymerase II transcription complex (Sections 1.4.4). The exact order (and even involvement) of assembly of the basal transcription factors remains controversial, and may differ according to the promoter, but the generally accepted view is outlined in Figure 1.10 and summarised below.

Figure 1.10 *Assembly of the basal transcription complex*

Schematic model of stepwise basal transcription complex assembly on a TATA containing promoter. The bent arrow indicates the site of transcription initiation. General transcription factors are indicated by bold letters.


TATA
D
D
TATA
A
B
F
pol II
A
D
B
F
TATA
pol II
E
H
A
D
B
F
pol II
TATA
E
H

The first step in assembly is probably binding of TFIID to the basal promoter; to both TATA-less and TATA containing promoters (Kaufmann & Smale, 1994; Purnell & Gilmore, 1993). Mammalian TFIID consists of the TATA binding protein (TBP) together with at least 8 TBP associated factors (TAF's) (Dynlacht et al., 1991; Tanese et al., 1991; Goodrich & Tjian, 1994a). Although, TBP alone can bind to the TATA element and is sufficient for basal transcription, some, or all of the TAFs are required for the activity of transcriptional activator proteins (Dynlacht et al., 1991; Hoey et al., 1993; Tanese et al., 1991; Zhou et al., 1992; Zhou et al., 1993), and some may be involved in the formation of nucleoprotein complexes at the transcription start. For example, TAF complexes resembling histone tetramers or octamers have been described (Xie et al., 1996; Hoffmann et al., 1996), and it has been suggested that one role of these TAFs is to induce a partial bend in DNA which may contribute to the stability of the initiation complex (Hoffmann et al., 1996).

The next step in the formation of the transcription initiation complex is the association of TFIIA. The requirement for TFIIA is controversial and it may play a stimulatory though not essential role in assembly of the basal transcription complex, possibly through interacting with one of the TAFs (Yokomori et al., 1993). In addition, TFIIA has been implicated as a target for certain transcriptional activator protiens (Kobayashi et al., 1995).

The next component of the transcription complex to join the initiation complex is likely to be TFIIB which binds directly via its C-terminal domain to DNA-bound TBP (Nikolov et al., 1995) (Figure 1.10), and makes weak contacts with the DNA phosphate backbone (Lee & Hahn, 1995; Nikolov et al., 1995). TFIIB and RNA polymerase II may be preassociated and enter the initiation complex together (Conaway & Conaway, 1993) ; N-terminus is a zinc finger domain that is essential to recruit RNA polymerase II to the initiation complex, possibly via an interaction with TFIIF (Buratowski, 1994), the factor that delivers RNA polymerase II to the initiation complex (Ha et al., 1993) (Figure 1.10). Transcriptional activators can bind TFIIB (Lin et al., 1991) and may be necessary for the stable interaction of TFIIB with TFIID (Choy & Green, 1993).

Therefore, through the combined activities of TFIID, TFIIB and TFIIF, RNA polymerase II is stably recruited into the preinitiation complex (Buratowski, 1994) (Figure 1.10). The carboxy terminal domain of RNA polymerase II is implicated in contacting DNA (Suzuki, 1990) and TBP (Usheva et al., 1992). It contains a seven peptide repeat (YSPTSPS) which is phosphorylated *in vivo*, and the polymerase exists in at least two distinguishable forms that differ in the extent of this phosphorylation (Conaway and Conaway, 1993). The hypophosphorylated form of the polymerase is preferentially recruited into the preinitiation complex, whilst the transcribing enzyme is hyperphosphorylated (reviewed in Buratowski, 1994). TFIIH which can phosphorylate the carboxy terminal domain, has been implicated in the conversion of the initiation complex into an elongation complex (Goodrich & Tjian, 1994b).

TFIIE and TFIIH are in general required to form the preinitiation complex (Wang et al., 1992a). TFIIE is a 34/56kDa heterodimer (Inostroza et al., 1991), whereas TFIIH is a multisubunit complex made up of at least eight polypeptides (Schaeffer et al., 1994). It is associated with several activities, including a DNA dependent and a Cdk dependent protein kinase activity capable of phosphorylating the carboxy terminal domain of RNA polymerase II (Lu et al., 1992; Serizawa et al., 1992; Serizawa et al., 1995; Shiekhatter et al., 1995; Liao et al., 1995), a DNA-dependent ATPase activity and an ATP-dependent DNA helicase activity (Drapkin et al., 1994; Roy et al., 1994). Evidence suggests that TFIIE may regulate the activities of TFIIH (Drapkin et al., 1994; Ohkuma and Roeder, 1994). TFIIH has been implicated as a target for the transcriptional activators VP16 and p53 which interact *in vitro* with TFIIH (Xiao et al., 1994). TFIIH also has an essential role in excision repair of DNA (Drapkin et al., 1994; van Vuuren et al., 1994; Mu et al., 1995).

It should be emphisised that the above scheme outlined in Figure 1.10 is based on experiments carried out *in vitro* and it is possible that *in vivo* active transcription complexes may be formed in pre-assembled manner rather than by stepwise recruitment of individual complexes (Koleske & Young, 1994; Ossipow et al., 1995). The assembly of the basal transcription complex and the conversion into an actively transcribing complex can be influenced, either positively or negatively by the action of transcription factors.

1.4.2 Transcription factors

The structure of transcription factors is modular. In general, transcription factors have a domain required for tethering close to the promoter, either a DNA binding domain or a domain required to bind to a DNA-bound protein, and a domain involved in transcriptional control, the 'activation' domain (Ptashne M & Gann AF, 1990). Transcriptional activation domains have been much less well characterised than those involved in DNA binding and may be much more diverse. However, some activation domains appear to fall into particular classes; the acidic activation domains e.g. VP16, the glutamine-rich activation domains e.g. Sp-1 and the proline rich activation domains (Tjian & Maniatis, 1994). These activation domains interact with the basal transcription machinery to alter the rate of transcription initiation (Section 1.4.3).

The DNA binding domain of transcription factors have been much better characterised. The majority of proteins which bind directly to DNA use one of two structures in DNA binding; an α-helix or a ß-sheet. The ß-sheet has so far only been described in a limited number of prokaryotic DNA binding proteins (Breg et al., 1990; Phillips, 1991; Raumann et al., 1994) and so will not be further discussed in this thesis. Below I will briefly describe eukaryotic DNA binding motifs containing an α-helix.

1.4.2.1 α-helix-containing DNA binding domains

An α-helix is the most common mode of DNA binding and occurs in several 'motifs' including the helix-turn-helix (HTH) / homeodomain motif, $(Cys)_4$ and $(Cys)_2(His)_2$ zinc fingers, the leucine zipper motif and the helix-loop-helix (HLH) motif.

1.4.2.1.1 Helix-turn-helix and homeodomains

The HTH DNA binding motif was first described in prokaryotic DNA binding proteins and was later found to occur in a related eukaryotic motif, (reviewed in Burley, 1994). Structural studies (2D NMR and X-ray crystallography) have shown that the overall fold of the homeodomain shows strong similarity to the prokaryotic HTH motif (Anderson et al., 1981; Klemm et al., 1994; Ohlendorf et al., 1982). Both use an α-helix (the 'recognition' helix) to specifically recognise the exposed edges of the base pairs in the major groove (Figure 1.11a).

The amino acid 60 residue homeodomain forms a stably folded structure containing 3 α-helices, and can bind DNA by itself as a monomer (Affolter et al., 1990; Qian et al., 1989; Sauer et al., 1988) in contrast to the prokaryotic HTH proteins which all bind DNA as dimers (Anderson et al., 1981; Ohlendorf et al., 1982). The main DNA contacts are made by helix 3 of the homeodomain which fits into the major groove (as in the prokaryotic HTH protein (Wharton et al., 1984)). In addition, the N-terminal arm of the homeodomain (Kissinger et al., 1990) makes contacts in the minor groove of the DNA. The homeodomain therefore contacts both faces of DNA (major and minor groove interactions) giving a stable monomer interaction (Garcia-Blanco et al., 1989).

1.4.2.1.2 The leucine zippers and helix-loop-helix proteins

The leucine zipper family of transcription factors is a large group of proteins, which bind to DNA as either homo- or heterodimers. This family includes the liver-enriched transcription factor C/EBP (reviewed in Section 1.5), which is the subject of a large part of the work contained in this thesis. Like the leucine zipper proteins, HLH transcription factors all bind to DNA as homo- and heterodimers and are typified by the prototype HLH dimer myc/max. Both contain an N-terminal region rich in basic amino acids which bind to DNA, and an α-helical segment (the leucine zipper or the HLH region) involved in dimerisation.

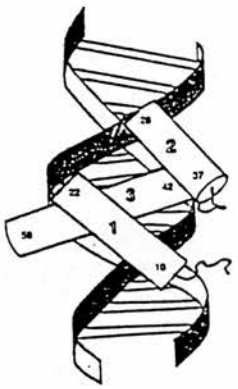
Crystal structures of the DNA binding domains of several leucine zipper containing transcription factors and HLH proteins have been determined (O'Shea et al., 1991; Ferre-D'Amare et al., 1993) and show some similarities; representations of the DNA binding domains are shown in Figure 1.11b and Figure 1.11c respectively.

The structure of the basic leucine zipper of GCN4 (a yeast leucine zipper transcription factor) complexed to either a CRE or an AP-1 binding site (Ellenberger et al., 1992; Konig & Richmond, 1993) showed that the basic region forms an α-helix which contacts the major groove of a half site, contacting both the phosphate back bone and the edges of the base pairs (Ellenberger et al., 1992; Konig & Richmond, 1993). The 30-40 amino acid C-terminal 'leucine zipper' region contains an α-helix with a heptad repeat of leucines ($(leuX_6)_N$, where X is any amino acid) which forms the dimerisation interface, with two parallel α-helices in a coiled-coil

Figure 1.11 *Transcription factor DNA binding domains*

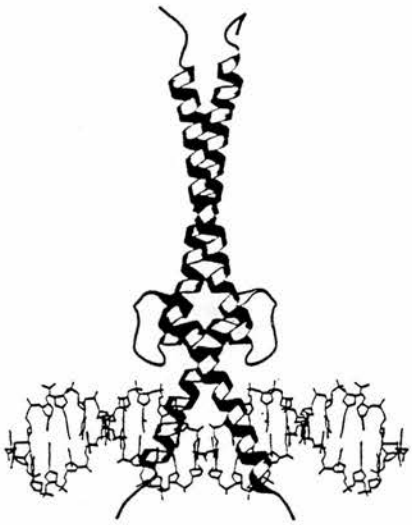
Models of the DNA binding domains of (a) The *Drosophila* homeodomain protein *engrailed*, (b) a dimer of the yeast leucine zipper protein GCN4, (c) a homodimer of the mammalian helix-loop-helix protein, MAX, (d) the mammalian steroid receptor protein and (e) the mammalian zinc finger protein zif 268. Engrailed, GCN4, MAX and zif 268 are shown bound to DNA. Taken from Pabo & Sauer, 1992; Ellenberger, 1994; Schmiedeskamp & Klevit, 1994.

a

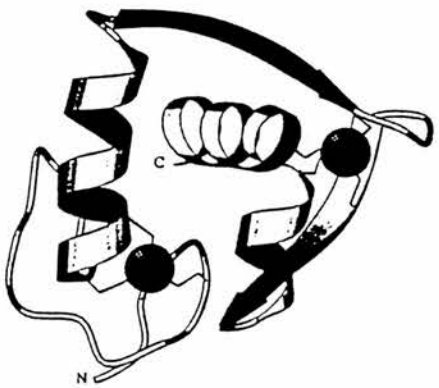


b

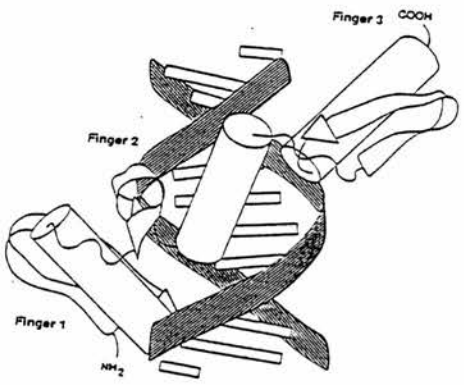
c



d



e



arrangement and one α-helix contributed from each subunit of the dimer (O'Shea et al., 1989).

The crystal structure of the Max homodimer/DNA complex shows that the DNA binding surface of Max is formed by a straight α-helix oriented parallel to the major groove. The HLH region is involved in dimerisation and forms a left handed parallel four helix bundle with a loop connecting helices 1 and 2 of each subunit of the dimer (Ferre-D'Amare et al., 1993).

1.4.2.1.3 Zinc fingers

At least 10 zinc-containing DNA-binding motifs exist and include the classical zinc finger (Cys_2His_2 type), nuclear hormone receptor zinc fingers (Cys_4 type), GAL4, Ring Finger and MetRS (reviewed in Klug & Schwabe, 1995). All contain zinc coordinated to Cys/His which holds together the structure in a form able to recognise and bind specifically to DNA. The Cys_4 type of zinc finger, formed in nuclear receptors is described in Section 1.2.1 and is depicted schematically in Figure 1.11d, and will not be further discussed here. In this section I limit the discussion to the classical zinc finger motif.

'Classical' zinc were first described in the *Xenopus* transcription factor IIIA and contain tandem repeats of the 30 amino acid zinc finger motif containing cysteine and histidine (O-X-Cys-X_{2-5}-Cys-X_3-O-X_5-O-X_2-His-X_{2-5}-His); where O is a hydrophobic amino acid and X is variable in character (Miller et al., 1985). The first structural data on Cys_2His_2 zinc finger came from the crystal structure of Zif268 (a mammalian transcription factor) bound to DNA (Pavletich and Pabo, 1991). The structure revealed that zinc fingers are modular structures with each finger recognising a 3 bp unit in the DNA recognition sequence (Pavletich and Pabo, 1991). Each zinc finger contains an α-helix which sits in the major groove, held in place by an antiparallel ß-sheet (Pavletich and Pabo, 1991).

1.4.3 Activation of transcription

Increased transcription occurs when RNA production is stimulated in response to activators binding to the promoter DNA or components of the basal transcription machinery (Emili et al., 1994; Kerr et al., 1993; Lee et al., 1991; Lieberman & Berk,

1991; Lin et al., 1991; Xiao et al., 1994). Transcription factors can alter transcription initiation, either by increasing recruitment of basal transcription factors to the promoter, for example, TFIID, TFIID/A or TFIIB (Klein & Struhl, 1994; Lieberman & Berk, 1994; Lin & Green, 1991; Sauer et al., 1995; Wang et al., 1992b), thereby increasing formation of the preinitiation complex, or by interacting with the preinitiation complex to cause conformational changes resulting in conversion of the preinitiation complex into an initiated complex (Roeder, 1991).

The precise mechanisms by which activators increase the rate of transcription are not clear and are likely to be diverse. Interactions between 'acidic' activators (VP16 in particular) and TBP (Ingles et al., 1991; Stringer et al., 1990), TFIIB (Lin et al., 1991; Roberts et al., 1993) and TFIIH (Xiao et al., 1994) have been described. However, many of these results were obtained *in vitro*, with partially purified preparations of proteins and it remains open to question the extent to which these interactions may occur *in vivo*.

Coactivators, or mediators e.g. PC4 transduce the activation signal from the DNA bound transcriptional activator to the basal transcription machinery, they do not themselves activate transcription (Ge and Roeder, 1994). Coactivators have also been identified for steroid receptors; the nuclear factor RIP140 interacts only with ligand-occupied ER; mutations within the ligand binding domain of ER which abolish transactivation function also eliminate the interaction with RIP140 (Cavailles et al., 1995).

1.4.4 Repression of transcription

Selective repression of gene transcription is of importance in generating the required pattern of tissue specific gene expression. Various mechanisms of repression exist, ranging from interference with transcriptional activation via activators, co-activators or the basal transcription machinery, active repression by 'co-repressors' to modification of chromatin structure or methylation of promoter DNA. Only the last 3 will be described here in any detail as evidence suggests they are likely to be important *in vivo* in tissue-specific control of gene expression.

1.4.4.1 Co-repressors of transcription

Co-repressors are proteins that interact with the repressor and the target of the repressor, probably the transcription initiation complex, and have recently been described for members of the nuclear receptor superfamily. In the absence of ligand, T_3R is a repressor of transcription. Two recent studies have shown that T_3R and RARα in the absence of ligand interact with a 'co-repressor' protein, called thyroid-retinoic-acid-receptor associated co-repressors (TRACs) (Chen & Evans, 1995; Horlein et al., 1995; Kurokawa et al., 1995), or silencing mediator for retinoic and thyroid hormone receptors (SMRT) (Chen & Evans, 1995). Comparison of protein sequences show homology between these factors in their receptor interacting domains, implying the existance of a family of co-repressor molecules (Perlmann & Vennstrom, 1995).

1.4.4.2 The role of chromatin in transcriptional regulation

Nucleosomes play a central role in repressing basal transcription *in vivo* and disruption of chromatin structure is needed for activation of transcription *in vivo*. Perhaps the best studied example is the activation of the MMTV promoter by GR, where positioned nucleosomes on the MMTV promoter prevent access of ubiquitous and general transcription factors to the promoter DNA (Beato et al., 1996). Binding of GR to MMTV DNA results in the displacement of a nucleosome on the MMTV promoter, allowing access of transcription factors to the promoter and leads to transcription initiation (Beato et al., 1996). Many other studies are demonstrating the importance of chromatin structure in gene activation and repression *in vivo*, for example, the action of steroid receptors in yeast requires SW1 (Yoshinga et al., 1992) and recent evidence shows the SWI/SNF complex, a component of the yeast RNA polymerase II holoenzyme, has ATP-dependent chromatin disruption capabilities which allows RNA polymerase II access to DNA encoding genes to be transcribed (Wilson et al., 1996). Histone acetylation plays a role in repression/activation of genes embedded in chromatin (Hebbes et al., 1994; Braunstein et al., 1993); acetylation of histones results in a change in nucleosome conformation, rendering nucleosomal DNA more accessible to transcription factors (Garcia-Ramirez et al., 1995). It is becoming increasingly clear that some generally acting transcription factors may act by acetylating histones, an example is the yeast GCN5 protein (Brownell et al., 1996). The importance of chromatin structure in

regulating transcription is also illustrated by the recently described conservation of structure between histones and certain basal transcription factors (Hoffmann et al., 1996; Xie et al., 1996); and the demonstration that TAFs probably form a nucleosome-like structure at the promoter in the pre-initiation complex at the transcription start (Hoffmann et al., 1996; Xie et al., 1996).

1.4.4.3 The role of methylation in gene repression

Vertebrate genomic DNA is methylated, at CpG dinucleotides, on the 5' position of cytosine (Bird, 1986). CpG dinucleotides are under-represented in the bulk of vertebrate DNA and occur in clusters, 'CpG islands', generally at the 5' end of genes (Bird, 1995). Methylation is essential for viability as a targeted disruption of the MeCP2 gene encoding a CpG methylase is lethal in early mouse embryogenesis (Tate et al., 1996). It has been suggested that methylation of DNA acts as a global repressor of transcription, reducing noise background, thereby allowing transcription of important genes (Bird, 1995). The effect of methylation appears to be modulated at any one site by the number of methylated sites; high densities of methyl-CpG repress even strong promoters, low densities of methyl-CpG only repress weak promoters (Bird, 1992; Boyes & Bird, 1992).

1.5 CCAAT/enhancer binding protein

11ß-HSD1 expression in liver is a major focus of the work I have carried out towards this thesis. I have explored the role of C/EBP in binding to the 11ß-HSD1 promoter and here I review the properties and functions of the C/EBP family of transcription factors.

1.5.1 Structure and characterisation of the CCAAT/enhancer binding protein

The CCAAT/Enhancer Binding Protein (C/EBP) was first identified in crude rat liver nuclear extract by virtue of its ability to bind to a CCAAT motif located approximately 80bp upstream of the transcription start of the herpes simplex virus thymidine kinase gene (HSV-TK), and to the enhancer of the Moloney murine sarcoma virus (MSV) located in the MSV long terminal repeat (LTR) (Graves et al., 1986; Johnson et al., 1987). C/EBP was purified from rat liver, and the encoding cDNA cloned (Landschultz et al., 1988). C/EBP was found to show homology to other transcription factors e.g. GCN4 and was one of the first leucine zipper

containing transcription factors (Section 1.4.2.1.2) to be identified. With the subsequent discovery of other related proteins (Table 1.3), the original C/EBP cDNA clone from rat liver was designated C/EBPα. C/EBPα encodes a 42kDa protein which is susceptible to proteolytic cleavage (Landschultz, 1988). Proteolytic cleavage of C/EBPα results in a 14kDa fragment consisting of the C-terminal 106 amino acids, including the basic leucine zipper region (Section 1.4.2.1.2), which retains DNA binding activity (Landschultz et al., 1988).

1.5.2 C/EBP related proteins

C/EBP related proteins (summarised in Table 1.3) all form homo- and heterodimers with other members of the family, but do not hetero-dimerise with other leucine zipper proteins such as Fos or Jun. All share very similar DNA binding domains and hence sequence specificity (the structure the DNA binding domain of leucine zipper proteins is reviewed in section 1.4.2.1.2 and the structure of C/EBP is shown in Figure 1.12). C/EBP related proteins can bind to a wide range of related DNA sequences which diverge considerably from the consensus binding site T(T/G)NNG(T/C)AA(T/G) (Locker, 1993) including ATF and CREB binding sites (Bakker & Parker, 1991) and binding sites for the PAR subgroup of leucine zipper transcription factors (VBP/TEF, DBP and HLF) (Mueller et al., 1990).

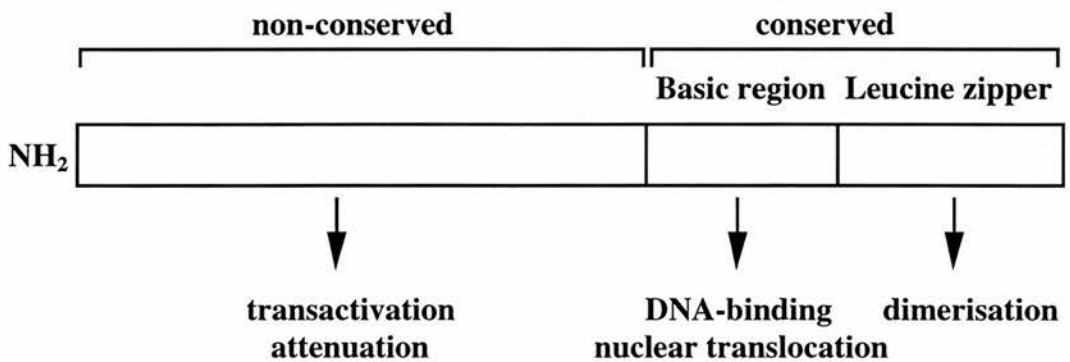


Figure 1.12 *Diagrammatic view of the structure of C/EBPs*
Domain structure of C/EBP and related factors. The basic/leucine zipper region is conserved and the N-terminal domain is non-conserved.

1.5.3 Inhibitors of C/EBP action

Shorter translation products of C/EBPα and C/EBPß (termed C/EBPα (30kDa) and LIP respectively) exist which are truncated at the N-terminal domain and lack part of

Protein	Synonyms	MWt (kDa)	Source	Reference
C/EBPα	C/EBP	42	rat	(Landschultz et al., 1988a)
			human	(Antonson et al., 1995)
C/EBPß	NF-IL6,	38	human	(Haas et al., 1995; Akira et al., 1990)
	IL-6DBP, LAP, CRP2,	32	rat	(Poli et al., 1990; Descombes et al., 1990; Williams et al., 1991)
	GPE-BP, AGP/EBP,	35	mouse	(Cao et al., 1991; Nishizawa et al., 1992; Chang et al., 1990)
	NF-M	45	chicken	(Katz et al., 1993)
C/EBPδ	NF-IL6ß	28	human	(Kinoshita et al., 1992)
	CRP3	29	mouse	(Cao et al., 1991; Williams et al., 1991)
C/EBPγ			rat	(Thomassin et al., 1992)
	Ig/EBP-1	45	mouse	(Roman et al., 1990)
	α1/EGP		chicken	(Bowers et al., 1992)
			human	(Davydov et al., 1995)
crp1		27	rat	(Williams et al., 1991)
d/C/EBP		49	drosophila	(Montell et al., 1992)
CHOP10	GADD153	29	mouse	(Ron et al., 1992)

Table 1.3 *Cloned cDNAs encoding members of the C/EBP family*

the transactivation domains (and therefore do not activate transcription), but are still capable of dimerisation and DNA binding (Calkhoven et al., 1994; Descombes & Schibler, 1991; Lin et al., 1993; Ossipow et al., 1993). It is possible that these factors form heterodimers with full length C/EBPs therefore sequestering the transcriptionally active subunits in inactive heterodimeric complexes. Another C/EBP related factor is CHOP-10 (C/EBP homologous protein), which dimerises with C/EBP's, but is unable to bind C/EBP consensus binding sites (Ron & Habener, 1992), and thus acts as a dominant negative regulator of C/EBP. CHOP is induced by a variety of cellular stresses including exposure to toxins, nutrient deprivation and the acute phase response (Carlson et al., 1993; Fornace et al., 1989; Ron and Habener, 1992; Sylvester et al., 1994; Ubeda et al., 1996).

1.5.4 Distribution of C/EBPα and C/EBPß mRNAs

C/EBPα and C/EBPß mRNA's show similar distributions, with highest levels in liver, fat and lung (Williams et al., 1991). The tissue distributions of C/EBPα and C/EBPß mRNA in rat are summarised in Table 1.4.

Tissue Distribution	C/EBPα mRNA	C/EBPß mRNA
Liver	+++	+++
Brown fat	+++	+
Small intestine	+	+
Lung	++	++
Kidney	+	+
Brain	-/+	+
Testis	-/+	+
Spleen	-/+	++

Table 1.4 Summary of the tissue specific distribution of C/EBPα and C/EBPß in rats. +++, highly expressed; ++, moderately expressed; +, weakly expressed; -/+ very weakly expressed (Birkenmeier et al., 1989; Descombes et al., 1990; Williams et al., 1991).

In liver, C/EBPα and C/EBPß expression is dependent on differentiation of hepatocytes. No expression of C/EBPα or C/EBPß mRNA is seen in liver before E12 and E14 respectively (Van den Hoff et al., 1994). Between E14 and birth, mRNA levels rise to reach adult levels by birth (Van den Hoff et al., 1994). However, no C/EBPα protein is detectable before birth despite the expression of C/EBPα mRNA in foetal liver, but C/EBPß protein is expressed (Diehl et al., 1994a). The regeneration of liver after partial hepatectomy has been used to examine the developmental regulation of C/EBPα and C/EBPß. Partial hepatectomy of adult liver in rodents induces the remaining liver cells to divide in a synchronised manner (Johansson & Andersson, 1990) while retaining almost all metabolic and hepatic functions (Friedman et al., 1984; Michalopoulos, 1990). A complex pattern of C/EBP related protein expression occurs after partial hepatectomy, with reduced C/EBPα and increased C/EBPß and C/EBPδ expression (Flodby et al., 1993), consistent with a role for C/EBPα only in fully differentiated post-mitotic cells.

Other C/EBP mRNA are widely distributed. C/EBPδ is found in liver, lung, kidney, adipose, brain, heart, testes, uterus and submaxillary gland (Williams et al., 1991). C/EBPγ mRNA is ubiquitously expressed with high levels found in early B cells (Roman et al., 1990).

1.5.5 Roles of C/EBP related proteins

Members of the C/EBP family of transcription factors are reported to have many roles and regulate networks of genes involved in terminal cell differentiation, arrest of mitotic growth, energy metabolism and the immune response (Darlington et al., 1995; Yeh & Mcknight, 1995; McKnight, 1993; Tanaka et al., 1995; Screpanti et al., 1995). An insight into some of the major roles of C/EBPα and C/EBPß have been provided by mice lacking either C/EBPα or C/EBPß.

1.5.5.1 The role of C/EBPα in the regulation of energy metabolism

C/EBPα was suggested to be a central regulator of energy metabolism (McKnight et al., 1989) and several lines of evidence support this hypothesis. C/EBPα is expressed in tissues that are involved in fuel metabolism (Section 1.5.4). Key enzymes involved in energy homeostasis such as PEPCK, acetyl CoA carboxylase, GLUT4, the insulin receptor and SCD1 all have C/EBP binding sites within their promoters (Park et al.,

1993; Tae et al., 1994; Kaestner et al., 1990; Kaestner et al., 1989; McKeon & Pham, 1991; Christy et al., 1989). Most recently, mice which lack C/EBPα have been described; the phenotype of these mice strongly supports the involvement of C/EBPα in the regulation of energy metabolism (Wang et al., 1995). Homozygous mice lacking the C/EBPα gene develop normally and are born in similar numbers to their wild type litter mates, demonstrating that C/EBPα does not play an essential role during development. However, they become profoundly hypoglycaemic and die several hours after birth (Wang et al., 1995). Injecting glucose into these mice enables them to survive for up to 40 hours after birth (Wang et al., 1995). Mutant animals showed many metabolic defects (Wang et al., 1995). They lacked hepatic glycogen at birth and glycogen synthase mRNA was undetectable. Also at birth mRNA's encoding the key gluconeogenic enzymes PEPCK, G6Pase and tyrosine amino transferase were undetectable in liver. However, the levels of PEPCK and G6Pase mRNA returned to normal 7 hours after birth, suggesting that expression of these enzymes relies on C/EBPα expression at a specific time during development, and that 7 hours after birth either compensatory mechanisms come into effect or other transcription factors are normally expressed which regulate the expression of these gluconeogenic enzymes. In addition to defects in liver metabolism these mice failed to accumulate lipid in brown and white adipose tissue (Darlington et al., 1995; Wang et al., 1995). C/EBPα gene knock-out mice also have defects in their immune response with a component of complement, C3, and γ-fibrinogen levels being reduced compared to wild type mice (Burgess-Beusse & Darlington, 1996).

1.5.5.2 The role of C/EBPß, C/EBPγ and C/EBPδ in the acute phase response

C/EBPß was first described as part of the interleukin-6 (IL-6) signalling pathway in liver (Akira et al., 1990). Roles for C/EBPα, C/EBPγ and C/EBPδ have also been proposed in the immune system (Burgess-Beusse and Darlington, 1996; Chang et al., 1990; Roman et al., 1990). Binding sites for these factors are found in the promoters of genes expressed in monocytes and macrophages including TNF-α (Pope et al., 1994), IL-6 (Bretz et al., 1994; Matsusaka et al., 1993; Tanaka et al., 1995), and lysozyme (Ness et al., 1993). C/EBPß and C/EBPδ are themselves induced by lipopolysaccaride (LPS), an inducer of the acute phase response (Section 1.6.5) (Kinoshita et al., 1992; Natsuka et al., 1992), and in peripheral blood monocytes C/EBPß is translocated to the nucleus upon LPS stimulation (Bretz et al., 1994).

C/EBPγ is found in early B cells and both C/EBPß and C/EBPγ are expressed in mature B cells (Cooper et al., 1992; Roman et al., 1990). C/EBPs bind to the promoter of immunoglobin genes, and C/EBPß and C/EBPγ mediate LPS and IL-4 responsiveness of the unrearranged V_H genes (Cooper et al., 1992; Lundgren et al., 1994).

A role for C/EBPß in the immune response is supported by the phenotype of mice lacking the C/EBPß gene. Two studies have described the construction of lines of C/EBPß knock-out mice (Screpanti et al., 1995; Tanaka et al., 1995). One study (Tanaka et al., 1995) reports that elimination of C/EBPß compromised viability, with fewer homozygous 'knock-out' mice reaching the weaning stage than predicted (Tanaka et al., 1995). Both lines of C/EBPß 'knock-out' mice are highly susceptible to yeast or bacterial infection (Screpanti et al., 1995; Tanaka et al., 1995) having defective activation of the immune response and mice lacking C/EBPß showed impaired induction of G-CSF, a cytokine involved in the immune response (Tanaka et al., 1995). Unexpectedly, C/EBPß 'knock-out' mice had increased interleukin 6 (IL-6) levels, which contradicted earlier reports that C/EBPß acts as a positive regulator of IL-6 production (Akira et al., 1990).

1.5.5.3 A role for C/EBPs in controlling cell proliferation

Additional roles of C/EBP's not addressed by the 'knock-out' mice are their developmental and antiproliferative activities which have been studied in adipocytes and liver. Mice lacking either the C/EBPα or C/EBPß have normal livers and have adipose tissue (Wang et al., 1995; Screpanti et al., 1995; Tanaka et al., 1995)., therefore C/EBPα and C/EBPß may not be essential for cell differentiation. However, the long term effects of loss of C/EBPß have not been studied (the long term effects on C/EBPα 'knock-out' mice cannot be studies because of perinatal lethality). Evidence for a role in proliferation came from studies on certain fibroblast cell lines which can be induced, *in vitro*, to differentiate into adipocytes all of which were cloned from mouse 3T3 cells (Green & Kehinde, 1974; Green & Kehinde, 1975). Much of the biology of C/EBPs involvement in adipose differentiation and gene expression has been elucidated in the model cell line 3T3-L1. This cell line (and the related cell line 3T3-F442A) can be induced to differentiated *in vitro*, from fibroblast-like cells into cells exhibiting many of the properties of post mitotic

adipocytes including expression of differentiated adipocyte genes and lipid accumulation (MacDougald & Lane, 1995). Constitutive expression of antisense C/EBPα RNA blocked expression of C/EBPα, the differentiation of preadipocytes into adipocytes and the expression of adipocyte specific genes e.g. 422(aP2), SCD1 and GLUT4 (Lin & Lane, 1992). The adipocyte phenotype could be rescued by expressing a 'sense' C/EBPα RNA (Lin and Lane, 1992). C/EBPα therefore appears to be sufficient to induce differentiation of 3T3-L1 adipocytes. Inappropriate expression of C/EBPα in 3T3-L1 preadipocytes, however inhibited mitosis in the cells, demonstrating the antimitotic activity of C/EBPα (Freytag & Geddes, 1992). As discussed earlier (Section 1.5.3) there are two translation products of C/EBPα mRNA; a 42kDa and a 30kDa protein. The 42kDa product is anti-mitotic but the 30 kDa product is not. Constitutive expression of the 30kDa translation product of C/EBPα in transfected preadipocytes is insufficient to trigger differentiation, but when hormonal stimuli are used differentiation occurs (Lin et al., 1993). C/EBPß and C/EBPδ are also involved in adipocyte differentiation (Cao et al., 1991). These are expressed at high levels in proliferating cells but the level of expression decreases when cells stop dividing as they reach confluence and differentiate (Cao et al., 1991).

1.5.6 Regulation of C/EBPα and related proteins

C/EBPα is most highly expressed in fully differentiated liver and adipose cells (Williams et al., 1991). Several lines of evidence suggest that C/EBPα is autoregulatory, stimulating expression of its own gene. In 3T3-L1 cells C/EBPα antisense RNA blocks transcription of the C/EBPα gene (Lin and Lane, 1992). This effect may be indirect; C/EBPα does not bind to the promoter of the human gene encoding C/EBPα but induces the ubiquitously expressed transcription factor USF which in turn regulates the C/EBPα promoter (Timchenko et al., 1995). Another factor which regulates C/EBPα expression is C/EBPα undifferentiated protein (CUP) which may repress transcription in cells in which C/EBPα is not expressed including a cell line derived from mouse 3T3 cells 3T3-C2, which does not differentiate into adipocytes (Vasseur-Cognet & Lane, 1993). CUP binds to the C/EBPα promoter in preadipocytes and its expression decreases as preadipocytes differentiate into adipocytes and C/EBPα expression increases (Vasseur-Cognet and Lane, 1993).

1.5.6.1 Regulation of C/EBPα and C/EBPβ in liver

In hepatocytes C/EBPα gene expression is down regulated by epidermal growth factor (EGF) at both the transcriptional and postranslational levels (Mischoulon et al., 1992) Insulin and growth hormone do not affect C/EBPα expression (Bosch et al., 1995; Potter et al., 1993), however, C/EBPß mRNA is altered by these hormones; insulin inhibits (Bosch et al., 1995) and growth hormone increases (Potter et al., 1993) the expression of C/EBPß mRNA. Glucagon and dexamethasone induce C/EBPß gene expression in primary cultures of rat hepatocytes (Matsuno et al., 1996).

1.5.6.2 Regulation of C/EBPα and C/EBPβ in adipocytes

There is not much data on C/EBPα gene regulation in adipocytes as it is only expressed in fully differentiated post mitotic cells. As expected, in its role as a global regulator of energy homeostasis, the C/EBPα gene is hormonally regulated by glucocorticoids, which decrease expression (MacDougald et al., 1994), by insulin and cAMP which both increase expression (Guerra et al., 1994; Tae et al., 1995). C/EBPß expression in adipocytes is controlled by similar factors to C/EBPα. It is regulated by cytokines and agents which induce the acute phase response e.g. TNFα and LPS administration to adipocyte cells is associated with increased C/EBPß levels (Alam et al., 1992; Ron et al., 1992). Hormonal manipulations also alter C/EBPß expression, with growth hormone and cAMP increasing expression (Clarkson et al., 1995; Tae et al., 1995).

1.5.6.3 Postranslational regulation

In chicken C/EBPß (NF-M), the N-terminally located transactivation domain interacts with an inhibitory domain within the protein. Phosphorylation of the inhibitory domain liberates the transactivating domain allowing activation of transcription (Kowenz-Leutz et al., 1994). One of the signal pathways leading to derepression of the C/EBPß includes MAP kinases that phosphorylate MAP-kinase site in the C/EBPß protein (Nakajima et al., 1993). Other phosphorylation sites include a serine in the leucine zipper dimerisation region of C/EBPß that can be phosphorylated by Ca_2^+-calmodulin-dependent protein kinase II (CaMKII), conferring calcium regulated stimulation of transcriptional activity (Wegner et al., 1992), and a serine in the DNA-binding region of all C/EBP family members which

when phosphorylated by protein kinase C attenuates DNA-binding of C/EBPα *in vitro* (Mahoney et al., 1992). C/EBPδ is also activated by phosphorylation and this modification is required for its DNA binding activity (Ray & Ray, 1994). Regulation of transcriptional activity by phosphorylation provides a mechanism by which hormonal signals can alter expression of C/EBP-regulated genes.

1.5.6.4 Synergistic transcriptional activation of by C/EBP and other factors

Synergistic activation of genes by C/EBP-related and non-related transcription factors can occur as well as synergy between members of the C/EBP family. Members of the C/EBP family synergistically activate transcription, for example, C/EBPδ-C/EBPß heterodimers are more potent in activating transcription than homodimers of C/EBPß (Kinoshita et al., 1992). Several groups have reported an interaction between the Rel protein NFκB, and C/EBP family members. Heterodimers of NFκB and C/EBP bind to C/EBP but not to Rel consensus sites (Diehl & Hannink, 1994b). Another group found that activation from both the NFκB and C/EBP binding sites in the serum ameloid A gene (Ray et al., 1995). NFκB, like C/EBPß, is a global regulator of cytokine encoding genes and genes regulated by cytokines. The interaction between these two factors probably modulates cytokine gene expression. A three way interaction of transcription factors involving NFκB, C/EBPß and ER represses the IL-6 promoter (Stein & Yang, 1995). Inhibition of IL-6 by oestrogens was shown to be mediated by binding sites on the IL-6 promoter for NFκB and C/EBP, and may involve the formation of heteromeric ER-NFκB and ER-C/EBPß complexes which reduces the amount of NFκB and C/EBP heterodimers and therefore transcription (Stein and Yang, 1995).

C/EBPß and GR synergistically activate transcription of the acute phase response protein α_1–acid glycoprotein (Nishio et al., 1993) and interact during chick neural retina development (Ben-Or & Okret, 1993). Other examples of synergistic activation involving C/EBP related proteins and non-related proteins include C/EBPα with HNF1 (Wu et al., 1994; Yanuka-Kashles et al., 1994) and HNF4 (Metzger et al., 1993); C/EBPß interacting with AP1 (Hsu et al., 1994), CREB (Vallejo et al., 1995) and SP1 (Lee et al., 1994) and T_3 binding to a response element in the PEPCK gene which is dependent upon binding of C/EBP (Park et al., 1995).

1.6 Regulation of hepatic gene expression

1.6.1 Liver function

The liver has many functions that can be broadly classified into three groups. Firstly, a major role of liver is maintaining blood glucose homeostasis, through the metabolism of carbohydrates, proteins and lipids. It is also the major site of detoxification in the body, and is the main organ responsible the synthesis of plasma proteins (Guyton, 1986).

In liver, many metabolic pathways operate to maintain glucose homeostasis. These pathways include glycogenesis (the synthesis of glycogen from glucose), glycogenolysis (the conversion of glycogen to glucose), glycolysis, (the oxidation of glucose or glycogen to pyruvate and lactate), and gluconeogenesis (the formation of glucose from non-carbohydrate sources). Metabolism of fat occurs throughout the body, but liver performs some specific metabolic functions such as ß-oxidation of lipid, formation of lipoproteins, synthesis of cholesterol and conversion of carbohydrate and proteins into fat. Liver performs many functions required for protein metabolism, including deamination of amino acids, formation of urea for removal of ammonia, formation of plasma proteins and interconversion among different amino acids (Guyton, 1986).

Detoxification occurs to render toxic substances more polar so that they can be removed from the body and includes processes such as conjugation or oxidation. Conjugation is a process by which potentially harmful substances are removed from the body. The unwanted substance is rendered more hydrophilic by complexing with another molecule or chemical group such as sulphate, glycine, glucuronic acid or acetic acid eventually resulting in excretion in the urine. Many substances are destroyed by the liver by complete oxidation e.g. nicotine and the short acting barbiturates with partial oxidation often precedes conjugation (Guyton, 1986).

Liver forms part of the reticulo-endothelial system which includes bone marrow, lymph nodes and spleen and has a role in regulating the formation and destruction of red blood cells, is a major site of blood storage in the body and synthesises plasma proteins such as albumin, fibrinogen and prothrombin (Guyton, 1986).

1.6.2 Hormonal control of hepatic gene expression

Networks of genes are involved in metabolism, detoxification and haematopoiesis which are coordinately regulated by extracellular signals, namely hormones and cytokines. Networks of genes involved in metabolism of lipids, carbohydrates and proteins are regulated chiefly by insulin, glucagon and glucocorticoids.

If blood glucose levels increase then the rate of insulin secretion from the pancreas increases to promote uptake of glucose into the liver, glycogen synthesis and storage, and if in excess storage in adipose tissue. Insulin binds to cell surface receptors, initiating a cascade of events culminating in the phosphorylation or dephosphorylation of transcription factors and hence regulation of insulin target genes. Glucagon is secreted when blood glucose levels fall and its functions oppose those of insulin. It promotes the breakdown of glycogen stores in liver and lipids in adipose tissue for glucose production and gluconeogenesis. Glucagon binds to cell surface receptors resulting in increased adenylate cyclase activity, increased cAMP production and hence regulates metabolic gene transcription by phosphorylation of transcription factors. Cortisol is secreted in response to hypoglycaemia promoting breakdown of fat and gluconeogenesis. Glucocorticoid activation of genes is described in Section 1.2 and its effects on liver metabolism in Section 1.1.5.1.

1.6.3 Transcriptional control of hepatic gene expression

There are four main transcription factor sub-families which have been identified as regulating liver specific genes, these do not include all liver transcriptional regulators but are representative of factors controlling liver specific gene expression and include hepatocyte nuclear factor 1 (HNF1) (Derman et al., 1981), C/EBP, HNF3 and members of the nuclear hormone receptor family including HNF4 and COUP-TF (Cereghini, 1996).

HNF1 binds to the promoters of the ß-fibrinogen, albumin and α_1-antitrypsin genes, and was one of the first liver-enriched transcription factors to be identified (Cereghini et al., 1988; Courtios et al., 1987; Hardon et al., 1988). The genes regulated by HNF1 generally encode proteins involved in a wide range of metabolic functions, including carbohydrate metabolism and detoxification including PEPCK and C-reactive protein (Cereghini, 1996). HNF1 is dispensable for normal

development but it does seem to be required for the maintenance of metabolic functions. Mice which lack HNF1 die after 1 month from an acute wasting syndrome (Pontoglio et al., 1996). These animals lack phenylalanine hydroxylase (metabolises phenylalanine to tyrosine), and develop phenylketonuria, although expression of other genes is not significantly altered (Pontoglio et al., 1996).

HNF3 related proteins were originally identified as binding to the promoter of the transthyretin and α1-antitrypsin genes (Costa et al., 1989). Three HNF3 transcription factors exist; α, ß and γ (Lai et al., 1991). All three are essential for normal development (Cereghini, 1996).

The orphan receptors HNF4 and COUP-TF also regulate liver gene expression. Like HNF3, HNF4 was first identified as binding to the promoter of the transthyretin and α1-antitrypsin genes (Costa et al., 1989) and is essential for development as disruption of HNF4 leads to early embryonic death (Chen et al., 1994). HNF4 interacts with the promoter of genes involved in a variety of functions, including cholesterol and amino acid metabolism, gluconeogenesis and blood coagulation (Cereghini, 1996). The regulatory functions of HNF4 usually depend upon its synergistic interaction with other transcription factors (Cereghini, 1996). COUP-TF acts as a negative regulator of transactivation by HNF4.

Members of the C/EBP transcription factor family (see Section 1.5) activate many liver specific genes including albumin (Friedmann et al., 1989), PEPCK (Park et al., 1990), transferrin (Theisen et al., 1993) and factor IX (Crossley & Brownlee, 1990), and the role of C/EBP related proteins in hepatic gene expression is described in Section 1.5.5. C/EBPα 'knock-out' mice have defects in glucose metabolism and do not survive for long after birth (Wang et al., 1995) and mice lacking C/EBPß have defects in their immune systems (Screpanti et al., 1995; Tanaka et al., 1995).

These transcription factors seem to affect a wide range of genes with different functions, but they also regulate genes with related functions with some affecting primarily development (HNF3 and HNF4), others metabolism (HNF1, HNF4, COUP-TF and C/EBPα) and others the immune response (C/EBPß).

1.6.4 Regulation of phosphoenolpyruvate carboxykinase

The enzymes involved in glucose homeostasis are all regulated by a similar array of transcription factors. As an example of gene regulation in liver I will focus on the regulation of the gluconeogenic enzyme PEPCK, which synthesises phosphoenolpyruvate from oxaloacetate (Cryer, 1992).

PEPCK is expressed predominantly in liver, kidney and adipose tissue and at lower levels in other tissues including mammary gland, colon, heart and lung (Lemaigre & Rousseau, 1994). Liver extracts produce 8 DNase I footprints on the promoter between -500 and +1, termed cyclic AMP response element (CRE)1, CRE2 and P1 to P6 (Quinn et al., 1988; Roesler et al., 1989). The proteins in liver which bind to these sites include C/EBPα (Park et al., 1990; Roesler et al., 1992), C/EBPß (Park et al., 1993), DBP (Roesler et al., 1992), HNF-1 (Roesler et al., 1989), HNF-4 (Hall et al., 1992) and the ubiquitous transcription factors CREB (Quinn et al., 1988) and AP-1 (Gurney et al., 1992). C/EBPα, C/EBPß and DBP bind to several promoter binding sites including CRE1, although C/EBPß binds with highest affinity to the CRE1 site (Park et al., 1993). CREB, a mediator of cAMP action, and AP-1 also binds to the CRE1 site (Gurney et al., 1992).

PEPCK gene expression is regulated by carbohydrate intake; fasting increases PEPCK expression (Lemaigre & Rousseau, 1994). It is hormonally regulated by the factors controlling blood glucose; insulin, glucagon and glucocorticoids (Lemaigre & Rousseau, 1994). Insulin decreases the transcription rate of the PEPCK gene (Granner et al., 1983) and inhibits cAMP and glucocorticoid induction of PEPCK (Magnuson et al., 1987; Sasaki et al., 1984). Two insulin responsive sites exist on the promoter; one of these binds AF-2 (an accessory factor involved in glucocorticoid gene regulation (Imai et al., 1990). However, expression of AF2 is not affected by insulin (Iami et al., 1990), and it has been suggested that insulin could block glucocorticoid activity by disabling the function of AF-2 (O'Brian et al., 1990). Glucagon acting through cAMP stimulates PEPCK gene expression. Several cis-acting sequences in the promoter mediate the effect of cAMP including CRE1, (but not CRE2) (Short et al., 1986). CRE1 is a target for CREB, C/EBPα, C/EBPß and DBP (Park et al., 1990; Park et al., 1993; Roesler et al., 1993). CREB and potentially C/EBPα (Mahoney et al., 1992; Quinn et al., 1988), but not C/EBPß or DBP

(Roesler et al., 1993), are phosphorylated by cAMP-dependent protein kinase, and potentially mediate the effects of glucagon on PEPCK gene transcription (Mahoney et al., 1992; Quinn et al., 1988). Glucocorticoids stimulate PEPCK gene transcription in liver and repress it in adipocytes (Nechushtan & al, 1987; Sasaki et al., 1984). The mechanism of glucocorticoid activation of the PEPCK gene in liver requires accessory factors AF1 and AF2, in addition to GR which are individually unable to stimulate transcription in response to glucocorticoids (Lemaigre & Rousseau, 1994).

1.6.5 Regulation of the acute phase response

An early reaction occurs in liver in response to injury, trauma or infection, during the process of 'inflammation', - the Acute Phase Response (APR).

Macrophages and monocytes are activated by trauma, injury or infection and are the primary initiators of the APR cascade (Baumann & Gauldie, 1994). Activated macrophages release cytokines belonging to the interleukin and tumour necrosis factor families. These factors act locally to release additional cytokines which then initiates a 'second wave' of cellular and cytokine cascades involved in the APR. The mediators of the APR can be classified into 4 groups; IL-6-type cytokines (e.g. IL-11, IL-6), IL-1-type (IL-1α, IL-1ß, TNF-α and TNFß), glucocorticoids and growth factors (e.g. insulin, hepatocyte growth factor) (Baumann and Gauldie, 1994). IL-6, is the major regulator of APR genes and in general synergistically enhances genes induced by IL-1 type cytokines such as serum amyloid A and C-reactive protein (a component of complement C3) (Baumann & Gauldie, 1990). Glucocorticoids can stimulate expression of some APR proteins directly although its main action is to synergistically enhance IL-1 and IL-6 type actions (Baumann & Gauldie, 1990). The fourth group of mediators modulates the response of liver to the IL-1 and IL-6 type cytokines, for example, insulin attenuates the effects of IL-1 and IL-6 type cytokines on gene expression (Baumann & Gauldie, 1990). The cytokines bind to cell surface receptors activating intracellular pathways resulting in regulation of transcription factor activity, for example, IL-1 and IL-6 activate the JAK2 and mitogen-activated kinase pathway (MAPK), to mediate phosphorylation of transcription factors (Baumann & Gauldie, 1990). The cooperative interaction between transcription factors is required to achieve the expression of genes for the APR e.g. IL-6, is regulated by C/EBPß and an NF-KB-like proteins. The promoter for IL-6 also

contains binding sites for other factors including GR and AP-1, but these do not directly regulate the IL-6 gene although they may regulate other secondary cytokines (Baumann & Gauldie, 1990).

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and solvents

All chemicals and solvents were of analytical grade and were obtained from either BDH Chemicals Ltd, Poole, UK or Sigma Chemicals Ltd, Poole, UK with the following exceptions:

Aprotinin	Bayer UK Ltd, Pharmaceutical Business Group, Bayer House, Strawberry Hill, Newbury, Berkshire. RG13 1JN. UK.
Ethanol	Hayman Ltd, 70, Eastways Industrial Park, Witham, Essex CM8 3YE. UK.
Agarose	Gibco BRL, (Life Technologies Ltd), Trident House, Renfrew Road, Paisley. PA3 4EF. UK.
SeaPlaque agarose NuSieve agarose	FMC Bioproducts, Flowgen Instruments Ltd, Broad Oak Enterprise Village, Broad Oak Road, Sittingbourne, Kent. ME9 8AQ. UK.
Nucleotide triphosphates	Pharmacia Biosystems Ltd, Davy Avenue, Knowlhill, Milton Keynes. MK5 8PH. UK.
O-methyl-GTP	Pharmacia Biosystems Ltd, Davy Avenue, Knowlhill, Milton Keynes. MK5 8PH. UK.
Proteinase K	Boehringer Mannheim UK (Diagnostics/ Biochemicals) Ltd, Bell Lane, Lewes. BNY 1LG. UK.
Yeast tRNA	Gibco BRL, (Life Technologies Ltd), Trident House, Renfrew Road, Paisley. PA3 4EF. UK.
Poly (dI-dC).(dI-dC)	Pharmacia Biosystems Ltd, Davy Avenue, Knowlhill, Milton Keynes. MK5 8PH. UK.
Caesium chloride	Boehringer Mannheim UK (Diagnostics/ Biochemicals) Ltd, Bell Lane, Lewes. BNY 1LG. UK.
Dimethyl sulphate (DMS)	Aldrich Chemical Company Ltd, The Old Brick Yard, New Road, Gillingham. SP5 4BR. UK.

2.1.2 Radiochemicals

All radiochemicals were supplied by Amersham International plc (Amersham, UK.).

Compound	Specific Activity
[α^{32}P]-dATP [α^{32}P]-dCTP [α^{32}P]-dGTP [α^{32}P]-dTTP	3000 Ci/mmol, 10mCi/ml
[α^{32}P]-UTP	800 Ci/mmol, 20mCi/ml

2.1.3 Enzymes

Restriction enzymes, DNase I (RNase free) and the Klenow fragment of DNA polymerase I were routinely supplied by Promega Ltd, Delta House, Chilworth Research Centre, Southampton. SO16 7NS. UK.

RNase A (20µg/ml)	was freshly prepared by dilution in TE buffer a stock (10mg/ml), made by dissolving RNase A (Sigma Chemicals Ltd, Poole, UK) in water, and boiling for 10min (to inactivate DNase I).

2.1.4 Miscellaneous

DNA size markers	Routinely used 1kb DNA ladder. Gibco BRL, (Life Technologies Ltd), Trident House, Renfrew Road, Paisley. PA3 4EF. UK.
Dried skimmed milk powder	Marvel, Premier Beverages, Knighton, Adbaston, Stafford. ST20 0QJ. UK.
Film (DuPont NEF 485)	DuPont UK Ltd, Wedgwood Way, Stevenage. SG1 4QN. UK.
Oligonucleotides	Synthesised by Oswell DNA Service, Southampton. UK.
Bovine gamma globulin	BioRad Laboratories Ltd, Maylands Avenue, Hemel Hampstead. HP2 7TD. UK.
BioRad protein assay solution	BioRad Laboratories Ltd, Maylands Avenue, Hemel Hampstead. HP2 7TD. UK.

2.1.5 DNA's

2.1.5.1 Plasmids

The following plasmids were used for the preparation of DNA fragments (Figure 2.1) containing regions of the rat 11ß-HSD1 gene promoter. The plasmids pKC 302, pKC 303, pKC 304, pKC 305 and pKC 306 have 11ß-HSD1 promoter DNA inserted into the vector pGem 3 (Promega Ltd). pDelta 10 and pDelta 47 were from a 5' deletion series produced by exonucleaseIII/mung bean nuclease digestion of pVL116, containing a PstI fragment (-966/+47) from the rat 11ß-HSD1 gene (the deletion series was made by Val Lyons; unpublished data) (Figure 2.1). A 185 base pair EcoRI/EcoRV pBR 322 fragment was used as non-specific competitor in gel mobility shift analyses.

2.1.5.2 Oligonucleotides

Sequences of oligonucleotides used in investigations of protein interaction with the rat 11ß-HSD1 gene are shown in Table 2.1.

2.1.5.3 Plasmids used for in vitro transcription reactions

A plasmid in which the adenovirus major late promoter is linked to a 370bp G-free cassette, pML(C_2AT) (Sawadogo & Roeder, 1985) was a gift from R. Roeder. Plasmids in which the adenovirus major late promoter is linked to a 270bp G-free cassette, pAdM(270) or a 170bp G-free cassette, pAdM(170) (Vaulont et al., 1989) were a gift from S. Vaulont. p11ß1(-599)-(C_2AT)$_{19}$ and p11ß1(-88)-(C_2AT)$_{19}$, were constructed by Dr. K. Chapman as follows. To construct p11ß1(-599)-(C_2AT)$_{19}$ an SstI fragment encoding -599 to +76 of the rat 11ß-HSD1 gene was subcloned into the unique SstI site of the G-free cassette vector, p(C_2AT)$_{19}$ (Sawadogo & Roeder, 1985). An EcoRI-HindIII fragment containing the 11ß-HSD1 sequence and the G-free cassette was subcloned between the EcoRI and HindIII sites of M13mp18. Oligonucleotide-directed mutagenesis was carried out to remove 11ß-HSD1 DNA between +9 and +76 (which contained G residues) using an oligonucleotide corresponding to sequences between -22 of 11ß-HSD1 and the first 12 nucleotides of p(C_2AT)$_{19}$ immediately following the second G of the SstI site (5'-AGCCTCCC CCGTCCCTGATGTCACAATTCACTCCATACCCTT-3'; the transcriptional start is underlined). The altered sequence was verified by DNA sequencing and the EcoRI-HindIII fragment recloned into p(C_2AT)$_{19}$ to create p11ß1(-599)-(C_2AT)$_{19}$.

Table 2.1 *Sequences of synthetic double stranded oligonucleotides*

Oligonucleotides encoding parts of the 11ß-HSD1 gene are designated 11ß-HSD1 with the nucleotide numbers of the 5' and 3' ends of the oligonucleotides shown in brackets. The 11ß-HSD1 transcription start site nucleotide is in bold type in the oligonucleotides O_A, O_G, O_H, all of which span the transcription start. 11ß-HSD1 promoter oligonucleotides O_G and O_H encode -14 to +15 of the rat 11ß-HSD1 gene with mutations introduced at +2 (O_G) and +2, -1 (O_H) (underlined in Table 2.1). O_D encodes the P3(I) site from the PEPCK promoter shown to bind C/EBP (Park et al., 1993) and O_E encodes an optimal C/EBP site (Nye & Graves, 1990). Oligonucleotides O_I, O_J, O_K, O_L, O_M and O_N were a gift from Dr John Quinn, University of Edinburgh, O_I encodes the cAMP response element (CRE) of the somatostatin gene (Montminy et al., 1986); O_J encodes a functional CRE from the bovine preprotachykinin gene (Kageyama et al., 1991) which does not bind Fos/Jun or CREB (J. Quinn, personal communication); O_K encodes an AP-1 site which binds Fos/Jun (J. Quinn, personal communication); O_L encodes a Fos/Jun binding site from the arginine vasopressin gene (J. Quinn, personal communication); O_M encodes a putative E-box but doesn't bind anything (J. Quinn, personal communication); O_N is an E-box-like oligonucleotide, and has an AP-1 and CRE site (J. Quinn, personal communication). O_T encodes the TdT initiator element (Smale & Baltimore, 1989). O_{NS} encodes the oestrogen response element from the rat prolactin gene (Maurer & Notides, 1987) which was used as a non-specific competitor.
References: 1, this work; 2, (Park et al., 1993); 3, (Nye & Graves, 1990), 4, Dr. John Quinn, personal communication; 5, (Smale & Baltimore, 1989); 6, (Maurer & Notides, 1987).

NAME	SEQUENCE	IDENTITY	REF
O_A	GATCCGTCCCTGATGTC**A**CAATTCAGAGGCTG GCAGGGACTACAGTGTTAAGTCTCCGACTTAA	11ß-HSD1 (-14/+15)	1
O_B	GATCCCACCCAAAGCCAATCATTGC GGTGGGTTTCGGTTAGTAACGAGAC	11ß-HSD1 (-88/-57)	1
O_C	TTAATCATTGCTCTGACAGGG GTAACGAGACTGTCCCTAGG	11ß-HSD1 (-71/-52)	1
O_D	CTAGATCAAACGTTGTGTAAGG TAGTTTGCAACACATTCCGATC	PEPCK P3(I) site	2
O_E	GATCCCAATTGCCCAATCAG GGTTAACGGGTTAGTCCTAG	Optimal C/EBP site	3
O_F	AATTCTCTTTGATGTTGCAATGCTTTG GAGAAACTACAACGTTACGAAACTTAA	11ß-HSD1 (-682/-705)	1
O_G	GATCCGTCCCTGATGTC**A**AAATTCAGAGGCTG GCAGGGACTACAGTTTTAAGTCTCCGACTTAA	11ß-HSD1 (+2 MUT)	1
O_H	GATCCGTCCCTGATGTAA**A**AAATTCAGAGGCTG GCAGGGACTACATTTTTAAGTCTCCGACTTAA	11ß-HSD1 (-1,+2 MUT)	1
O_I	GATCCCTGACGTCAC GGACTGCAGTGCTAG	Somatostatin CRE	4
O_J	TCGAGAAATGACGCAATT CTTTACTGCGTTAAAGCT	PPT-A CRE	4
O_K	TCGATGATGAGTAATC ACTACTCATTAGAGCT	AP-1 SITE	4
O_L	TCGAGCAGTGATTCTGGCT CGTCACTAAGTCCGTAGCT	AVP AP-1 SITE	4
O_M	TCGACGTGGCTCTCCAGG GCACCGAGAGGTCCAGCT	PPT-A -60	4
O_N	TCGACCCTTTAAGAAGGCTACTGACGTGCCAAAGAGCC GGGAAATTCTTCCGATGACTGCACGGTTTCTCGGAGCT	E-box	4
O_O	CTAGAAACTCTGACCTCTCTGGCCTT TTTGAGACTGGAGAGACCGGAAGATC	11ß-HSD1 (+629/+650)	1
O_P	CTAGCCATTCTTGCAAGGCCATTGCTG GGTAAGAACGTTCCGGTAACGACGATC	11ß-HSD1 (-264/-239)	1
O_Q	CTAGGTGCGGCAAGGCTGAGATTTGG CACGCCGTTCCGACTCTAAACCGATC	11ßHSD1 (-540/-516)	1
O_R	CTAGCTGGTCTGACCTTCTTTTCCCAG GACCAGACTGGAAGAAAAGGGTCGATC	11ß-HSD1 (-919/-897)	1
O_S	AATTCTTTATTCCCTGTAGTAATAAGGGCACG GAAATAAGGGACATCATTATTCCCGTGCCTAG	11ß-HSD1 (-804/-776)	1
O_T	GATCCATCAGAGCCCTCATTCTGGAGACA GTAGTCTCGGGAGTAAGACCTCTGTTCTA	TdT initiator element	5
O_{NS}	GTCCAGGACATCGTGACATGC CAGGTCCTGTAGCACTGTACG	Prolactin ERE	6

Figure 2.1 *Plasmids containing 11ß-HSD1 gene promoter DNA*

▛ and ▜ indicate regions of polylinker derived from pGem3.

PLASMID	FRAGMENT
pKC 302	F_A (-88/+47)
pKC 302	F_A (-88/+47)
pKC 303	11ß(-599/-249)
pKC 304	11ß(-174/+74)
pKC 305	11ß(-249/-174)
pKC 306	11ß(-174/-88)
pDelta 10	F_B (-812/-599)
pDelta 47	11ß(-322/+47)

To create p11ß1(-88)-$(C_2AT)_{19}$, p11ß1(-599)-$(C_2AT)_{19}$ was restricted with BamHI, and blunt-ended using the Klenow fragment of DNA polymerase I, before restricting with SmaI. The resulting fragment containing -88 to +8 of the 11ß-HSD1 gene linked to the G-free cassette was subcloned into p$(C_2AT)_{19}$, which had been restricted with EcoRI and SmaI and blunt-ended using the Klenow fragment of DNA polymerase I.

2.1.6 Antibodies

Polyclonal C/EBPα antibody	Autogen Bioclear Ltd, Butts Farm, Pottern, Nr Devizes, Wiltshire. SN10 5LR. UK or a gift from Dr Steven L. McKnight, Tularic Inc. USA.
Polyclonal C/EBPß antibody	Autogen Bioclear Ltd, Butts Farm, Pottern, Nr Devizes, Wiltshire. SN10 5LR. UK.
Polyclonal COUP-TF antibody	Gift from Dr M. Parker, ICRF, Lincoln Inns Field, London. UK.

2.1.7 Buffers

2.1.7.1 General buffers and solutions

All solutions were prepared using distilled water and stored at room temperature unless otherwise stated.

DNA loading buffer	0.25%(w/v) bromophenol blue, 0.25%(w/v) xylene cyanol, 30% glycerol
Gel mobility shift assay buffer (5x)	20mM Tris-HCl (pH 7.5), 500mM KCl, 25mM $MgCl_2$, 5mM EDTA, 50% glycerol, 0.1%Triton X-100
DNase I protection buffer (5x)	50% glycerol, 50mM Tris-HCl (pH 7.5), 12.5mM $MgCl_2$, 5mM $CaCl_2$, 0.5mM EDTA, 375mM KCl, 20mM spermidine, 2.5mM DTT
Tris-saturated phenol (pH 8.0)	Frozen phenol was thawed by heating to 60°C. Phenol was then extracted 2-3 times with 0.2M Tris-HCl (pH 8.0) until buffer pH was >7.0. Phenol was extracted twice further with 0.1M Tris-HCl (pH 8.0) and finally stored at 4°C under a layer of 0.1M Tris-HCl (pH 8.0)
Chloroform:isoamylalcohol	(24:1, v/v)

Phenol:chloroform	(1:1, v/v)
Poly (dI-dC).(dI-dC)	Resuspended at 3μg/μl in water
GTE	50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA
Alkaline SDS solution	0.2M NaOH, 1% SDS
Potassium acetate	Prepared by combining 60ml 5M potassium acetate, 11.5ml glacial acetic acid, 28.5ml water. The final solution is 3M with respect to potassium, 5M with respect to acetate
Formamide gel loading buffer	Deionised formamide to which 0.03% xylene cyanol, 0.03% bromophenol blue, 20mM EDTA has been added
SpinBind DNA binding buffer	9 parts 8M NaI, 22mM DTT; 1 part 1M sodium phosphate buffer (pH 6.0). 8M NaI, 22mM DTT was made by dissolving 34mg DTT and 11.9g NaI at 50°C with stirring into a final volume of 10ml dH_2O. Sodium phosphate buffer was prepared by adding 1M Na_2HPO_4 to 10ml of 1M NaH_2PO_4 while stirring until the pH reaches 6.0
SpinBind high ethanol wash buffer	80ml ethanol, 1.0ml 1M Tris-HCl (pH 8.0), 40μl 0.5M EDTA (pH 8.0) made up to 100ml with dH_2O
DMS stop	1.5M sodium acetate (pH 7.0), 200μg/ml yeast tRNA, 7%(v/v) 2-mercaptoethanol
Maxam and Gilbert (G+A) stop	0.3M Sodium Acetate (pH 7.0), 0.1mM EDTA, 25μg/ml yeast tRNA
Maxam and Gilbert (G) stop	1.5M Sodium acetate (pH 7.0), 200μg/ml yeast tRNA, 7%(v/v) mercaptoethanol
TBE (10x)	108.9g Tris base, 55.7g boric acid, 4.7g EDTA made up to 1000ml
TE (1x)	10mM Tris-HCl (pH 8.0), 1mM EDTA

2.1.7.2 Buffers for making nuclei and nuclear extracts

Homogenisation buffer	10mM Hepes-KOH (pH 7.6), 15mM KCl, 0.15mM spermine, 0.5mM spermidine, 1mM EDTA, 2.1M sucrose, 1% low fat milk (MARVEL), 1% protease inhibitor mixture, 0.5mM DTT, 0.5mM PMSF
100% protease inhibitor	Made by mixing 5ml 100% aprotinin, 2ml mixture pepstatin (250µg/ml) and 50µl leupeptin (10mg/ml). Stored at -20°C in 1ml aliquots.
Nuclear lysis buffer (nuclear extracts used for *in vitro* transcription reactions (IVT)	10mM Hepes-KOH (pH 7.6), 0.1M KCl, 0.1mM EDTA, 10% glycerol, 3mM $MgCl_2$, 1mM DTT, 0.1mM PMSF, 1% aprotinin
Nuclear dialysis buffer (IVT)	25mM Hepes-KOH (pH 7.6), 0.1mM EDTA, 40mM KCl, 10% glycerol, 1mM DTT
4.0M ammonium sulphate	52.85g $(NH_4)_2SO_4$ was dissolved in 100ml dH_2O, pH was adjusted to 7.9 with NaOH and stored at 4°C
Nuclear resuspension buffer (for investigating protein-DNA interactions)	400mM NaCl, 10mM Hepes-KOH (pH 7.9), 1.5mM $MgCl_2$, 0.1mM EGTA, 0.5mM DTT, 5% glycerol, 0.5% PMSF
Dialysis buffer (for investigating protein-DNA interactions)	20mM HEPES-KOH (pH 7.9), 75mM NaCl, 0.1mM EDTA, 0.5mM DTT, 20% glycerol, 0.5mM PMSF

2.1.7.3 Buffers used for in vitro transcription

5x *In vitro* transcription reaction buffer	100µl 1.0M Hepes-KOH (pH 7.6), 350µl 87% glycerol, 85µl 3.0M KCl, 60µl 1.0M $MgCl_2$, 250µl 25mM CTP, 250µl 25mM ATP, 35µl 10mM UTP, 200ml 10mM O-methyl-GTP, 670µl dH_2O
Transcription stop buffer	0.25M NaCl, 1% SDS, 20mM Tris-HCl (pH 7.5), 5mM EDTA
Yeast tRNA	10mg/ml
Proteinase K	10mg/ml
DNA elution buffer	10mM Tris-HCl (pH8.0), 20mM acetic acid, 1mM EDTA (pH 8.0), 0.1% SDS

2.1.7.4 Restriction enzyme buffers

EcoRI, PstI restriction buffer (1x)	90mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 50mM NaCl
HindIII restriction buffer (1x)	6mM Tris-HCl (pH 7.5), 6mM $MgCl_2$, 60mM NaCl, 1mM DTT
SmaI restriction buffer (1x)	10mM Tris-HCl (pH 7.5), 7mM $MgCl_2$, 50mM KCl, 1mM DTT. DNA cleaved at 25°C
Multicore restriction buffer (1x)	25mM Tris-Acetate (pH 7.8), 100mM potassium acetate, 10mM magnesium acetate, 1mM DTT
Klenow buffer (10x)	500mM Tris-HCl (pH 7.2), 100mM $MgSO_4$, 1mM DTT

2.1.8 Bacterial media and agar

Bacto tryptone, yeast extract and bacto agar were supplied by Difco Laboratories, Michigan, USA. All media were made in dH_2O and autoclaved prior to use.

LB (Luria-Bertoni) broth	1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl
LB-agar	As for L-broth except with 1.5% (w/v) bacto agar
LB-agar plates	LB-agar was melted in the microwave, allowed to cool until warm, and if required, ampicillin (100mg/ml) was added prior to pouring plates in sterile plastic 100mm petri dishes.
M9 (5x)	64g $Na_2HPO_4.7H_2O$, 15g KH_2PO_4, 2.5g NaCl, 5g NH_4Cl to 1 litre with dH_2O
M9 Minimal medium	1xM9, 0.3% casamino acids, 0.5% glucose, 0.1mM $CaCl_2$, 0.00002% vitamin B_1, 4mM $MgSO_4$

2.2 Methods

2.2.1 Gel electrophoresis

2.2.1.1 Agarose gel electrophoresis

Agarose gels were prepared by adding agarose at 0.8-2% (w/v) to 0.5xTBE followed by boiling in a microwave oven. 1µg/ml of ethidium bromide was added to the

dissolved agarose and the solution was poured into a gel mould. Once set, the gel was submerged in 0.5xTBE in a gel tank and DNA samples, containing agarose loading buffer, were loaded into the wells. Samples were electrophoresed at 100-200V until the DNA fragments were resolved. DNA was visualised on a UV transilluminator and photographed. The sizes of DNA fragments were estimated by comparison of their mobility to that of DNA size markers.

2.2.1.2 Vertical polyacrylamide gel electrophoresis

2.2.1.2.1 Gel plates

Gel plates used in polyacrylamide gel electrophoresis were scrubbed in detergent, rinsed with dH_2O and wiped with ethanol before use.

2.2.1.2.2 Non-denaturing polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis was used in gel mobility shift analysis (Section 2.2.5.1) and methylation interference analysis (Section 2.2.5.3). DNA fragments were electrophoresed on 4% polyacrylamide gels and oligonucleotides on 5% polyacrylamide gels.

The gel mix contained 2.5ml of 10xTBE, 300µl 10% ammonium persulphate and either 5ml of 40% acrylamide:bis-acrylamide (19:1) (for 4% gels) or 6.25ml of 40% acrylamide:bis-acrylamide (19:1) (for 5% gels), in a final volume of 50ml. Polymerisation was initiated by addition of 40µl TEMED and the gel was poured into the gel cast. The gel cast for gel mobility shift analysis consisted of 15cm x 17cm glass plates sandwiching spacers 1.5mm thick, which was sealed by 1% agarose (w/v). The gel cast for methylation interference non-denaturing gel consisted of 16.5cm x 28cm plates which sandwiched 1.5mm spacers. Once the gel was poured, a 20 tooth comb was inserted into the gel cast to form wells for use in gel mobility shift assays and a 6 tooth comb inserted for the methylation interference gel. The gel was allowed to set for at least 2h prior to electrophoresis on a Gibco BRL Model V16 apparatus for gel mobility shift analysis, or a Sigma Model E5514 apparatus for methylation interference. Gels were pre-run at 100V in 0.5xTBE for at least 30min before samples were loaded. Gels were run at 200V for approximately 90min. After electrophoresis the gel was transferred to Whatman 3MM paper, covered with Saranwrap and dried under vacuum at 80°C in a gel drier (BioRad 583

Gel Drier). Protein-DNA complexes were visualised by exposing the dried gel for 24h to autoradiographic film (2.2.2).

2.2.1.2.3 Denaturing polyacrylamide gel electrophoresis

An 8% or 10% denaturing polyacrylamide gel was used in DNase I protection analysis and methylation interference analysis, depending on the size of fragment under investigation, and a 4% denaturing gel was used in *in vitro* transcription analysis. For a 10% denaturing gel, 42g of urea was dissolved in 25ml 40% acrylamide:bis-acrylamide (19:1), 10ml 10xTBE, 600µl ammonium persulphate, made up to 100ml with dH_2O and filtered through Whatman #1 filter paper. The solution was degassed, and polymerisation started by the addition of 40µl TEMED, and then poured into the gel cast. The gel cast was made using 0.3mm spacers sandwiched between glass plates (45cm x 35cm) and sealed with 1% agarose (w/v). Once the gel was poured the comb was inserted, and the gel allowed to polymerise for at least 2h before assembly into the electrophoresis apparatus (IBI model STS45i). The gel was pre-run at 1800V for 30min, samples in formamide loading buffer were heated to 90°C for 3min, centrifuged and loaded onto the gel. Gels were run at 1800V until the bromophenol blue had reached the bottom of the gel. For single nucleotide resolution of DNA fragments greater than 100 nucleotides, a second loading of sample was applied to the gel and electrophoresed until the bromophenol blue had reached the bottom, at which time the xylene cyanol had electrophoresed approximately 3/4 of the way down the gel. After electrophoresis the gel was transferred to Whatman 3MM paper and dried under vacuum at 80°C on a BioRad 583 Gel Drier. The gel was exposed to autoradiographic film overnight at -80°C to visualise the DNA.

For a 4% denaturing gel, 21g of urea was dissolved in 5ml 40% acrylamide:bis-acrylamide (19:1), 5ml 10xTBE, 300µl ammonium persulphate, made up to 50ml with dH_2O and filtered through Whatman #1 filter paper. The solution was degassed, polymerisation started by addition of 20µl TEMED and then poured in the gel cast. To make the gel cast, glass plates (15cm x 17cm) were put together using 0.8mm spacers and sealed with 1% agarose (w/v). Once the gel was poured, a 20 tooth comb was inserted and the gel allowed to polymerise for at least 2h before assembly into the electrophoresis apparatus (Gibco BRL Model V16). The gel was pre-run at 150V

for 30min, samples (in formamide loading buffer) were heated to 90°C for 3min, centrifuged and loaded onto the gel. After electrophoresis at 200V for 1.5h the gel was fixed in 20% methanol, 10% acetic acid for 20min and transferred to Whatman 3MM paper and dried under vacuum at 80°C on a BioRad 583 Gel Drier. The gel was visualised by exposure to autoradiographic film overnight at -80°C.

2.2.2 Autoradiography

Dried gels were exposed to autoradiographic film (DuPont NEF 485) at -80°C inside cassettes containing a single intensifying screen (DuPont). Film was exposed for an appropriate length of time then developed for 3mins in D19 developer (Kodak) and fixed in Amfix fixative (1 in 5 dilution) (Champion), rinsed in tap water and left to dry before analysis.

2.2.3 DNA techniques

2.2.3.1 Propagation of DNA in E. coli

2.2.3.1.1 Preparation of competent bacteria

A single colony of *Escherichia coli* (HB101 or JM109) was routinely used for transformations and was inoculated into 2ml of L-Broth and grown overnight with shaking at 37°C. These cells were then diluted into 50ml L-Broth and were grown at 37°C to mid-log phase (A_{600}=0.4-0.8). Cells were harvested at 6,000rpm in a Beckman JA20 rotor in a J2MC centrifuge at 4°C for 10min. Cells were resuspended in 10ml ice cold 0.1M $CaCl_2$ and left on ice for 10min to 1h. Centrifugation was repeated and cells were finally resuspended into 2ml ice cold 0.1M $CaCl_2$. Cells were kept on ice for up to 4 days until use.

2.2.3.1.2 Transformations

Competent cells were transformed as outlined in Sambrook et al., 1989. 100µl of competent cells were mixed with 10-100ng of plasmid DNA in TE. The DNA and cells were left on ice for 10min prior to heat shock at 42°C for 2min. Transformed cells were then plated out onto LB agar plates containing 100µg/ml ampicillin using a glass spreader. Plates were inverted and incubated overnight at 37°C. Control plates included pGEM3 as a positive control, or no plasmid as a negative control. Cells which grew on the plates represented colonies which had been transformed by

plasmids containing ß-lactamase, the gene encoding resistance to ampicillin. Plates were stored at 4°C for up to 3 months.

2.2.3.1.3 Large scale preparation of plasmid DNA

2ml of L-broth containing 100µg/ml ampicillin was inoculated with a single bacterial colony and grown overnight at 37°C with shaking. The overnight culture was diluted into 40ml of L-broth or M9 minimal medium containing 100µg/ml ampicillin, grown at 37°C to A_{600} 0.4-0.7, then further diluted into 460ml LB medium or M9 minimal medium and grown overnight in a 2 litre flask with shaking. Cells were harvested at 6,000rpm for 5min at 4°C using a Beckman JA-14 rotor in a Beckman J2-MC centrifuge. The bacterial pellet was resuspended in 12ml of GTE, mixed immediately with 24ml of freshly prepared alkaline-SDS solution, then stored on ice for 5min. 16ml of ice cold potassium acetate was added, mixed well, and stored on ice for a further 10min, before centrifugation in the JA-14 rotor at 6,000rpm, 4°C for 10min. The supernatant was strained through gauze into 250ml centrifuge pots and 32ml of isopropanol added to precipitate DNA at room temperature for 30min. Plasmid DNA was pelleted by centrifugation at 10,000 rpm for 5min at 4°C. The DNA pellet was dried and resuspended in 2.2ml TE, then 1g of CsCl was added for each ml of DNA solution and dissolved. 50µl of ethidium bromide (10mg/ml) was added; the resulting solution was then transferred into a 3ml Beckman Quickseal tube and centrifuged either for 4h at 100,000rpm, or overnight at 70,000rpm in a TLA100.3 rotor in a Beckman Optima TLX Ultracentrifuge. Banded plasmid DNA was removed using a needle and syringe through the tube wall and transferred to a new ultracentrifuge tube. Fresh CsCl solution (prepared by adding 100g CsCl to 100ml TE) was added to the recovered plasmid DNA in the tube and centrifuged as before. The plasmid DNA was collected and ethidium bromide removed by extraction using isopropanol, until the isopropanol was no longer pink. The plasmid preparation was dialysed against three 1 litre changes of TE at 4°C. Concentrations of recovered plasmid DNA were calculated by measuring the A_{260} in an Shimadzu UV-160A spectrophotometer (50µg/ml DNA gives an absorbance of 1.0 at A_{260}). Plasmid DNA was stored at -20°C.

2.2.3.2 Manipulation of DNA

2.2.3.2.1 Restriction endonuclease digests

Typically, 1-10µg of plasmid DNA was cut using 1-10 units of restriction endonuclease at 37°C for 1h in 1x recommended restriction buffer, in a final volume of 10-30µl. A tenth of the digest was saved for comparison with the recovered DNA fragment after purification from a low melting point agarose gel.

2.2.3.2.2 DNA fragment purification

Fragments were separated by electrophoresis on a 1-2% SeaPlaque low melting point agarose gel run at 100-150V. The gel was examined using a UV transilluminator and a photograph taken. The appropriate DNA fragment was excised in a gel slice for further DNA purification. DNA was purified by melting the gel slice at 70°C; for every 100µl of melted gel slice 100µl of Tris-saturated phenol was added, vortexed, samples left on dry ice for 10min, then spun in a microcentrifuge for 10min. The upper aqueous layer was extracted and added to Tris-saturated phenol (pH 8.0) in the same ratio as above, vortexed, centrifuged for 2min and the aqueous layer again removed. This was then extracted using 100µl Tris-saturated phenol (pH 8.0) and 100µl chloroform:isoamylalcohol. The aqueous layer was finally extracted by chloroform:isoamylalcohol. DNA from the aqueous layer was recovered by ethanol precipitation and resuspension in 10µl dH_2O. Recovery was checked using 1µl of recovered DNA compared against an aliquot retained from the original restriction digest.

2.2.3.2.3 Ethanol precipitation of DNA

Precipitation of DNA was routinely carried out by addition of 15µl 5M NaCl and 250µl 100% ethanol per 100µl of DNA, incubation on dry ice for 10min followed by centrifugation in an Eppendorf centrifuge for 10min. Ethanol was removed using a drawn-out pasteur pipette, and the DNA pellet was air dried for 15min before resuspension in 10µl dH_2O.

2.2.3.2.4 Preparation of [^{32}P]-labelled double stranded oligonucleotides

Pairs of complementary single stranded oligonucleotides were heated to 65°C for 10min, annealed by cooling slowly to room temperature, and end-labelled using the Klenow fragment of DNA polymerase I by incubation for 10min at room

temperature in a 10µl reaction containing 100pmol of complementary oligonucleotides, 1x Klenow buffer, 0.2mM each of dGTP, dCTP, dTTP, 0.5µl Klenow (10 units/µl), 3µl [α^{32}P]-dATP (3000Ci/mmol). Labelled oligonucleotides were purified using SpinBind columns. The reaction was mixed with 2x the labelling reaction volume of SpinBind DNA binding buffer, applied to the column, and spun for 10s in an Eppendorf centrifuge. SpinBind DNA binding buffer was applied twice more to the column, spun and the eluate discarded. The SpinBind high ethanol wash buffer was then applied three times to the column, spun each time and the eluate discarded. End-labelled oligonucleotides were eluted into 50µl dH_2O to give 1.4pmol/µl, assuming a 70% recovery from purification through the column. Labelled oligonucleotides were stored at -20°C for up to 3 weeks. Generally, specific activity was 5000cpm/pmol double-stranded oligonucleotides.

2.2.3.2.5 Preparation of [^{32}P]-labelled DNA restriction fragments

10µg of plasmid was cut with the appropriate restriction enzymes; for DNase I and methylation interference analysis restriction enzymes were chosen so that only one end would be labelled (at a 5' overhang), with the other end being blunt or having a 3' overhang. The fragment was purified (Section 2.2.3.2.2) and end-labelled using the Klenow fragment of DNA polymerase I by incubation for 10min at room temperature in a 10µl reaction containing DNA restriction fragment at a concentration equivalent to 1µg plasmid, 1x Klenow buffer, 2mM each dGTP, dCTP, dTTP, 0.5µl Klenow (10units/µl), 3µl [α^{32}P]-dATP (3000 Ci/mmol). Fragments were purified through SpinBind columns as described (Section 2.2.3.2.4) and eluted into 50µl dH_2O. Purified labelled fragments were stored at -20°C for up to 3 weeks. Generally, the specific activity was 2000-5000cpm/fmol.

2.2.3.2.6 Maxam and Gilbert DNA sequencing reactions

Two types of Maxam and Gilbert reactions were used as size markers in DNase I footprinting and in methylation interference reactions; G+A and G reactions. G+A reaction: 5µl of [^{32}P]-end labelled DNA fragment, labelled at a single end, was incubated with 25µl formic acid to chemically modify G and A bases. The length of incubation depended on the length of the labelled DNA (the longer the DNA fragment, the shorter the exposure) for fragment F_B (-812 to -599), 45s. G reaction: 190µl TE was added to 10µl of [^{32}P]-labelled DNA fragment which was then

incubated with DMS to chemically modify G bases. The concentration of DMS added and length of incubation depended on the length of the labelled DNA; for fragment F_A, 1µl of DMS diluted 1 in 5 in dH_2O for 30s; for the fragment encoding -599 to -249 of the 11ß-HSD1 promoter, 20µl of DMS diluted 1 in 15 in dH_2O for 30s. Both G+A and G reactions were then processed in the same way. Reactions were stopped by the addition of 200µl of the appropriate Maxam and Gilbert stop solution. DNA was precipitated by the addition of 750µl ethanol, placed on dry ice for 5min, and spun in an Eppendorf centrifuge for 5min at 14,000rpm. Pelleted DNA was washed by resuspending in 250µl 0.3M sodium acetate (pH 7.0) and 750µl ethanol, precipitated on dry ice and centrifuged as before. DNA pellets were resuspended in 75µl of 1M piperidine (diluted fresh from the bottle 1:9 with dH_2O) and heated to 90°C for 20min to cleave chemically modified bases. Cleaved DNA was precipitated by the addition of 50µl 0.3M sodium acetate (pH 7.0) and 400µl ethanol, then placed on dry ice for 10min and centrifuged for 10min at 14,000rpm in an Eppendorf centrifuge. The ethanol precipitation was repeated by resuspending the DNA pellet in 50µl 0.3M sodium acetate (pH 7.0), adding 400µl ethanol, and placing on dry ice for 10min followed by centrifugation as before. The supernatant was removed and DNA pellets resuspended in formamide loading buffer.

2.2.4 Protein techniques

2.2.4.1 Recombinant C/EBPα protein synthesis

Recombinant C/EBPα protein (rC/EBPα) was produced in bacteria as described (Landschultz et al., 1988). pT5 plasmid (a gift of S. L. McKnight) directs expression of the rat C/EBPα gene under the control of the T7 promoter. pT5 was transformed into *E. coli.* strain BL21(DE3)pLysS which contains the bacteriophage T7 gene encoding the bacteriophage RNA polymerase, under control of the *E. coli lac* promoter (Studier et al., 1990). Cells were grown in M9 minimal medium supplemented with antibiotics to mid-log phase (A_{600} 0.6-1.0), then 0.4mM IPTG was used to induce T7 RNA polymerase synthesis which in turn, initiates transcription from the T7 promoter present in the pT5 plasmid. Cells were grown for a further 2-3h after addition of IPTG and harvested by centrifugation, and resuspended in PBS containing 5M urea, 1mM benzamidine, 1mM EDTA, 0.2% Triton X-100. Cells were then frozen at -80°C, quick thawed at 37°C, and sonicated. The resulting lysate was passed over a DEAE-cellulose column that had been

equilibrated with PBS containing 5M urea, 1mM benzamidine, 1mM EDTA. The flowthrough was collected in fractions and dialysed against 2 changes of PBS. Fractions were snap frozen and stored at -80°C in 50µl aliquots. Protein concentrations were determined (Section 2.2.4.2.4) and C/EBPα assayed in each fraction by gel mobility shift analysis (Section 2.2.5.1) using a [^{32}P]-labelled double-stranded C/EBP-binding oligonucleotide (O_E, Table 2.1). Fractions with most rC/EBPα activity binding were used as the source of rC/EBPα.

2.2.4.2 Preparation of nuclear extracts

2.2.4.2.1 Purification of nuclei

Nuclei were made using freshly isolated adult male rat liver (Han/Wistar). Approximately 10g was chopped finely and homogenised in homogenisation buffer by 10 strokes with an all glass dounce homogeniser. The homogenate was layered on top of pre-chilled (placed at -20°C for at least 1h) 5ml cushions of homogenisation buffer from which the low fat milk was omitted, then centrifuged at 21,000rpm for 1h in a Sorvall AH627 swing-out rotor in a DuPont Sorvall OTD centrifuge; this gave clean pelleted nuclei from which nuclear extracts were made. Two methods were employed in making nuclear extracts; a standard method was used for making extracts for use in gel mobility shift analysis and DNase I protection assays and a lengthier procedure, involving further purification steps was used to make extracts for *in vitro* transcription experiments.

2.2.4.2.2 Preparation of nuclear extracts for analysis of protein-DNA interactions

Nuclear extracts were prepared essentially as described (Dignam et al., 1983). Nuclei were resuspended using a glass homogeniser in a volume of Nuclear resuspension buffer equivalent to using 2.5 x vol. of pelleted nuclei. Resuspended nuclei were stirred slowly for 30min at 4°C then centrifuged for 60min at 42,000rpm in a Beckman Optimal TLX Ultracentrifuge using a TLA100.3 rotor. The supernatant was then dialysed for 3h against 50 vol. of Dialysis buffer. The protein concentration of the nuclear extract was determined (Section 2.2.4.2.4) and was routinely 2-10µg/ml; 15µl aliquots were snap frozen in liquid N_2.

2.2.4.2.3 Preparation of nuclear extracts for in vitro transcription

Freshly prepared nuclei were resuspended in 5ml Nuclear lysis buffer (IVT) and homogenised by several strokes with an all glass Dounce homogeniser. 18ml of Nuclear lysis buffer (IVT) was added, then 0.1 vol. of 4.0M $(NH_4)_2SO_4$, followed by gentle mixing for 30min by shaking on ice, then centrifugation for 1h at 42,000rpm at 2°C in a Beckman Optimal TLX Ultracentrifuge using a TLA100.3 rotor. The supernatant was transferred to a fresh centrifuge tube and 0.3g solid $(NH_4)_2SO_4$ per ml of supernatant was slowly added and gently shaken on ice for 30min before centrifuging as above for 20min at 42,000rpm. The supernatant was discarded and the pellet resuspended in 600µl Nuclear dialysis buffer (IVT) by pipetting up and down every 15min for 2h to ensure complete recovery of pelleted proteins. Resuspended proteins were dialysed twice for a total of 4h at 4°C against 100 vol. of Nuclear dialysis buffer (IVT). After dialysis, a white precipitate formed which was removed by centrifugation in a microcentrifuge for 2min. The protein concentration of the nuclear extract was determined (Section 2.2.8) and the supernatant was aliquoted and snap frozen in liquid N_2.

2.2.4.2.4 Estimation of protein concentration of extracts

Protein concentrations were estimated using a commercially available assay (BioRad) based on the Bradford method (Bradford, 1976). A working solution of BioRad Protein assay reagent was prepared by diluting 1 part BioRad Protein Assay concentrate in 5 parts dH_2O and filtering through Whatman #1 paper. A standard curve was constructed in duplicate as follows, standards containing 0, 1.5, 3, 6, 12 or 18µg of bovine gamma globulin were made up to 20µl with dH_2O. A range of sample concentrations were prepared in duplicate in which the final volume was made up to 20µl with dH_2O. 1ml of diluted BioRad protein assay reagent was added to each standard and sample and vortexed. The colour was allowed to develop for 5min before being transferred to 1ml plastic cuvettes for measurement of A_{595} in a Shimadzu UV-160 spectrophotometer. A calibration curve was obtained from the standards, from which the protein concentration of the samples could be calculated.

2.2.5 Analysis of protein-DNA interactions

2.2.5.1 Gel mobility shift assays

Gel mobility shift assays were carried out as described (Singh et al., 1986). Each reaction contained 30fmol of [^{32}P]-labelled DNA fragment or 0.1pmol of [^{32}P]-labelled double-stranded oligonucleotide, 4µl of 5x Gel mobility shift assay buffer, 3µg of the non-specific DNA competitor poly (dI-dC).(dI-dC) and 6-10µg nuclear extract or 0.5µg bacterial extract containing rC/EBPα protein, in a final reaction volume made up to 20µl with dH_2O. Reactions were pre-incubated for 15min at room temperature prior to addition of [^{32}P]-labelled DNA. A further 15min incubation at room temperature preceded loading onto either a 4% or 5% non-denaturing polyacrylamide gel (Section 2.2.1.2.2) which had been pre-run for 30min at 100V. Gels were loaded and electrophoresed at 200V in 0.5xTBE to resolve the protein-DNA complexes from unbound DNA. The gel was dried for 1h at 80°C under vacuum in a BioRad 583 Gel Drier and protein-DNA complexes were observed after exposure at -80°C to autoradiographic film overnight.

Competition gel mobility shift assays used approximately 10- or 100-fold molar excess of unlabelled specific or non-specific competitor fragment or oligonucleotide, which were included in the pre-incubation reaction prior to addition of [^{32}P]-labelled DNA. Where antibodies to specific transcription factors were used in gel retardation assays, 1µl antibody was included in the pre-incubation prior to the addition of [^{32}P]-labelled DNA.

2.2.5.2 DNase I protection analysis

DNase I protection analysis was carried out essentially as described (Galas & Schmitz, 1978). Each reaction contained 5µl of 5x DNase I protection buffer, 1µg of poly (dI-dC).(dI-dC), 50-100fmol of [^{32}P]-labelled DNA and up to 80µg of protein extract (either nuclear extract or bacterial extract containing rC/EBPα) in a final reaction volume of 25µl. Reactions were pre-incubated on ice for 15min prior to the addition of [^{32}P]-labelled DNA. This was followed by a further 15min incubation on ice before addition of DNase I. The amount of DNase I and time of exposure to DNase I varied, depending on the batch and was determined in prior experiments using a range of DNase I concentrations and times of digestion. Reactions were stopped by addition of EDTA to 0.1M and NaCl to 400mM (which dissociates most

proteins from DNA). Each reaction was added to a protein removal cartridge (NBL), the membrane washed with 400mM NaCl and eluate collected. 1µl of 10µg/µl of yeast tRNA was added to the eluted DNA, which was then ethanol precipitated, and washed in 70% ethanol before resuspension in formamide loading buffer. Samples were heated to 90°C for 3min prior to loading on a denaturing polyacrylamide gel which had been pre-run at 1000V for 30min. The gel was electrophoresed at 1800V until the bromophenol blue had reached the bottom.

2.2.5.3 Methylation interference analysis

1-2x10^6cpm of single end-labelled DNA was partially methylated using 1µl dimethyl sulphate (DMS) in 200µl TE buffer for 2.5min. The reaction was terminated by the addition of 50µl DMS stop mixture and 750µl 95% ethanol. Methylated DNA was precipitated by placing on dry ice for 5min followed by centrifugation at 14,000rpm for 10min in an Eppendorf centrifuge. The supernatant was discarded and the pellet washed with 70% ethanol, air dried and resuspended in TE to give 20,000cpm/µl. The partially methylated DNA was then incubated with protein, either from nuclear extract or bacterial extract containing rC/EBPα, then electrophoresed at 200V on a non-denaturing polyacrylamide gel to separate free DNA from complexed DNA. After electrophoresis the top plate of the gel was removed and the gel covered with Saranwrap and exposed to autoradiographic film overnight at 4°C. The autoradiograph was developed, and once dry this was lined up with the original gel; the protein/DNA complexes and free DNA were cut out of the gel using a scalpel. DNA was eluted from gel slices by electroelution in a BioRad Electro-Eluter (Model 422) using membrane caps with a molecular weight cut-off of 3,500Da. Gel slices were placed in the electroeluting apparatus containing DNA elution buffer and eluted onto membranes by electrophoresis at 10mA per chamber used, for 30min. The current was then reversed and the eluted DNA was recovered. DNA was extracted using equal volumes of phenol and chloroform:isoamylalcohol, then ethanol precipitated. Pelleted DNA was washed in 70% ethanol and resuspended in 50µl dH_2O. DNA was cleaved at methylated guanine residues by incubation with 50µl of 20% piperidine at 90°C for 30min. DNA was precipitated by addition of 100µl 0.6M sodium acetate (pH 7.0) and 500µl of 95% ethanol, left on dry ice for 5min and spun in an Eppendorf centrifuge for 20min. Pelleted DNA was washed in 70% ethanol and resuspended in 10µl of formamide loading dye. Samples were denatured by heating

to 90°C for 3min and loaded onto a pre-run 10% denaturing polyacrylamide gel. Once run, gels were dried under vacuum and exposed to autoradiographic film.

2.2.6 In vitro transcription

In vitro transcription analysis made use of the G-free cassette method (Sawadogo & Roeder, 1985). The G-free cassette assay relies on the use of a synthetic DNA template (of defined length) which does not contain any G residues in the non-coding strand. When a promoter is cloned in front of the G-free cassette, the *in vitro* transcription reaction can be performed in the absence of GTP and in the presence of a GTP-analogue (O-methyl-GTP) which does not support chain elongation; transcripts of defined length are produced at the insertion of O-methyl-GTP at the first G after the G-free cassette. Transcripts can then be visualised on a gel.

Reactions were carried out essentially as described (Sawadogo & Roeder, 1985). Construction of plasmids used (p11ß-599-C_2AT and p11ß-88-C_2AT) for *in vitro* transcription are described in section 2.1.5.3. Control plasmids used for *in vitro* transcription were Ad370, Ad270, Ad170 (Vaulont et al., 1989) and p$(C_2AT)_{19}$ (Sawadogo and Roeder, 1985). *In vitro* transcription reactions were carried out in a final volume of 20µl. 12µl of nuclear extract and 1µl (0.8-1.0µg) of template plasmid DNA were incubated on ice for 10min. To each sample, 7µl of transcription mixture (4µl 5x transcription buffer, 1µl [α^{32}P]-UTP (800Ci/mmol), 1µl RNasin (40U/µl)) was added followed by incubation at 30°C for 45min. To each sample, 274µl of Transcription stop buffer containing 2µl 10mg/ml tRNA and 4µl 10mg/ml proteinase K was added, followed by further incubation for 30min at 37°C. *In vitro* transcribed RNA was extracted with 100µl phenol:chloroform and centrifuged at 14,000rpm in an Eppendorf centrifuge to separate organic and aqueous phases. The aqueous phase was then precipitated by addition of 750µl ethanol, left on dry ice for 15min, and RNA pelleted at 14,000rpm in an Eppendorf centrifuge. Pelleted RNA was resuspended in 5µl formamide loading dye, the sample denatured at 90°C for 3min and electrophoresed on a pre-run 4% denaturing polyacrylamide gel.

CHAPTER 3

C/EBPα and C/EBPß bind to the proximal promoter of the rat 11ß-HSD1 gene

3.1 Introduction

The major site of 11ß-HSD1 expression is liver, where it may play an important role in glucocorticoid action by maintaining high intrahepatic levels of active glucocorticoid through its reductase activity (Jamieson et al., 1995). Glucocorticoids play a major role (with insulin and glucagon) in regulating blood glucose homeostasis, and recent evidence suggests that elimination of 11ß-HSD1 activity in transgenic mice leads to decreased fasting glucose levels and decreased glucose 6-phosphatase activity (Y. Kotelevtsev, personal communication). The regulation of 11ß-HSD1 expression is therefore potentially important in energy homeostasis. Regulation of 11ß-HSD1 mRNA levels by glucocorticoids, sex steroids and growth hormone are described in Chapter 1. However, the mechanism of transcriptional regulation has not previously been investigated, in particular, the basis for tissue specific expression of 11ß-HSD1.

Part of the rat 11ß-HSD1 gene, including the promoter and 5'-flanking region, has been cloned and sequenced, and putative regulatory elements within the promoter identified (Moisan et al., 1992). In liver, one major transcription initiation site is utilised, but in kidney two additional transcription starts are used as well as the major one found in liver (Moisan et al., 1992). The 11ß-HSD1 gene lacks a TATA box, but putative transcription factor binding sites are present, including CACCC boxes at -80, -280, -290 and -325, a CCAAT sequence at -73, glucocorticoid response element half sites at -375 and +62 and predicted binding sites for the liver enriched transcription factor C/EBP (Figure 3.1).

In this chapter I have investigated (a) whether the liver enriched transcription factor C/EBP is able to bind to the promoter region of the 11ß-HSD1 gene (the GCAAT sequence at -67 is a predicted C/EBP site) and (b) the nature of the factors which bind to the proximal promoter. Surprisingly, I show that C/EBP does not bind significantly to the GCAAT sequence at -67 but that it binds with highest affinity to the transcription start.

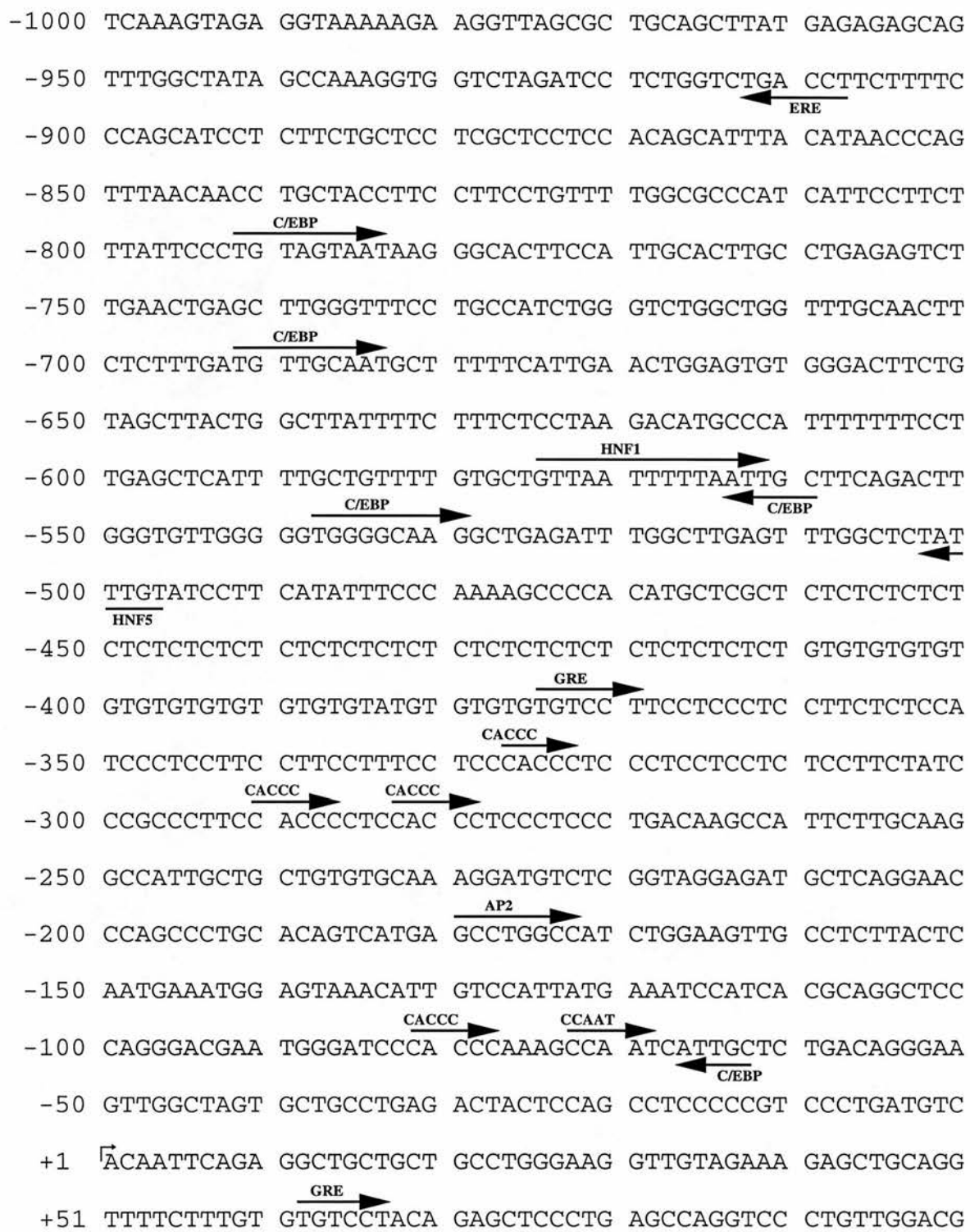


Figure 3.1 *Sequence of the proximal promoter of the rat 11ß-HSD1 gene showing putative transcription factor binding sites*

Nucleotide sequence of 1kb of the rat 11ß-HSD1 gene promoter showing putative transcription factor binding sites; CACCC boxes, a CCAAT box, oestrogen and glucocorticoid response element half sites, identified by (Moisan et al., 1992) are shown, as well as putative binding sites for C/EBP, HNF-1, HNF-5 and AP2 identified by comparison of known transcription factor binding sequences (Locker, 1993).

3.2 Results

3.2.1 The 11ß-HSD1 promoter binds rat liver nuclear proteins

To investigate potential transcription factor interaction with the rat 11ß-HSD1 promoter, DNase I protection analysis and gel mobility shift assays were carried out on a fragment of the rat 11ß-HSD1 gene encoding -88 to +47 (fragment F_A) (for details of all DNA fragments used, refer to Chapter 2, Figure 2.1). In gel mobility shift assays, 4 or 5 specific complexes (the exact number varied from experiment to experiment) were formed by rat liver nuclear extract on fragment F_A (Figure 3.2). Competition analysis was used to demonstrate the specificity of these complexes; all of the complexes were competed by a 10- or 100-fold molar excess of unlabelled fragment F_A, but not by a 10- or 100-fold molar excess of a similar sized non-specific fragment, F_{NS} (Figure 3.2).

To identify where transcription factors were binding on the 11ß-HSD1 promoter, DNase I protection analysis was carried out on fragment F_A encoding -88 to +47 (^{32}P-label was incorporated at the 3' end of the fragment, on the lower strand) (Figure 3.3). Three regions of protection were observed. At the lowest concentration of liver nuclear proteins (5µg), a single, clear region of protection was observed over the transcription start (+1) from -8 to +9 (Figure 3.3). At higher concentrations of liver nuclear extract additional footprints were formed between +27 to +39, and weaker footprints at -54 to -36 (Figure 3.3). In some experiments a footprint was formed between -74 and -61 (encompassing the GCAAT sequence in the C/EBP binding site) but was not apparent in all DNase I protection experiments (Figure 3.18). In addition to the three regions that were protected from DNase I digestion by liver nuclear extract (Figure 3.3), a number of sites showed increased sensitivity to DNase I in the presence of rat liver nuclear extract; these were located primarily at the edges of the stronger footprints (at -8 to +9 and at +27 to +40) and also between the two footprints (Figure 3.3). Footprint analysis of the top strand revealed protection at the transcription start between -10 and +12, however no footprint was apparent between +27 and +39 (Figure 3.4).

Figure 3.2 *Factors in rat liver nuclear extract bind to the rat 11ß-HSD1 promoter*

Gel mobility shift assay using the 11ß-HSD1 promoter fragment, F_A (-88 to +47) and rat liver nuclear extract. 8µg rat liver nuclear extract was incubated with 30fmol of [^{32}P]-labelled F_A in the absence of added competitor DNA fragment, or in the presence of 10- or 100-fold molar excess of unlabelled competitor DNA, either F_A (lanes 3, 4) or a non-specific competitor, F_{NS}, (lanes 5, 6). Lane 1 contained no nuclear extract. Arrows indicate specific protein-DNA complexes.

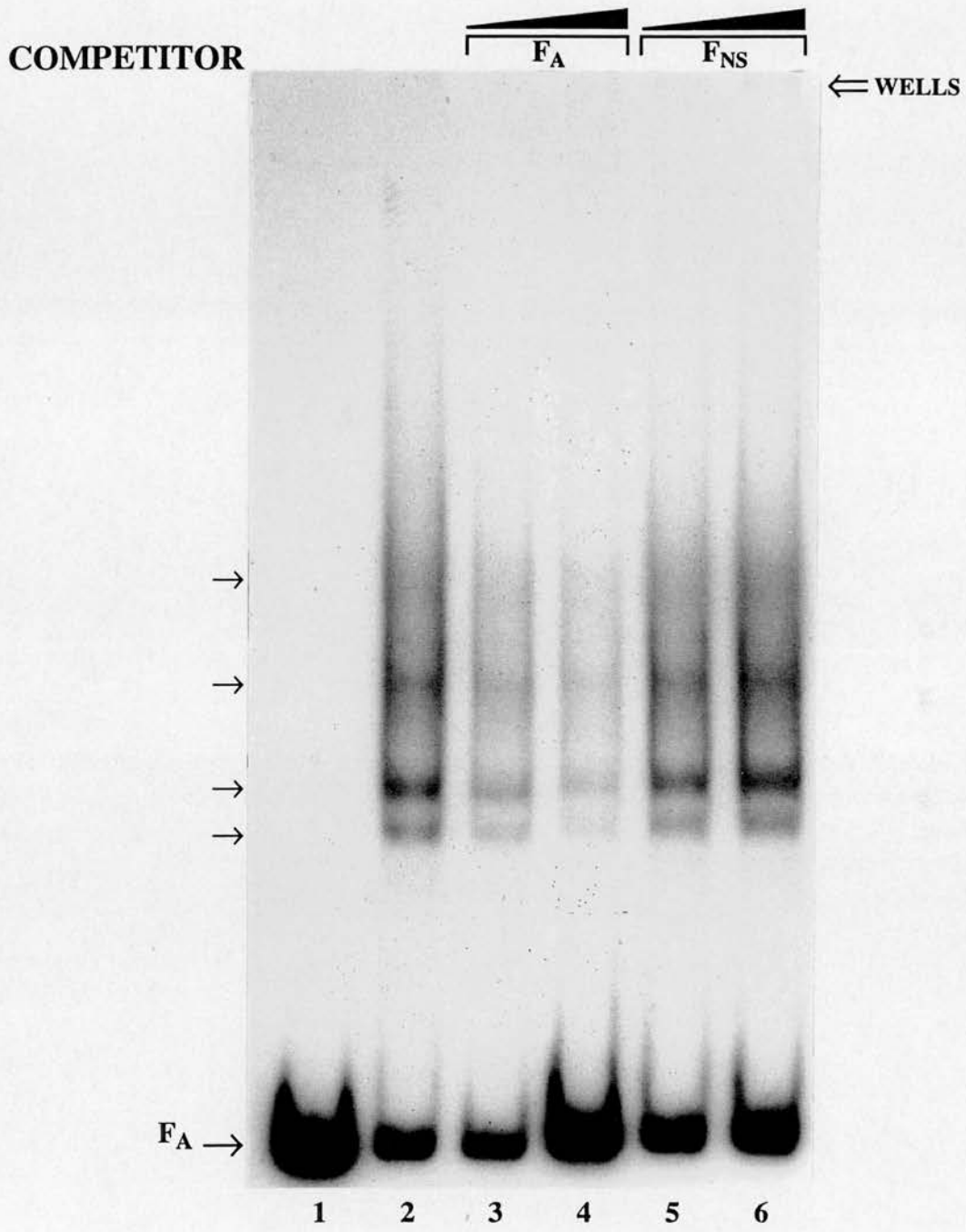
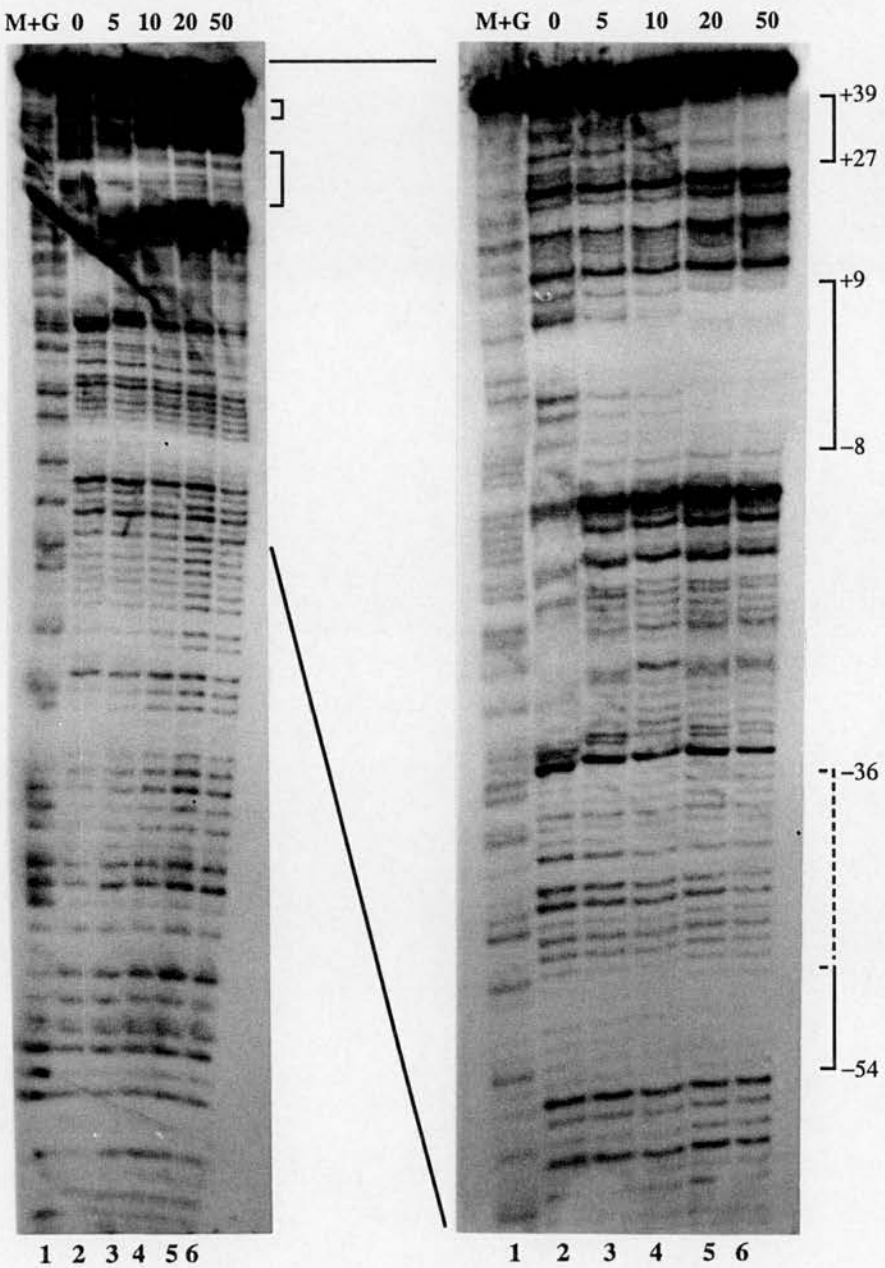

COMPETITOR
F_A
F_{NS}
⇐ WELLS
→
→
→
→
F_A →
1
2
3
4
5
6

Figure 3.3 *Factors in rat liver nuclear extract bind to the transcription start of the 11ß-HSD1 promoter (lower strand)*

DNase I protection analysis of the lower strand of the rat 11ß-HSD1 gene promoter between -88 and +47 (F_A). Panel A and panel B change show the same reactions, with panel B having been electrophoresed for longer. Lane 1, G+A Maxam and Gilbert sequencing reactions. Lane 2, no nuclear extract added. Lanes 3-6 contain increasing amounts of rat liver nuclear extract; 5µg (lane 3), 10µg (lane 4), 20µg (lane 5) and 50µg (lane 6). Brackets indicate regions of DNA protected from DNase I digestion by rat liver nuclear extract. Dashed bracket indicates weak region of protection.


M+G 0 5 10 20 50
M+G 0 5 10 20 50
+39
+27
+9
–8
–36
–54
1 2 3 4 5 6
1 2 3 4 5 6

3.2.2 C/EBP-related factors in rat liver nuclear extract bind to the 11ß-HSD1 promoter (-88 to +47)

To explore the identity of factors producing footprints on F_A, gel mobility shift analysis was carried out using a series of oligonucleotides, either as labelled binding sites or unlabelled competitors. The oligonucleotides used are detailed in Chapter 2, Table 2.1 and include O_A, corresponding to the strong footprint over the transcription start; overlapping oligonucleotides O_B and O_C, encompassing the GCAAT and CCAAT sequences at -73 and -67 respectively, and oligonucleotide O_E encoding an optimal binding site for the liver enriched transcription factor C/EBP. O_E corresponds to an extended version of a consensus sequence devised by Nye and Graves (Nye & Graves, 1990) and binds bacterially expressed recombinant C/EBPα (rC/EBPα) in gel mobility shift assays (data not shown; but see also Figure 3.17). This oligonucleotide was included to assess the possible involvement of C/EBP-related proteins in binding either to the CCAAT sequence at -73 or to the transcription start (which shows a weak resemblance to a consensus C/EBP site). O_E was used initially to demonstrate the presence of C/EBP or C/EBP-related proteins in rat liver nuclear extract. Competition gel mobility shift analysis of complexes formed by liver nuclear extract on the C/EBP-binding oligonucleotide, O_E, identified 2 specific complexes which were fully competed by a 10-fold molar excess of specific competitor oligonucleotide (O_E), but not by a 100-fold molar excess of the non-specific oligonucleotide, O_{NS}, encoding the oestrogen response element (ERE) from the rat prolactin gene (Maurer & Notides, 1987) (Figure 3.5). This result confirmed that C/EBP or C/EBP-related proteins are present in rat liver nuclear extract. Some members of the C/EBP family, including C/EBPα, C/EBPß and C/EBPδ are resistant to heat treatment (Chang et al., 1990; Johnson et al., 1987; Roman et al., 1990). When gel mobility shift assays were performed using rat liver nuclear extract which had been heated to 65°C for 10min, the same pattern of binding to F_A was produced for both heat-treated and non-heat treated extracts (with the possible exception of the lowest mobility complex which was lost upon heat treatment) (Figure 3.6), suggesting that members of the C/EBP family resistant to heat treatment are present in the complexes formed by rat liver nuclear extract on the proximal 11ß-HSD1 promoter (-88 to +47).

Figure 3.4 *Factors in rat liver nuclear extract bind to the transcription start of the 11ß-HSD1 promoter (upper strand)*

DNase I protection analysis of the upper strand of the rat 11ß-HSD1 gene promoter between -88 and +47 (F_A). Lane 1, no nuclear extract added. Lanes 2-6 contain increasing amounts of rat liver nuclear extract; 3µg (lane 2), 6µg (lane 3), 12µg (lane 4), 18µg (lane 5) and 40µg (lane 6). Brackets indicate regions of DNA protected from DNase I digestion by rat liver nuclear extract. Nucleotide postions were estimated by comparison with similar gels on which molecular size standards were run.

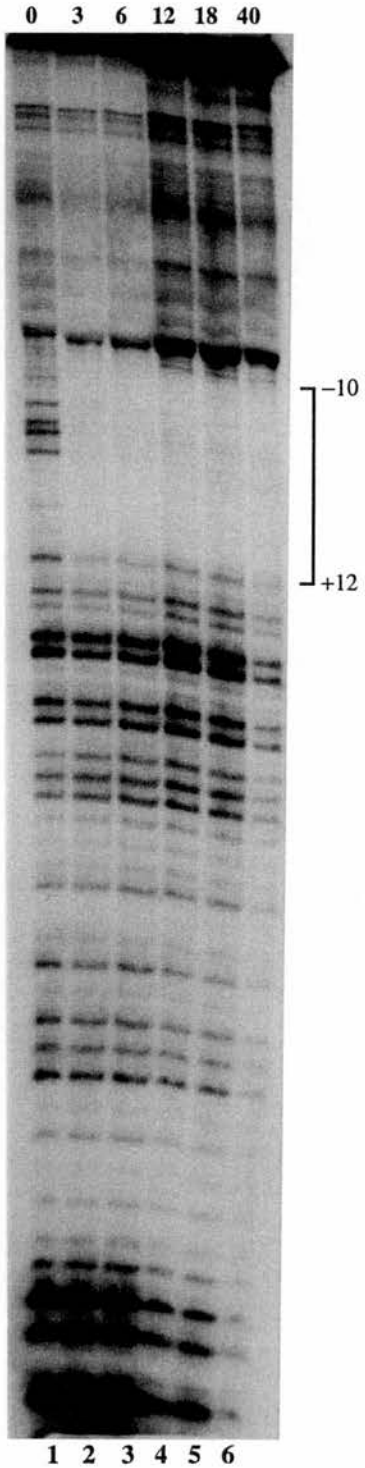

0 3 6 12 18 40
−10
+12
1 2 3 4 5 6

Figure 3.5 *Factors from liver nuclear extract bind to a synthetic C/EBP binding oligonucleotide*

Gel mobility shift analysis of rat liver nuclear extract binding to O_E, encoding an optimal C/EBP binding site. 8μg rat liver nuclear extract was incubated with 0.1pmol of [^{32}P]-labelled O_E in the absence of added competitor oligonucleotide (lane 2) or in the presence of 10- or 100-fold molar excess of competitor oligonucleotide (lanes 3-6), as indicated above the lanes. Lane 1 contained no nuclear extract. Arrows indicate specific protein-DNA complexes.

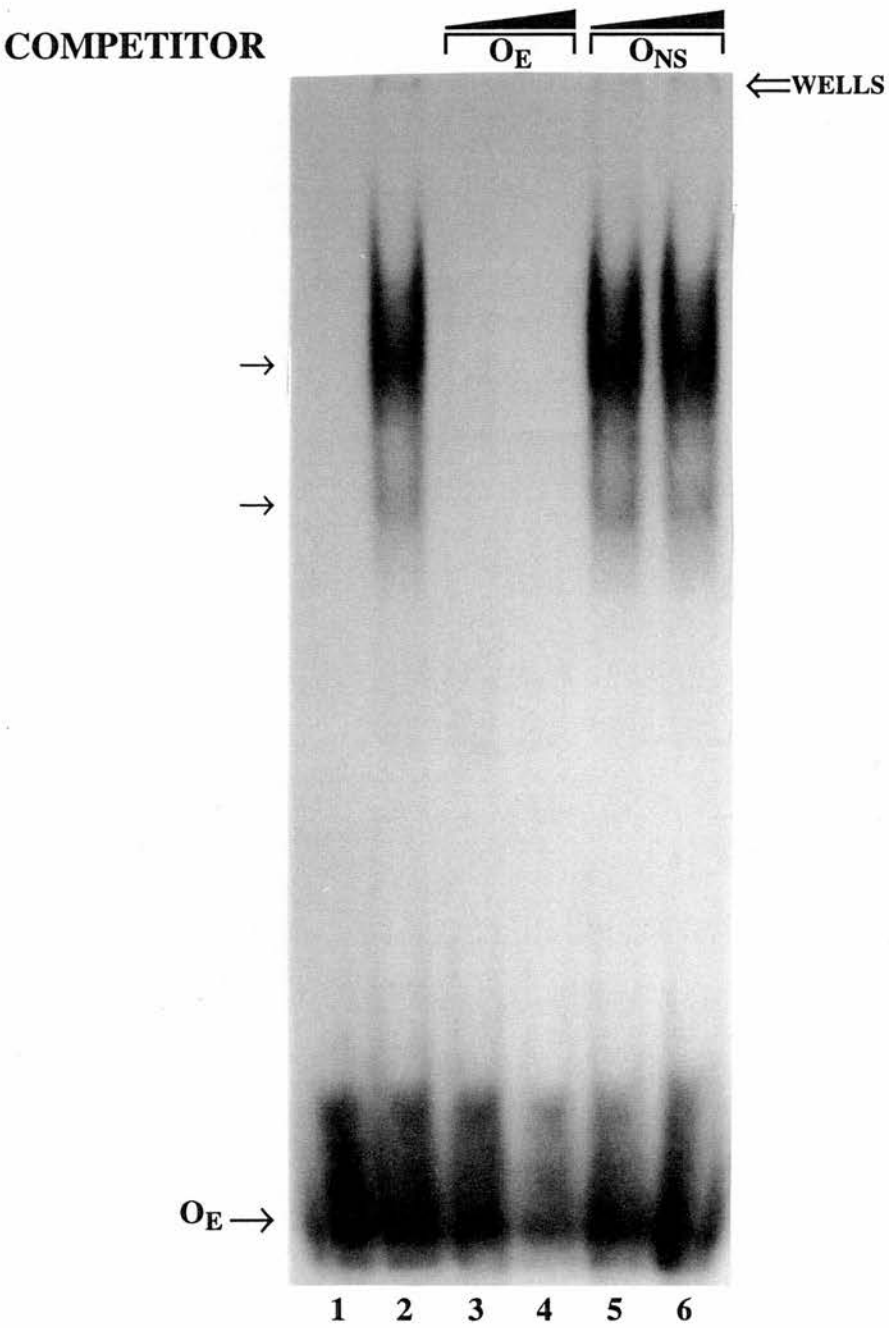
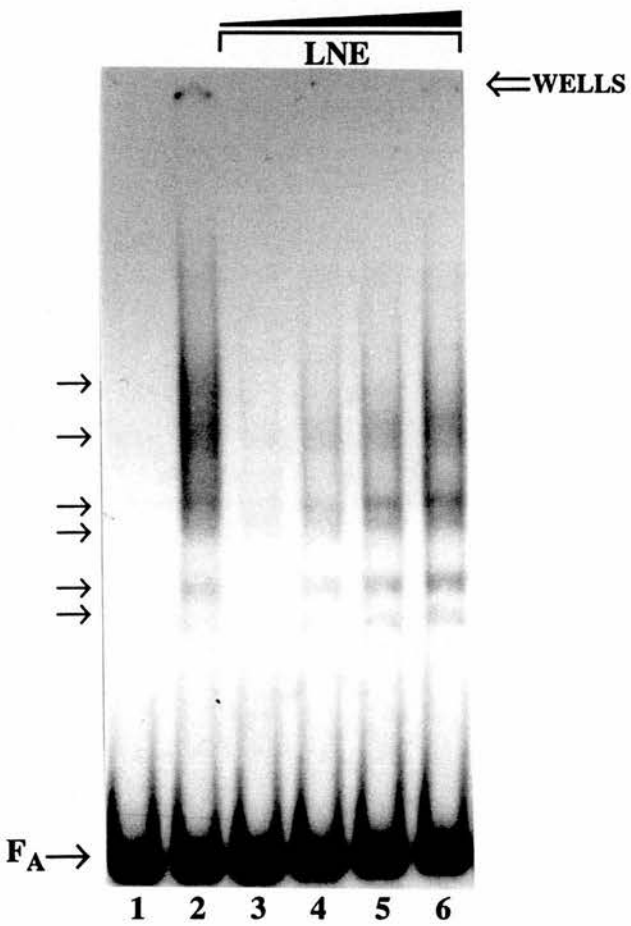

COMPETITOR
O_E
O_{NS}
⇐WELLS
→
→
O_E →
1 2 3 4 5 6

Figure 3.6 *Heat treatment of rat liver nuclear extract does not alter complex formation on fragment F_A, encoding -88 to +47 of the 11ß-HSD1 promoter*

Gel mobility shift analysis of heat treated rat liver nuclear extract binding to [^{32}P]-labelled F_A. Lane 2 contains 5µg of liver nuclear extract which has not been heat treated. Lanes 3-6 contains increasing amounts (5, 10, 15 and 20µg) of heat treated liver nuclear extract.


LNE
⇐WELLS
→
→
→
→
→
→
F_A→
1 2 3 4 5 6

Initially, it seemed probable that an initiator-binding protein may be responsible for the footprint formed at the transcriptional start, which displayed some similarity to an initiator element (a comparison of the 11ß-HSD1 gene from -10 to +10 with the TdT initiator binding sequence (Smale and Baltimore, 1989) and a C/EBP binding sequence (Nye and Graves, 1990) are shown in Figure 3.7). However, no competition of complexes formed on F_A was seen by oligonucleotide O_T, corresponding to the TdT initiator element (Smale and Baltimore, 1989) (Figure 3.8), suggesting that TdT initiator binding proteins are not involved in binding to the 11ß-HSD1 initiator element, and consistent with the results of heat treatment demonstrating that all protein binding to F_A was relatively heat stable. In contrast, O_E, encoding an optimal C/EBP site, proved a strong competitor, eliminating complex formation on F_A at 10-fold molar excess of competitor (Figure 3.8) again suggesting the involvement of C/EBP or C/EBP related proteins in binding to F_A.

The DNA binding specificity of the liver nuclear protein(s) which bind to the transcription start site were examined in gel mobility shift assays using a double stranded oligonucleotide O_A, corresponding to the protected region and the flanking sequences (-14 to +15). At least four protein-DNA complexes were formed on O_A by rat liver nuclear extract (Figure 3.9), indicating heterogeneity of protein binding to this site. The identity of the protein(s) binding to the transcriptional start were explored using a series of oligonucleotides (for oligonucleotide sequences see Chapter 2, Table 2.1) as competitors in gel mobility shift assays. Competition with unlabelled oligonucleotide O_A demonstrated the specificity of these complexes (Figure 3.9). O_E, encoding an optimal binding site for the C/EBP family of transcription factors competed for rat liver nuclear protein binding as effectively as oligonucleotide O_A. Furthermore, a 100-fold molar excess of an oligonucleotide corresponding to the P3(I) C/EBP binding site in the PEPCK gene, O_D (Park et al., 1990), also competed for liver nuclear protein binding to oligonucleotide O_A, although not as effectively as oligonucleotides O_A or O_E (Figure 3.9), implicating the C/EBP family of proteins in binding to the transcription start of the 11ß-HSD1 gene. Oligonucleotides O_B and O_C (encoding the CCAAT sequence at -73 and the GCAAT sequence at -67) showed little or no competition (Figure 3.9). When the same oligonucleotides were used as competitors for liver nuclear protein binding to fragment F_A (-88 to +47 of the 11ß-HSD1 gene) the same rank order of competition

Figure 3.7 *Comparison of the 11ß-HSD1 transcription start region with TdT initiator and C/EBP binding sequences*

Sequence comparison of -10 to +10 of the 11ß-HSD1 gene (Green), the transcription start is underlined, with a known C/EBP binding site (orange) (Nye & Graves, 1990), and the TdT initiator binding element (blue) (Smale & Baltimore, 1989). Matching nucleotides are indicated by the boxes.


CAGAGCCCTCATTCTGGAGA
TdT
CCCTGATGTCACAATTCAGA
11ß-HSD1
TCCCAATTGCCCAATCAGGA
C/EBP

Figure 3.8 *The TdT initiator element does not compete for complex formation on* F_A

Competition gel mobility shift assay using fragment F_A (encoding -88 to +47 of the 11ß-HSD1 gene) and rat liver nuclear extract. 8µg rat liver nuclear extract was incubated with 30fmol of [^{32}P]-labelled F_A in the absence of added competitor oligonucleotide (lane 2) or in the presence of 10- or 100-fold molar excess of competitor oligonucleotides (lanes 3-12), as indicated above the lanes. Lane 1 contained no nuclear extract. Arrows indicate specific protein-DNA complexes.

Oligonucleotides used were: O_B, 11ß-HSD1 (-88 to -57); O_C, 11ß-HSD1 (-71 to -52); O_E, optimal C/EBP site; O_T, TdT initiator element; O_{NS}, prolactin ERE (non-specific control).

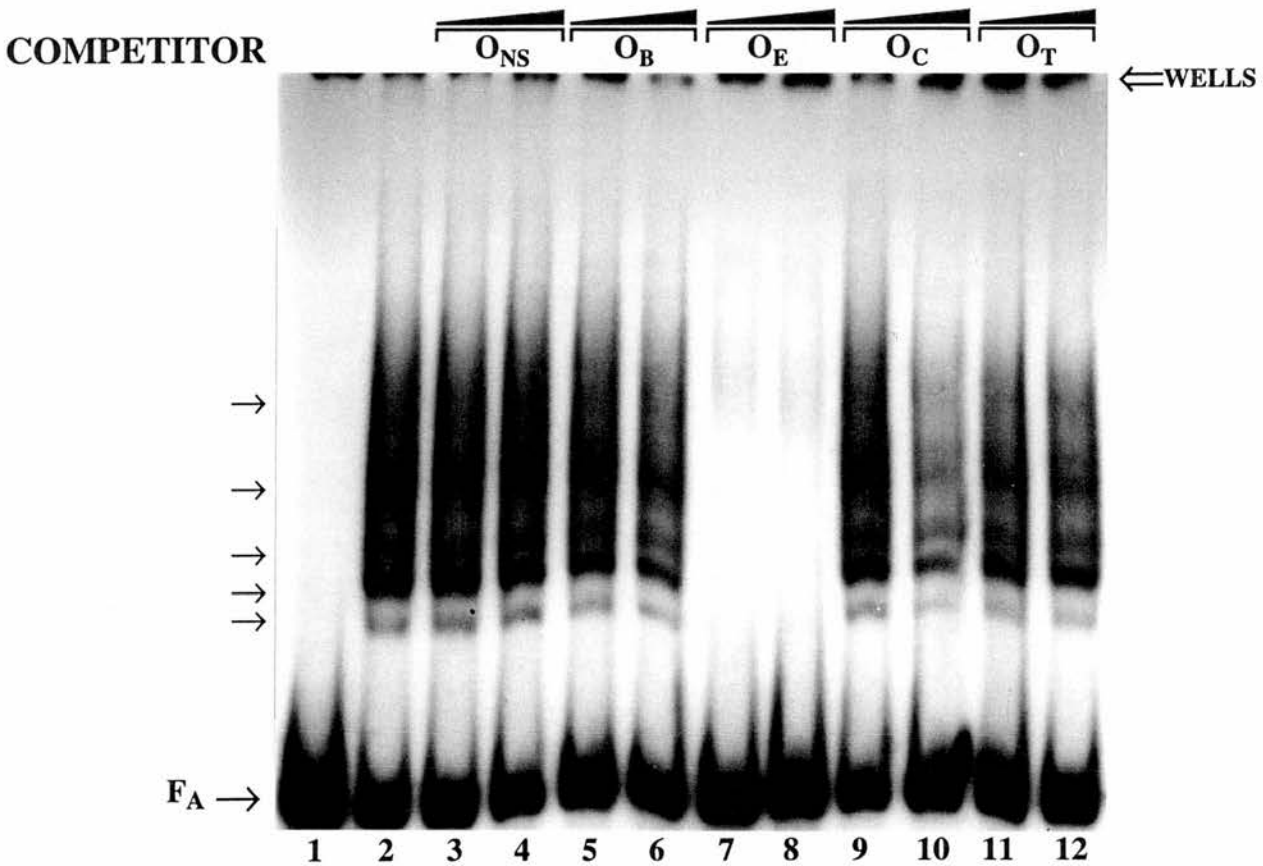
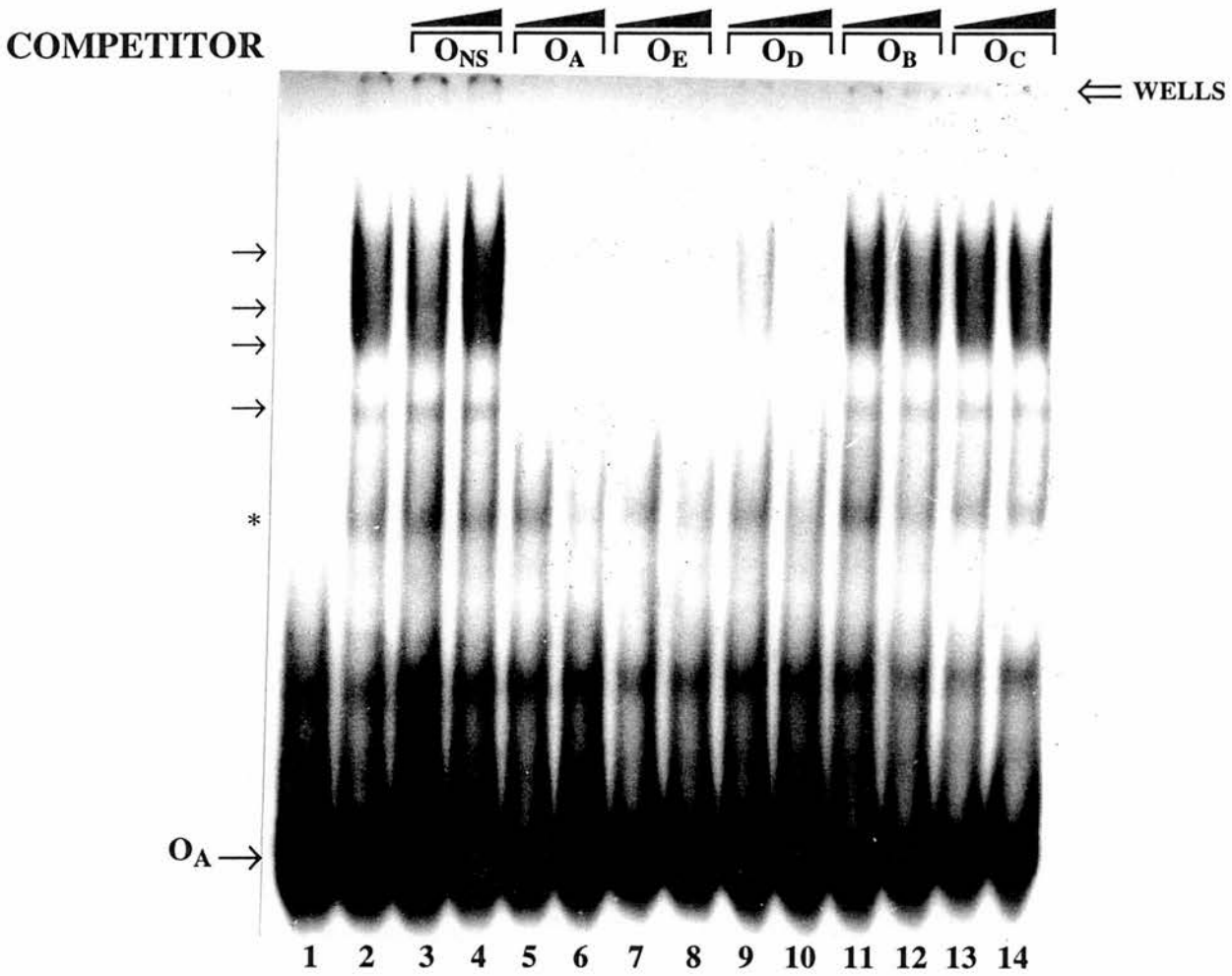

COMPETITOR
O_{NS}
O_B
O_E
O_C
O_T
⇐WELLS
F_A →
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3.9 *Liver nuclear proteins form complexes with the transcription start oligonucleotide O_A*

Gel mobility shift analysis demonstrating binding of rat liver nuclear extract to O_A, encoding -14 to +15 of the 11ß-HSD1 gene. 10µg rat liver nuclear extract was incubated with 0.1pmol of [^{32}P]-labelled O_A in the absence of added competitor DNA fragment (lane 2) or in the presence of 10- or 100-fold molar excess of competitor oligonucleotide (lanes 3-14), as indicated above the lanes. Lane 1 contained no nuclear extract. Arrows indicate specific protein-DNA complexes; * indicates a non-specific complex.

Oligonucleotides used were: O_A, 11ß-HSD1 (-14 to +15); O_B, 11ß-HSD1 (-88 to -57); O_C, 11ß-HSD1 (-71 to -52); O_D, PEPCK P3(I) site; O_E, optimal C/EBP site; O_{NS}, prolactin ERE (non-specific control).


COMPETITOR
O_{NS}
O_A
O_E
O_D
O_B
O_C
⟸ WELLS
→
→
→
→
*
O_A →
1 2 3 4 5 6 7 8 9 10 11 12 13 14

was observed, with $O_A \geq O_E > O_D >> O_B \geq O_C$ (Figure 3.10). The lack of competition by O_B and O_C (encoding the CCAAT box at -73 or the GCAAT sequence at -67 respectively of the 11ß-HSD1 gene) (Figure 3.9 and Figure 3.10), is consistent with the absence of a footprint over these sequences in DNase I protection analysis, and suggests that these sequences, present in a position typical of such regulatory elements, play little or no role in the expression of 11ß-HSD1 in rat liver. These results suggest that all the nuclear proteins which bind to fragment F_A and oligonucleotide O_A have identical or very similar DNA-binding specificity, and are probably members of the C/EBP family of transcription factors.

Bakker and Parker have reported that C/EBP binds to oligonucleotides encoding binding sites for Activating Transcription Factor (ATF)/CREB, but binds weakly or not at all to oligonucleotides encoding AP-1 sites (Bakker and Parker, 1991). To see if a similar pattern of specificity existed for rat liver nuclear proteins which bind to F_A and O_A, a series of oligonucleotides encoding CREs or AP-1 sites were used as competitors in gel mobility shift assays (refer to Chapter 2, Table 2.1 for oligonucleotide sequences) (Figure 3.11). O_J, encoding a CRE from the bovine preprotachykinin A gene (PPT-A) (Kageyama et al., 1991) proved the strongest competitor, fully competing complex formation on F_A at 100-fold molar excess (Figure 3.11). Both O_I and O_K (encoding respectively a somatostatin CRE (Montminy et al., 1986) and an AP-1 site which binds Fos/Jun, J. Quinn, Personal communication) effectively competed at 100-fold molar excess. Either very little or no competition was observed with the other oligonucleotides used; O_L (encoding a sequence in the arginine vasopressin (AVP) gene which binds Fos/Jun, J. Quinn, Personal communication), O_M (encoding a sequence at -60 of the rat PPT-A gene promoter which does not bind anything, J. Quinn, personal communication) and O_N (encoding an E-box-like sequence, J. Quinn, personal communication). None of the oligonucleotides were as effective as O_A at competing complex formation; this pattern of specificity is consistent with the results of Bakker & Parker, 1991 who found that ATF sites competed approximately 5-fold less well than C/EBP sites and that AP-1 sites competed weakly, if at all. The same rank order of competition was seen with liver nuclear extract binding to O_A, with $O_A > O_J > O_I \geq O_K > O_L = O_M = O_N = O_{NS}$ (Figure 3.12).

Figure 3.10 *Protein-DNA complexes formed on the 11ß-HSD1 promoter (-88/+47) contain C/EBP-related transcription factors*

Competition gel mobility shift assay using fragment F_A (encoding -88 to +47 of the 11ß-HSD1 gene) and rat liver nuclear extract. 8µg rat liver nuclear extract was incubated with 30fmol of [^{32}P]-labelled F_A in the absence of added competitor oligonucleotide (lane 2) or in the presence of 10- or 100-fold molar excess of competitor oligonucleotides (lanes 3-14), as indicated above the lanes. Lane 1 contained no nuclear extract. Arrows indicate specific protein-DNA complexes.

Oligonucleotides used were: O_A, 11ß-HSD1 (-14 to +15); O_B, 11ß-HSD1 (-88 to -57); O_C, 11ß-HSD1 (-71 to -52); O_D, PEPCK P3(I) site; O_E, optimal C/EBP site; O_{NS}, prolactin ERE (non-specific control).

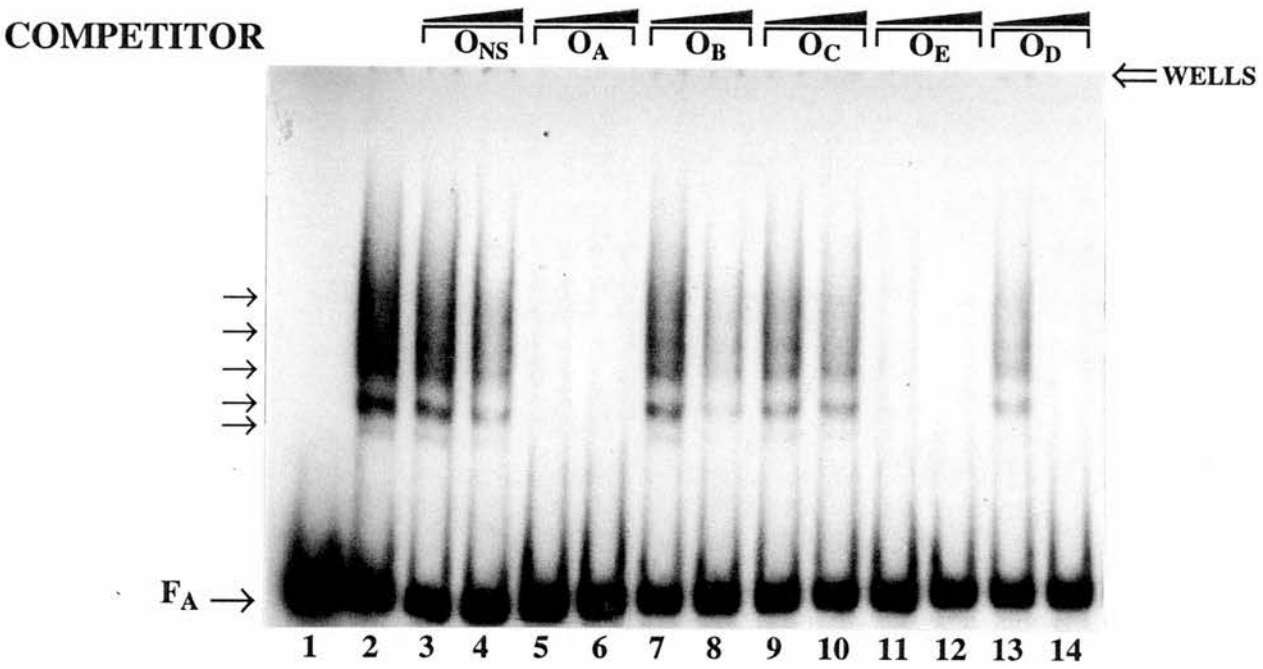

COMPETITOR
O_{NS}
O_A
O_B
O_C
O_E
O_D
WELLS
F_A
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 3.11 *ATF/CREB binding oligonucleotides compete complex formation on the 11ß-HSD1 promoter fragment*

Gel mobility shift analysis using rat liver nuclear extract and the 11ß-HSD1 promoter fragment -88 to +47 (F_A). 10µg rat liver nuclear extract was incubated with 30fmol of [^{32}P]-labelled F_A in the absence or in the presence of 10- or 100-fold molar excess of competitor oligonucleotide (lanes 3-18), as indicated above the lanes. Lane 1 contained no nuclear extract. Arrows indicate specific protein-DNA complexes.

Oligonucleotides used were: O_A, 11ß-HSD1 (-14 to +15); O_I, somatostatin CRE; O_J, PPT-A CRE; O_K, AP-1 site; O_L, AVP AP1 site; O_M, PPT-A (-60); O_N, E-box; O_{NS}, prolactin ERE (non-specific control).

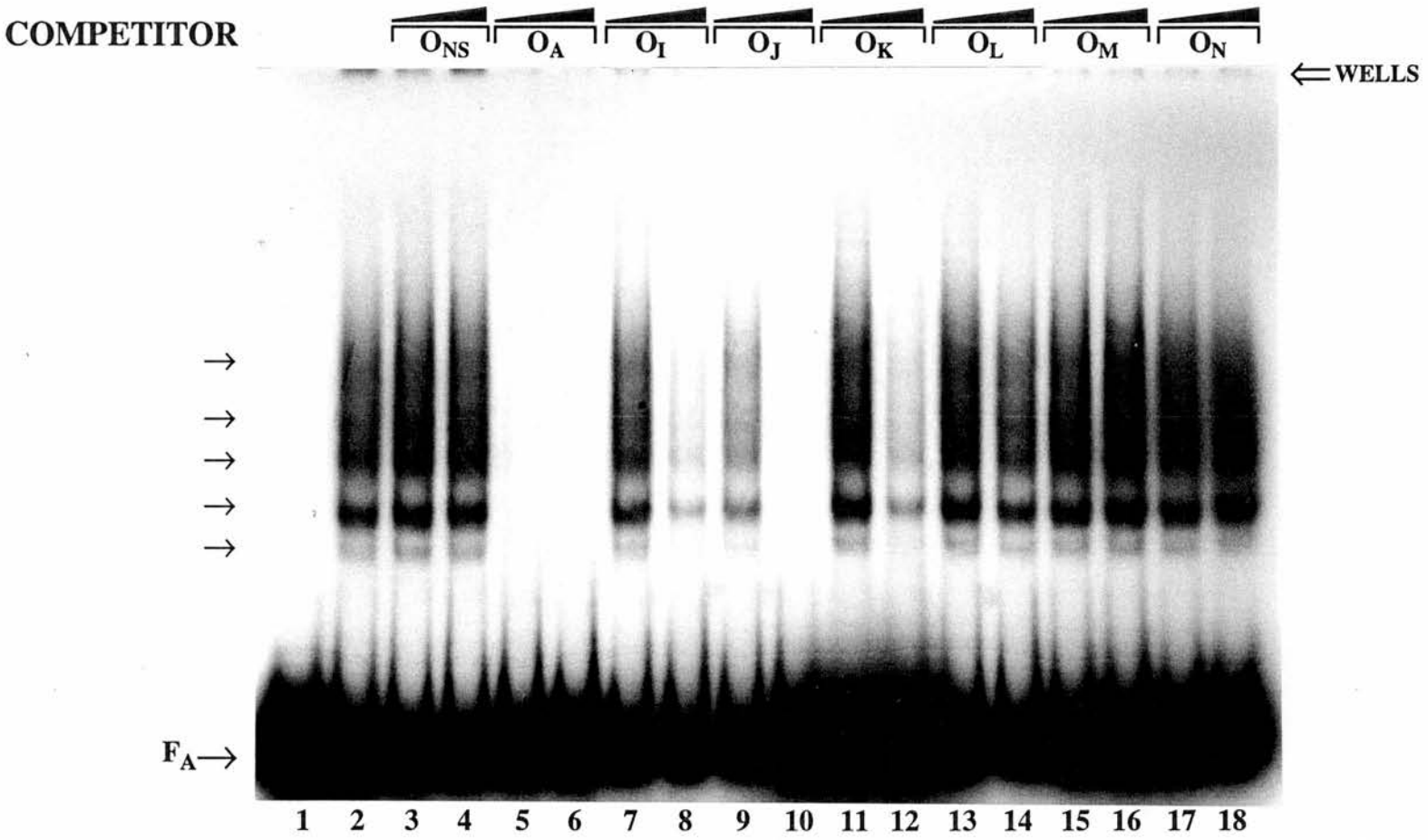
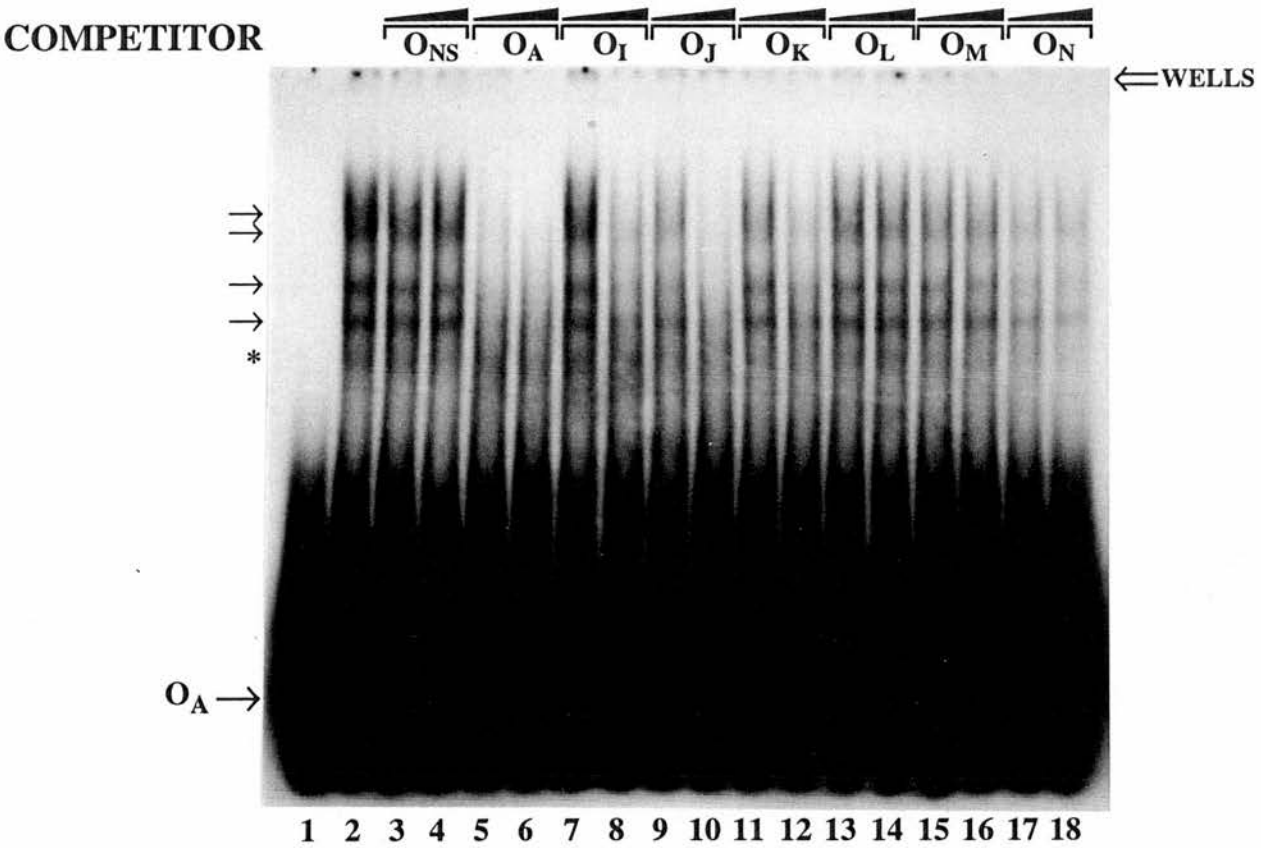

COMPETITOR
O_{NS}
O_A
O_I
O_J
O_K
O_L
O_M
O_N
⇐WELLS
→
→
→
→
→
F_A→
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 3.12 *ATF/CREB binding oligonucleotides compete complex formation on the 11ß-HSD1 transcription start*

Gel mobility shift analysis using rat liver nuclear extract and the 11ß-HSD1 promoter oligonucleotide (O_A). 10µg rat liver nuclear extract was incubated with 30fmol of [^{32}P]-labelled F_A in the absence or in the presence of 10- or 100-fold molar excess of competitor oligonucleotide (lanes 3-18), as indicated above the lanes. Lane 1 contained no nuclear extract. Arrows indicate specific protein-DNA complexes; * indicates a non-specific complex.

Oligonucleotides used were: O_A, 11ß-HSD1 (-14 to +15); O_I, somatostatin CRE; O_J, PPT-A CRE; O_K, AP-1 site; O_L, AVP AP1 site; O_M, PPT-A (-60); O_N, E-box; O_{NS}, prolactin ERE (non-specific control).


COMPETITOR
O_{NS}
O_A
O_I
O_J
O_K
O_L
O_M
O_N
WELLS
*
O_A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Members of the C/EBP family include C/EBPα (Johnson et al., 1987; Landschultz et al., 1988), C/EBPß (Akira et al., 1990; Chang et al., 1990; Poli et al., 1990), C/EBPγ (Roman et al., 1990), C/EBPδ (Cao et al., 1991; Williams et al., 1991) and C/EBPε (Williams et al., 1991), all of which contain a basic-leucine zipper domain required for dimerisation and DNA binding, and which can form homo- or heterodimers through the leucine zip region (reviewed in Chapter 1, Section 1.4.2.1.2). Most of the C/EBP DNA-binding activity in rat liver under normal conditions consists of C/EBPα and C/EBPß (Williams et al., 1991). We have used antisera specific for C/EBPα or C/EBPß in gel mobility supershift analysis to determine the relative contributions of C/EBPα and ß in binding to the transcription start of the rat 11ß-HSD1 gene. Incubation of C/EBPα antiserum with rat liver nuclear extract and fragment F_A resulted in decreased intensity of the upper two protein-DNA complexes (the lower two complexes were unaffected), and the appearance of additional lower-mobility 'supershifted' complexes (Figure 3.13). Similarly, when antiserum against C/EBPß was added, a reduction was seen in the intensity of the complexes formed on F_A, and an additional low-mobility supershifted complex became apparent (Figure 3.13). Addition of C/EBPα and C/EBPß antisera together virtually eliminated three of the four complexes formed on F_A and attenuated the remaining complex, whereas control antiserum (raised against COUP-Transcription Factor; COUP-TF) did not affect nuclear protein binding to F_A (Figure 3.13). This demonstrates the presence of either C/EBPα or C/EBPß in the majority of the complexes formed on F_A. Qualitatively similar results were seen with oligonucleotide O_A (Figure 3.14), which spans the transcription start. Most of the complexes formed on O_A contained either C/EBPα and/or C/EBPß (Figure 3.14). The one, high mobility, specific complex which was not supershifted when both antisera were added together, was competed in gel mobility shift analysis by C/EBP binding oligonucleotides. This indicates that the factor giving rise to this complex may also be related to C/EBP, but that it is not C/EBPα or C/EBPß.

3.2.3 Recombinant C/EBPα protein binds to the 11ß-HSD1 transcription start

To further investigate the involvement of C/EBPα in binding to the transcription start of 11ß-HSD1, a 35kDa fragment of rat C/EBPα lacking the N-terminal 60 amino acids (encoded by pT5; (Landschulz et al., 1989)) expressed in *E. coli*, was used, in gel mobility shift assays, DNase I protection and methylation interference

Figure 3.13 *C/EBPα and C/EBPβ from rat liver nuclear extract bind to the 11β-HSD1 promoter*

Gel mobility supershift analysis of complexes formed on the 11ß-HSD1 promoter by rat liver nuclear extract. 10μg rat liver nuclear extract was incubated with 30fmol of [^{32}P]-labelled F_A in the absence of antisera (lane 2), or with antisera added (lane3, 1μg of C/EBPα antisera; lane 4, 1μg of C/EBPß antisera; lane 5, 1μg C/EBPα antisera and 1μg C/EBPß antisera; lane 6, 1μg COUP-TF antisera). Lane 1 contained no nuclear extract. Supershifted complexes are indicted by arrows.

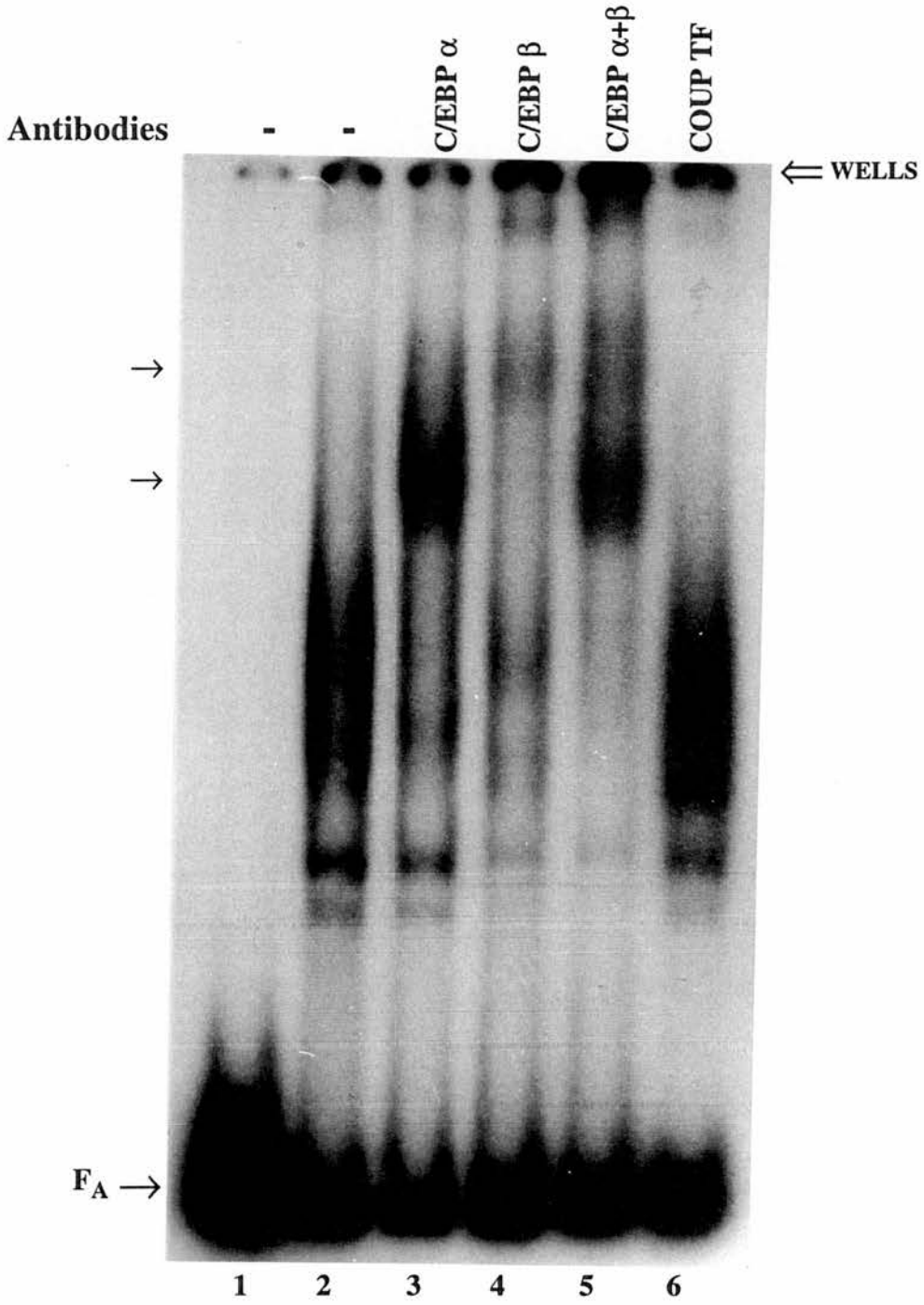

Antibodies
-
-
C/EBP α
C/EBP β
C/EBP α+β
COUP TF
WELLS
F_A
1
2
3
4
5
6

Figure 3.14 *C/EBPα and C/EBPß form complexes on the transcription start of the 11ß-HSD1 promoter*

Gel mobility supershift analysis of complexes formed on the 11ß-HSD1 promoter by rat liver nuclear extract. 10μg rat liver nuclear extract was incubated with 0.1pmol of [^{32}P]-labelled O_A in the absence of antiserum (lane 2), or with antiserum added; (lane 3, 1μg of C/EBPα antiserum; lane 4, 1μg of C/EBPß antiserum; lane 5, 1μg C/EBPα antiserum and 1μg C/EBPß antiserum; lane 6, 1μg COUP-TF antiserum). Lane 1 contained no nuclear extract. Supershifted complexes are indicted by arrows; ∗ indicates a non-specific complex.

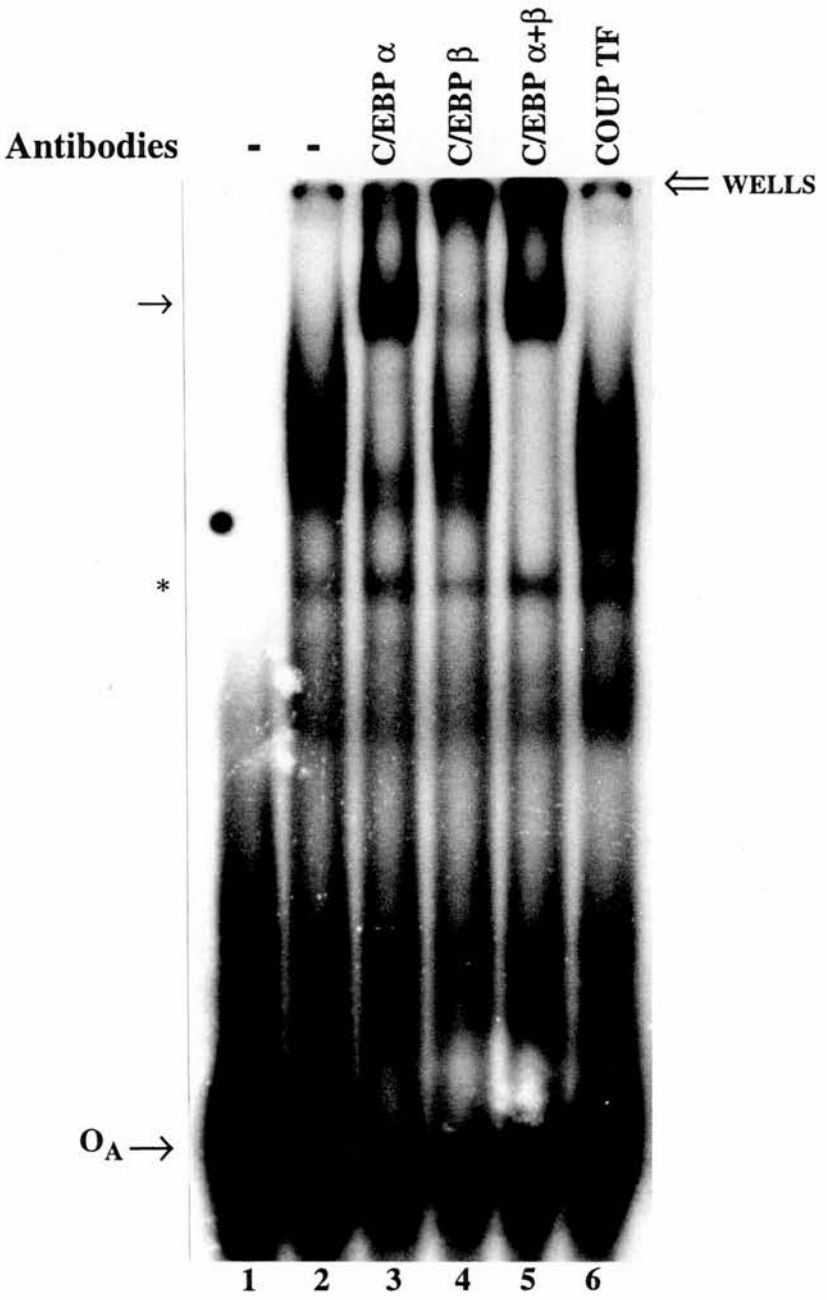
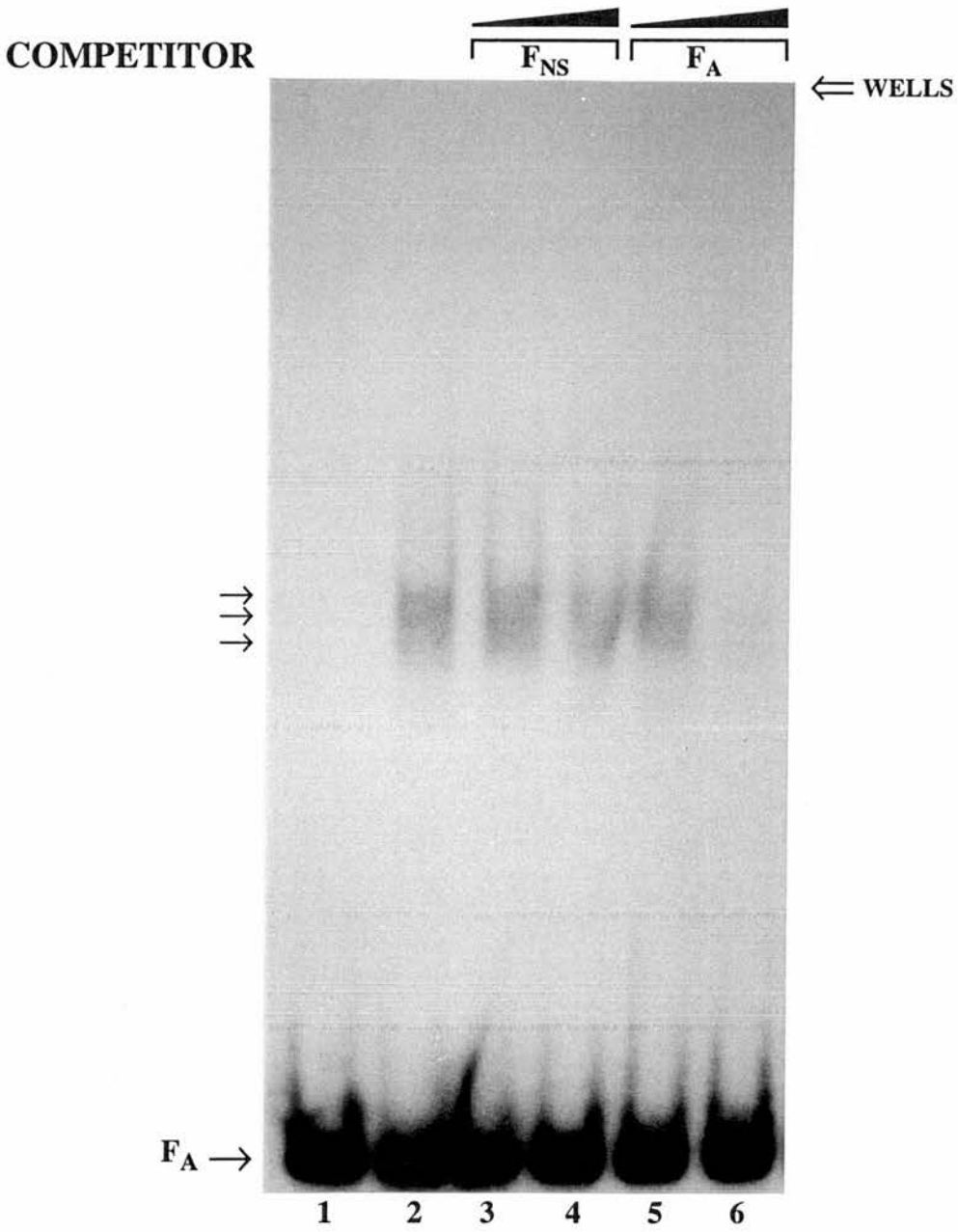

Antibodies
-
-
C/EBP α
C/EBP β
C/EBP α+β
COUP TF
⇐ WELLS
→
*
O_A →
1
2
3
4
5
6

Figure 3.15 *Bacterially expressed rC/EBPα binds to the 11ß-HSD1 promoter*

Gel mobility shift analysis using recombinant C/EBPα protein and 11ß-HSD1 promoter fragment F_A, encoding -88 to +47. 0.1μg bacterial extract containing rC/EBPα protein was incubated with 30fmol of [^{32}P]-labelled F_A in the absence or in the presence of 10- or 100-fold molar excess of competitor DNA fragment (lanes 3-6), as indicated above the lanes. Lane 1 contained no nuclear extract. Arrows indicate specific protein-DNA complexes.


COMPETITOR
F_{NS}
F_A
⇐ WELLS
F_A →
1
2
3
4
5
6

experiments. Bacterially expressed rC/EBPα formed 3 discrete specific complexes on fragment F_A (Figure 3.15). Addition of competitor oligonucleotides at 10- or 100-fold molar excess gave the same pattern of competition as seen with liver nuclear extract, with O_A (-14 to +15 of 11ß-HSD1) ≥ O_E (optimal C/EBP site) > O_D (PEPCK P3(I) C/EBP site) >> O_B, O_C and O_{NS} (respectively, the 11ß-HSD1 CCAAT box at -70, the 11ß-HSD1 GCAAT sequence at -65 and the non-specific control) (Figure 3.16). The mobility of the complexes produced on fragment F_A by rC/EBPα was similar to that of the liver nuclear protein complexes which were supershifted by the C/EBPα antiserum (Figure 3.16; compare with Figure 3.13). A single specific complex was formed by rC/EBPα on oligonucleotide O_A, of similar electrophoretic mobility to that of the major complex (containing C/EBPα) formed by liver nuclear proteins on O_A, which again showed the same specificity with competitor oligonucleotides as seen with liver nuclear proteins (Figure 3.17).

Comparison of the footprints produced by rat liver nuclear proteins and rC/EBPα on fragment F_A showed clearly that both protect very similar regions from digestion by DNase I, including the strongly protected region (-8 to +9) over the transcription start site (Figure 3.18). Similar weak footprints were generated by liver nuclear proteins and rC/EBPα at -54 to -36 and over the CCAAT box at -73 (Figure 3.18) (this very weak footprint was not observed in some experiments). rC/EBPα, however, only weakly protected the site at +27 to +39, indicating that C/EBP-related factors are capable of binding to the region between +27 and +39, but suggesting that the protein in liver nuclear extracts which binds to this site is probably not C/EBPα. In addition, rC/EBPα did not generate the DNase I hypersensitive sites observed in the presence of rat liver nuclear extract (Figure 3.18).

3.2.4 Additional evidence for C/EBP binding to the transcription start

Methylation interference analysis was used to compare the specific contacts made to fragment F_A by rC/EBPα and liver nuclear proteins. Methylated DNA was incubated with liver nuclear extract or bacterial extract containing rC/EBPα and subjected to preparative gel mobility shift analysis. Five complexes (C1-C5) produced by liver nuclear extract, and three complexes (C1-C3) produced by rC/EBPα were excised from the gel, as well as the free DNA not bound by protein. Methylated DNA was eluted, cleaved at methylated G residues, and electrophoresed on a denaturing

Figure 3.16 *Recombinant C/EBPα-11ß-HSD1 promoter complexes are competed by C/EBP-binding oligonucleotides*

Gel mobility shift analysis using recombinant C/EBPα protein and 11ß-HSD1 promoter fragment F_A. 0.1µg bacterial extract containing rC/EBPα protein was incubated with 30fmol of [^{32}P]-labelled F_A in the absence or in the presence of 10- or 100-fold molar excess of competitor oligonucleotide (lanes 3-14), as indicated above the lanes. Lane 1 contained no nuclear extract. Lane 15 contains 10µg of liver nuclear extract instead of recombinant C/EBPα. Arrows indicate specific protein-DNA complexes.

Oligonucleotides used were: O_A, 11ß-HSD1 (-14 to +15); O_B, 11ß-HSD1 (-88 to -57); O_C, 11ß-HSD1 (-71 to -52); O_D, PEPCK P3(I) site; O_E, optimal C/EBP site; O_{NS}, prolactin ERE (non-specific control).

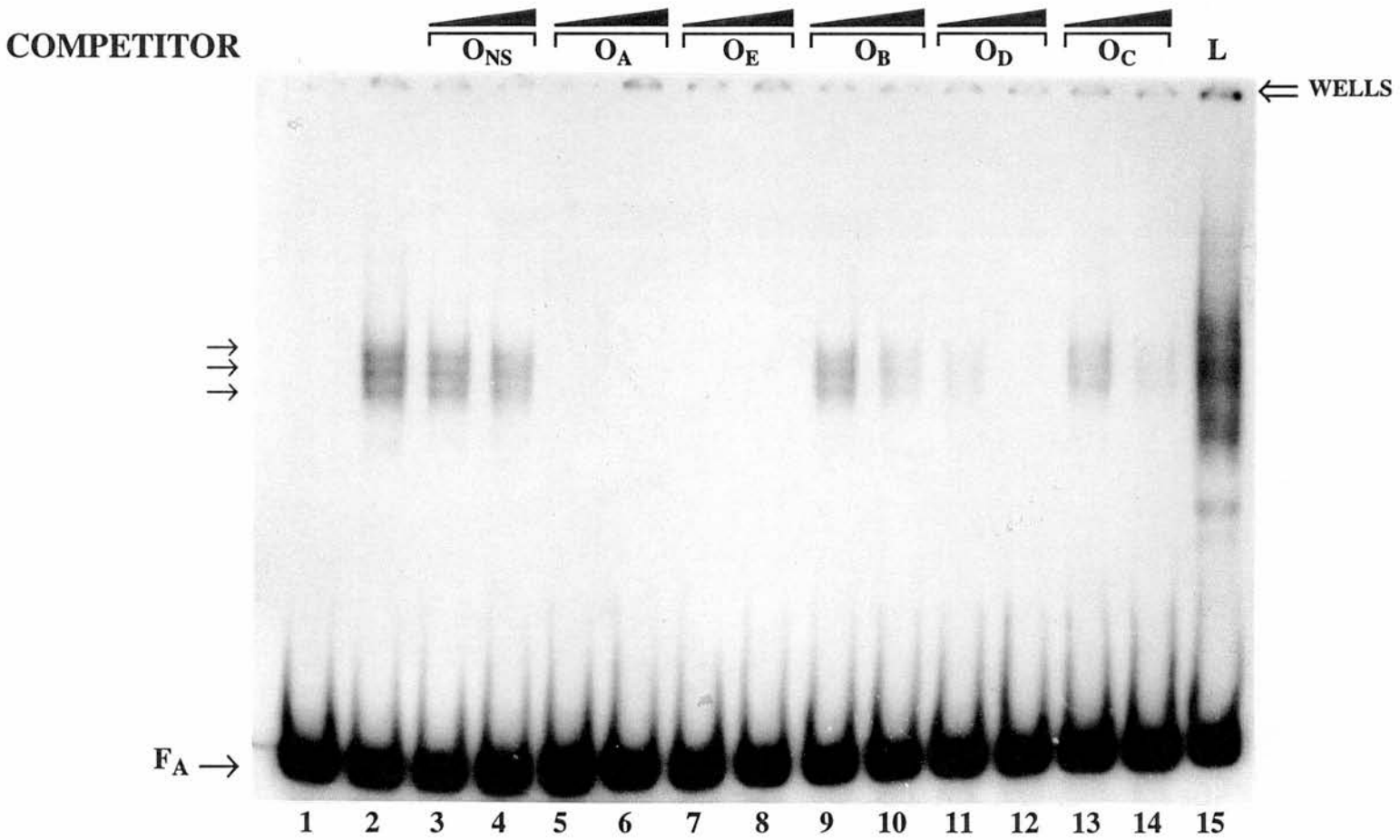

COMPETITOR
O_{NS}
O_A
O_E
O_B
O_D
O_C
L
WELLS
F_A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 3.17 *Recombinant C/EBPα protein forms a complex with the 11ß-HSD1 transcription start site oligonucleotide, O_A*

Gel mobility shift analysis using rC/EBPα protein and the oligonucleotide encompassing the 11ß-HSD1 transcription start O_A. 0.1μg bacterial extract containing rC/EBPα protein was incubated with 0.1pmol of [^{32}P]-labelled O_A in the absence or in the presence of 10- or 100-fold molar excess of competitor DNA fragment (lanes 3-14), as indicated above the lanes. Lane 1 contained no nuclear extract. Lane 15 contains 10μg of rat liver nuclear extract added instead of rC/EBPα. Arrows indicate specific protein-DNA complexes.

Oligonucleotides used were: O_A, 11ß-HSD1 (-14 to +15); O_B, 11ß-HSD1 (-88 to -57); O_C, 11ß-HSD1 (-71 to -52); O_D, PEPCK P3(I) site; O_E, optimal C/EBP site; O_{NS}, prolactin ERE (non-specific control).

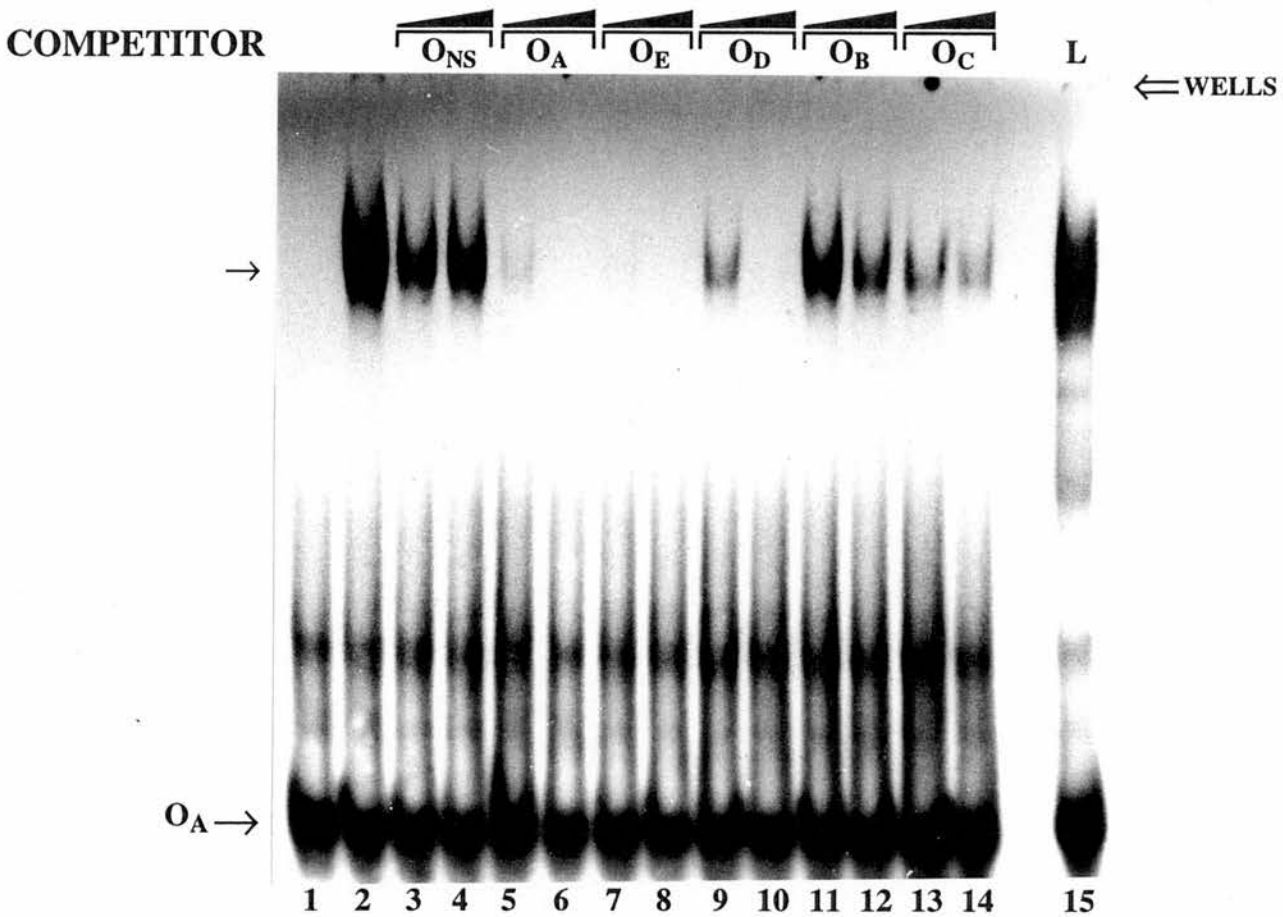
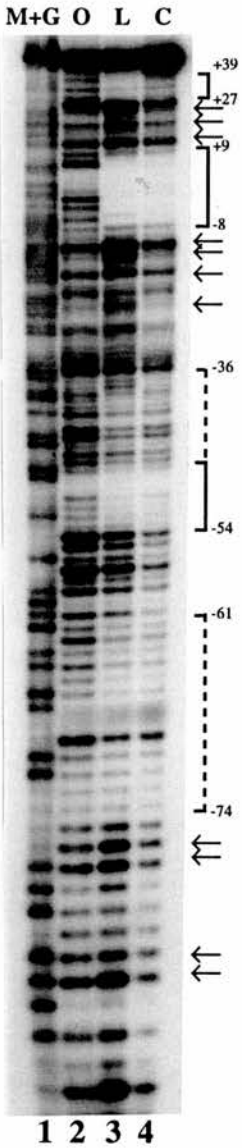

COMPETITOR
O_{NS}
O_A
O_E
O_D
O_B
O_C
L
⟸WELLS
→
O_A →
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 3.18 *Recombinant C/EBPα binds similar sites to liver nuclear proteins on the 11ß-HSD1 promoter*

DNase I protection analysis of the 11ß-HSD1 promoter fragment F_A, encoding -88 to +47 using rat liver nuclear extract and recombinant C/EBPα protein. Lane 1, Maxam and Gilbert sequencing reaction used as size markers; lane 2, no added protein; lane 3, 50µg of rat liver nuclear extract; lane 4, 0.5µg of bacterial extract containing rC/EBPα. Brackets indicate regions of DNA protected from DNase I digestion by rat liver nuclear extract. Arrows indicate nucleotides hypersensitive to DNase I. Dashed bracket indicates weak region of protection.


M+G O L C
+39
+27
+9
-8
-36
-54
-61
-74
1 2 3 4

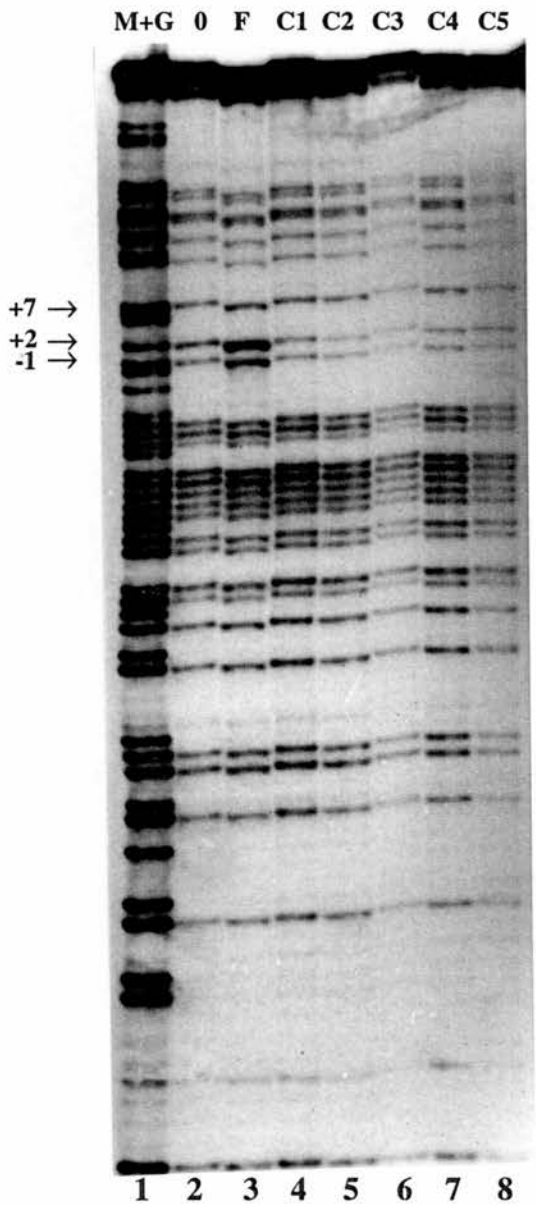
polyacrylamide gel. DNA methylated at positions -1 and +2 and to a lesser extent at position +7, was under-represented in the DNA recovered from complexes C1-C5 produced by liver nuclear proteins on fragment F_A (Figure 3.19 and Table 3.1). In contrast, DNA methylated at these three positions, -1, +2 and +7 was enriched in the free DNA, not bound to protein (Table 3.1 and Figure 3.19). Similarly, DNA methylated at -1, +2 and, to a lesser extent, +7 prevented binding of rC/EBPα to fragment F_A (Figure 3.20). Mutation of oligonucleotide O_A at -1 (O_G) or -1 and +2 (O_H) reduced (O_G), or virtually eliminated (O_H), binding of both liver nuclear extract and rC/EBPα in gel mobility shift assays (Figure 3.21). Furthermore, the use of O_G (mutated at -1) and O_H (mutated at -1 and +2) as competitors in gel mobility shift analysis of complexes formed by liver nuclear extract or rC/EBPα (Figure 3.22 and 3.23 respectively) on oligonucleotide (O_A) revealed an affinity order $O_A > O_G > O_H$. These data demonstrate the importance of the G residues at -1 and +2 for C/EBP binding to the 11ß-HSD1 transcription start.

3.3 Discussion

In this chapter I have described binding by members of the C/EBP transcription factor family to the proximal promoter of the rat 11ß-HSD1 gene. DNase I protection analysis identified similar sites of binding by rat liver nuclear proteins and rC/EBP. The main binding site, apparent at the lowest concentrations of liver nuclear extract, was between -8 and +9, encompassing the transcription start. Gel mobility shift assays using oligonucleotide competitors identified C/EBP-related factors binding between -88 to +47, and more specifically, to the transcription start. G residues at -1 and +2 on the lower strand were shown to be important for binding of liver nuclear proteins and rC/EBPα to the transcription start. At higher concentrations of liver nuclear extract a second footprint became apparent within exon 1, between +27 and +39. Additionally, two partially protected regions were observed 5' of the transcription start, at -74 to -61 and -54 to -36. DNase I hypersensitive sites were generated by liver nuclear extract but not by rC/EBPα. The hypersensitive nucleotides were located at borders of footprints and between footprints with spacing at approximately 10 base pair intervals, a pattern of hypersensitivity associated with DNA bending (Hochschild & Ptashne, 1986). This may reflect an interaction between C/EBP (and possibly associated proteins) at the transcription start with

Figure 3.19 *Specific nucleotide contacts are made at the transcription start by factors from rat liver nuclear extract*

Methylation interference analysis of the rat 11ß-HSD1 gene promoter using rat liver nuclear extract. Lane 1, Maxam and Gilbert G+A sequencing reaction used as size marker; lane 2, cleavage pattern of total methylated DNA with no extract added; lane 3, free DNA excised from a gel mobility shift reaction that included rat liver nuclear extract; lanes 4-8, DNA eluted from complexes excised from preparative non-denaturing gels (C1=lowest mobility complex, C5=highest mobility complex). Arrows indicate G residues that, when methylated, interfere with binding by rC/EBPα protein.


M+G
0
F
C1
C2
C3
C4
C5
+7 →
+2 →
-1 →
1
2
3
4
5
6
7
8

	Total DNA	Complexed DNA (mean ± SEM)	Free DNA
+16	100	100	100
+13	54	55.8 ± 2.5	48
+7	59	52.3 ± 4.6	75
+2	77	59.3 ± 3.3	185
-1	36.8	36.8 ± 3.3	139
-7	32.5	32.5 ± 2.5	85
-9	71.3	71.3 ± 5.0	

Table 3.1 *Quantitation of interference by DNA methylation of liver nuclear extract binding to the transcription start of 11ß-HSD1*

Densitometric scanning of the autoradiograph shown in Figure 3.19. Positions of guanine nucleotides which have been analysed are indicated in the left hand column. For each lane the optical density of the G residue at +16 has been arbitrarily set to 100%, and the optical density of all other G bands in that lane is expressed as a percentage of the +16 signal. Total DNA; results from the densitometric scanning of lane 2 in Figure 3.19, containing total DNA (which had not been excised from a gel). Complexed DNA; combined data from the densitometric scanning of lanes 4-8 in Figure 3.19 expressed as mean ± SEM. Free DNA; results from the densitometric scanning of lane 3 in Figure 3.19 corresponding to DNA that did not bind nuclear extract.

Figure 3.20 *Recombinant C/EBPα forms specific nucleotide contacts at the 11ß-HSD1 transcription start*

Methylation interference analysis of the rat 11ß-HSD1 gene promoter using rC/EBPα. Lane 1, Maxam and Gilbert G+A sequencing reaction used as size marker; lane 2, free DNA excised from a gel mobility shift reaction that included rC/EBPα protein. Lanes 3-5, DNA eluted from complexes excised from preparative non-denaturing gels (C1=lowest mobility complex, C3=highest mobility complex); lane 6, cleavage pattern of total methylated DNA with no extract added. Arrows indicate G residues that, when methylated, interfere with binding by rC/EBPα protein.

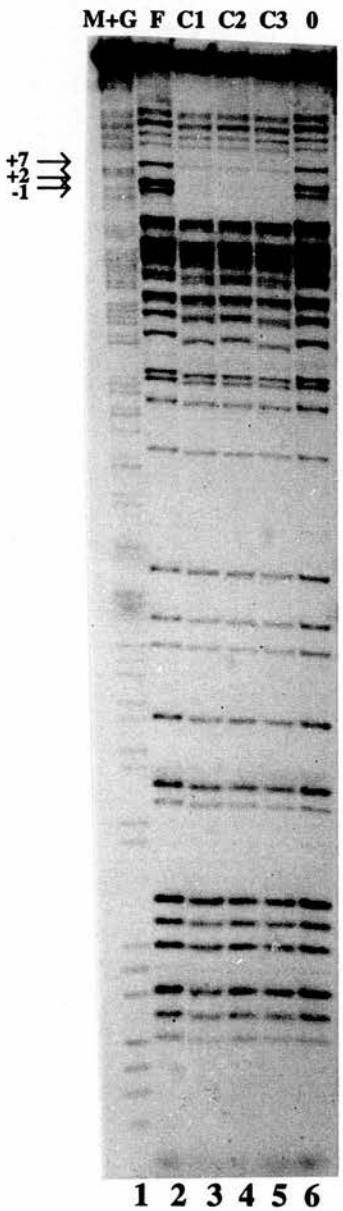

M+G F C1 C2 C3 0
+7
+2
-1
1 2 3 4 5 6

Figure 3.21 *Effect of mutation at -1 and +2 on binding of rat liver nuclear extract and rC/EBPα to the transcription start*

Gel mobility shift analysis comparing complexes formed by rat liver nuclear extract and rC/EBPα protein on the 11ß-HSD1 transcription start oligonucleotide, O_A and derivatives mutated at -1 (O_G) or -1 and +2 (O_H). All lanes contain 0.1pmol of [^{32}P]-labelled oligonucleotide. Lanes 1, 4 and 7 contain O_A; lanes 2, 5 and 8 contain O_G; lanes 3, 6 and 9 contain O_H. Lanes 4-6 contain 10µg of rat liver nuclear extract and lanes 7-9 contain 0.1µg bacterial extract containing rC/EBPα. Arrows indicate specific protein-DNA complexes; ∗ indicates a non-specific band.

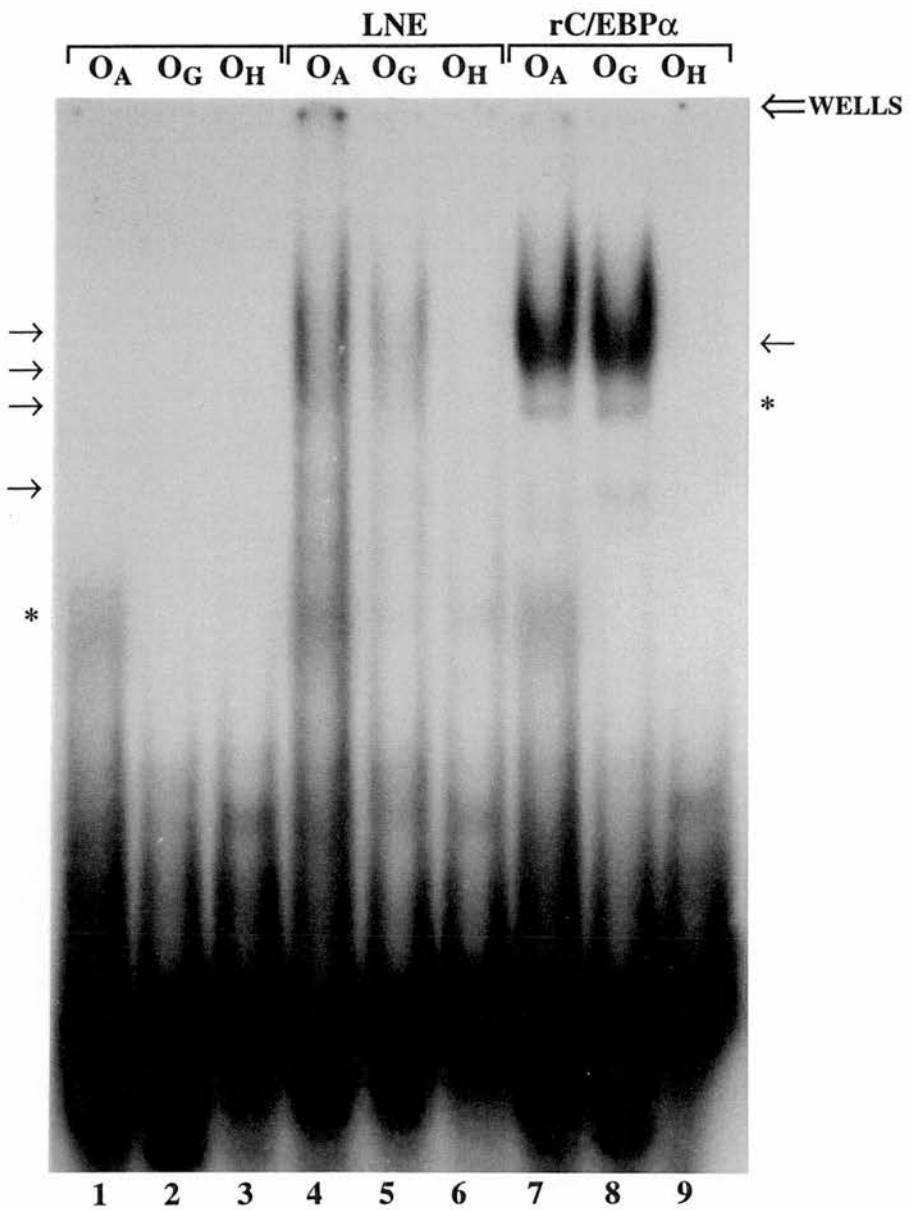

LNE
rC/EBPα
O_A O_G O_H O_A O_G O_H O_A O_G O_H
⇐WELLS
→
→
→
→
*
←
*
1 2 3 4 5 6 7 8 9

Figure 3.22 *Mutation at -1 and +2 reduces binding of rat liver nuclear proteins to the transcription start*

Gel mobility shift analysis using rat liver nuclear extract and the 11ß-HSD1 promoter oligonucleotide, O_A. 10µg rat liver nuclear extract was incubated with 1pmol of [^{32}P]-labelled O_A in the absence or in the presence of 10- or 100-fold molar excess of competitor oligonucleotide (lanes 3-10), as indicated above the lanes. Lane 1 contained no nuclear extract. Arrows indicate specific protein-DNA complexes; * indicates a non-specific band.

Oligonucleotides used were: O_A, 11ß-HSD1 (-14 to +15); O_G, 11ß-HSD1 (-14 to +15 mutated at -1); O_H, 11ß-HSD1 (-14 to +15 mutated at -1 and +2); O_{NS}, prolactin ERE (non-specific control).

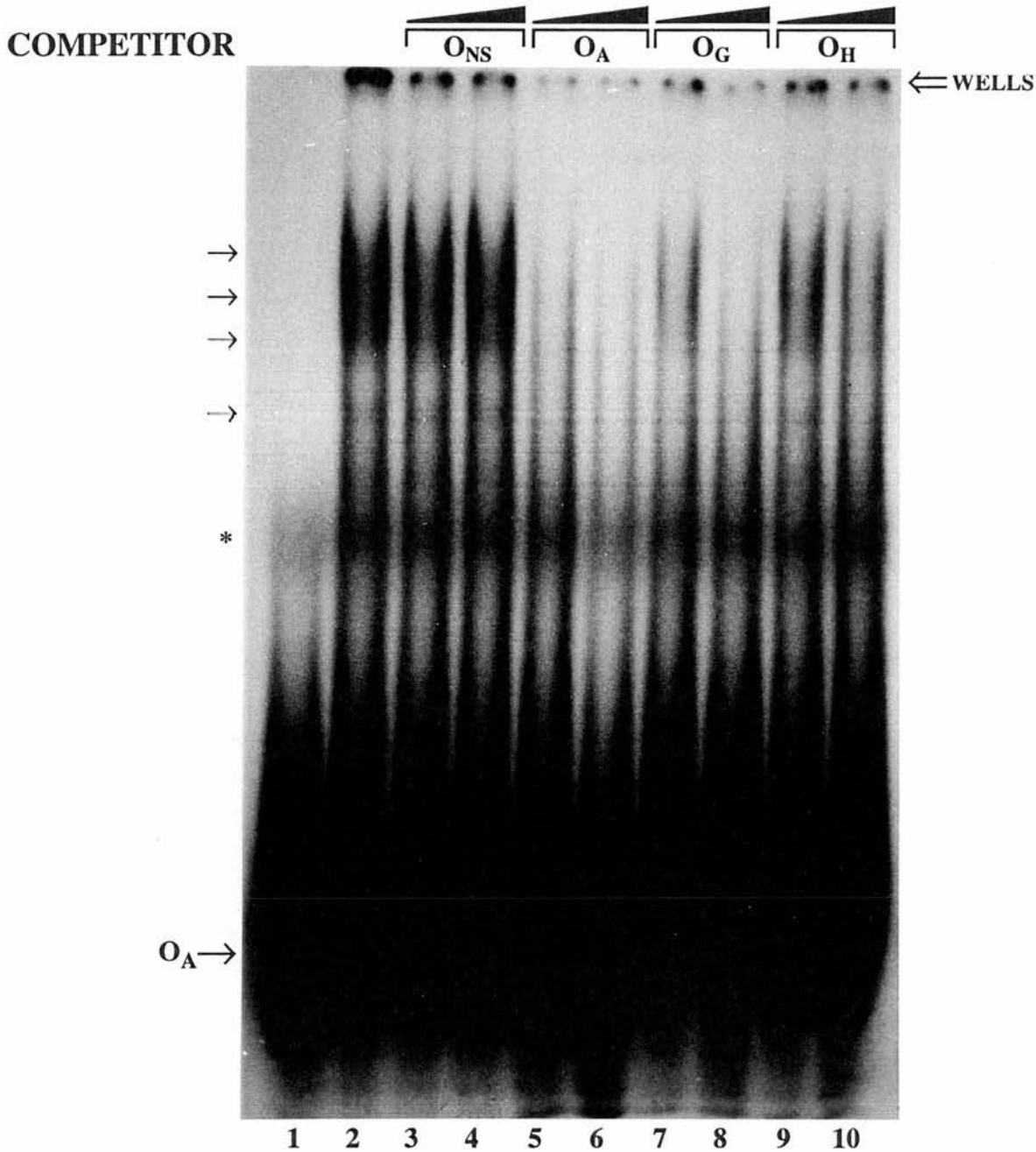
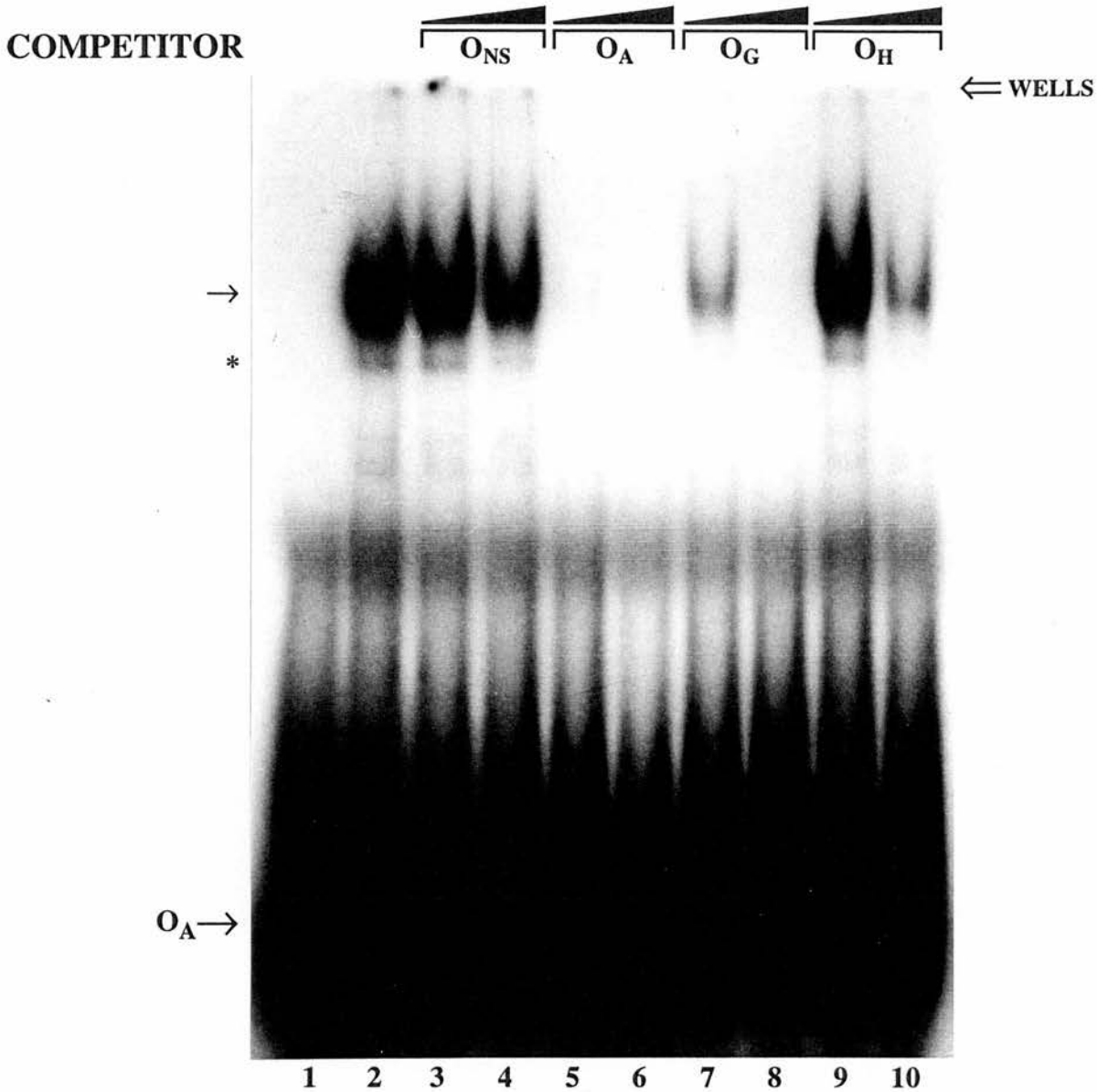

COMPETITOR
O_{NS}
O_A
O_G
O_H
WELLS
*
O_A
1 2 3 4 5 6 7 8 9 10

Figure 3.23 *Mutation at -1 and +2 reduces binding of rC/EBP to the transcription start*

Gel mobility shift analysis using rC/EBPα and the 11ß-HSD1 promoter oligonucleotide, O_A. 0.1μg rC/EBPα was incubated with 0.1pmol of [^{32}P]-labelled F_A in the absence or in the presence of 10- or 100-fold molar excess of competitor oligonucleotide (lanes 3-10), as indicated above the lanes. Lane 1 contained no nuclear extract. Arrows indicate specific protein-DNA complexes; ∗ indicates a non-specific band.

Oligonucleotides used were: O_A, 11ß-HSD1 (-14 to +15); O_G, 11ß-HSD1 (-14 to +15 mutated at -1); O_H, 11ß-HSD1 (-14 to +15 mutated at -1 and +2); O_{NS}, prolactin ERE (non-specific control).


COMPETITOR
O_{NS}
O_A
O_G
O_H
⟸ WELLS
→
*
O_A→
1 2 3 4 5 6 7 8 9 10

proteins bound elsewhere on the promoter forming a nucleoprotein complex. The absence of hypersensitive sites when rC/EBPα is bound to the promoter suggests that other proteins (or possibly the N-terminal 60 amino acids of C/EBPα, missing from the protein used here (Landschultz et al., 1988)) are necessary to generate the hypersensitive sites. C/EBPα and C/EBPß were identified in complexes formed at the transcription start and it is likely that they are involved in the regulation of 11ß-HSD1 transcription in liver.

The C/EBP family of transcription factors is widely expressed and shows a differential pattern of expression (Williams et al., 1991). C/EBPα and C/EBPß mRNAs show a similar distribution, with highest levels in liver, moderate expression in lung, kidney, adipose tissue, placenta and specific regions of brain, and lower levels elsewhere (Kuo et al., 1990; Williams et al., 1991), a pattern of expression resembling that of 11ß-HSD1 (Agarwal et al., 1989; Moisan et al., 1990a). In contrast, C/EBPδ is expressed at highest levels in lung (Williams et al., 1991), and low levels in liver under basal conditions (Cao et al., 1991), although it is induced in liver under certain pathological conditions (Alam et al., 1992). The majority of the complexes formed on the 11ß-HSD1 promoter fragment F_A and transcription start oligonucleotide O_A contained C/EBPα and/or C/EBPß. The two lowest mobility complexes formed on F_A and O_A contained predominantly C/EBPα, and faster migrating complexes possibly contained heterodimers of C/EBPα and C/EBPß or other C/EBP-like factors. Only one, high mobility, specific complex was not supershifted when both antisera were added together to gel mobility shift assays. This complex was, however, competed on gel mobility shift analysis by C/EBP-binding oligonucleotides, indicating that it is likely to involve a member of the C/EBP family. It is possible that the high mobility specific complex contains (i) a degradation product of either C/EBPα or C/EBPß that has lost the epitope for the antibody, (ii) an alternative translation product of C/EBPα or C/EBPß protein with a truncated N-terminus which lacks part of the transactivation domains and consequently binds DNA but does not activate transcription, (Descombes & Schibler, 1991; Ossipow et al., 1993), (iii) that it contains the C/EBP-related protein, DBP (Mueller et al., 1990). However, all of these possibilities are unlikely. DBP has a molecular size of 39kDa (Mueller et al., 1990) and would be expected to migrate through the gel with a mobility comparable to, or only slightly faster than, C/EBPα.

The peptide against which the C/EBPß antiserum was raised includes part of the leucine zipper region, and corresponds to the C-terminal 19 amino acids of the protein, which is present in LIP (Descombes and Schibler, 1991), a shorter C/EBPß translational product, and which would be present in any degradation products of C/EBPß still able to bind to DNA. The C/EBPα antiserum was raised against a peptide corresponding to the N-terminus of a 14kDa degradation product of C/EBPα, which was the major degradation product when C/EBPα was isolated from liver (Landschultz et al., 1988). It is possible that the high mobility specific complex formed at the transcription start of 11ß-HSD1 contains homodimers of C/EBPδ. At 28kDa (Cao et al., 1991), C/EBPδ is smaller than either C/EBPα (predominantly 42kDa and 30kDa) and C/EBPß (32kDa), and would not be recognised by the antisera used, which were specific for either C/EBPα or C/EBPß.

The relative levels of C/EBP family members varies according to cell type, developmental stage and pathological state (Alam et al., 1992; Descombes & Schibler, 1991; Raught et al., 1995; Williams et al., 1991; Yeh et al., 1995). The ratio of C/EBPα and C/EBPß in liver changes in different conditions; thus, under conditions in which the acute-phase response is activated, C/EBPα expression is decreased and C/EBPß and C/EBPδ expression is dramatically increased (Alam et al., 1992). C/EBPß is a weak transcriptional activator compared to other members of the family (Poli et al., 1990), and it is likely that, by changing the ratio of C/EBPα/ß, expression of C/EBP-regulated genes is altered. Evidence suggests that this occurs; on induction of the acute-phase response, albumin expression is transiently decreased, possibly due to reduced expression of C/EBPα (Burke et al., 1994), and expression of α_1-acid glycoprotein is rapidly induced with the concomitant exchange of C/EBPα by C/EBPß/C/EBPδ (Alam et al., 1992). In addition, evidence suggests that transcriptional activation, and probably DNA-binding, by members of the C/EBP family is influenced by phosphorylation (Macdougald et al., 1995; Ray and Ray, 1994). Alteration of the ratio of C/EBPα to C/EBPß, or the phosphorylation state of either, may alter transcription initiation at the 11ß-HSD1 promoter.

The position of the C/EBP site, spanning the transcription start of 11ß-HSD1 is unusual. Many C/EBP binding sites have been described in other genes, both upstream and, in some cases, downstream from the transcription start. However,

there is a binding site for C/EBP at +1 to +18 of the gene encoding the clotting factor IX (Crossley & Brownlee, 1990). A naturally occurring mutation at +13 which results in haemophilia B Leyden both disrupts binding of C/EBP to this site and prevents transactivation of the promoter by C/EBP in transfected cells (Crossley and Brownlee, 1990). Binding of C/EBP to the site at -8 to +9 of the 11ß-HSD1 gene promoter may be important in the basal expression of the gene possibly functioning as an initiator-binding protein. Initiator sequences overlap the transcription start site and determine the start of transcription in promoters which lack a TATA box, or increase the strength of a TATA-containing promoter. A variety of proteins, including TFII-I, USF, YY1 and HIP1, have been found to bind to initiator elements of specific genes (Du et al., 1993; Means & Farnham, 1990; Roy et al., 1991; Seto et al., 1991). In addition to binding to Inr elements at the transcriptional start sites of specific genes, these proteins also act as transcription factors binding to sites upstream or downstream of regulated genes (Kovesdi et al., 1986; Means and Farnham, 1990; Riggs et al., 1991; Roy et al., 1991). C/EBPα has been shown to interact with TBP *in vivo*, and *in vitro*, C/EBPα interacts with TBP and TFIIB (Nerlov & Ziff, 1995). The 11ß-HSD1 promoter lacks a TATA box; it is possible that C/EBP binding to the transcription start site of this gene performs the function of an initiator-binding protein, although this remains to be directly tested.

CHAPTER 4

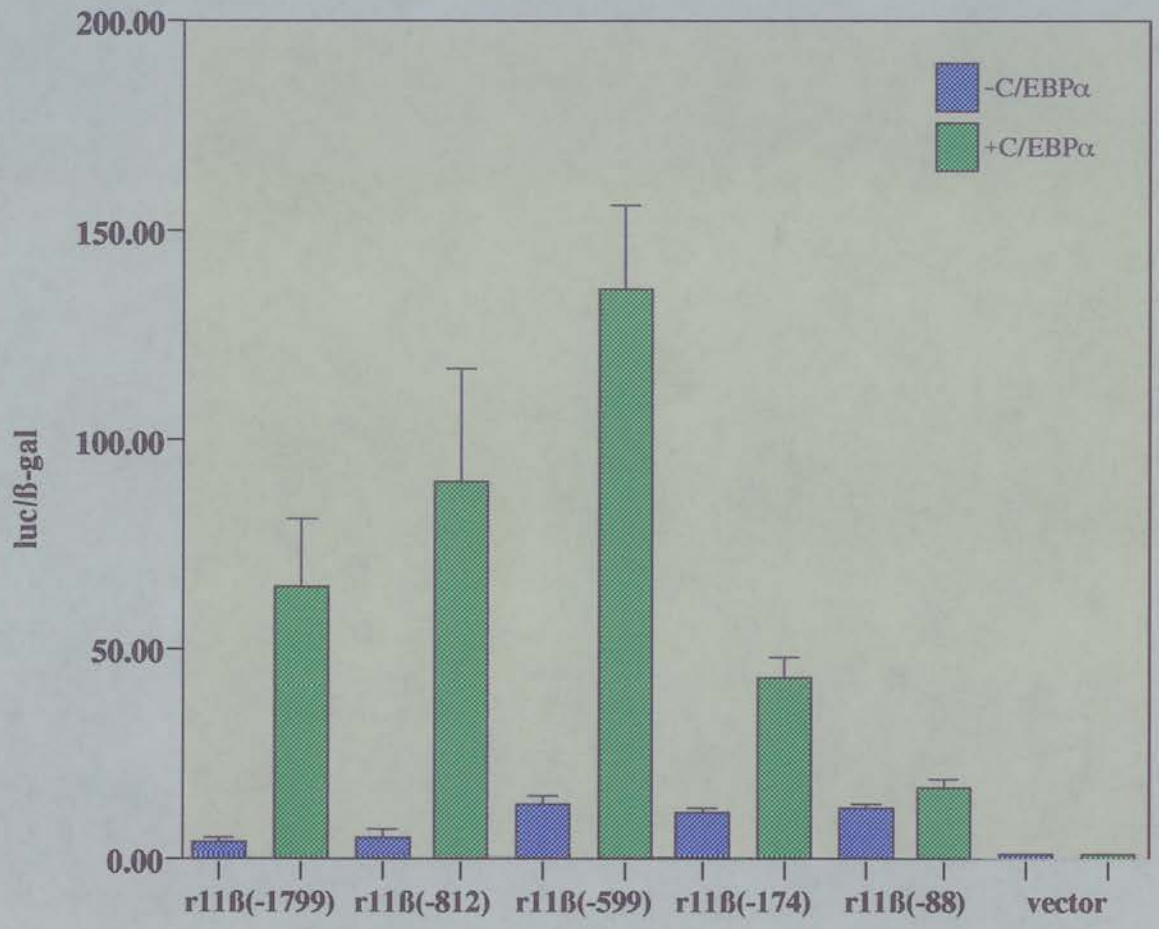
Transcription factor interaction with the rat 11ß-HSD1 gene between -812 and +74

4.1 Introduction

The rat 11ß-HSD1 gene promoter contains consensus binding sites for C/EBP and other factors (Chapter 3, Figure 3.1) within its 5' flanking region, and is regulated *in vivo* by glucocorticoids, growth hormone and oestrogen (reviewed in Chapter 1, Section 1.3.1.3). Transient transfections using a deletion series of the rat 11ß-HSD1 promoter fused to a luciferase reporter gene (deleted 5' from -1799 and fused within exon 1 at +47 with the luciferase reporter) have been carried out on HepG2 cells and 2S-FAZA cells to examine whether the 11ß-HSD1 promoter is indeed regulated by C/EBP, and to localise the DNA sequence elements required for the responsiveness to hormones. HepG2 cells are a human hepatoma-derived cell line which express a limited range of liver specific genes (Aden et al., 1979; Knowles et al., 1980), have very little C/EBP (Becker et al., 1991; Williams et al., 1991), and no 11ß-HSD1 (Y. Koteletsev, personal communication). 2S-FAZA cells are derived from rat liver and have a number of properties typical of differentiated liver (Deschatrette et al., 1980; Malawista & Weiss, 1974), and they express endogenous 11ß-HSD1 in a hormonally-regulated fashion (Voice et al., 1996). Cotransfection of HepG2 cells with C/EBPα and the 11ß-HSD1-luciferase deletion series demonstrated the responsiveness of the promoter to C/EBPα (Figure 4.1). Deletion of 11ß-HSD1 promoter DNA from -1799 to -812 had little effect on either basal expression of luciferase, or on the induction of luciferase activity by cotransfected C/EBPα (approximately 15-20 fold induction). Deletion of DNA between -812 and -599 resulted in an increase of basal level expression (approximately 2.5 fold), but had little or no effect on the level of C/EBPα-stimulated activity (with approximately 10-fold induction caused by C/EBPα) (Figure 4.1). Further deletion between -599 and -88 had no effect on basal promoter activity in HepG2 cells, but dramatically reduced the response to C/EBPα; deletion to -174 reduced the C/EBP-induction to approximately 4-fold, and further deletion to -88 virtually eliminated the effect of C/EBP, with a residual 1.5-fold stimulation by C/EBPα (Figure 4.1). Transfections of 2S-FAZA cells using the 11ß-HSD1 promoter deletion series fused to a luciferase reporter gene demonstrated that the rat 11ß-HSD1 promoter between -1799 and +47 is responsive to insulin and glucocorticoids (Voice et al., 1996). Upon deletion of promoter DNA from -599 to -249 the responsiveness to insulin was lost (M. Voice, personal communication). The responsiveness to glucocorticoids was retained to -174 but was lost upon deletion to -88 (M. Voice, personal communication).

Figure 4.1 *The 11ß-HSD1 gene is activated by C/EBPα in transfected HepG2 cells*

HepG2 cells were cotransfected with a series of plasmids containing 11ß-HSD1 promoter DNA, linked to luciferase at +47 within exon 1. $5x10^5$ HepG2 cells in a 60mm dish were cotransfected with a total of 10µg DNA comprising 5µg 11ß-HSD1-luciferase plasmid, 1µg pCHII0 encoding ß-galactosidase, 1µg pMSV-C/EBPα (where appropriate) made to 10µg with pGEM3, the calcium phosphate procedure. 48h after transfection, cells were harvested and lysed to assay luciferase and ß-galactosidase activities; the ratio of luciferase to ß-galactosidase activity as calculated. Data shown are means ± SEM, calculated from 4 experiments, each carried out in quadruplicate; data are normalised to the basal (-C/EBP) ratio of luciferase/ß-galactosidase. The units shown are arbitrary units. (Figure contributed by K. Chapman).


200.00
150.00
100.00
50.00
0.00
luc/ß-gal
-C/EBPα
+C/EBPα
r11ß(-1799)
r11ß(-812)
r11ß(-599)
r11ß(-174)
r11ß(-88)
vector

However, no sequences exist that exactly match the consensus half site GRE sequence TGTYCT (Beato, 1987) or the insulin response element, CCCGCCTC (Locker, 1993), in the 11ß-HSD1 gene between -599 and -88.

These transfection experiments indicate that C/EBPα can act on the 11ß-HSD1 promoter to regulate 11ß-HSD1 expression. I was therefore interested in identifying the sites to which C/EBP binds, and the identity of the proteins binding to the regions responsive to hormonal signals.

4.2 Results

4.2.1 Rat liver nuclear proteins bind to multiple sites on the 11ß-HSD1 gene between -812 and +74

DNase I protection analysis was used to identify sites of liver nuclear protein interaction with the 11ß-HSD1 promoter between -812 and +74. Parallel reactions were carried out with rC/EBPα protein to identify the footprints which may be due to C/EBP-related factors. A series of overlapping restriction fragments covering the 11ß-HSD1 promoter between -812 and +74 were each labelled at the 5' end on the lower strand (Figure 4.2). In most cases the fragment was first subcloned into pGEM in order to generate suitable restriction sites for end-labelling with the Klenow fragment of DNA polymerase I. Two fragments, (-812 to -599 and -322 to +47) were derived from a series of 5' deletion plasmids in which a SalI linker was introduced at the deletion end point (V. Lyons, personal communication). I shall describe the results of the DNase I protection analysis by splitting the 11ß-HSD1 promoter between -812 and +74 into 3 sections; (i) -812 to -599; (ii) -599 to -130; (iii) -130 to +74.

4.2.1.1 Proteins from rat liver nuclei bind to the 11ß-HSD1 gene between -812 and -599

Four footprints were formed by liver nuclear extract on the fragment extending from -812 to -599. Figure 4.3 shows a representative experiment (of four carried out). An identical footprint was formed by liver nuclear extract and rC/EBPα between -792 and -780. This site was characterised in more detail (Section 4.2.4). A region between -771 and -747 was protected by liver nuclear extract but not rC/EBPα. A

Figure 4.2 *11ß-HSD1 gene fragments used in DNase I protection analysis*

Summary of 11ß-HSD1 gene fragments used for analysis of liver nuclear extract and rC/EBPα interaction with the promoter.

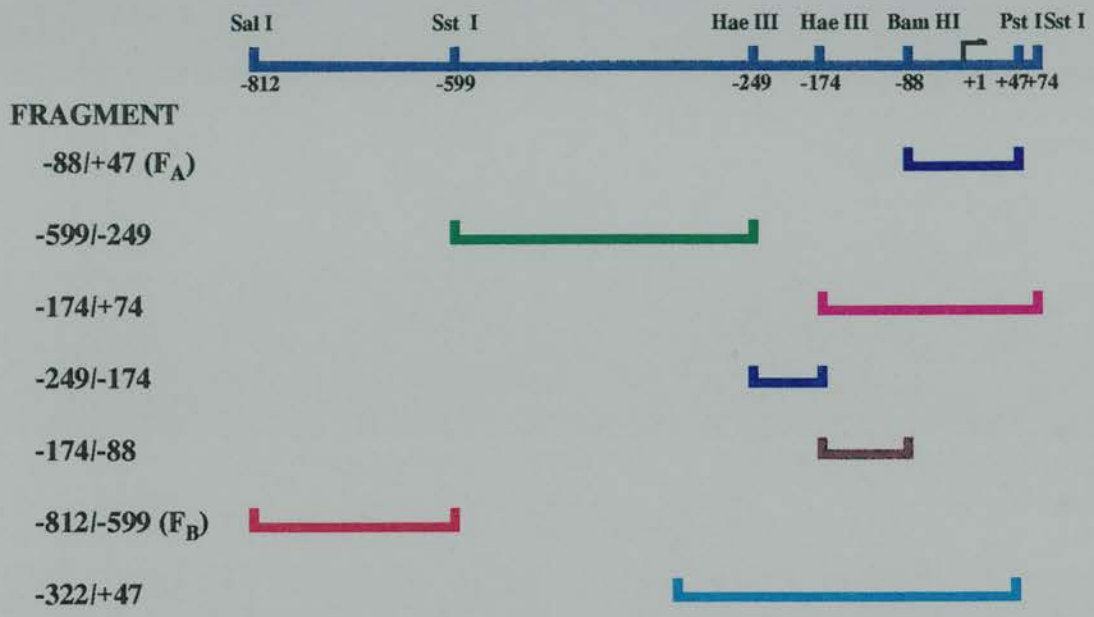

Sal I
Sst I
Hae III
Hae III
Bam HI
Pst I
Sst I
-812
-599
-249
-174
-88
+1
+47
+74
FRAGMENT
-88/+47 (F_A)
-599/-249
-174/+74
-249/-174
-174/-88
-812/-599 (F_B)
-322/+47

Figure 4.3 *DNase I footprints formed by liver nuclear extract and rC/EBPα on the 11ß-HSD1 promoter between -812 and -599*

DNase I protection analysis of the 11ß-HSD1 promoter fragment encoding -812 to -599 using liver nuclear extract and rC/EBPα. Panels were from a single gel with multiple loadings. Each panel shows the results from the same reactions run for different lengths of time. Lane 1 shows a Maxam and Gilbert sequencing reaction used as a molecular size marker (M+G); lane 2 has no extract added; lane 3 contains 100μg of liver nuclear extract (L) and lane 4 has 1.3μg of rC/EBPα (C) added. Strong regions of protection are indicated by brackets; dashed bracket indicates a region of protection for which the boundaries have not been defined; inner brackets refer to regions protected by liver nuclear extract; outer brackets, protection by rC/EBPα. Nucleotides showing enhanced sensitivity to DNase I are indicated by arrows.

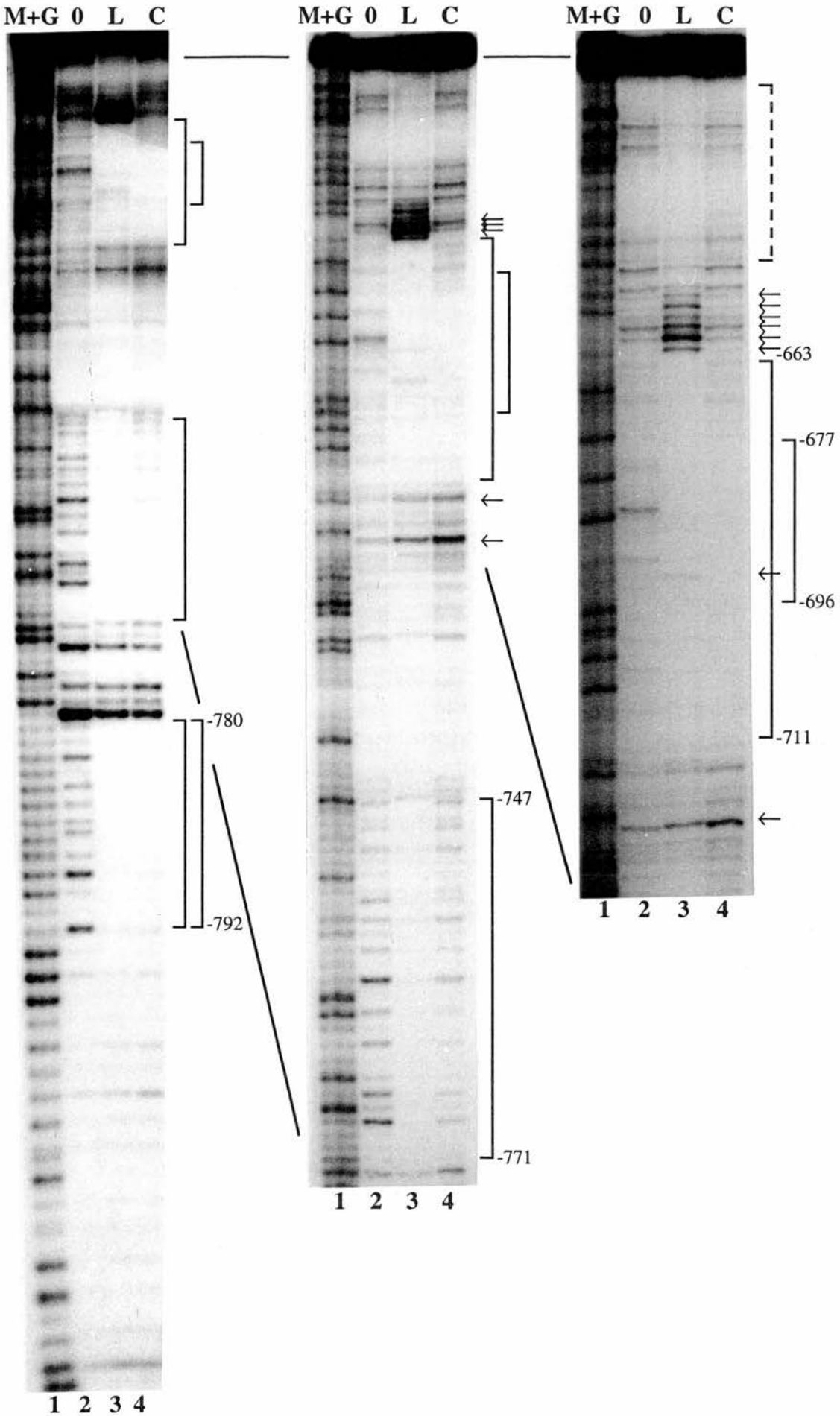
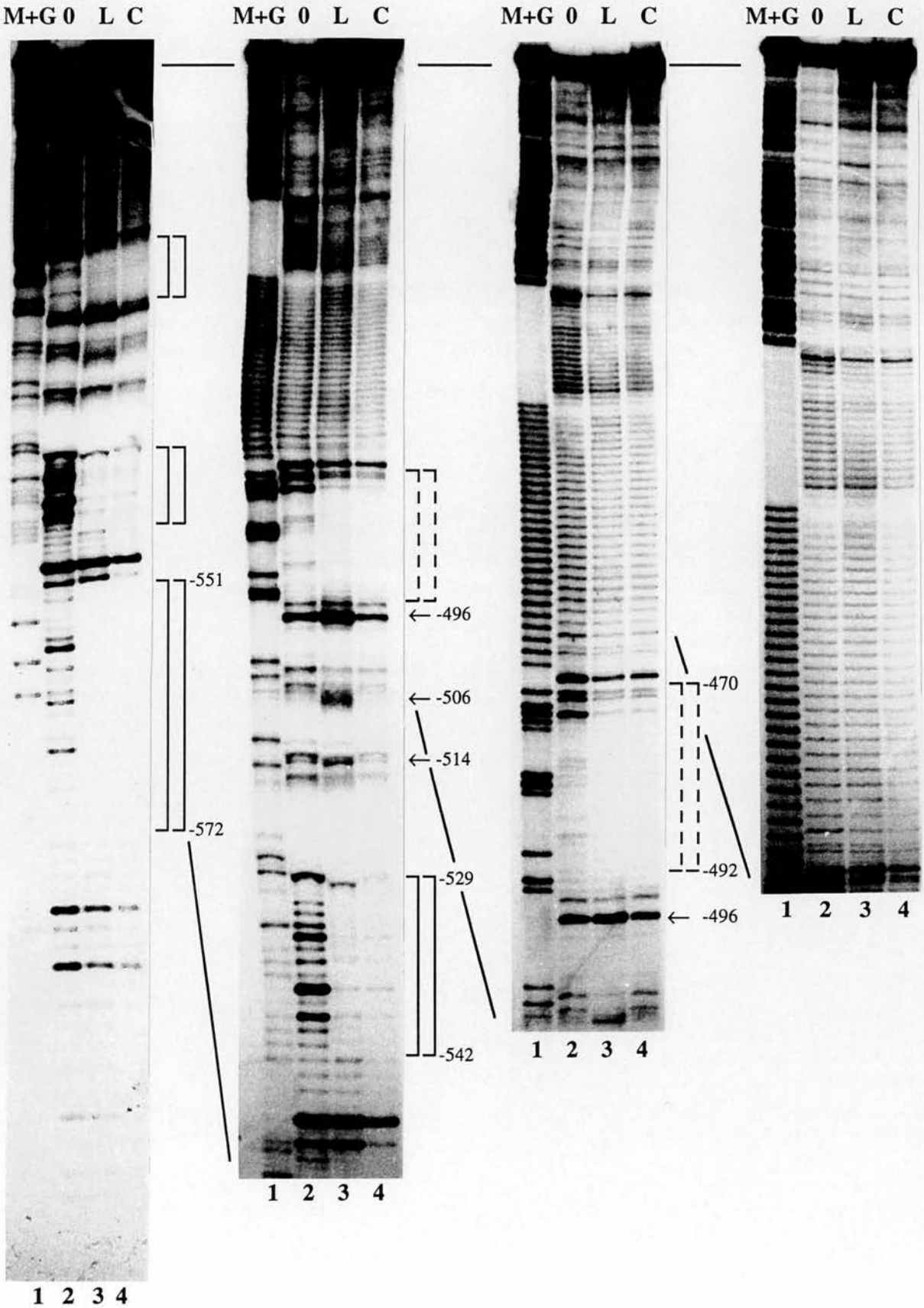

M+G 0 L C
M+G 0 L C
M+G 0 L C
-780
-792
-747
-771
-663
-677
-696
-711
1 2 3 4
1 2 3 4
1 2 3 4

Figure 4.4 *DNase I footprints formed by liver nuclear extract and rC/EBPα on the 11ß-HSD1 promoter fragment encoding -599 to -249*

DNase I protection analysis of the 11ß-HSD1 promoter encoding -599 to -249 using liver nuclear extract and rC/EBPα. All panels were from a single gel with multiple loadings, run for different lengths of time. Lane 1 shows a Maxam and Gilbert sequencing reaction used as molecular size markers (M+G); lane 2 has no extract added; lane 3 contains 100µg of liver nuclear extract (L) and lane 4 has 1.3µg of rC/EBPα (C) added. Strong regions of protection are indicated by solid brackets, weak regions of protection by dashed lines; inner brackets refer to regions protected by liver nuclear extract; outer brackets, protection by rC/EBPα. Nucleotides showing enhanced sensitivity to DNase I are indicated by arrows.


M+G 0 L C
M+G 0 L C
M+G 0 L C
M+G 0 L C
-551
-572
-496
-506
-514
-529
-542
-470
-492
-496
1 2 3 4
1 2 3 4
1 2 3 4
1 2 3 4

third footprint was formed by liver nuclear extract between -711 and -663, and within this region a rC/EBPα footprint was formed covering -696 to -677. This latter site was characterised in more detail by gel mobility shift analysis (refer to Section 4.2.3). Liver nuclear extract induced a strong region of hypersensitivity at the 3' boundary of this footprint which was not observed with recombinant C/EBPα. Downstream of the hypersensitive region, an additional footprint was formed by liver nuclear extract only, although it was not seen in all footprinting reactions using this fragment. However, on this gel (Figure 4.3) using this promoter fragment it was not possible to accurately read the sequence of this protected region, and the exact boundaries of this site remain to be defined.

4.2.1.2 Proteins from rat liver nuclei bind to the 11ß-HSD1 gene between -599 and -130

Two overlapping fragments, encoding -599 to -249 and -322 to +47, were used to analyse nuclear protein binding sites between -599 and -130. The results of DNase I protection experiments using fragment -599/-249 are shown in Figure 4.4. Three regions were protected from DNase I digestion by rat liver nuclear extract, all of which were similarly protected by rC/EBPα protein. Two of these sites, at -572 to -551 and -542 to -529, were strongly protected and the third site at -492 to -470, was partially protected from DNase I digestion (Figure 4.4). No binding of either liver nuclear proteins or rC/EBPα was observed to the $(CT)_{26}(GT)_{19}$ repeat at -462 to -373. Hypersensitive sites induced by liver nuclear extract (but not by rC/EBPα) were observed at -496, -506 and -514, a spacing interval close to 10 base pairs. The limit of single base resolution on the gel shown in Figure 4.4 was about -300. Therefore, to identify protein binding sites 3' of -300, the fragment encoding -322 to +47 was used, from which data could be obtained to approximately -130 (Figure 4.5). Part of this region is also covered by the smaller 11ß-HSD1 promoter fragments, -249/-174 (Figure 4.6), -174/-88 (Figure 4.7) and -174/+74 (Figure 4.8). Between -322 and -130, two regions were similarly protected from DNase I digestion by liver nuclear extract and rC/EBPα protein. These footprinted regions were located at nucleotides -244 to -226 (Figure 4.5 and Figure 4.6) and at -180 to -139 (Figure 4.5). When the fragments -174/-88 (Figure 4.7) and -174 to +74 (Figure 4.8) were used the latter footprint extended from -146 to -164 (Figure 4.7) or from -146 to -163 (Figure 4.8), possibly as a consequence of the fragment not extending the full extent

Figure 4.5 *DNase I footprints formed by liver nuclear extract and rC/EBPα on the 11ß-HSD1 promoter fragment encoding -322 to +47*

DNase I protection analysis of the 11ß-HSD1 promoter encoding -322 to +47 using liver nuclear extract and rC/EBPα. Panels were from a single gel with multiple loadings. Each panel shows the results of the same reactions run for different lengths of times. Lane 1 shows a Maxam and Gilbert sequencing reaction used as a molecular size marker (M+G); lane 2 has no extract added; lane 3 contains 100µg of liver nuclear extract (L) and lane 4 has 1.3µg of rC/EBPα (C) added. Strong regions of protection are indicated by brackets; inner brackets refer to regions protected by liver nuclear extract; outer brackets, protection by rC/EBPα.

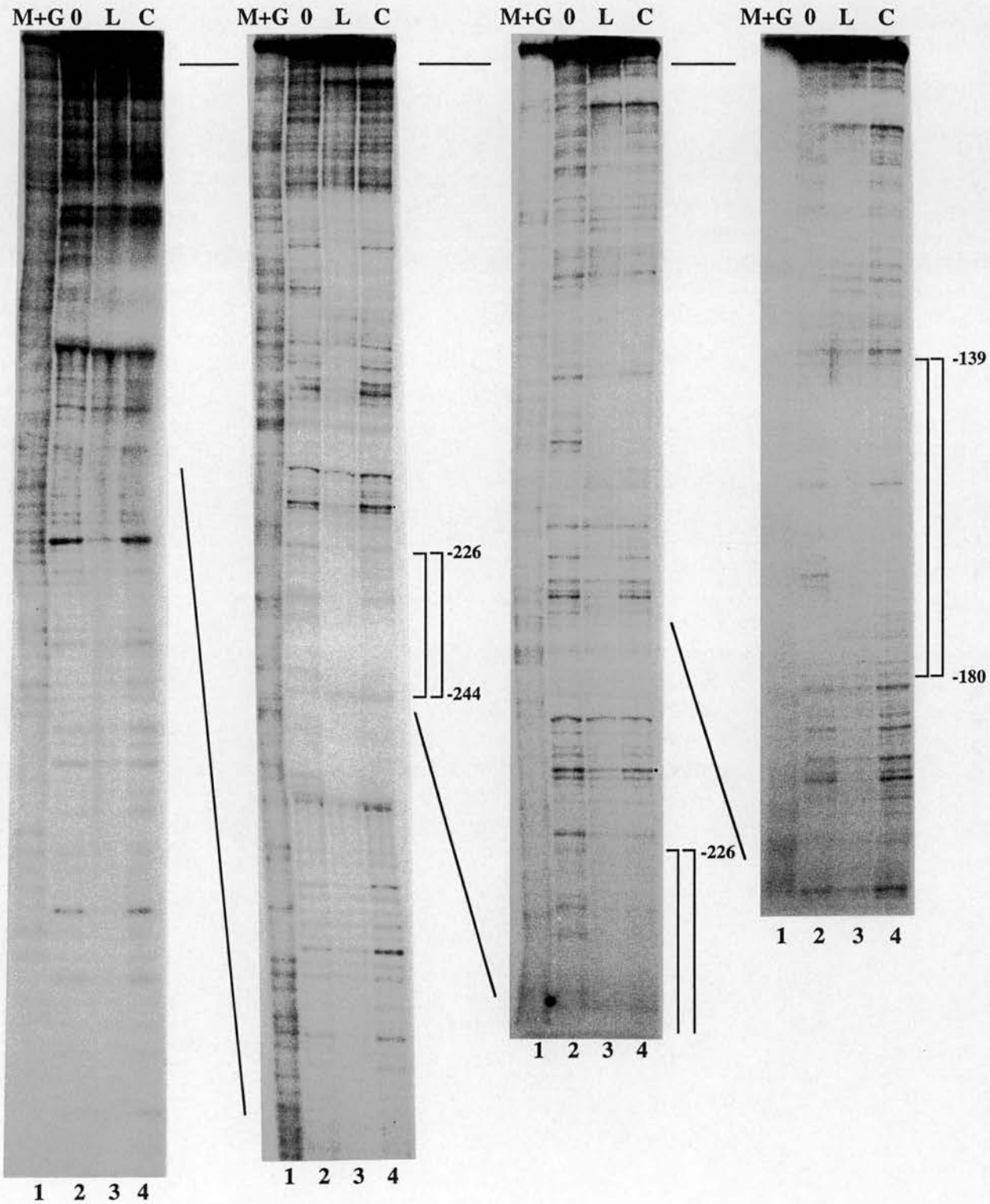

M+G 0 L C
M+G 0 L C
M+G 0 L C
M+G 0 L C
-226
-244
-226
-139
-180
1 2 3 4
1 2 3 4
1 2 3 4
1 2 3 4

Figure 4.6 *DNase I footprints formed by liver nuclear extract and rC/EBPα on the 11ß-HSD1 promoter fragment encoding -249 to -174*

DNase I protection analysis of the 11ß-HSD1 promoter encoding -249 to -174 using liver nuclear extract and rC/EBPα. Panels were from a single gel with multiple loadings. Each panel show the results of the same reactions run for different lengths of times. Lane 1 shows a Maxam and Gilbert sequencing reaction used as a molecular size marker (M+G); lane 2 has no extract added; lane 3 contains 100µg of liver nuclear extract (L) and lane 4 has 1.3µg of rC/EBPα (C) added. Strong regions of protection are indicated by brackets; inner brackets refer to regions protected by liver nuclear extract; outer brackets, protection by rC/EBPα.

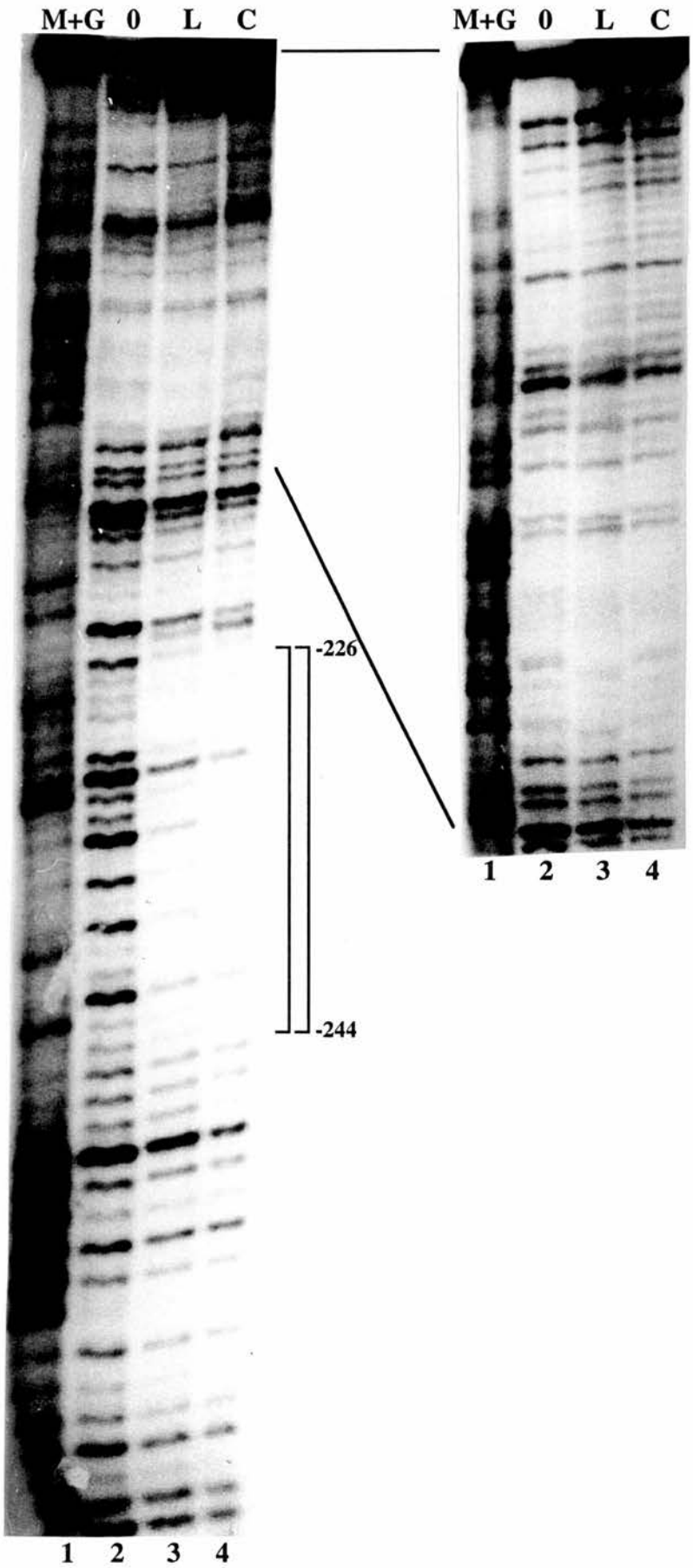
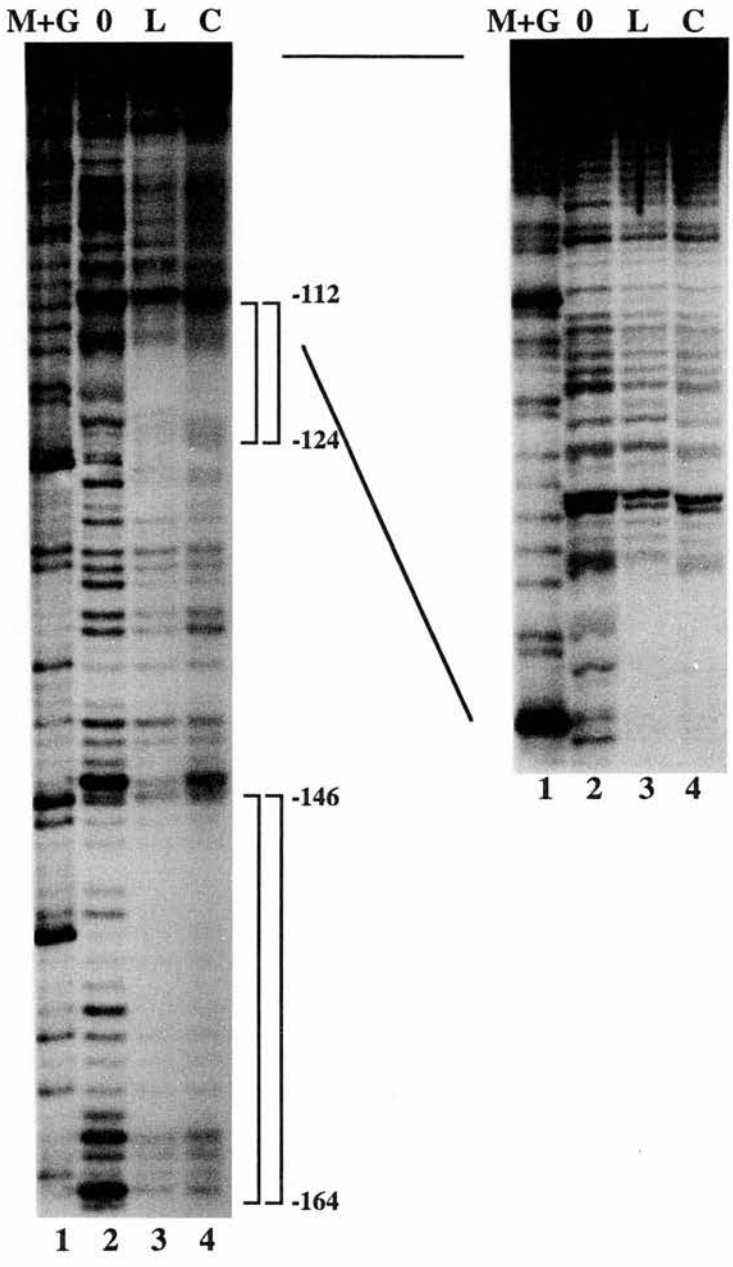

M+G
0
L
C
M+G
0
L
C
-226
-244
1
2
3
4
1
2
3
4

Figure 4.7 *DNase I footprints formed by liver nuclear extract and rC/EBPα on the 11ß-HSD1 promoter fragment encoding -174 to -88*

DNase I protection analysis of the 11ß-HSD1 promoter fragment encoding -174 to -88 using liver nuclear extract and rC/EBPα. Panels were from a single gel with multiple loadings. Each panel show the results of the same reactions run for different lengths of times. Lane 1 shows a Maxam and Gilbert sequencing reaction used as a molecular size marker (M+G); lane 2 has no extract added; lane 3 contains 100µg of liver nuclear extract (L) and lane 4 has 1.3µg of rC/EBPα (C) added. Strong regions of protection are indicated by brackets; inner brackets refer to regions protected by liver nuclear extract; outer brackets, protection by rC/EBPα.


M+G 0 L C
-112
-124
-146
-164
1 2 3 4
M+G 0 L C
1 2 3 4

of the footprint produced on the -322/+47 fragment (Figure 4.5). Clusters of hypersensitive sites were occasionally seen to appear at the boundaries of footprinted regions (Figure 4.4 and Figure 4.8).

4.2.1.3 Proteins from rat liver nuclei bind to the 11ß-HSD1 gene between -130 and +74

To identify binding sites for liver nuclear proteins in the remaining promoter region between -130 and +74, fragments encoding -174 to +74 (Figure 4.8) and -174 to -88 (Figure 4.7) were used in DNase I protection analysis. Between -130 and -88 there was one strong region of transcription factor interaction with the promoter, at -125 to -112 (Figure 4.8) (in Figure 4.7 the protected region is between -124 and -112), which was seen with both liver nuclear extract and rC/EBPα protein (Figure 4.7 and Figure 4.8). The remaining footprints seen between -88 and +47 have already been discussed in Chapter 3. Hypersensitive sites are again formed at the boundaries of footprinted regions (Figure 4.8).

The results of the DNase I protection analysis over the whole promoter region, between -812 to +74 are summarised in Figure 4.9. These results show that liver nuclear proteins bind to at least 14 sites on the 11ß-HSD1 promoter between -812 and +47, including the site at around -600 (Figure 4.3) for which the boundaries are not known, and that 12 of these sites can be wholly or partially occupied by C/EBP related proteins.

Further characterisation was carried out on the liver nuclear proteins which bind to the promoter between -812 and -599, described below (Section 4.2.2), and, in addition two sites of C/EBP binding within this region, between -702 to -679 and -804 to-776 were further characterised (Sections 4.2.3 and 4.2.4).

4.2.2 Further characterisation of the proteins in liver nuclear extract which form complexes on the 11ß-HSD1 gene between -812 to -599

Although the footprints generated by liver nuclear proteins and rC/EBPα between -599 and +47 were very similar, a number of differences were observed in binding to the fragment encoding -812 to -599. Deletion of 5'-flanking DNA from -812 to -599 increased basal expression of luciferase activity in transfected HepG2 cells,

Figure 4.8 *DNase I footprints formed by liver nuclear extract and rC/EBPα on the 11ß-HSD1 promoter fragment encoding -174 to +74*

DNase I protection analysis of the 11ß-HSD1 promoter fragment encoding -174 to +74 using liver nuclear extract and rC/EBPα. Panels were from a single gel with multiple loadings. Each panel show the results of the same reactions run for different lengths of times. Lane 1 shows a Maxam and Gilbert sequencing reaction used as a molecular size marker (M+G); lane 2 has no extract added; lane 3 contains 100µg of liver nuclear extract (L) and lane 4 has 1.3µg of rC/EBPα (C) added. Strong regions of protection are indicated by solid brackets; inner brackets refer to regions protected by liver nuclear extract; outer brackets, protection by rC/EBPα. Nucleotides showing enhanced sensitivity to DNase I are indicated by arrows.

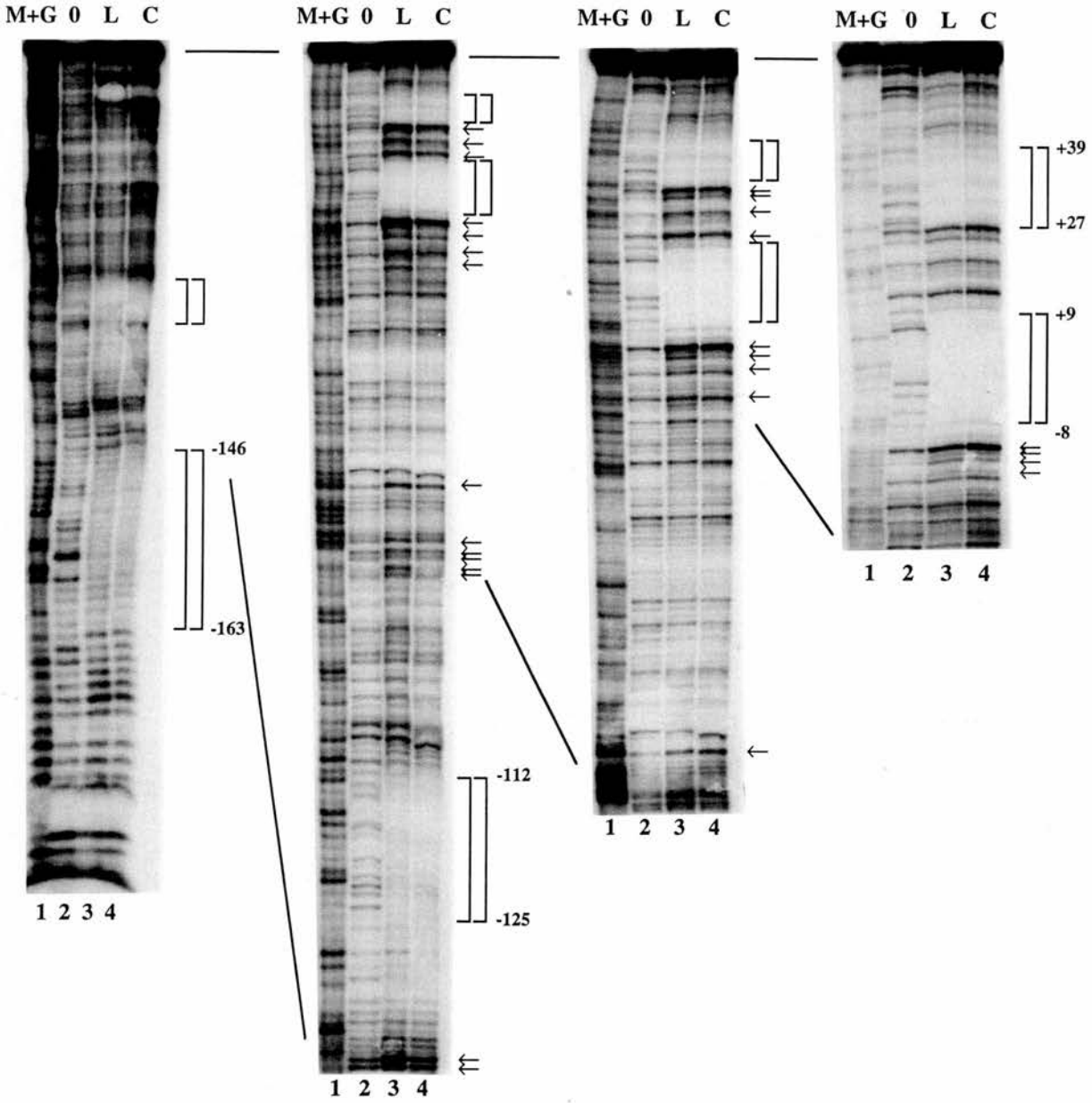
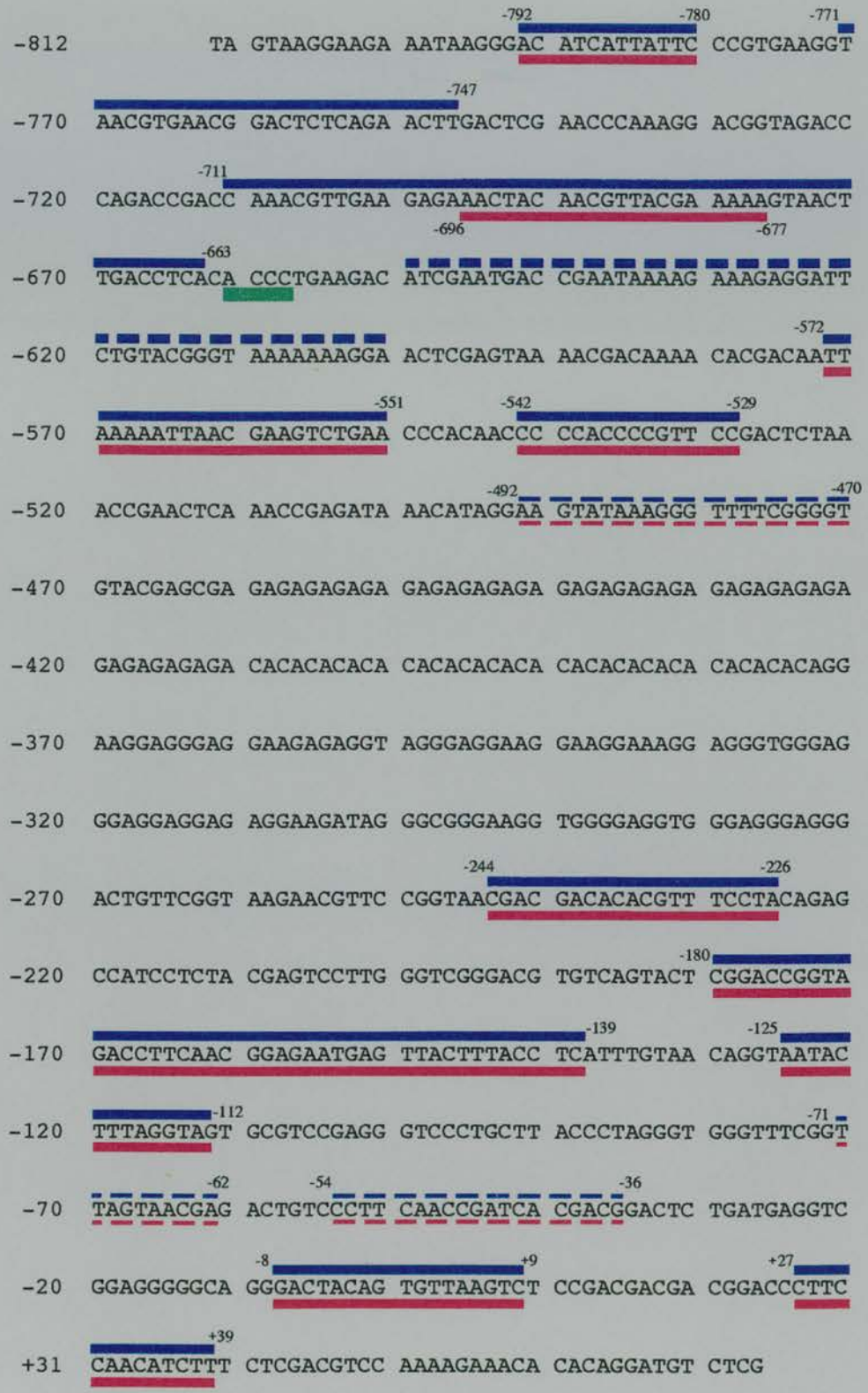

M+G 0 L C
M+G 0 L C
M+G 0 L C
M+G 0 L C
-146
-163
-112
-125
+39
+27
+9
-8
1 2 3 4
1 2 3 4
1 2 3 4
1 2 3 4

Figure 4.9 *Summary of the regions of the 11ß-HSD1 promoter protected from DNase I digestion by liver nuclear extract and recombinant C/EBPα protein*

Blue lines refer to promoter regions protected by liver nuclear extract; red lines show regions protected by rC/EBPα. Solid lines indicate strong regions of protection, thin, dashed lines indicate weak regions of protection, a thick dashed line indicates uncertainty about the exact boundary of the footprint. The strong DNase I hypersensitive site at -660 is indicated by a green line.


-792
-780
-771
-812 TA GTAAGGAAGA AATAAGGGAC ATCATTATTC CCGTGAAGGT
-747
-770 AACGTGAACG GACTCTCAGA ACTTGACTCG AACCCAAAGG ACGGTAGACC
-711
-720 CAGACCGACC AAACGTTGAA GAGAAACTAC AACGTTACGA AAAAGTAACT
-696
-677
-663
-670 TGACCTCACA CCCTGAAGAC ATCGAATGAC CGAATAAAAG AAAGAGGATT
-572
-620 CTGTACGGGT AAAAAAAGGA ACTCGAGTAA AACGACAAAA CACGACAATT
-551
-542
-529
-570 AAAAATTAAC GAAGTCTGAA CCCACAACCC CCACCCCGTT CCGACTCTAA
-492
-470
-520 ACCGAACTCA AACCGAGATA AACATAGGAA GTATAAAGGG TTTTCGGGGT
-470 GTACGAGCGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA
-420 GAGAGAGAGA CACACACACA CACACACACA CACACACACA CACACACAGG
-370 AAGGAGGGAG GAAGAGAGGT AGGGAGGAAG GAAGGAAAGG AGGGTGGGAG
-320 GGAGGAGGAG AGGAAGATAG GGCGGGAAGG TGGGGAGGTG GGAGGGAGGG
-244
-226
-270 ACTGTTCGGT AAGAACGTTC CGGTAACGAC GACACACGTT TCCTACAGAG
-180
-220 CCATCCTCTA CGAGTCCTTG GGTCGGGACG TGTCAGTACT CGGACCGGTA
-139
-125
-170 GACCTTCAAC GGAGAATGAG TTACTTTACC TCATTTGTAA CAGGTAATAC
-112
-71
-120 TTTAGGTAGT GCGTCCGAGG GTCCCTGCTT ACCCTAGGGT GGGTTTCGGT
-62
-54
-36
-70 TAGTAACGAG ACTGTCCCTT CAACCGATCA CGACGGACTC TGATGAGGTC
-8
+9
+27
-20 GGAGGGGGCA GGGACTACAG TGTTAAGTCT CCGACGACGA CGGACCCTTC
+39
+31 CAACATCTTT CTCGACGTCC AAAAGAAACA CACAGGATGT CTCG

decreasing the fold induction by C/EBPα (Figure 4.1). Therefore, competition gel mobility shift analysis was carried out on fragment F_B, encoding -812 to -599, to determine the relative involvement of C/EBP-related proteins in binding to this region. Several specific complexes were formed on F_B (the exact number varied between experiments and was difficult to determine, as lower-mobility complexes predominantly ran as a smear) (Figure 4.10). However, all of the complexes were specific as demonstrated by competition with a 100-fold molar excess of specific competitor fragment F_B, but not by the non-specific fragment competitor, F_{NS} (Figure 4.10).

Oligonucleotides were used as competitors in gel mobility shift analysis to further explore the DNA-binding specificity of the factors in liver nuclear extract which bind to F_B. Oligonucleotides O_A, O_E, O_F and O_D (see Table 2.1 for sequences) encoding respectively, -14 to +15 of the 11ß-HSD1 promoter, an optimal C/EBP binding site, -702 to -679 of the 11ß-HSD1 gene and the P3(I) C/EBP binding site from the PEPCK gene, at 100-fold molar excess over F_B proved the best competitors, dramatically reducing the intensity of the highest mobility complexes, and diminishing the intensity of lower mobility complexes (Figure 4.11). The pattern of competition with all the oligonucleotides (with the exception of O_F, see below) was similar to that described for the -88 to +47 fragment (described in Chapter 3, Figure 3.8), with $O_A=O_E>O_D>O_B=O_C=O_{NS}$ (Figure 4.11). For O_A, O_E and O_D, the competition at 100-fold molar excess was more complete than at 10-fold molar excess. O_F showed similar competition at both 10- and 100-fold molar excess suggesting that effective competition was complete at a 10-fold molar excess of oligonucleotide. All of the oligonucleotides which competed (Chapter 3 and see Section 4.2.3 for O_F) encode binding sites for C/EBP-related proteins. In contrast to the competition with F_B (Figure 4.10), the oligonucleotides used failed to compete all of the complexes formed, several were either not competed, or were competed poorly by the oligonucleotides, suggesting that either these complexes are specific but do not contain C/EBP related proteins, or that they are non-specific. However, as the complexes appeared to be fully competed by F_B these complexes are probably specific. Clearly the 3 highest mobility complexes contain C/EBP-related proteins as they are completely competed (note incomplete competition by 10-fold molar excess

Figure 4.10 *Liver nuclear extract binds to the 11ß-HSD1 gene fragment encoding -812 to -599*

Competition gel mobility shift assay using the 11ß-HSD1 promoter fragment encoding -812 to -599 (F_B) and liver nuclear extract. 10µg of rat liver nuclear extract was incubated with 30fmol of [^{32}P]-labelled F_B in the absence of added competitor (lane 2) or in the presence of 10- or 100-fold molar excess of competitor DNA, as indicated above the lanes (lanes 3-6). Lane 1 contains no nuclear extract. Arrows indicate specific protein-DNA complexes.

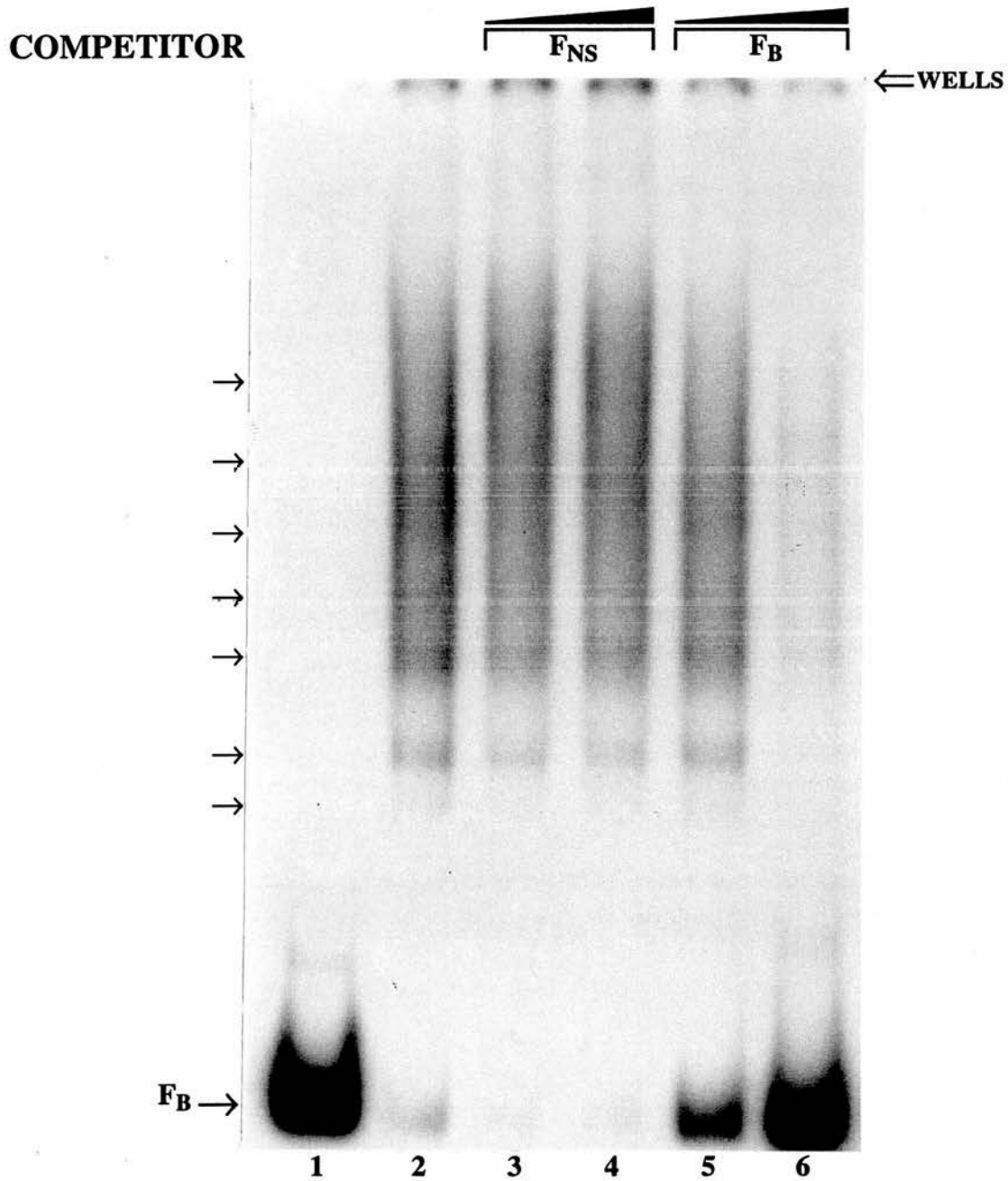
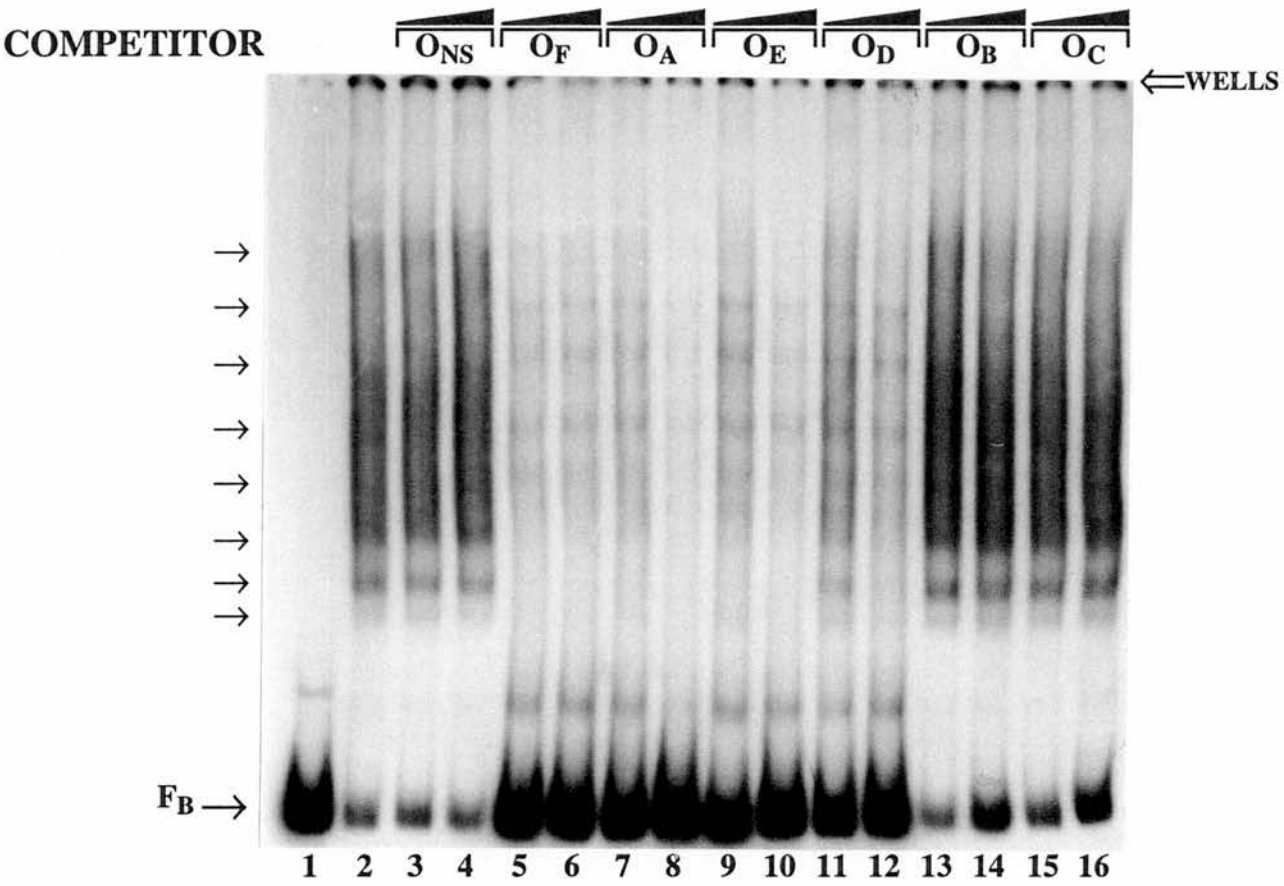

COMPETITOR
F_{NS}
F_B
⇐WELLS
F_B →
1
2
3
4
5
6

Figure 4.11 *C/EBP-related proteins from rat liver nuclear extract bind to the 11ß-HSD1 gene between -812 and -599*

Competition gel mobility shift assay using the 11ß-HSD1 promoter fragment encoding -812 to -599 (F_B) and liver nuclear extract. 10µg of rat liver nuclear extract was incubated with 30fmol of [^{32}P]-labelled F_B in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-16). Lane 1 contains no nuclear extract. Arrows indicate specific protein-DNA complexes.

Oligonucleotides used were, O_A, 11ß-HSD1 (-14 to +15); O_B, 11ß-HSD1 (-88 to -57); O_C, 11ß-HSD1 (-71 to -52); O_D, PEPCK P3(I) site; O_E, optimal C/EBP site; O_F, 11ß-HSD1 (-682 to -705); O_{NS}, prolactin ERE (non-specific control).


COMPETITOR
O_{NS}
O_F
O_A
O_E
O_D
O_B
O_C
⇐WELLS
F_B →
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

of O_D, which is the weakest C/EBP competitor). O_F appears to be the strongest C/EBP site based on competition experiments (Figure 4.11).

Of the four footprints generated on this fragment by liver nuclear extract (Figure 4.3), one (-792 to -780) was reproduced by rC/EBPα, and a second (-711 to -663) may be due, in part, to a C/EBP-related protein binding at -696 to -677. When rC/EBPα protein was examined for its ability to bind to F_B, 4 specific complexes were formed which were partially competed by a 10-fold molar excess, and fully by a 100-fold molar excess, of competitor F_B (Figure 4.12). All of the complexes formed by rC/EBPα on F_B were competed similarly by a 10-fold molar excess of C/EBP binding oligonucleotides with $O_A=O_E=O_F>O_D$ (Figure 4.13). O_C proved a very weak competitor at 100-fold molar excess and no competition was observed for O_{NS} or O_B. This was similar to that described for the -88 to +47 fragment, described in Chapter 3, with $O_A=O_E>O_D>O_B=O_C=O_{NS}$.

To specifically identify whether complexes formed on F_B by liver nuclear proteins included C/EBPα and/or C/EBPß, monospecific polyclonal antisera were included in gel mobility supershift experiments (Figure 4.14). C/EBPα antibody supershifted the lower mobility complexes (Figure 4.14). Complexes were also supershifted using C/EBPß antisera. When C/EBPα and C/EBPß were used in the same supershift reaction all complexes except one were supershifted. This complex was, however, competed by the C/EBP binding oligonucleotides (Figure 4.13), suggesting that this complex contains a C/EBP related factor. These results suggest therefore that all of the protein complexes formed on this fragment involved members of the C/EBP family. However, not all of the protein-DNA complexes were competed by C/EBP-binding oligonucleotides (Figure 4.11). It is possible that in the experiment shown in Figure 4.14 residual complexes (not readily apparent) were not supershifted by the antibodies. Alternatively the complexes which were not competed by C/EBP-binding oligonucleotides may be associated with C/EBPα and/or C/EBPß and were therefore supershifted, or alternatively these complexes are maybe masked by the supershifted complexes. These results demonstrate clearly that C/EBP-related factors from liver nuclear extract bind to F_B which is consistent with footprinting results. Of the 2 sites protected by rC/EBPα on -812 to -599, one was protected identically by proteins

Figure 4.12*Recombinant C/EBPα binds to the 11ß-HSD1 gene fragment encoding -812 to -599*

Competition gel mobility shift assay using the 11ß-HSD1 promoter fragment encoding -812 to -599 (F_B) and rC/EBPα. 0.2µg of rC/EBPα was incubated with 30fmol of [^{32}P]-labelled F_B in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor DNA, as indicated above each lane (lanes 3-6). Lane 1 contains no nuclear extract. Lane 7 contained 10µg of rat liver nuclear extract instead of rC/EBPα. Arrows indicate specific protein-DNA complexes.

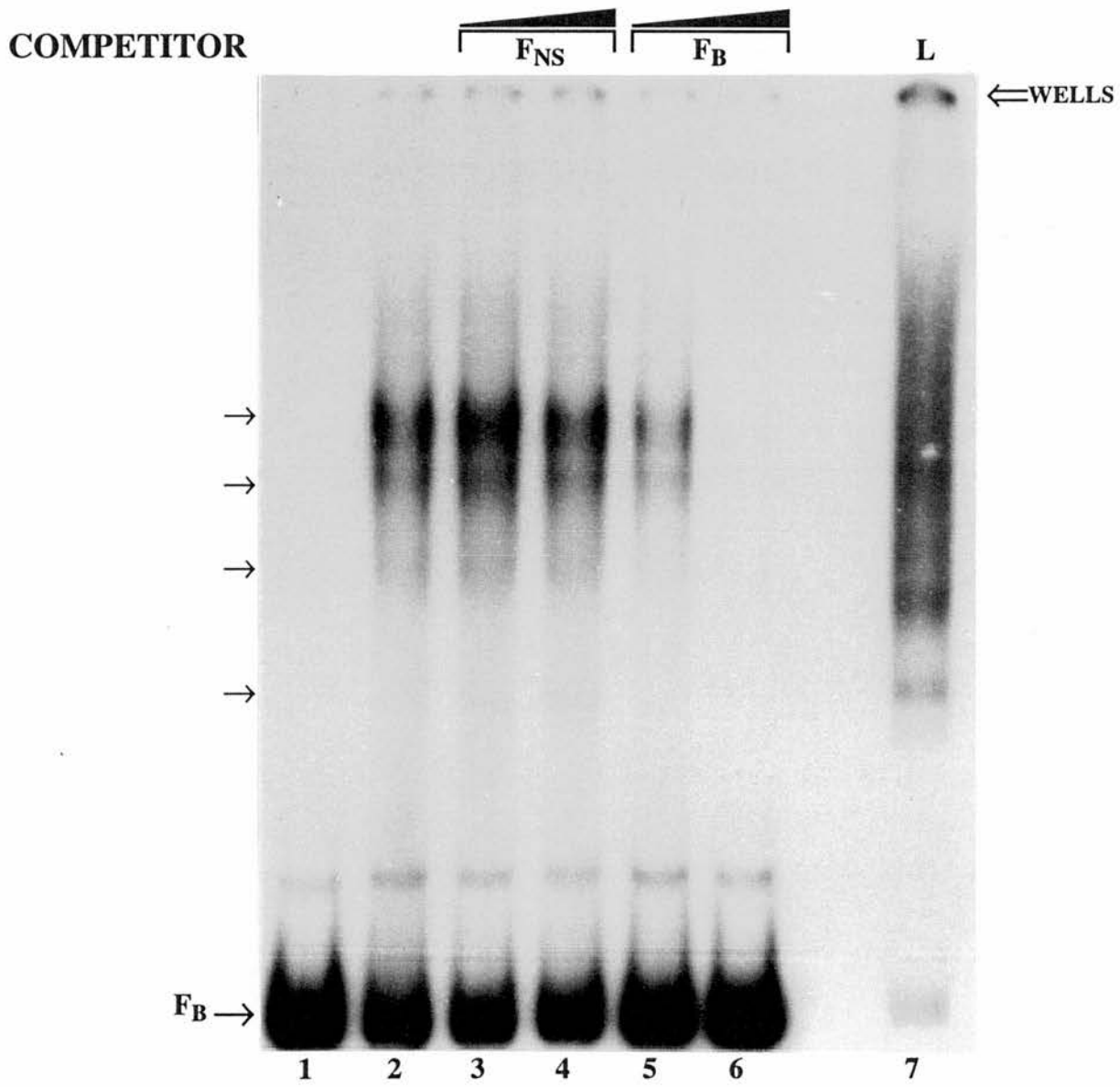

COMPETITOR
F_{NS}
F_B
L
⇐WELLS
→
→
→
→
F_B →
1
2
3
4
5
6
7

Figure 4.13 *Complexes are formed by recombinant C/EBPα on the 11ß-HSD1 gene fragment encoding -812 to -599*

Competition gel mobility shift assay using the 11ß-HSD1 promoter fragment encoding -812 to -599 (F_B) and rC/EBPα. 0.2μg of rC/EBPα was incubated with 30fmol of [^{32}P]-labelled F_B in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-16). Lane 1 contains no nuclear extract. Lane 17 contains 10μg of rat liver nuclear extract instead of rC/EBPα. Arrows indicate specific protein-DNA complexes.

Oligonucleotides used were, O_A, 11ß-HSD1 (-14 to +15); O_B, 11ß-HSD1 (-88 to -57); O_C, 11ß-HSD1 (-71 to -52); O_D, PEPCK P3(I) site; O_E, optimal C/EBP site; O_F, 11ß-HSD1 (-682 to -705); O_{NS}, prolactin ERE (non-specific control).

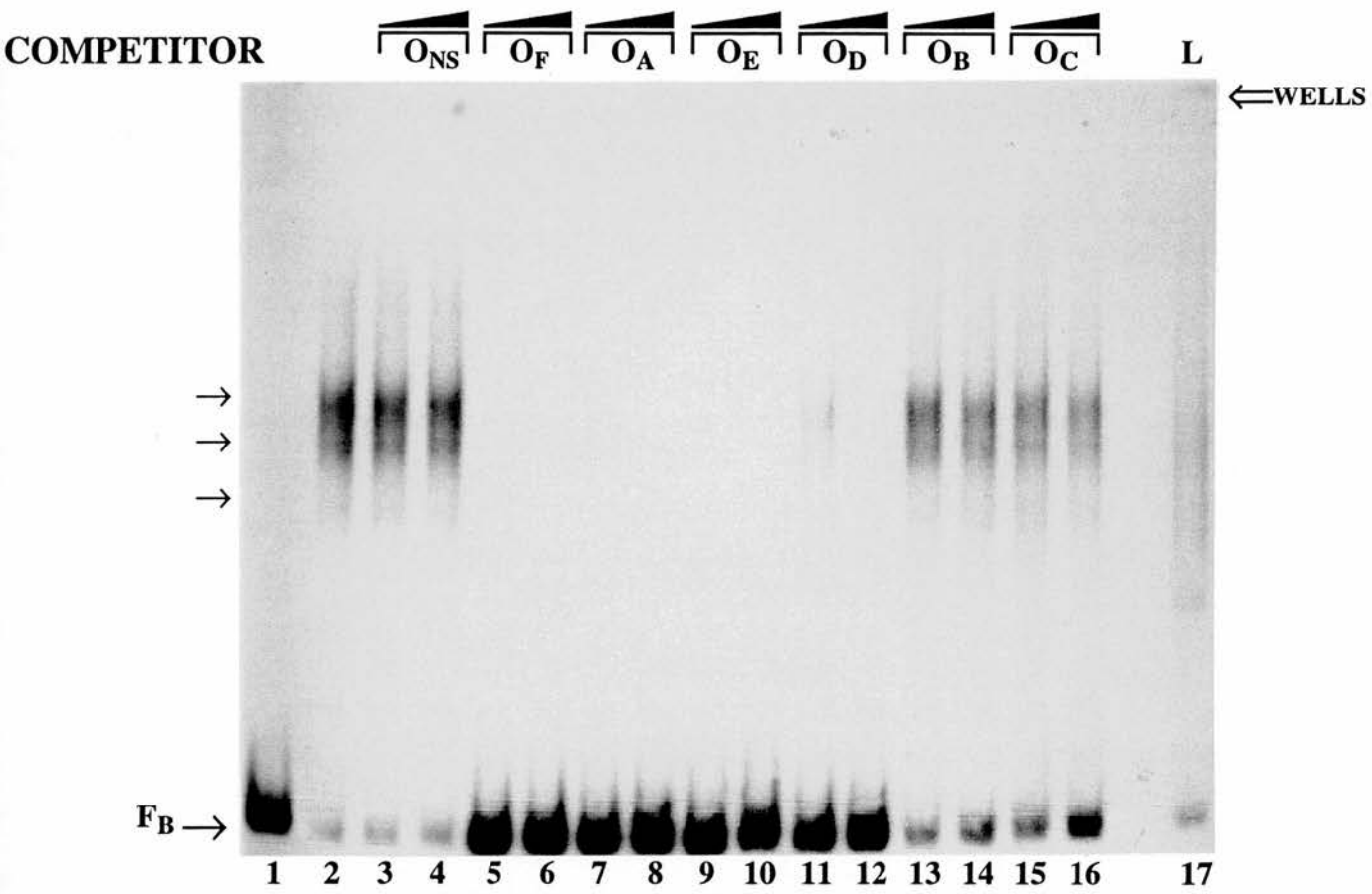
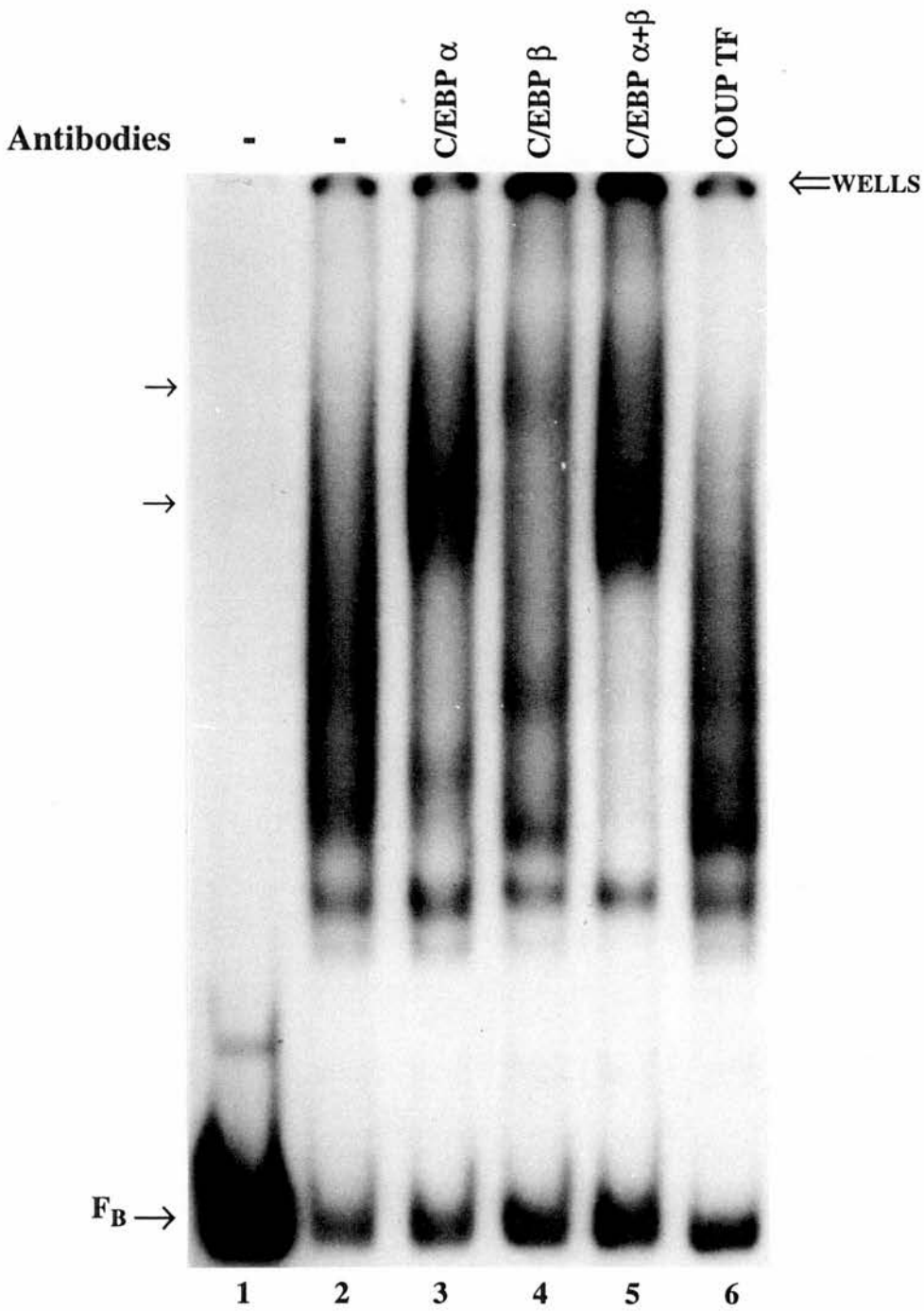

COMPETITOR
O_{NS}
O_F
O_A
O_E
O_D
O_B
O_C
L
⇐WELLS
→
→
→
F_B →
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 4.14 *C/EBPα and C/EBPβ are present in the complexes formed on the 11ß-HSD1 gene fragment encoding -812 to -599*

Gel mobility supershift assay using the 11ß-HSD1 promoter fragment encoding -812 to -599 (F_B) and liver nuclear extract. 10µg of liver nuclear extract was incubated with 30fmol of [^{32}P]-labelled F_B in the absence of antibody (lane 2) or with antisera added as indicated above each lane (lanes 3-6). Lane 1 contains no nuclear extract. Supershifted complexes are indicated by arrows.


Antibodies
-
-
C/EBP α
C/EBP β
C/EBP α+β
COUP TF
⇐WELLS
→
→
F_B →
1
2
3
4
5
6

from liver nuclear extract, the other corresponded to part of a footprint formed by liver nuclear extract. Both of these binding sites were further characterised.

4.2.3 Proteins from rat liver nuclei bind an oligonucleotide encoding -702 to -679 of the 11ß-HSD1 gene

Oligonucleotide O_F encodes -702 to -679 of the rat 11ß-HSD1 gene, encompassing the recombinant C/EBPα footprint at -696 to -677 which may form part of the larger footprint seen with liver nuclear extract (-711 to -663). In competition gel mobility shift analysis in which liver nuclear extract binding to O_F was competed by a series of oligonucleotides, the strongest competitor was O_F, completely competing complex formation at a 10-fold molar excess (Figure 4.15). Other C/EBP-binding oligonucleotides also competed, with $O_E>O_A>O_D>>O_B=O_C=O_{NS}$ (Figure 4.15). Competition analysis of rC/EBPα binding to O_F showed that O_F was a very high affinity site, with similar rank order of competition as seen for liver nuclear extract ($O_F \geq O_E>O_A>O_D>> O_B=O_C=O_{NS}$) (Figure 4.16). These gel mobility shift experiments therefore show that the footprint between -702 and -679 represents a high affinity site for C/EBP related proteins, with at least as high an affinity for rC/EBPα as O_E.

Addition of antisera to gel mobility shift experiments demonstrated that the majority of the complexes formed on O_F by liver nuclear extract include C/EBPα, as most of the complexes were supershifted by the addition of C/EBPα antibody (Figure 4.17). Addition of C/EBPß antiserum alone supershifted a small proportion of the complexes, with all but one complex supershifted by addition of both antisera together (Figure 4.17). The remaining complex was unaffected by addition of the antisera. There is probably minimal involvement of heterodimer formation between C/EBPα and C/EBPß on this site as the amount of complexed DNA not supershifted by C/EBPα is about equal to the amount supershifted by C/EBPß (Figure 4.17).

4.2.4 Weak complex formation by liver nuclear extract on the oligonucleotide encoding -804 to -776 of the rat 11ß-HSD1 gene

Liver nuclear extract and C/EBPα formed similar footprints between -792 and -780 (Figure 4.3 and 4.9). However, when an oligonucleotide, O_S, corresponding to -804 to -776 was used in gel mobility shift analysis, although complexes were formed by liver nuclear extract on O_S, there was no distinct pattern of binding as had been

Figure 4.15 *C/EBP-related transcription factors from liver nuclear extract bind to oligonucleotide O_F, encoding -702 to -679 of the 11ß-HSD1 promoter*

Competition gel mobility shift assay using the 11ß-HSD1 oligonucleotide encoding -712 to -679 (O_F) and liver nuclear extract. 10µg of liver nuclear extract was incubated with 0.1pmol of [^{32}P]-labelled O_F in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-16). Lane 1 contains no nuclear extract. Arrows indicate specific protein-DNA complexes, ∗ indicates a non-specific band.

Oligonucleotides used were, O_A, 11ß-HSD1 (-14 to +15); O_B, 11ß-HSD1 (-88 to -57); O_C, 11ß-HSD1 (-71 to -52); O_D, PEPCK P3(I) site; O_E, optimal C/EBP site; O_F, 11ß-HSD1 (-682 to -705); O_{NS}, prolactin ERE (non-specific control).

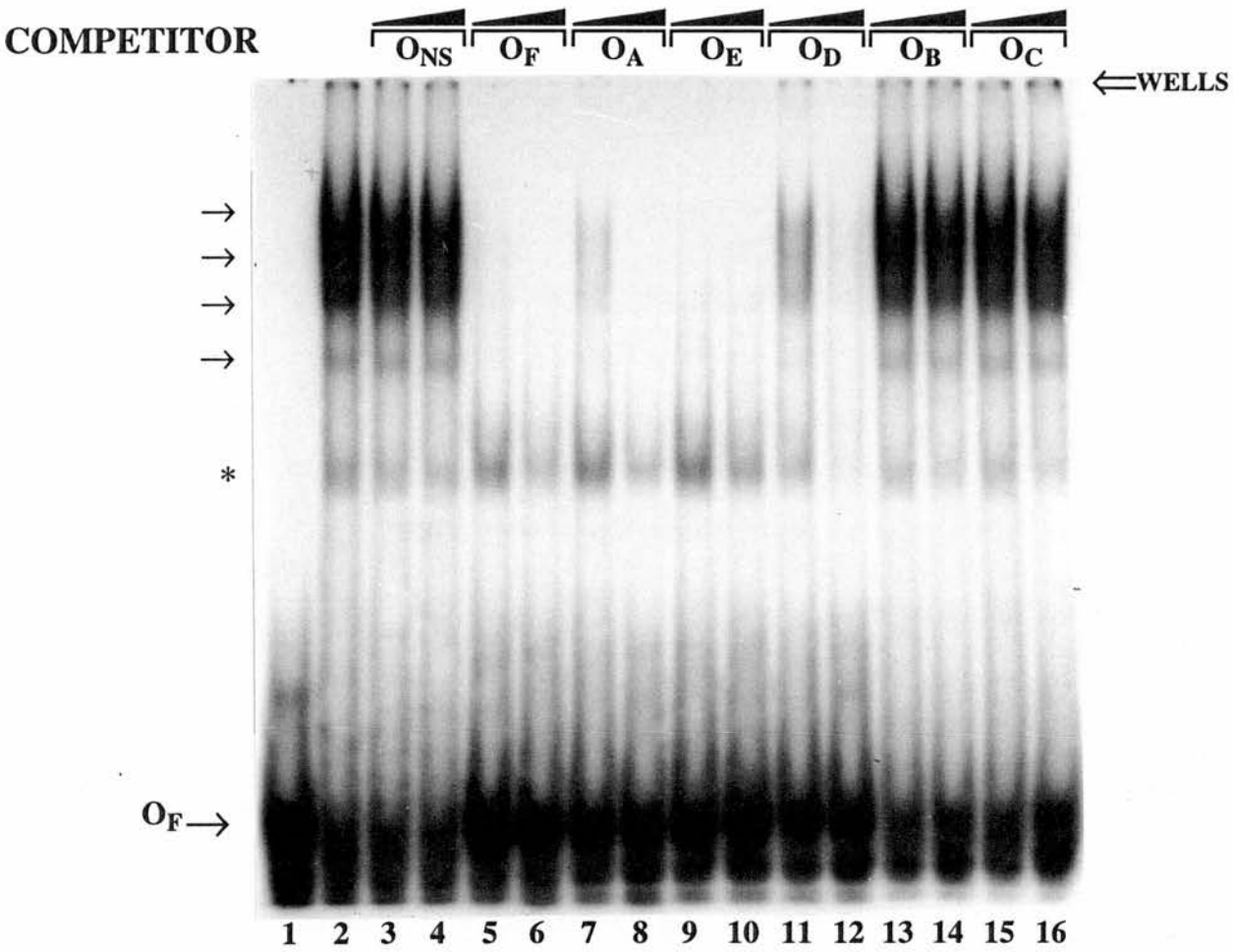
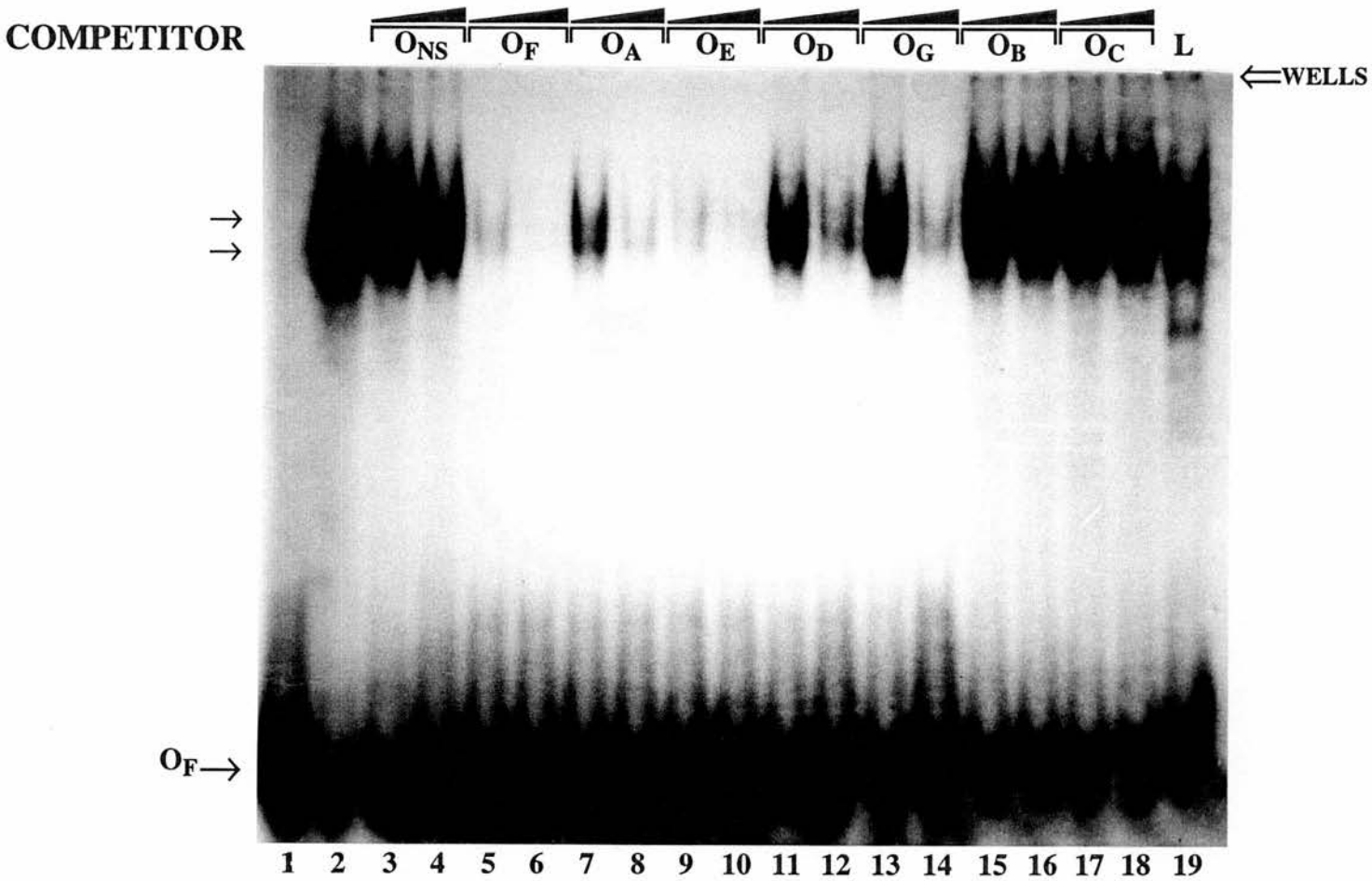

COMPETITOR
O_{NS}
O_F
O_A
O_E
O_D
O_B
O_C
⟸WELLS
→
→
→
→
*
O_F→
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 4.16 *Recombinant C/EBPα binds specifically to oligonucleotide O_F, encoding -702 to -679 of the 11ß-HSD1 gene*

Competition gel mobility shift assay using the oligonucleotide O_F encoding -712 to -679 and rC/EBPα. 0.2µg of rC/EBPα was incubated with 0.1pmol of [^{32}P]-labelled O_F in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-18). Lane 1 contains no nuclear extract. Lane 19 contained 10µg of rat liver nuclear extract instead of rC/EBPα. Arrows indicate specific protein-DNA complexes.

Oligonucleotides used were, O_A, 11ß-HSD1 (-14 to +15); O_B, 11ß-HSD1 (-88 to -57); O_C, 11ß-HSD1 (-71 to -52); O_D, PEPCK P3(I) site; O_E, optimal C/EBP site; O_F, 11ß-HSD1 (-682 to -705); O_{NS}, prolactin ERE (non-specific control).


COMPETITOR
O_{NS}
O_F
O_A
O_E
O_D
O_G
O_B
O_C
L
⟸WELLS
→
→
O_F→
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

observed previously with other oligonucleotides; instead, a smear of complexes was formed (Figure 4.18). The complexes formed on O_S appeared to be predominantly non-specific as excess O_S only weakly competed for complex formation, even at a 100-fold molar excess. Furthermore, only weak competition was observed with O_A, O_F, O_E and O_D (Figure 4.18). In addition, rC/EBPα failed to form specific complexes on O_S (Figure 4.19). It therefore appears that, despite the fact that this region is protected from DNase I digestion by liver nuclear extract and rC/EBPα in footprinting experiments, an oligonucleotide encompassing the footprinted region is unable to specifically bind proteins when analysed using gel mobility shift analysis.

4.3 Discussion

The importance of C/EBPα in regulating 11ß-HSD1 promoter activity has been demonstrated by transient co-transfections of HepG2 cells (a liver derived cell line) using C/EBPα and a plasmid deletion series in which 11ß-HSD1 promoter DNA is fused to a luciferase reporter gene. The basal activity of the reporter gene in the absence of C/EBPα, was lower for a plasmid encoding -812 to +47 of 11ß-HSD1 than for the -599 to +47 plasmid (K. Chapman, personal communication), suggesting that deletion from -812 to -599 removed a repressor element. This region contained 4 sites protected by rat liver nuclear extract from digestion by DNase I, only one of which was similarly bound by rC/EBPα. Of the other 3 sites the boundaries of 2 were determined (-771 to -663 and -773 to -747), but comparison with known transcription factor binding sites did not reveal any strong similarities; the identity of the liver nuclear factors protecting these sites is therefore not known. A region footprinted by rC/EBPα within the large footprint at -711 to -663 bound C/EBPα, C/EBPß and an unidentified C/EBP-related factor(s) in a similar ratio to C/EBP binding to the whole fragment encoding -812 to -599. These results clearly demonstrate high affinity C/EBP binding to a part of the larger footprint at -711 to -663 and furthermore suggest that the vast majority of the C/EBP binding to the -812 to -599 fragment may be attributed to the binding to this high affinity site. The binding of C/EBP to this region suggests that there may be an interaction between C/EBP and the unidentified liver nuclear factor(s) giving rise to the larger footprint. Alternatively, the larger footprinted region may be due to C/EBP from liver nuclear extract distorting DNA, giving less access to DNase I and therefore a larger footprint.

Figure 4.17 *C/EBPα and C/EBPβ bind to oligonucleotide O_F, encoding -702 to -679 of the 11β-HSD1 promoter*

Gel mobility supershift assay using oligonucleotide O_F and liver nuclear extract. 10μg of liver nuclear extract was incubated with 0.1pmol of [^{32}P]-labelled O_F in the absence of antibody (lane 2) or with antisera added as indicated above each lane (lanes 3-6). Lane 1 contains no nuclear extract. Supershifted complexes are indicated by an arrow; ∗ indicates a non-specific band.

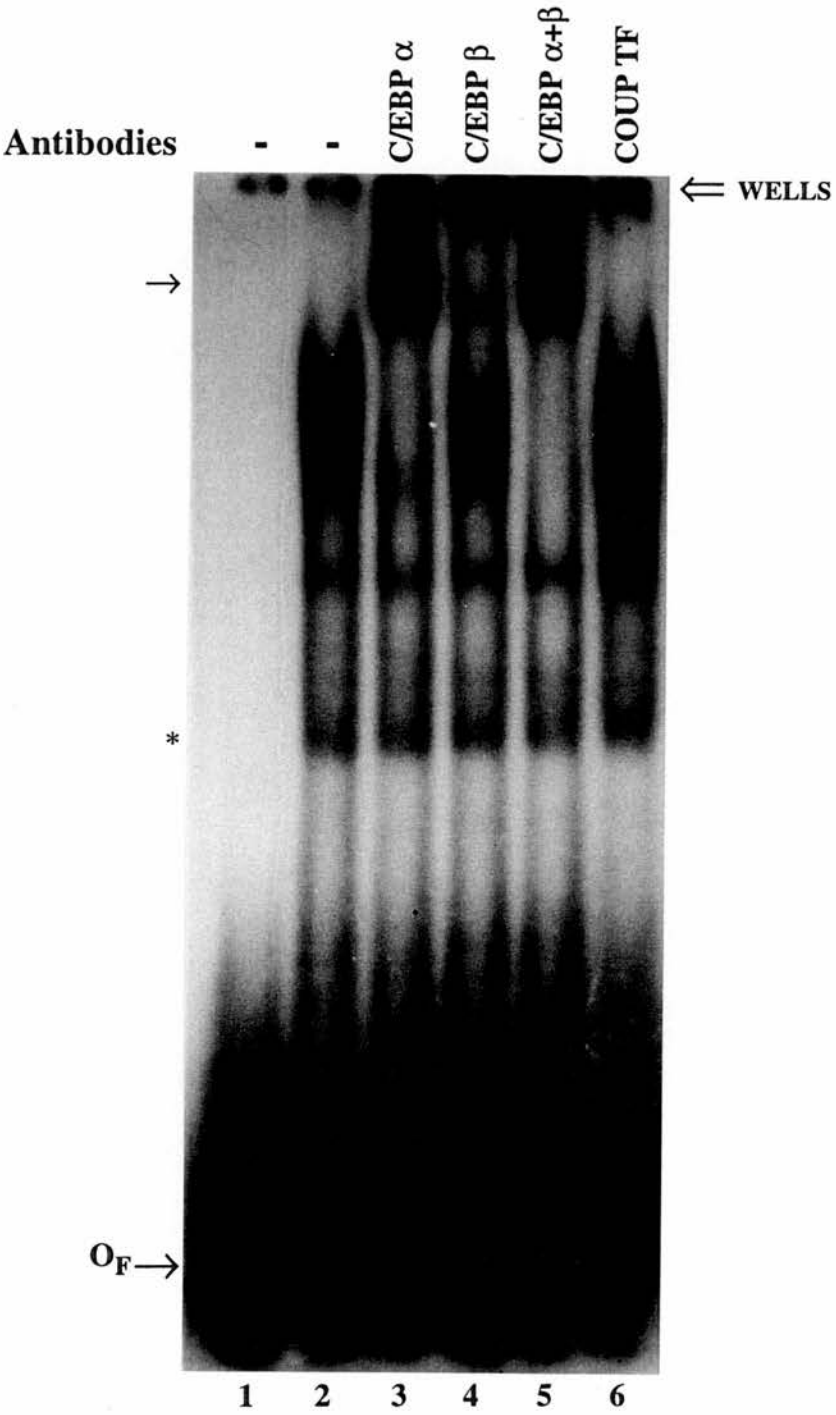

Antibodies
-
-
C/EBP α
C/EBP β
C/EBP α+β
COUP TF
⇐ WELLS
→
*
O_F →
1
2
3
4
5
6

Figure 4.18 *Liver nuclear extract binds weakly to oligonucleotide O_S, encoding -804 to -776 of the 11ß-HSD1 promoter*

Competition gel mobility shift assay using oligonucleotide O_S encoding -804 to -776 and liver nuclear extract. 10µg of liver nuclear extract was incubated with 0.1pmol of [^{32}P]-labelled O_S in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-18). Lane 1 contains no nuclear extract. Complexes are indicated by the bracket.

Oligonucleotides used were, O_A, 11ß-HSD1 (-14 to +15); O_B, 11ß-HSD1 (-88 to -57); O_C, 11ß-HSD1 (-71 to -52); O_D, PEPCK P3(I) site; O_E, optimal C/EBP site; O_F, 11ß-HSD1 (-682 to -705); O_S, 11ß-HSD1 (-804 to -776); O_{NS}, prolactin ERE (non-specific control).

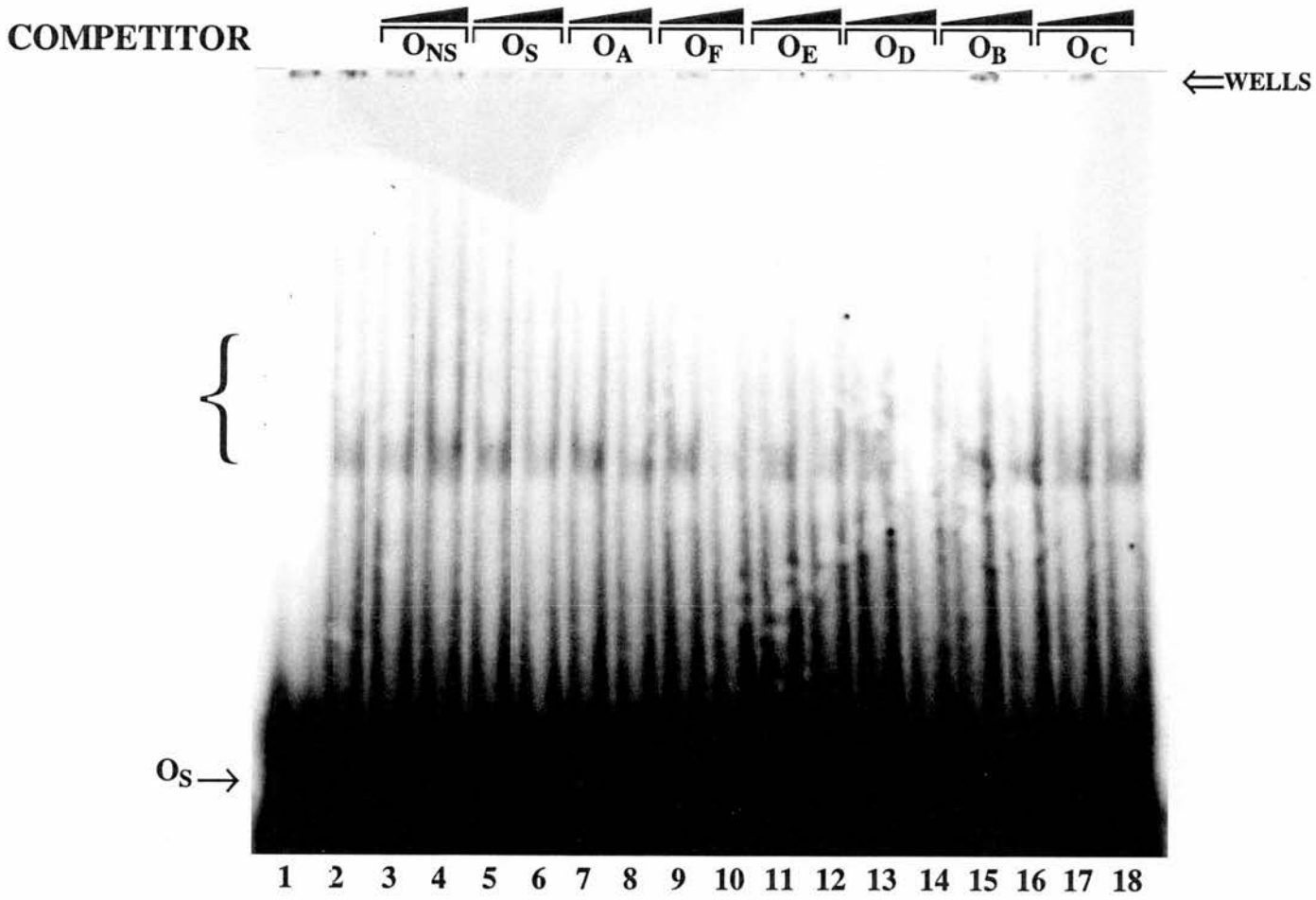
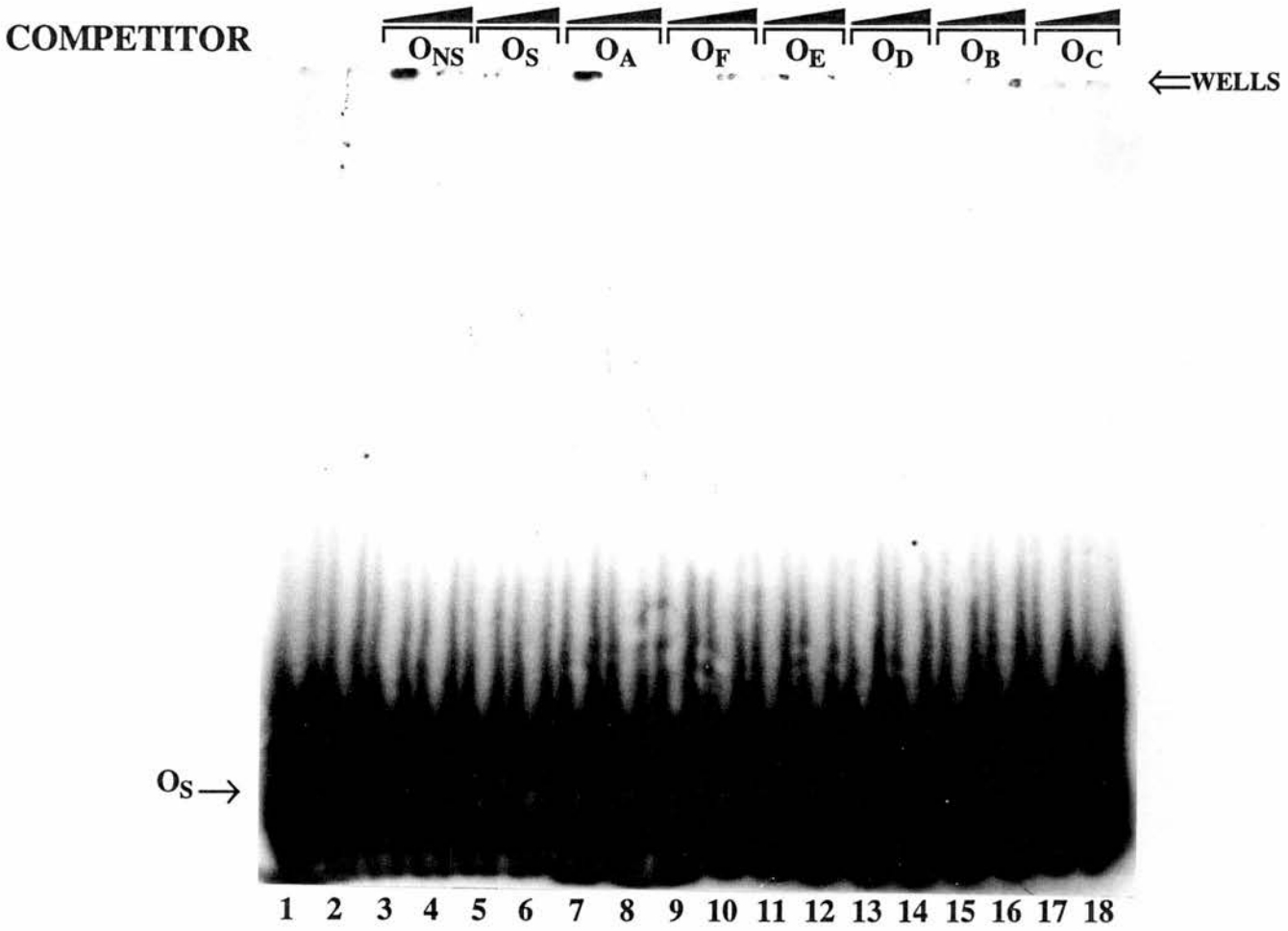

COMPETITOR
O_{NS}
O_S
O_A
O_F
O_E
O_D
O_B
O_C
⇐WELLS
O_S →
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 4.19 *Recombinant C/EBPα does not bind to oligonucleotide O_S, encoding -804 to -776 of the 11ß-HSD1 promoter*

Competition gel mobility shift assay using oligonucleotide O_S encoding -804 to -776 and rC/EBPα. 0.2μg of rC/EBPα was incubated with 0.1pmol of [^{32}P]-labelled O_S in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-18). Lane 1 contains no nuclear extract.

Oligonucleotides used were, O_A, 11ß-HSD1 (-14 to +15); O_B, 11ß-HSD1 (-88 to -57); O_C, 11ß-HSD1 (-71 to -52); O_D, PEPCK P3(I) site; O_E, optimal C/EBP site; O_F, 11ß-HSD1 (-682 to -705); O_S, 11ß-HSD1 (-804 to -776); O_{NS}, prolactin ERE (non-specific control).


COMPETITOR
O_{NS} O_S O_A O_F O_E O_D O_B O_C
⇐WELLS
O_S →
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

At the 3' boundary of this footprint at -711 to -663 was a strong region of hypersensitivity to DNase I induced by rat liver nuclear extract but not by rC/EBPα. This hypersensitive region seen *in vitro* is probably identical to a DNase I hypersensitive site formed at -650 of the 11ß-HSD1 gene seen in intact nuclei from rat liver (only 2 hypersensitive regions are seen in the 11ß-HSD1 gene between -5kb and +1.5kb in rat liver nuclei; at -650 and close to the transcription start; K. Chapman, personal communication). DNase I hypersensitive sites generally correspond to important gene regulatory regions (Kornberg & Lorch, 1995). These results implicate this region as important for transcriptional control of 11ß-HSD1 in rat liver. Liver nuclear proteins and rC/EBPα formed a strong footprint between -792 and -780, however, but they failed to form distinct complexes on an oligonucleotide encompassing this site; it may be that the length of the oligonucleotide needs to be extended to allow adequate binding by liver nuclear proteins, or that complex formation at this site may require factors to bound at other sites on the promoter.

Further deletion of the 11ß-HSD1 promoter, from -599 to -174 or -88 decreased the degree of induction of luciferase activity by C/EBPα (K. Chapman personal communication). Between -599 and -88, 6 sites were bound by factors from liver nuclear extract (Figure 4.9) all of which are similarly bound by rC/EBP suggesting that C/EBP is the only factor in liver nuclear extract binding to this region and that it is therefore responsible for the activity of these minimal 11ß-HSD1 promoters in liver cells. No binding of liver nuclear proteins was seen to the promoter region containing the $(CT)_{26}(GT)_{19}$ repeats and the CT rich region. Although widespread in mammalian genes, the functions (if any) of these repeat sequences, especially $(GT)_n$ repeats is unknown (Hamada & Kakunaga 1982). In *Drosophila*, repeated sequences have been associated with chromatin formation and nucleosome positioning (Miller et al., 1985) GT repeats form left-handed Z DNA *in vitro* (van Holde & Zlatanova, 1993), and have been suggested to inhibit transcription *in vivo* in mammalian systems. Deletion of the repeat sequence in 11ß-HSD1 gene has little or no effect on luciferase activity in transiently transfected cells therefore, the physiological significance of the repeats (if any) remains to be determined.

In rat liver and in cultures of liver derived cells (2S FAZA and primary cultures of hepatocytes), 11ß-HSD1 expression is hormonally regulated. Glucocorticoids

increase 11ß-HSD1 expression (Jamieson et al., 1995; Low et al., 1994c; Voice et al., 1996) and insulin decreases 11ß-HSD1 expression (Jamieson et al., 1995; Voice et al., 1996), which is in keeping with a role for glucocorticoids in maintaining glucose homeostasis. Preliminary data from transfections of 2S FAZA cells have suggested that the region required for responsiveness to glucocorticoids lies between -174 to -88 and that the region required for insulin responsiveness lies between -599 and -174 (M. Voice, personal communication). However, the region between -599 and -88 does not contain any sequences which resemble binding sites for either GR or a transcription factor known to mediate signalling by insulin. Furthermore, the only footprints generated between -599 and +47 appear to be generated by C/EBP-related proteins. However, there is a match to a half site GRE consensus sequence (TGTYCT) (Beato, 1987) at -370 (TGTCCT), which is outside the region implicated in the response to glucocorticoids and there is also a possible GR binding site at -140 to -126, located between the 2 C/EBP binding sites at -180 to -139 and -125 to -117. This site (AGTAAACATTGTCCA) matches the consensus GRE half site at 5 of the 6 nucleotides in one half site, and 4 out of 6 in the other. No footprint over this region is observed, however, the lack of detectable GR binding to this region may be due to a rapid dissociation of GR from DNA, allowing DNase I access to cleave the promoter DNA. GR may be mediating its effect by an interaction with adjacent C/EBP; the synergistic activation of gene transcription by C/EBPß and GR has been described (Nishio et al., 1993); as has synergy between GR and C/EBPα where chick neuronal retina development by glucocorticoids is dependent upon C/EBPα (Ben-Or & Okret, 1993). It is also possible that the glucocorticoid effect on 11ß-HSD1 expression is indirect and mediated by C/EBP. Glucocorticoids, regulate the expression of C/EBPα and C/EBPß genes; dexamethasone increases C/EBPß expression in primary cultures of rat hepatocytes cultures (Matsuno et al., 1996). In differentiated 3T3-L1 adipocyte cells C/EBPα expression is repressed by administration of glucocorticoids (MacDougald et al., 1994) although in rats, glucocorticoids have no effect on the expression of C/EBPα mRNA in liver (Ruppert et al., 1990). Regulation of 11ß-HSD1 by insulin could also be mediated by C/EBP, as insulin treatment is known to result in the phosphorylation of C/EBPß (Wegner et al., 1992) as well as decreasing hepatic C/EBPß mRNA and protein levels *in vivo*. Insulin has no effect on C/EBPα expression in liver (Bosch et al., 1995), however, in cultured foetal brown adipose tissue, insulin induced expression of C/EBPα and

C/EBPδ but had no effect on C/EBPß (Guerra et al., 1994). Alteration of the ratio of C/EBPα to C/EBPß, or the phosphorylation state of either may alter transcription initiation at the 11ß-HSD1 promoter. The effects of hormones on activity and expression of C/EBPs therefore appears complex and is probably tissue specific and may depend on differentiation status of the cells.

Hepatic 11ß-HSD1 expression is also regulated by growth hormone and by oestrogen. Oestrogen dramatically represses expression of 11ß-HSD1 mRNA in rat liver and kidney, but not in hippocampus (Low et al., 1993). Although this effect is due, in part, to the sex steroid specific pattern of growth hormone secretion (Low et al., 1993), it is also likely to involve a mechanism independent of growth hormone as administration of female specific pattern of growth hormone to males only reproduced the fold difference in hepatic 11ß-HSD1 expression seen between male and female rats (Low et al., 1994b). It is possible that the effects of both growth hormone and oestrogen are mediated through C/EBP. The growth hormone regulation of Class I alcohol dehydrogenase (ADH) is mediated via C/EBP binding to the ADH promoter (Potter et al., 1993). Cross talk between oestrogen receptor and C/EBP has been described, with oestrogen administration causing decreased expression of C/EBP regulated genes (Stein and Yang, 1995), possibly through a direct interaction between ER and C/EBP (Stein and Yang, 1995).

Members of the C/EBP family play a role in, or have been implicated in, the expression of numerous hormonally regulated genes in liver, and regulate genes involved in glucose homeostasis (Park et al., 1993; Park et al., 1990) and the APR (Alam et al., 1992; Cappelletti et al., 1996). Glucocorticoids also regulate similar sets of genes to C/EBP and in liver, together with the metabolic hormones insulin and glucagon, they co-ordinately regulate the expression of genes involved in the maintenance of blood glucose homeostasis, and together with cytokines (principally IL-6 and IL-1), induce the network of genes required for the acute-phase response. Hepatic glucocorticoid regulated genes include those encoding enzymes involved in the regulation of glucose homeostasis such as PEPCK and G6Pase (Garland, 1986; Sasaki et al., 1984), and attenuation of hepatic 11ß-HSD1 expression (by treatment with oestrogen) is associated with decreased expression of mRNA encoding enzymes involved in gluconeogenesis, including PEPCK (Jamieson et al., 1996). A role for

11ß-HSD1 in expression of glucocorticoid inducible hepatic genes is also demonstrated by transgenic mice lacking 11ß-HSD1, which have reduced fasting glucose levels and decreased expression of G6Pase (Kotelevtsev et al., 1996a).The key role of C/EBPα in regulating genes which regulate blood glucose levels is apparent in mice lacking the C/EBPα gene which die of hypoglycaemia shortly after birth (see Chapter 1, Section 1.5.5.1) (Wang et al., 1995). In liver, glucocorticoids also coordinately induce a network of genes involved in the acute phase response (APR) (reviewed in Baumann and Gauldie, 1994). C/EBPß and C/EBPδ have both been implicated as key regulators of the APR genes, and probably form part of an intracellular signalling pathway for IL-6 (Baumann and Gauldie, 1994). C/EBPß plays an important role in regulating the immune response, and mice which lack the C/EBPß gene have defective immune responses and impaired induction of APR genes (Tanaka et al., 1995; Screpanti et al., 1995). During the APR, hepatic expression of C/EBPα decreases and expression of C/EBPß and C/EBPδ increases (Alam et al., 1992) and glucocorticoids themselves induce expression of C/EBPß and C/EBPδ. The binding of both C/EBPα and C/EBPß (and possibly other members of the C/EBP family) to the promoter of the 11ß-HSD1 gene suggests that C/EBP may indirectly regulate the level of active intracellular glucocorticoids in liver by governing the transcription of hepatic 11ß-HSD1. This therefore provides a mechanism for 'cross-talk' between the hormonally responsive glucocorticoid signalling pathway permitting a complex coordinated control of the networks of genes involved in energy metabolism, and the cellular response to stress.

CHAPTER 5

The proximal promoter of the 11ß-HSD1 gene is transcriptionally active in vitro

5.1 Introduction

The rat 11ß-HSD1 gene contains 9 binding sites for C/EBP between -599 and +8 with the major binding site at the transcription start (Chapters 3 & 4). Binding of C/EBP to the transcription start is unusual, but C/EBPα binding at the transcription start has been reported and shown to be important for expression of the gene encoding clotting factor IX in liver (Crossley and Brownlee, 1990). The functional relevance of C/EBP binding to the transcription start of the 11ß-HSD1 gene could not be determined by transfection experiments, where cotransfection of a C/EBPα expression plasmid had little effect on the activity of an 11ß-HSD1(-88/+47)-luciferase construct in HepG2 cells (see Chapter 4). HepG2 cells contain low levels of C/EBPα (Friedman et al., 1989) which may be predominantly bound to the relatively high affinity site at +1 resulting in only a minimal stimulation by exogenous C/EBPα. It is also possible that C/EBPß may play an essential role in stimulating transcription of the minimal promoter of the 11ß-HSD1 gene. Alternatively, in transfected cells increased 11ß-HSD1 transcription by C/EBPα may require upstream C/EBP sites in addition to the site at +1. It is difficult to look at the effects of mutating nucleotides close to the transcription start in transfected cells, as these mutations may themselves interfere with the interaction of the basal transcription machinery with the transcription initiation site, making interpretation of results difficult.

Therefore, to show the functional relevance of C/EBP binding to the transcription start, *in vitro* transcription experiments were carried out using either -599/+8 or -88/+8 of the 11ß-HSD1 gene linked to a G-free cassette (Sawadogo and Roeder, 1985), with comparison made between activity produced in nuclear extracts made from rat liver, which expresses C/EBP, or from HeLa cells which do not express C/EBP (Williams et al., 1991).

5.2 Results

5.2.1 The 11ß-HSD1 promoter is transcriptionally active in vitro

Nuclear extracts produced from liver (in which 11ß-HSD1 enzyme activity is highly expressed) and from HeLa cells (in which 11ß-HSD1 activity is undetectable; S. Low, personal communication) were used to *in vitro* transcribe two different

plasmids, p11ß1(-599)-$(C_2AT)_{19}$ and p11ß1(-88)-$(C_2AT)_{19}$, containing 11ß-HSD1 DNA from -599 to +8 or -88 to +8 respectively fused to a 370bp G-free cassette (Sawadogo and Roeder, 1985) (Described in Chapter 2, Section 2.1.5.3). G-free cassettes (encoding transcripts of 370, 270 or 170 nucleotides) linked to the adenovirus major late promoter (AdMLP) (Sawadogo and Roeder, 1985; Vaulont et al., 1989) were used as size markers (Figure 5.1, lanes 1-3). *In vitro* transcription of either p11ß1(-599)-$(C_2AT)_{19}$ or p11ß1(-88)-$(C_2AT)_{19}$ in liver nuclear extracts produced the predicted 370 nucleotide transcript, with both plasmids showing a similar level of activity (Figure 5.1). However, neither p11ß1(-599)-$(C_2AT)_{19}$ nor p11ß1(-88)-$(C_2AT)_{19}$ were transcriptionally active in nuclear extracts from HeLa cells (Figure 5.1). In contrast, the internal control plasmid, pAdM(170), which does not require tissue-specific factors for transcriptional activity, was active both in rat liver nuclear extract and in HeLa nuclear extract. This demonstrates that the proximal promoter of 11ß-HSD1, between -88 and +8 is sufficient to confer both tissue-specificity upon a linked transcript and to direct transcription in liver nuclear extracts.

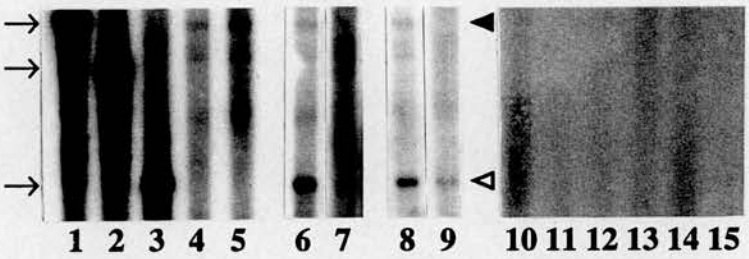
Attempts were made to directly demonstrate a role for C/EBP in the *in vitro* transcription of the 11ß-HSD1 promoter, using p11ß1(-599)-$(C_2AT)_{19}$. Addition of oligonucleotide O_E (encoding an optimal C/EBP site; Chapter 2, Table 2.1) at greater than 50-fold molar excess over the DNA template p11ß1(-599)-$(C_2AT)_{19}$ did indeed reduce transcription *in vitro* from p11ß1(-599)-$(C_2AT)_{19}$ in rat liver nuclear extract, but in addition, also reduced transcription from the internal control plasmid, pAdML(170) (data not shown). Furthermore, depletion of C/EBP by incubation of rat liver nuclear extract with biotinylated O_E linked to streptavidin-coated magnetic beads similarly reduced transcription *in vitro* of p11ß1(-599)-$(C_2AT)_{19}$ and pAdM(170) (data not shown).

5.3 Discussion

Here I show that only 96 base pairs of the rat 11ß-HSD1 promoter region (between -88 and +8) are sufficient to direct transcription *in vitro* in rat liver nuclear extracts, but not in nuclear extracts made from HeLa cells (which do not express 11ß-HSD1 or C/EBP). These results suggest that factors present in liver, but not in HeLa cells,

Figure 5.1 *The 11ß-HSD1 promoter is transcriptionally active in vitro*

In vitro transcription analysis of the 11ß-HSD1 promoter using liver and HeLa nuclear extracts. Lanes 1-3 contain respectively, 1µg pML(C_2AT), 1µg pAdM(270) or 1µg pAdM(170) transcribed in 100µg of liver nuclear extract (used as size markers). Lanes 4 & 5 contain 1µg of p11ß1(-599)-(C_2AT)$_{19}$ (lane 4) or 1µg p11ß1(-88)-(C_2AT)$_{19}$ (lane 5) transcribed in 100µg of liver nuclear extract. Lanes 6 & 7 contain 1µg of p11ß1(-599)-(C_2AT)$_{19}$ together with 0.1µg of pAdM(170) (as an internal control) transcribed in 100µg of liver nuclear extract (lane 6) or 40µg HeLa nuclear extract (lane 7). Lanes 8 & 9 contain 1µg of p11ß1(-88)-(C_2AT)$_{19}$ together with 0.1µg of pAdM(170) transcribed in 100µg of liver nuclear extract (lane 8) or 40µg HeLa nuclear extract (lane 9). Reactions were carried out in the absence of nuclear extract (lanes 10-13); in addition, plasmid DNA was omitted from the reaction in lane 10. Lanes 11-13 contain respectively 1µg of pAdM(170), 1µg of p11ß1(-599)-(C_2AT)$_{19}$ and 1µg of p11ß1(-88)-(C_2AT)$_{19}$. Lane 14 contains 100µg of liver nuclear extract only, lane 15 contains 40µg of HeLa nuclear extract only and lane 16 contains 100µg of liver nuclear extract and 1µg of p(C_2AT)$_{19}$. The predicted transcripts of approximately 370, 270 and 170 nucleotides produced by the control plasmids are indicated on the left side of the figure. A solid arrowhead indicates the 370 nucleotide transcript produced from p11ß1(-599)-(C_2AT)$_{19}$ and p11ß1(-88)-(C_2AT)$_{19}$ and an open arrowhead indicates the 170 nucleotide transcript produced from the internal control plasmid pAdM(170).


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

are responsible for the transcriptional activity of this minimal promoter *in vitro*. In contrast, the AdMLP, which requires ubiquitously expressed transcription factors for activity, is active in both rat liver and HeLa nuclear extracts. Within the region of 11ß-HSD1 DNA present in p11ß1(-88)-$(C_2AT)_{19}$ are 3 sites to which rat liver nuclear proteins bind, all of which correspond to C/EBP sites (Chapter 3).

The three regions to which C/EBP binds on the 11ß-HSD1 promoter (between -88 and +8) are the transcription start (the major site of interaction), between -54 and -36 (weak binding) and between -71 and -62 (very weak binding which was occasionally observed) (Chapter 3). It is therefore likely that the factors responsible for the differential expression of 11ß-HSD1 in liver and HeLa cells include C/EBP, as it is the only protein in rat liver nuclear extract which binds to the proximal promoter. However, it was not possible to determine whether addition of rC/EBP to HeLa nuclear extract activated transcription from p11ß1(-599)-$(C_2AT)_{19}$, as background radioactivity levels in the *in vitro* transcription reactions were increased by addition of rC/EBP (data not shown), resulting in smear formation, making it impossible to observe distinct transcripts on gels. Activation of the promoter is unlikely to directly involve factors other than C/EBP, although it is possible that transcription factors may recognise the 11ß-HSD1 promoter indirectly through C/EBP. It is also possible that transcriptional activation of the minimal 11ß-HSD1 promoter by C/EBP requires C/EBPß in addition to C/EBPα. C/EBPα binding to the transcription start may perform the function of an initiator binding protein; *in vivo*, C/EBPα interacts with TBP of the basal transcription complex (Nerlov and Ziff, 1995), and such an interaction may be essential for transcription of the 11ß-HSD1 gene. It is noteworthy that although rC/EBPα reproduced the footprint generated by rat liver nuclear extract at the transcription start site it was unable to reproduce the DNase I hypersensitive sites generated by rat liver nuclear extract in the DNase I protection analysis (Chapter 3). Therefore a direct role for C/EBP in influencing 11ß-HSD1 transcription through binding to the transcription start still remains to be demonstrated.

The failure to directly demonstrate involvement of C/EBP in 11ß-HSD1 expression was disappointing. Depletion of C/EBP available to transcribe the 11ß-HSD1 promoter *in vitro*, either by removal from the extracts or by addition of competitor oligonucleotide, did indeed result in a subsequent decrease in transcriptional activity

of p11ß1(-599)-$(C_2AT)_{19}$, but the activity of the internal control was also decreased. It is possible that expression of the AdMLP in liver nuclear extracts is modulated by C/EBP. There is little evidence to support this, although the AdMLP does contain a CCAAT sequence, to which C/EBP is capable of binding and which has been shown to play a role in governing transcription initiation *in vitro* (Maity et al., 1988; Connelly & Manley, 1989). Other workers have also reported data from *in vitro* transcription experiments in which addition of excess competitor oligonucleotides containing CCAAT sequences resulted in decreased tyrosine aminotransferase promoter activity, accompanied by a similar reduction in pAdML(270) activity (Schweizer-Groyer et al., 1994).

CHAPTER 6

Tissue specific transcriptional control of 11ß-HSD1 expression

6.1 Introduction

11ß-HSD1 is highly expressed in liver but it is also expressed at moderate levels in many other tissues including brain, adipose tissue and adrenal (Monder & White, 1993). Experiments described in this thesis, together with transfection experiments using hepatoma cells, have established the role played by C/EBP in controlling 11ß-HSD1 expression in liver. However, C/EBPα is widely expressed, being most highly expressed in liver, adipose tissue, lung (Williams et al., 1991) and hippocampus (Kuo et al., 1990). C/EBPß and C/EBPδ have similar distributions to C/EBPα but the levels of expression in tissues differs (Williams et al., 1991). C/EBP may therefore play a role in the expression of 11ß-HSD1 in other tissues, but it is possible that additional transcription factors may regulate 11ß-HSD1 expression in tissues other than liver.

The control of active intracellular glucocorticoids by 11ß-HSD1 in adipose cells is important as evidence suggests that glucocorticoids play a role in regulating adipose tissue distribution and metabolism (Orth et al., 1992). For example, in Cushings Disease, increased glucocorticoid levels are associated with enhanced lipoprotein lipase and glycero-phosphate dehydrogenase activities (Orth et al., 1992). Adipose tissue like liver, plays a central role in energy homeostasis and members of the C/EBP family play a major role in the differentiation of adipocytes and in regulating gene expression in adipose tissue (MacDougald & Lane. 1995) Adipocyte differentiation has been most extensively studied using the 3T3-L1 cell line, and involves the combined activities of members of the C/EBP transcription factor family; C/EBPα is expressed late in the differentiation process while C/EBPß and C/EBPδ are expressed early (Cao et al., 1991). C/EBPα in particular, has been implicated in the transcriptional regulation of genes in fully differentiated adipocytes (Tae et al., 1994) and abnormalities of lipid accumulation and metabolism are seen in mice which lack the C/EBPα gene (Wang et al., 1995). These experiments raise the possibility that C/EBPα plays a role in expression of 11ß-HSD1 in adipose tissue, as it does in liver.

In mammary adipose tissue, 11ß-HSD dehydrogenase activity has been reported (Quirk et al., 1990). Work in this laboratory has shown 11ß-HSD1 mRNA is present in adipocytes as well as in fully differentiated 3T3-F442A cells (A. Napolitano,

personal communication), an adipocyte cell line derived from mouse 3T3-cells (Green & Kehinde, 1974; Green & Kehinde, 1975). Furthermore, in intact, fully differentiated 3T3-F442A cells and in the related 3T3-L1 cell line, 11ß-HSD1 encodes an enzyme with 11ß-reductase activity and minimal dehydrogenase activity (A. Napolitano, personal communication). No 11ß-HSD1 mRNA is detectable in undifferentiated 3T3-F442A cells by northern analysis (A. Napolitano, personal communication) although it can be detected by RT-PCR (K. Chapman, personal communication). As 3T3-F442A cells have been widely used as a model for adipose differentiation and function, they therefore represent an attractive model system in which to identify the key transcription factors required for switching on expression of 11ß-HSD1 in adipocytes.

The adrenal gland is the site of corticosteroid synthesis (Chapter 1), and the expression of 11ß-HSD1 in adrenal suggests that it may potentially play a role in modulating output of glucocorticoids from the adrenal cortex. The presence and location of 11ß-HSD1 in adrenal appears to be species dependent. Immunohistochemistry shows 11ß-HSD1 is present throughout the bovine adrenal cortex (B. Williams, personal communication), while no 11ß-HSD1 mRNA was detected in sheep adrenals (Yang & Matthews, 1995). In rat adrenal, RT-PCR and northern analysis of rat adrenal total RNA demonstrated the presence of 11ß-HSD1 mRNA (data not shown). In rat, 11ß-HSD1 mRNA was localised to the adrenal cortico-medullary junction (Shimojo et al., 1996) and was shown to act as an oxo-reductase (Shimojo et al., 1996). Most if not all of the steroidogenic enzymes expressed in adrenal cortex are transcriptionally regulated by a member of the steroid hormone receptor superfamily, SF-1 (steroidogenic factor 1) (Rice et al., 1991). SF1 is highly expressed in steroidogenic tissues (Lala et al., 1992) and indeed is essential for their development; mice lacking SF-1 have no adrenal glands or gonads (Sadovsky et al., 1995). The rat 11ß-HSD1 gene contains DNA sequences which resemble predicted binding sites for SF1 (Table 6.1). Based on the sequences of the SF-1 binding sites in the cholesterol side chain cleavage enzyme, steroid 21-hydroxylase and aldosterone synthase genes (Lala et al., 1992) and the pattern of proteins from adrenocortical cells which bind to these sites, we have classified the putative SF-1 sites in the 11ß–HSD1 gene into 'complex' and 'simple' sites (Table 6.1). The 'complex' site contains the sequence AGGTCA (ERE half site, see Chapter

1, Section 1.2.5). Sites containing this sequence from the aldosterone synthase and cholesterol side chain cleavage genes formed multiple complexes in gel mobility shift assays using adrenal nuclear extract (Lala et al., 1992). The 'simple' sites deviate from the AGGTCA consensus sequence in the 3 nucleotides at the 3' end, but have a conserved CA dinucleotide immediately 5' to the AGGTCA hexanucleotide (Parker & Schimmer, 1993).'Simple sites' form a single complex with adrenal nuclear extract CAAGG (Lala et al., 1992). Because of the central role SF1 appears to play in steroid biosynthesis, I have investigated whether SF-1, from bovine adrenal gland, may play a role in regulating 11ß-HSD1 expression.

SF1 binding site	Sequence	Predicted complex formation	Oligonucleotide
11ß-HSD1 +655	AGAGAGGTCAGAGT	Complex	O_O
11ß-HSD1 -250	CTTGCAAGGCCAAT	Simple	O_P
11ß-HSD1 -530	GGGCAAGGCTGA	Simple	O_Q
11ß-HSD1 -910	AGAAGGTCAGACC	Complex	O_R
$P450_{C21}$ -210	ACAGAGAGGTCAGG	Complex	-
$P450_{C21}$ -140	TCTCCAAGGCTGA	Simple	-

Table 6.1 *Predicted SF-1 binding sites in the 11ß-HSD1 gene*
SF-1 binding sites were classified as 'simple' or 'complex' according to sequence and type of complex formation (see text for details). The match to the 'complex' AGGTCA, or 'simple' CAAGG consensus sequences are underlined. 'Complex' and 'simple' SF-1 sites from the $P450_{C21}$ gene are shown (Rice et al., 1991).

In this chapter I describe some preliminary data showing tissue specific proteins binding to the 11ß-HSD1 promoter from adrenal and 3T3-F442A cells.

6.2 Results

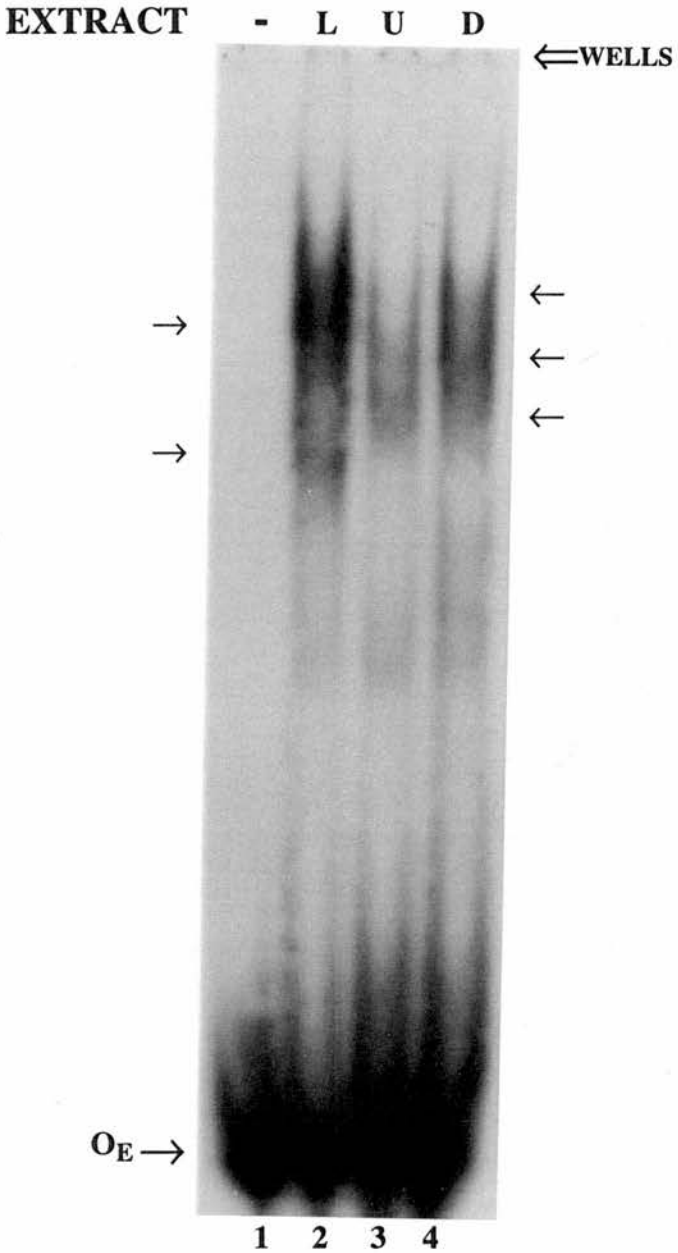
6.2.1 Nuclear proteins from 3T3-F442A cells bind to the 11ß-HSD1 proximal promoter between -88 and +47

6.2.1.1 Specific complexes are formed by undifferentiated and differentiated 3T3-F442A nuclear extracts

3T3-F442A cells, maintained in medium containing newborn calf serum, were grown to confluence then induced to differentiate by switching into growth medium

Figure 6.1 *Factors in nuclear extracts from rat liver, undifferentiated or differentiated 3T3-F442A cells bind to the optimal C/EBP binding oligonucleotide*

Gel mobility shift assay using the C/EBP optimal binding oligonucleotide, O_E and nuclear extracts made from rat liver and undifferentiated or differentiated 3T3-F442A cells. 10μg of nuclear extract from liver (lane 2) ,undifferentiated (lanes 3) or differentiated (lanes 4) 3T3-F442A cells was incubated with 30fmol of [^{32}P]-labelled F_A. Lane 1 contains no nuclear extract. Arrows indicate protein-DNA complexes.


EXTRACT
-
L
U
D
⇐WELLS
→
→
←
←
←
O_E →
1
2
3
4

containing foetal calf serum and insulin (this was carried out by A. Napolitano). Exposure to both foetal calf serum and insulin was necessary to achieve optimal differentiation into adipocytes. Nuclear extracts made from undifferentiated and differentiated 3T3-F442A cells bound to the optimal C/EBP binding oligonucleotide, O_E (Figure 6.1). Comparison of complexes formed by extracts from undifferentiated and differentiated 3T3-F442A cells showed a similar pattern of binding, although the extract from undifferentiated cells gave rise to more low mobility complexes than did the extract from undifferentiated cells (Figure 6.1) There also appeared to be more overall complex formation by extracts from differentiated cells. The mobility of the complexes formed by 3T3-F442A nuclear extracts was similar, but not identical to those fromed by liver nuclear extract (Figure 6.1). This pattern of binding is consistent with the presence of C/EBP-family members in both undifferentiated 3T3-F442A cells (C/EBPß and/or C/EBPδ), and differentiated 3T3-F442A cells, (C/EBPα; Cao et al., 1991).

Undifferentiated and differentiated 3T3-F442A cell nuclear extracts form similar specific complexes on the 11ß-HSD1 promoter fragment F_A, encoding -88 to +47 (Figure 6.2). No qualitative differences were observed between the two extracts, but quantitative differences exist with the intensity of complexes formed by extract from undifferentiated cells being less in comparison to those formed by extracts from differentiated cells (Figure 6.2). Both nuclear extracts form 5 complexes which migrate to the same positions on the gel and, which are partially competed by a 100-fold molar excess of specific competitor F_A, but not by non-specific competitor, F_{NS} (Figure 6.2). The intensity of the lowest mobility complex formed by differentiated 3T3-F442A nuclear proteins is much stronger than the equivalent complex formed by undifferentiated 3T3-F442A nuclear extracts.

6.2.1.2 C/EBP-related proteins from undifferentiated and differentiated 3T3-F442A cells bind -88 to +47 of the 11ß-HSD1 gene

The specificity of the 3T3-F442A nuclear proteins which bind to F_A was explored using a series of oligonucleotides as competitors in gel mobility shift assays. A similar pattern of competition was observed for nuclear extract from either undifferentiated (Figure 6.3) or differentiated (Figure 6.4) cells. Oligonucleotide O_A (encoding -14 to +15 of 11ß-HSD1 gene) and the C/EBP binding oligonucleotides

Figure 6.2 *Factors in nuclear extracts from undifferentiated or differentiated 3T3-F442A cells bind to the 11ß-HSD1 promoter fragment encoding -88 to +47*

Competition gel mobility shift assay using the 11ß-HSD1 promoter fragment encoding -88 to +47 (F_A) and nuclear extracts made from undifferentiated or differentiated 3T3-F442A cells. 10µg of nuclear extract from undifferentiated (lanes 1-6) or differentiated (lanes 8-12) 3T3-F442A cells was incubated with 30fmol of [^{32}P]-labelled F_A in the absence of added competitor (lanes 2 and 8) or in the presence of a 10- or 100-fold molar excess of competitor fragment, as indicated above each lane (lanes 3-6 and 9-12). Lanes 1 and 7 contain no nuclear extract. Arrows indicate specific protein-DNA complexes.

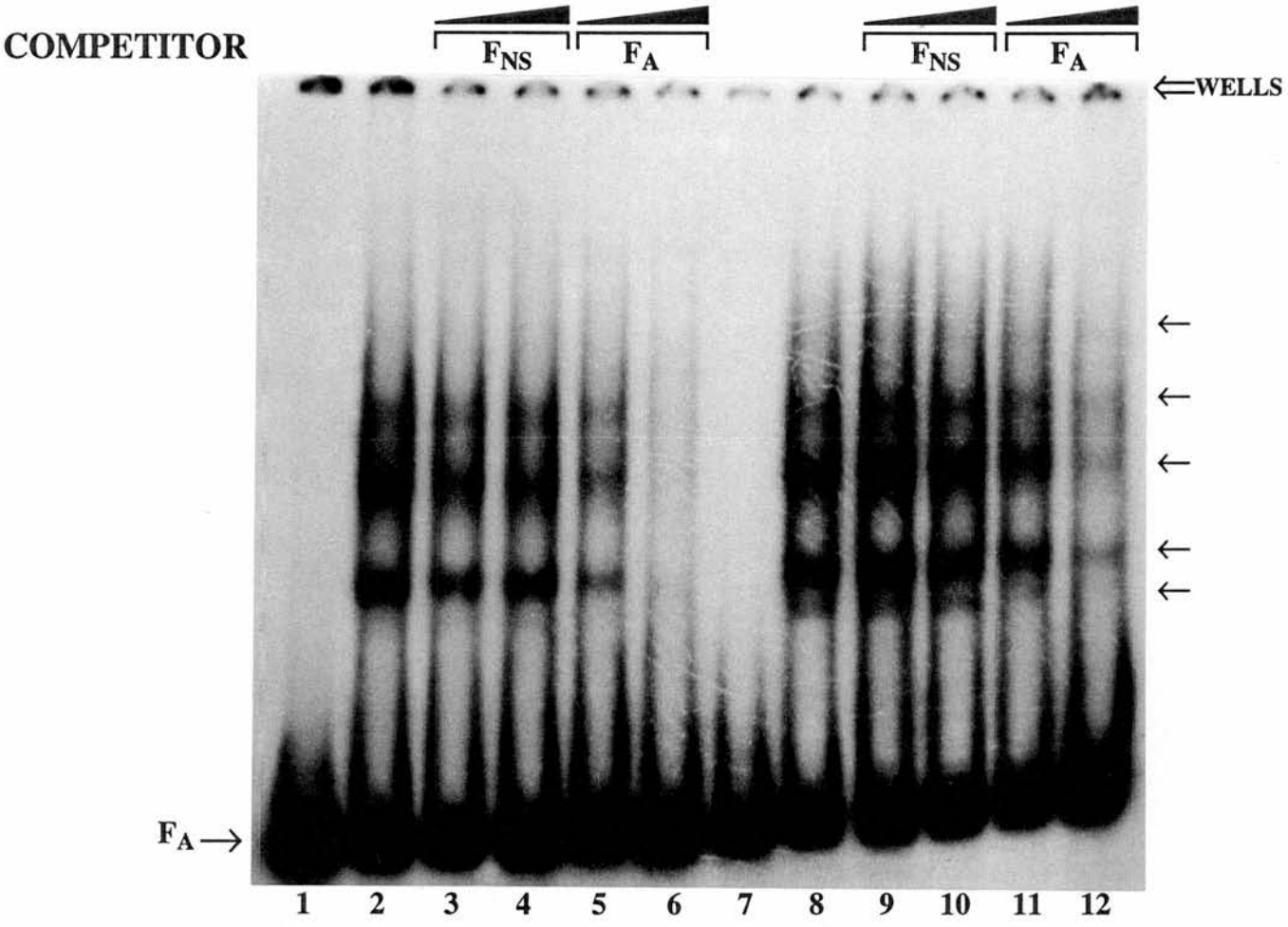

COMPETITOR
F_{NS}
F_A
F_{NS}
F_A
⇐WELLS
F_A →
1 2 3 4 5 6 7 8 9 10 11 12

Figure 6.3 *C/EBP-related factors from undifferentiated 3T3-F442A cells bind to the 11ß-HSD1 promoter fragment encoding -88 to +47*

Competition gel mobility shift using the 11ß-HSD1 promoter fragment encoding -88 to +47 (F_A) and nuclear extract from undifferentiated 3T3-F442A cells. 10µg of nuclear extract was incubated with 30fmol of [^{32}P]-labelled F_A in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-14). Lane 1 contains no nuclear extract. Arrows indicate specific protein-DNA complexes. * indicates a non-specific complex.

Oligonucleotides used were: O_A, 11ß-HSD1 (-14 to +15); O_B, 11ß-HSD1 (-88 to -57); O_C, 11ß-HSD1 (-71 to -52); O_D, PEPCK P3(I) site; O_E, optimal C/EBP site; O_{NS}, prolactin ERE (non-specific control).

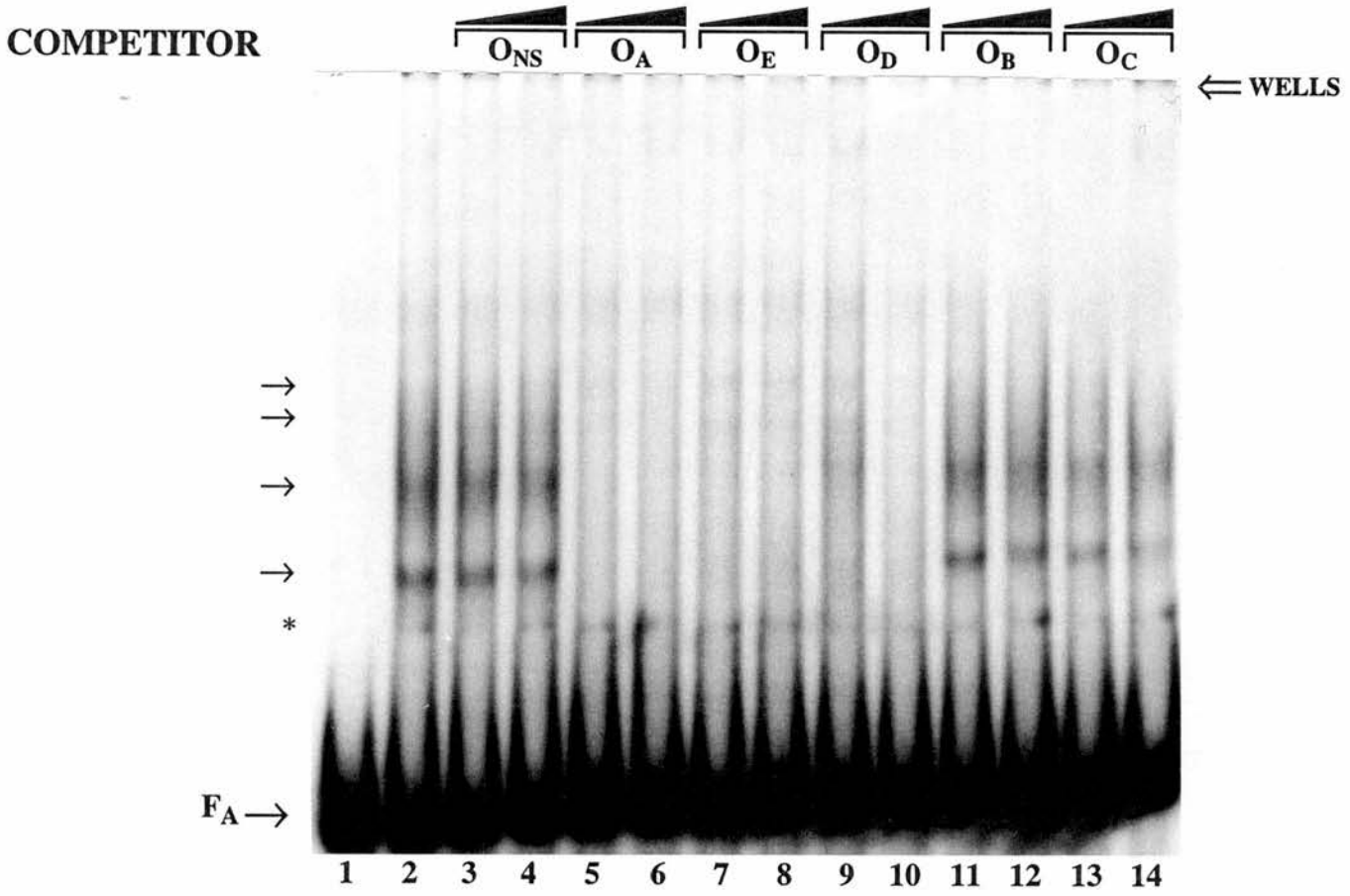
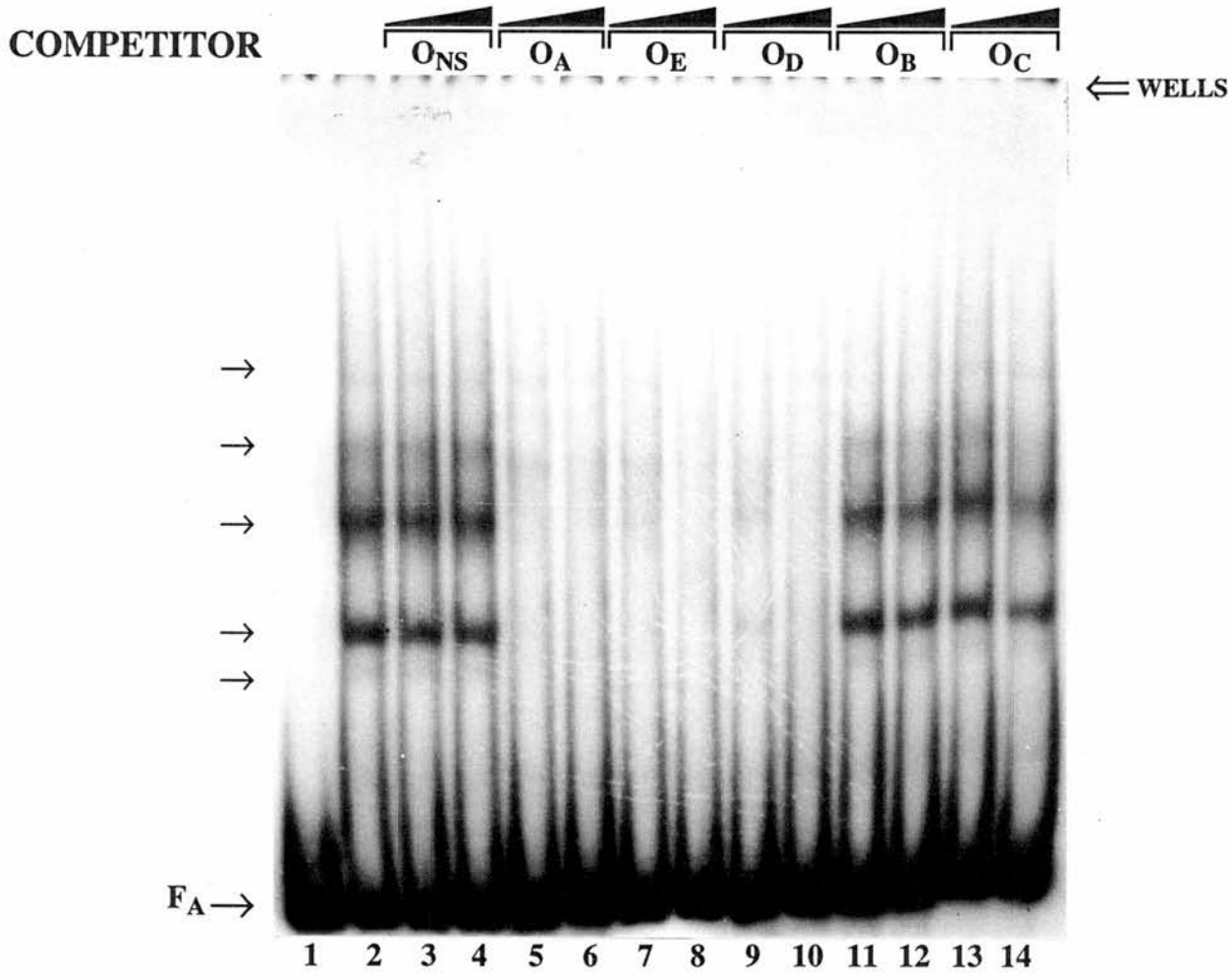

COMPETITOR
O_{NS}
O_A
O_E
O_D
O_B
O_C
⇐ WELLS
→
→
→
→
*
F_A →
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 6.4 *C/EBP-related factors from differentiated 3T3-F442A cells bind to the 11ß-HSD1 promoter fragment encoding -88 to +47*

Competition gel mobility shift assay using the 11ß-HSD1 promoter fragment encoding -88 to +47 (F_A) and nuclear extract from differentiated 3T3-F442A cells. 10µg of nuclear extract was incubated with 30fmol of [^{32}P]-labelled F_A in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-14). Lane 1 contains no nuclear extract. Arrows indicate specific protein-DNA complexes.

Oligonucleotides used were: O_A, 11ß-HSD1 (-14 to +15); O_B, 11ß-HSD1 (-88 to -57); O_C, 11ß-HSD1 (-71 to -52); O_D, PEPCK P3(I) site; O_E, optimal C/EBP site; O_{NS}, prolactin ERE (non-specific control).


COMPETITOR
O_{NS}
O_A
O_E
O_D
O_B
O_C
⇐ WELLS
→
→
→
→
→
F_A →
1 2 3 4 5 6 7 8 9 10 11 12 13 14

O_E and O_D, encoding respectively an optimal C/EBP binding site and the PEPCK P3(I) site (see Table 2.1 for sequences), competed complex formation at a 10-fold molar excess; the non-specific oligonucleotide O_{NS} and 11ß-HSD1 promoter oligonucleotides O_B and O_C did not compete complex formation (Figure 6.3 & Figure 6.4). These results show that C/EBP-related factors from undifferentiated and differentiated 3T3-F442A cells can bind to the 11ß-HSD1 promoter between -88 and +47.

6.2.2 Factors from adrenal cortex bind to the 11ß-HSD1 promoter

Four putative SF-1 binding sites were identified in the 11ß-HSD1 gene at +645, -250, -530 and -910 (Table 6.1). Oligonucleotides were designed to encompass these putative binding sites; O_O, +629 to +650; O_P, -264 to -239; O_Q, -540 to -516; O_R, -919 to -897 (see Table 2.1 for sequences of oligonucleotides) and were used in gel mobility shift analysis with nuclear extracts made from bovine adrenal cortex.

6.2.2.1 The SF-1 (complex) binding site at +645

A complex pattern of binding was observed when nuclear proteins from adrenal cortex were incubated with oligonucleotide O_O, encoding a 'complex' site at +650, within intron A of the rat 11ß-HSD1 gene (Moison et al., 1992). Bovine adrenal nuclear extract formed 4 complexes on O_O. However, only 2 appeared to be specific, being competed by O_O, but not by O_{NS} (Figure 6.5). The 2 specific complexes appeared to be competed to varying degrees by the other oligonucleotides O_P, O_Q, O_R (encoding putative SF-1 sites in the 11ß-HSD1 gene) but not by O_E, encoding an optimal C/EBP site (Figure 6.5). 2 weak complexes were formed on O_O by liver nuclear extract (Figure 6.6), both of which were specific and competed similarly by both O_O and O_R (encoding 'complex' sites). Interestingly, the upper complex was preferentially completed by oligonucleotides O_O and O_R (encoding the 2 complex sites) with weak or no competition by O_P, and O_Q (encoding the 'simple' sites) (Figure 6.6).

6.2.2.2 The SF-1 (simple) binding site at -250

Bovine adrenal nuclear extract formed a single specific complex on O_P, a putative 'simple' SF-1 binding site, which was competed most strongly by O_P at 10-fold molar excess of competitor, and was also competed by O_O, O_Q and O_R at 100-fold molar

Figure 6.5 *Factors in bovine adrenal nuclear extract bind to the oligonucleotide encoding +629 to +650 of the rat 11ß-HSD1 gene*

Competition gel mobility shift assay using the 11ß-HSD1 oligonucleotide encoding +629 to +650 (O_O) and bovine adrenal nuclear extract. 10µg of nuclear extract was incubated with 0.1pmol of [^{32}P]-labelled O_O in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-14). Lane 1 contains no nuclear extract. Lane 15 contains 10µg of liver nuclear extract instead of adrenal extract. Arrows indicate specific protein-DNA complexes.

Oligonucleotides used were: O_O, 11ß-HSD1 (+629 to +650); O_P, 11ß-HSD1 (-264 to -239); O_Q, 11ß-HSD1 (-540 to -516); O_R, 11ß-HSD1 (-919 to -897); O_E, optimal C/EBP site; O_{NS}, prolactin ERE (non-specific control).

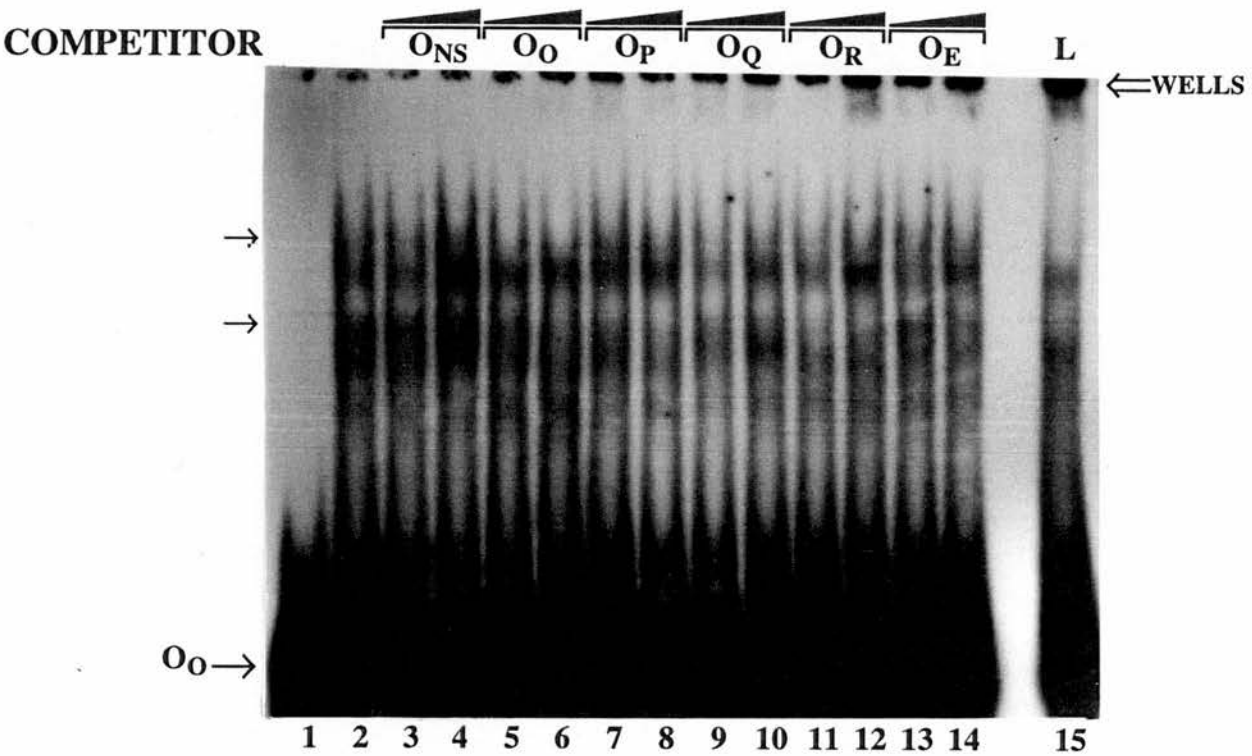

COMPETITOR
O_{NS}
O_O
O_P
O_Q
O_R
O_E
L
WELLS
O_O
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 6.6 *Rat liver nuclear proteins form complexes with the oligonucleotide encoding +629 to +650 of the rat 11ß-HSD1 gene*

Competition gel mobility shift assay using the 11ß-HSD1 oligonucleotide encoding +629 to +650 (O_O) and rat liver nuclear extract. 10µg of nuclear extract was incubated with 0.1pmol of [^{32}P]-labelled O_O in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-12). Lane 1 contains no nuclear extract. Arrows indicate specific protein-DNA complexes.

Oligonucleotides used were: O_O, 11ß-HSD1 (+629 to +650); O_P, 11ß-HSD1 (-264 to -239); O_Q, 11ß-HSD1 (-540 to -516); O_R, 11ß-HSD1 (-919 to -897); O_{NS}, prolactin ERE (non-specific control).

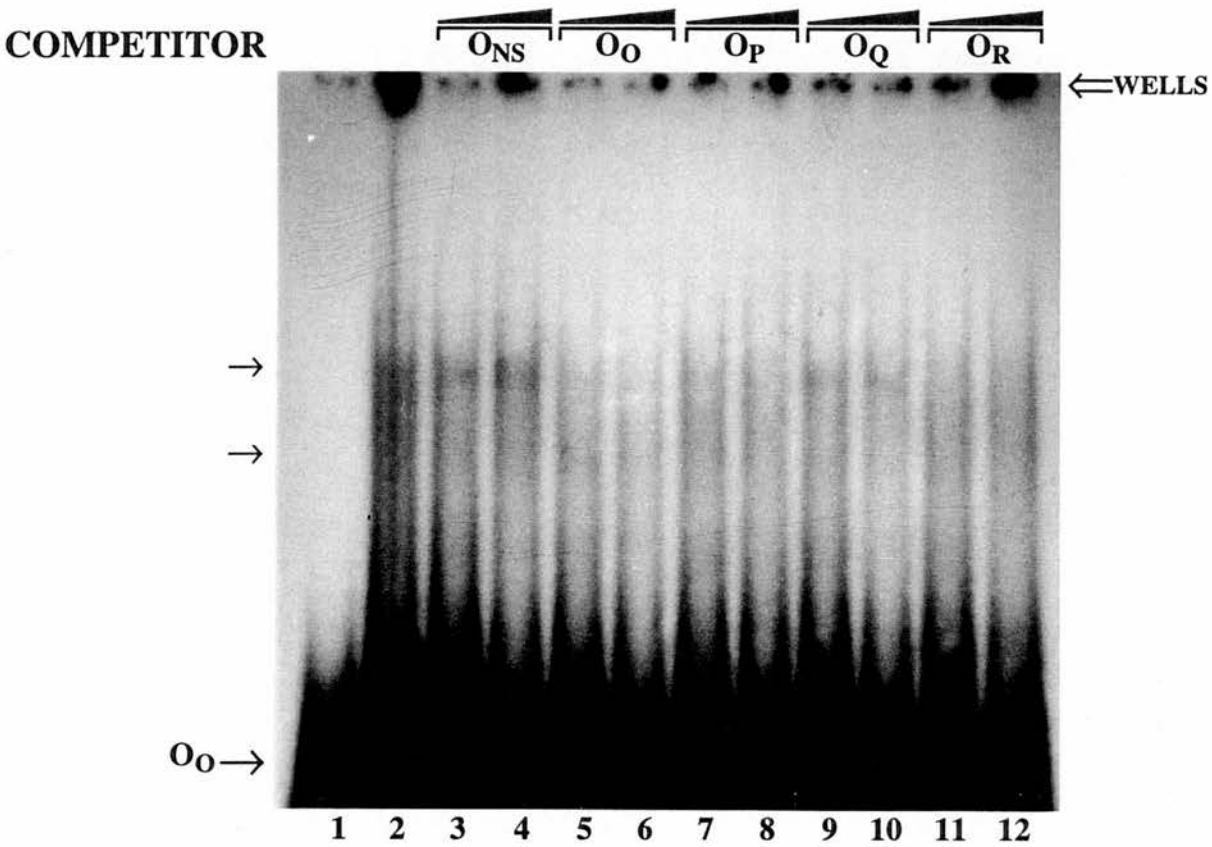

COMPETITOR
O_{NS}
O_O
O_P
O_Q
O_R
⟸WELLS
→
→
O_O→
1 2 3 4 5 6 7 8 9 10 11 12

Figure 6.7 *A single complex is formed by bovine adrenal nuclear extract and the oligonucleotide encoding -264 to -239 of the rat 11ß-HSD1 gene*

Competition gel mobility shift assay using the 11ß-HSD1 oligonucleotide encoding -264 to -239 (O_P) and bovine adrenal nuclear extract. 10µg of nuclear extract was incubated with 0.1pmol of [^{32}P]-labelled O_P in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-14). Lane 1 contains no nuclear extract. An arrow indicates the specific protein-DNA complex.

Oligonucleotides used were: O_O, 11ß-HSD1 (+629 to +650); O_P, 11ß-HSD1 (-264 to -239); O_Q, 11ß-HSD1 (-540 to -516); O_R, 11ß-HSD1 (-919 to -897); O_E, optimal C/EBP site; O_{NS}, prolactin ERE (non-specific control).

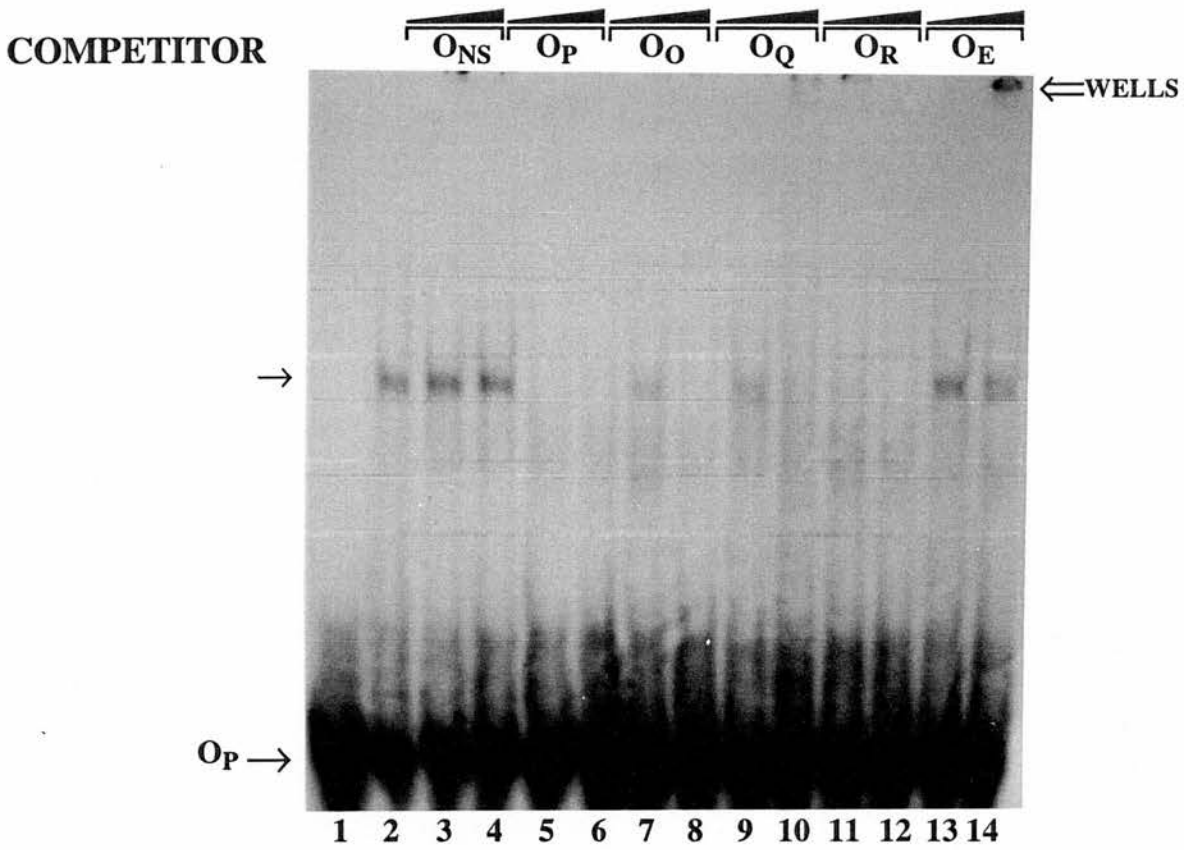
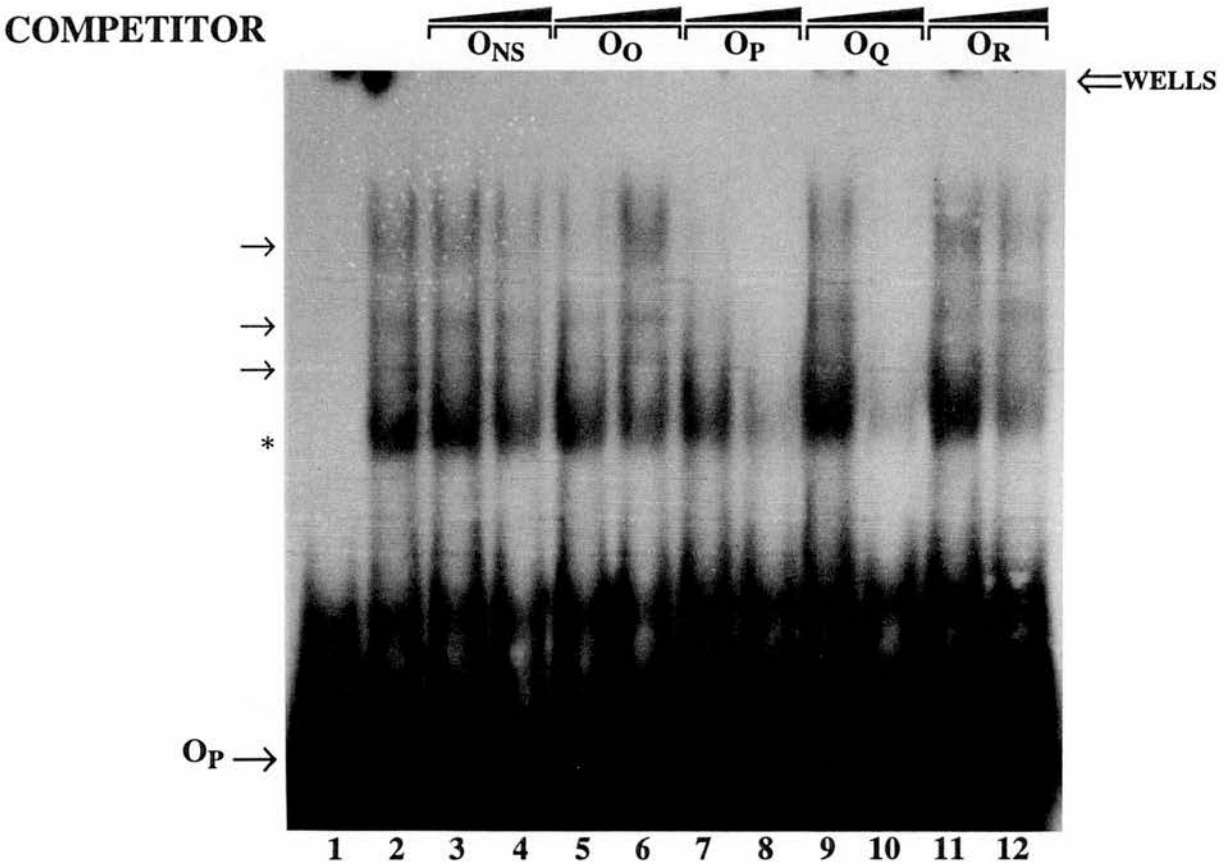

COMPETITOR
O_{NS}
O_P
O_O
O_Q
O_R
O_E
⇐WELLS
→
O_P →
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 6.8 *Factors in rat liver nuclear extract bind to the oligonucleotide encoding -264 to -239 of the rat 11ß-HSD1 gene*

Competition gel mobility shift assay using the 11ß-HSD1 oligonucleotide encoding -264 to -239 (O_P) and rat liver nuclear extract. 10µg of nuclear extract was incubated with 0.1pmol of [^{32}P]-labelled O_P in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-12). Lane 1 contains no nuclear extract. Arrows indicate specific protein-DNA complexes. * indicates a non-specific complex.

Oligonucleotides used were: O_O, 11ß-HSD1 (+629 to +650); O_P, 11ß-HSD1 (-264 to -239); O_Q, 11ß-HSD1 (-540 to -516); O_R, 11ß-HSD1 (-919 to -897); O_{NS}, prolactin ERE (non-specific control).


COMPETITOR
O_{NS}
O_O
O_P
O_Q
O_R
⟸WELLS
→
→
→
*
O_P →
1 2 3 4 5 6 7 8 9 10 11 12

excess of competitor, with a rank order of $O_P \geq O_R > O_O = O_Q$ (Figure 6.7). In this case, the 2 stronger competitors, O_P and O_R, encoded 'simple' (O_P) and 'complex' (O_R) sites (Table 6.1). Oligonucleotide O_E showed no competition, indicating that none of the complexes contain C/EBP-related factors (Figure 6.7). Liver nuclear extract formed several complexes on O_P (Figure 6.8). The fastest migrating complex was probably non-specific, showing only poor competition by a 100-fold molar excess of O_P. Only O_P and O_Q, encoding 'simple' sites competed complex formation (Figure 6.8); the 2 oligonucleotides encoding 'complex' sites, O_O and O_R showed no competition (Figure 6.8). This result suggests that members of the steroid hormone nuclear receptor superfamily, present in liver, are capable of binding to this site at -260. However, the relevance of liver nuclear protein binding to this site is not clear as no footprint was produced by liver nuclear extract over this sequence (Chapter 4).

6.2.2.3 The SF-1 (simple) binding site at -530

Adrenal nuclear extract formed 2 weak complexes on O_Q (Figure 6.9) which were non-specific as excess competitor unlabelled oligonucleotide O_Q failed to compete complex formation (Figure 6.9). Liver nuclear extract produced a similar result with no specific complexes formed on O_Q (Figure 6.10). This is consistent with the lack of a liver nuclear protein footprint over this region (Chapter 4). However, another possible explanation for this is that mistakes were identified in the original 11ß-HSD1 promoter sequence and unfortunately as a result of this there is a mistake in the oligonucleotide sequence at nucleotide -536 which has a C instead of a G residue.

6.2.2.4 The SF-1 (complex) binding site at -910

Bovine adrenal nuclear extract formed 3 specific complexes on oligonucleotide O_R, encoding the putative 'complex' SF-1 binding site at -910 of 11ß-HSD1. These complexes were competed most strongly by the two most related sequences, O_R and O_O, and overall the oligonucleotides showed differential competition of complexes (Figure 6.11). All of the complexes were competed by addition of excess unlabelled O_R, demonstrating the specificity of binding. However, the fastest and slowest migrating complexes were only partially competed by O_Q, and not competed at all by O_P or O_O and the middle complex showed partial competition by O_O and O_Q suggesting that different proteins with differing DNA-binding specificities may give rise to these complexes. Liver nuclear extract weakly bound to O_R, forming no

Figure 6.9 *No specific complexes are formed by bovine adrenal nuclear proteins on the oligonucleotide encoding -540 to -516 of the rat 11ß-HSD1 gene*

Competition gel mobility shift assay using the 11ß-HSD1 oligonucleotide encoding -540 to -516 (O_Q) and bovine adrenal nuclear extract. 10µg of nuclear extract was incubated with 0.1pmol of [^{32}P]-labelled O_Q in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-14). Lane 1 contains no nuclear extract. Lane 15 contains 10µg of liver nuclear extract instead of adrenal nuclear extract.

Oligonucleotides used were: O_O, 11ß-HSD1 (+629 to +650); O_P, 11ß-HSD1 (-264 to -239); O_Q, 11ß-HSD1 (-540 to -516); O_R, 11ß-HSD1 (-919 to -897); O_E, optimal C/EBP site; O_{NS}, prolactin ERE (non-specific control).

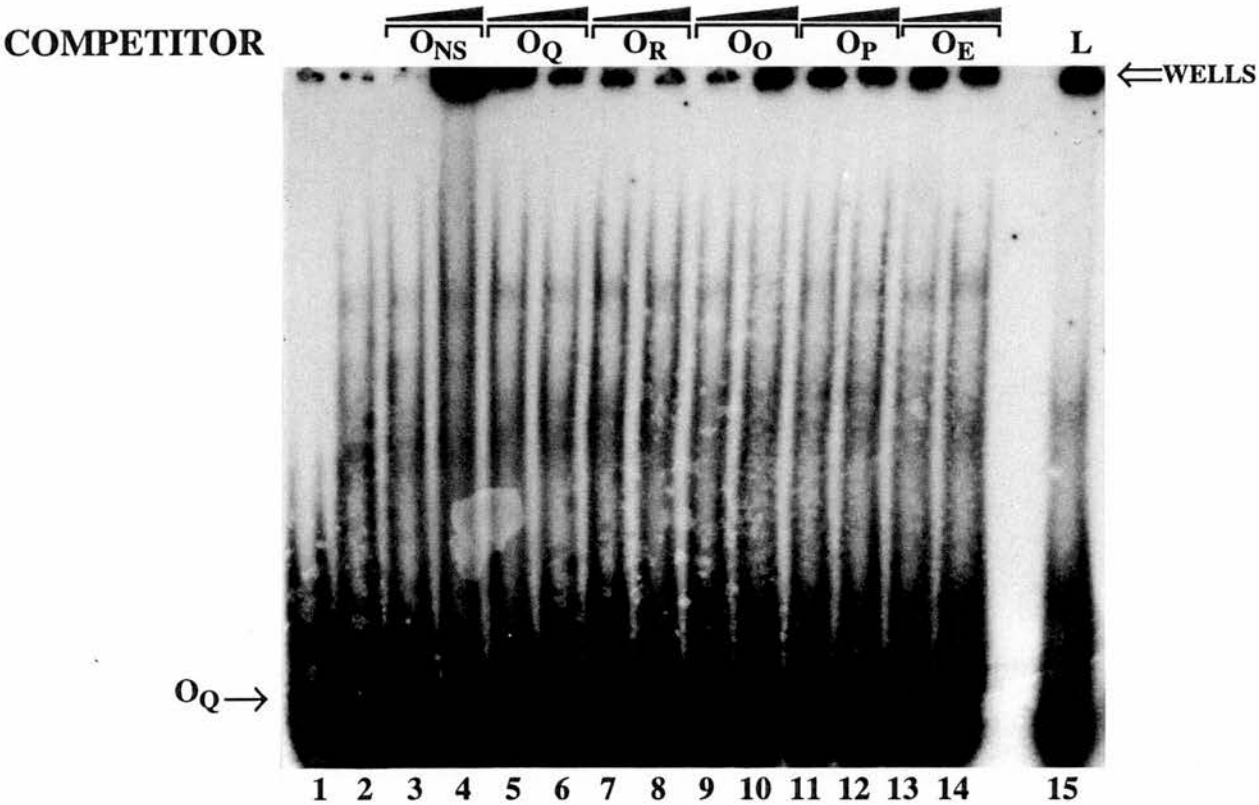

COMPETITOR
O_{NS}
O_Q
O_R
O_O
O_P
O_E
L
⟸WELLS
O_Q→
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 6.10 *No specific complexes are formed by rat liver nuclear proteins on the oligonucleotide encoding -540 to -516 of the rat 11ß-HSD1 gene*

Competition gel mobility shift assay using the 11ß-HSD1 oligonucleotide encoding -540 to -516 (O_Q) and rat liver nuclear extract. 10µg of nuclear extract was incubated with 0.1pmol of [^{32}P]-labelled O_Q in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-12). Lane 1 contains no nuclear extract.

Oligonucleotides used were: O_O, 11ß-HSD1 (+629 to +650); O_P, 11ß-HSD1 (-264 to -239); O_Q, 11ß-HSD1 (-540 to -516); O_R, 11ß-HSD1 (-919 to -897); O_{NS}, prolactin ERE (non-specific control).

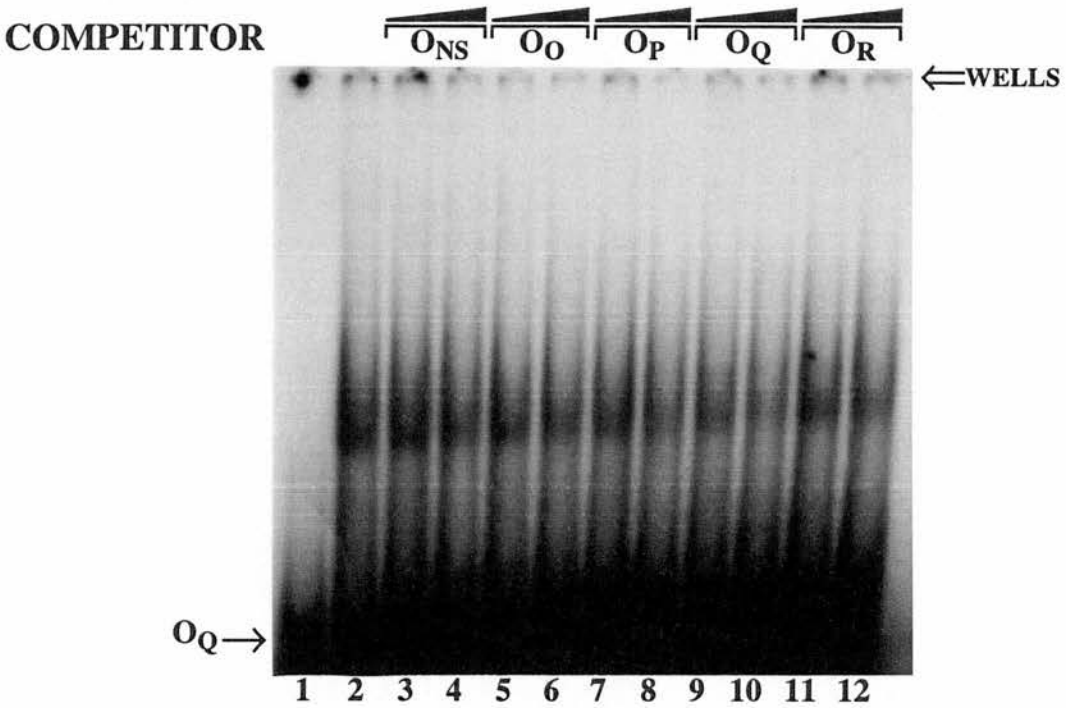
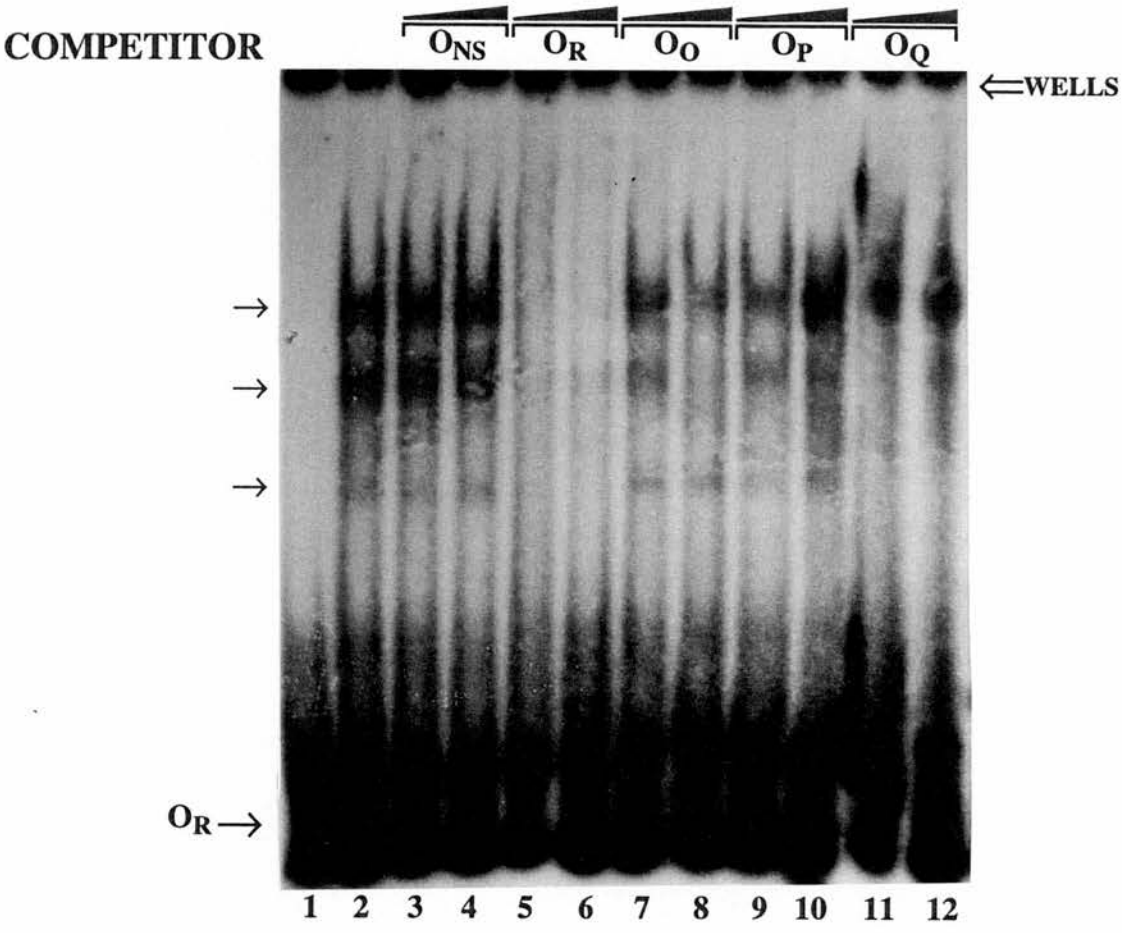

COMPETITOR
O_{NS}
O_O
O_P
O_Q
O_R
⇐WELLS
O_Q→
1 2 3 4 5 6 7 8 9 10 11 12

Figure 6.11 *Factors in bovine adrenal nuclear extract bind to the oligonucleotide encoding -919 to -897 of the rat 11ß-HSD1 gene*

Competition gel mobility shift assay using the 11ß-HSD1 oligonucleotide encoding -919 to -897 (O_R) and bovine adrenal nuclear extract. 10µg of nuclear extract was incubated with 0.1pmol of [^{32}P]-labelled O_R in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-12). Lane 1 contains no nuclear extract. Arrows indicate specific protein-DNA complexes.

Oligonucleotides used were: O_O, 11ß-HSD1 (+629 to +650); O_P, 11ß-HSD1 (-264 to -239); O_Q, 11ß-HSD1 (-540 to -516); O_R, 11ß-HSD1 (-919 to -897); O_{NS}, prolactin ERE (non-specific control).


COMPETITOR
O_{NS}
O_R
O_O
O_P
O_Q
⇐WELLS
→
→
→
O_R →
1 2 3 4 5 6 7 8 9 10 11 12

discrete complexes (Figure 6.12). However, the smear of complexes formed on O_R appeared to be specific as O_R and O_O were both effective competitors at 100-fold molar excess. O_P and O_Q competed less well (Figure 6.12).

6.3 Discussion

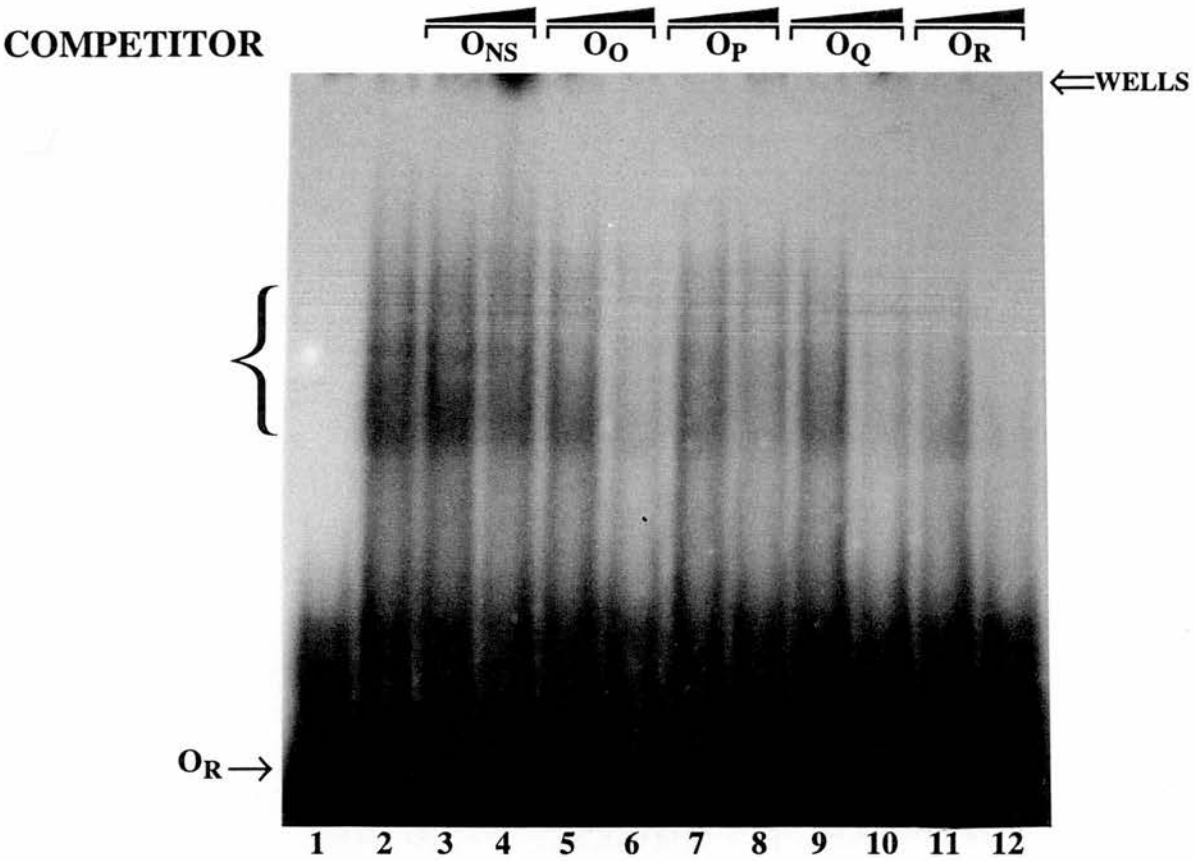
6.3.1 Differentiated and undifferentiated 3T3-F442A cells contain factors which specifically bind to the 11ß-HSD1 proximal promoter (-88/+47) and which are probably members of the C/EBP family of transcription factors

These results suggest that C/EBP-related factors from undifferentiated and differentiated 3T3-F442A cells bind to the 11ß-HSD1 promoter (between -88 and +47) producing similar patterns of C/EBP binding, with perhaps an overall slight reduction in binding by extracts from undifferentiated cells in comparison to extract from differentiated cells. The lack of a qualitative difference between the gel shift pattern produced by nuclear extracts from differentiated and undifferentiated cells indicates that binding of C/EBP-related transcription factors between -88 and +47 of the 11ß-HSD1 promoter is unlikely to be the main factor turning on 11ß-HSD1 expression. However, this does not rule out differentiation dependent binding of C/EBP or another factor upstream of -88. This shift pattern is not what was initially predicted as no binding by C/EBPα was expected using nuclear extracts made from undifferentiated cells. 3T3-L1 cells (a different subclone of mouse 3T3 cells), have been the most extensively characterised adipocyte cell line with regard to the role of the C/EBP family members during adipose differentiation. In 3T3-L1 cells, C/EBPα levels are low in undifferentiated cells and rise as cells differentiate (Cao et al., 1991). However, the binding observed in undifferentiated cells may be due to other members of the C/EBP family. C/EBPß and C/EBPδ are highly expressed in undifferentiated cells and levels decline as cells differentiate (Cao et al., 1991). The experiments described in this chapter used nuclear extracts made from differentiated cells 5 days after induction of differentiation, however, cells are not fully differentiated until 10 days after induction of differentiation. At 5 days after induction of 3T3-L1 cell differentiation C/EBPß and C/EBPδ levels are decreasing and C/EBPα levels are increasing, with the result that levels of C/EBPδ are low but C/EBPß and C/EBPα protein levels are higher and comparable (Cao et al., 1991). It is noteworthy that extracts from differentiated cells appeared to form more lower

Figure 6.12 *Factors in rat liver nuclear extract bind to the oligonucleotide encoding -919 to -897 of the rat 11ß-HSD1 gene*

Competition gel mobility shift assay using the 11ß-HSD1 oligonucleotide encoding -919 to -897 (O_R) and rat liver nuclear extract. 10µg of nuclear extract was incubated with 0.1pmol of [^{32}P]-labelled O_R in the absence of added competitor (lane 2) or in the presence of a 10- or 100-fold molar excess of competitor oligonucleotide, as indicated above each lane (lanes 3-12). Lane 1 contains no nuclear extract. Bracket indicates specific protein-DNA complexes.

Oligonucleotides used were: O_O, 11ß-HSD1 (+629 to +650); O_P, 11ß-HSD1 (-264 to -239); O_Q, 11ß-HSD1 (-540 to -516); O_R, 11ß-HSD1 (-919 to -897); O_{NS}, prolactin ERE (non-specific control).


COMPETITOR
O_{NS}
O_O
O_P
O_Q
O_R
⇐WELLS
O_R →
1 2 3 4 5 6 7 8 9 10 11 12

mobility complexes (possibly containing C/EBPα) than did extracts from undifferentiated cells. Alternatively, it is possible that the cultures of undifferentiated cells from which extracts were made contain a number of cells which had spontaneously differentiated, and therefore the 'undifferentiated extracts' were actually made from that mixture of undifferentiated and differentiated cells, accounting for the presence of C/EBPα in extracts from undifferentiated cells.

6.3.2 Factors from adrenal extract interact with the 11ß-HSD1 gene

The binding of an orphan member of the nuclear receptor family, SF-1, to the 11ß-HSD1 gene was examined using adrenal extract as an abundant source of SF-1 (Rice et al., 1991). 11ß-HSD1 contains a number of predicted sites or half sites for members of the nuclear receptor superfamily. The AGGTCA sequence, capable of binding SF-1, corresponds to a consensus half site for Class II nuclear receptors and forms the core element of Class III nuclear receptor binding sites (reviewed in Chapter 1, Section 1.2.5). AGGTCA sequences are found in the 11ß-HSD1 gene at +645 and -910. Some SF-1 sites bear only a loose resemblance to the consensus AGGTCA sequence, and more closely conform to a consensus CAAGG (Parker & Schimmer, 1993). Two such sequences occur within the sequenced region of the rat 11ß-HSD1 gene, at -250 and -530. Experiments performed using oligonucleotides encoding these 4 sites and *in vitro* translated SF-1 demonstrated that SF-1 is capable of binding to all 4 sites (A. Butler & M. Parker, personal communication). In contrast, another member of the steroid hormone receptor family, COUP-TF (translated *in vitro*) only bound to 2 of the oligonucleotides, those containing the AGGTCA sites (A. Butler & M. Parker, personal communication) indicating that at -250 and -530 there is a degree of transcription factor binding specificity. Nuclear extracts made from bovine adrenal cortex showed binding to 3 of the 4 oligonucleotides with different patterns of specificity for each, suggesting that different factors, probably including SF-1, are binding to each of the oligonucleotides. Only in one case was a single complex observed, which is likely to be due to SF-1 binding.

Proteins from liver nuclei also bound to 3 of the oligonucleotides, encoding the putative SF-1 sites at +655, -250 and -910. These data suggest that proteins in liver nuclear extract, probably members of the steroid hormone receptor superfamily, can

bind to these elements of the 11ß-HSD1 gene; liver contained several members of the nuclear receptor superfamily including COUP-TF, PPARα and HNF-4 (all of which were originally cloned from liver cDNA libraries; refer Chapter 1, Section 1.6.3). Proteins from rat liver nuclear extract bound to oligonucleotides encoding the AGGTCA sequence at both +655 and -910. Both sites were also bound by *in vitro* translated COUP-TF, and it is possible that one of the factors in rat liver nuclear extract which binds to these 2 sites is COUP-TF, although this remains to be tested. Neither of these 2 regions have yet been examined by footprinting experiments. If theses sites do represent bona fide targets for nuclear receptors, then they may be targets for binding of HNF-4, RXR (and related proteins), PPARα and COUP-TF in liver, as well as possibly PPARγ in adipocytes, a crucial regulator of adipocyte gene expression and differentiation (MacDougald & Lane, 1995) in the experiments described in Chapter 4. However, no footprint was generated over the corresponding region (Chapter 4), by rat liver nuclear extract, making the significance of the binding to the oligonucleotide questionable.

Glucocorticoid biosynthesis occurs primarily in the adrenal cortex where active glucocorticoids are produced via 11-deoxycortisol (man) and 11-deoxycorticosterone (rat) (see Chapter 1), but the biological function of 11ß-HSD1 in the adrenal is not known. Unstimulated, isolated bovine adrenal cortical cells secrete more cortisone than cortisol (Burt et al., 1991), but acute ACTH stimulation results in increased cortisol secretion with no change in cortisone production. (Burt et al., 1991). Chronic ACTH treatment of these cells resulted in decreased cortisol production but increased cortisone production (Williams et al., 1992). ACTH increases steroidogenic enzyme activity thereby increasing cortisol production (see Chapter 1) and it may possibly also increase the activity/expression of 11ß-HSD1, resulting in increased cortisol production. In humans, ACTH inhibited 11ß-HSD activity (Walker et al., 1992), suggesting that the adrenal is required for the inhibitory effects of ACTH. It was suggested that the adrenal produced an intermediate of cortisol production which inhibited 11ß-HSD activity (Walker et al., 1992). It is possible that another steroid biosynthetic pathway exists that results in the production of a precursor to cortisone or 11-dehydrocortisone on which 11ß-HSD1 could act to produce active glucocorticoids. Alternatively, adrenal 11ß-HSD2 may inactivate cortisol before

secretion by the adrenal, thereby modulating the amount of active glucocorticoid released.

Another possible biological effect of glucocorticoids in the adrenal is to modulate catacholamine release from the medulla (Shimojo et al., 1996); the gene encoding phenylethanolamine N-methyl transferase which converts noradrenaline into adrenaline (Shimojo et al., 1996). Blood passing through the adrenal cortex passes directly into the medulla which expresses some 11ß-HSD2 but not 11ß-HSD1 (Shimojo et al., 1996). However, 11ß-HSD1 is found at the cortico-medullary junction (at least in rats), (Shimojo et al., 1996). The combined action of the 11ß-HSD enzymes therefore may govern the concentration of active glucocorticoids reaching the medulla, and consequently affecting synthesis of adrenaline.

CHAPTER 7

Discussion

7.1 Discussion

Glucocorticoid receptors are present in virtually every cell type in the body and glucocorticoids modulate the action of many genes and gene networks, in particular those involved in maintaining blood glucose levels (Garland, 1986; Sasaki et al., 1984), and those involved in the response of the body to stress and infection (Baumann and Gauldie, 1994). 11ß-HSD1 plays a key role in the metabolism of glucocorticoids and through its reductase activity may enhance the induction of genes by glucocorticoid hormones (Jamieson et al., 1996). Expression of 11ß-HSD1 throughout the body is generally correlated with the level of expression of GR mRNA (Whorwood et al., 1992) and is highest in the liver and other tissues which are major targets of glucocorticoid action such as adipose tissue, kidney and brain (Orth et al., 1992). Reductase activity of 11ß-HSD1 predominates over its dehydrogenase activity in intact cells (Jamieson et al., 1995; Low et al., 1994a; Voice et al., 1996; Rajan et al., 1996), suggesting that the role of 11ß-HSD1 is to supply GR with ligand. In agreement with this, attenuation of hepatic 11ß-HSD1 by treatment with oestrogen led to a decrease in the expression of glucocorticoid inducible hepatic genes (Jamieson et al., 1996), and mice with targeted disruption of the 11ß-HSD1 gene have reduced fasting blood glucose levels (Kotelevtsev et al., 1996b) and decreased fasting levels of hepatic G6Pase (Y. Kotelevtsev, personal communication). Regulation of 11ß-HSD1 expression is therefore key to the normal actions of glucocorticoids. In this thesis I have shown the central role played by C/EBP in the regulation of 11ß-HSD1 expression in liver. C/EBP appears to be the predominant regulator of 11ß-HSD1 expression in liver, with at least 12 binding sites between -812 and +74, including one encompassing the transcription start. Using *in vitro* transcription I have shown that the binding of C/EBP to the transcription start of the 11ß-HSD1 gene is likely to be of functional importance in the tissue specific expression of 11ß-HSD1 in liver, and finally I have shown that C/EBP-related transcription factors may play a central role in expression of 11ß-HSD1 in adipocytes, but that in other tissues, e.g. adrenal gland, other transcription factors may play a role in regulating expression of this important glucocorticoid metabolising gene.

There was a strong correlation between the sites of hypersensitivity to DNase I induced by rat liver nuclear extract in the DNase I footprinting experiments on the rat

11ß-HSD1 promoter, and the sites which are hypersensitive to DNase I in intact rat liver nuclei. Within the region between -4000 and +1500 (relative to the transcription start) only two regions of the 11ß-HSD1 gene showed hypersensitivity in intact nuclei to DNase I; a discrete site at approximately -650 to 660, which reached a maximum of approximately 50% cleavage, and a region of hypersensitivity spanning approximately 200 nucleotides, including the transcription start, which was cleaved 100% by DNase I (K. Chapman, personal communication). The striking similarity between the site at -660 seen *in vitro* in DNase I footprinting experiments (Chapter 4) and that seen in intact nuclei suggests that a nucleoprotein complex may be formed by rat liver nuclear extract (at the region adjacent to the hypersensitive site), which causes a distortion of the DNA, allowing greater access of DNase I. Recombinant C/EBPα was unable to generate the hypersensitive sites and it is possible that a large protein or a protein complex from liver might bind here and be responsible for repressing expression (this region decreased basal levels of promoter activity), and that it is exchanged with C/EBPα with a concomitant release of repression. This might account for the incomplete cleavage seen at this site in intact nuclei. This hypothesis however, remains to be tested.

In future experiments it will be important to identify the protein(s) which bind to the region adjacent to the hypersensitive site as it may be a key regulatory factor repressing transcription of the 11ß-HSD1 gene. The factor could be further investigated by mutation of the nucleotides surrounding the C/EBP site which should decrease binding of the unknown factor but not binding by C/EBP. It will be interesting to examine the effect of mutation of this site on 11ß-HSD1 promoter activity in cells transfected with 11ß-HSD1-luciferase plasmids, in the absence or presence of C/EBP. Additionally, it will be important to determine whether the hypersensitive site is specific to liver to see if this is an important regulatory region in other tissues.

The 11ß-HSD1 promoter lacks a TATA box, furthermore, the transcription start 'initiator' of 11ß-HSD1 did not appear to bind a 'classical' initiator binding protein, instead, C/EBPα, C/EBPß and an unidentified C/EBP related factor bound to the transcription start (Chapter 3). The involvement of C/EBP in binding to the transcription start is unusual, but not unprecedented. C/EBP also interacts with the

transcription start of the factor IX gene between +1 and +18 and disruption of C/EBP binding by mutation at +13 results in haemophilia B Leyden (Crossley and Brownlee, 1990). C/EBPα has been shown to interact with TBP *in vivo* and *in vitro* (Nerlov and Ziff, 1995) demonstrating that it could potentially form part of the initiation complex, or interact with the transcription initiation complex, possibly acting as in initiator binding protein. Both *in vitro* transcription (Chapter 5) and transfection experiments (Chapter 4, Figure 4.2) show that a minimal promoter (-88 to +47) confers activity upon a linked reporter gene; basal activity from a short promoter containing just 88 base pairs of flanking sequence was the same, or higher, than that of longer constructs in transfected HepG2 cells and activity of the -599 and -88 constructs were also similar in *in vitro* transcription experiments. Interestingly, it is possible that rat liver nuclear extract forms a nucleoprotein complex with the fragment encoding -88 to +47. Rat liver nuclear extract induced the appearance of hypersensitive sites (not induced by rC/EBPα) at approximately ten base pair intervals. Again, this may correlate with the hypersensitive site seen in intact nuclei, although, in contrast to the discrete site at -660, in intact nuclei, a region of approximately 200 base pairs including the transcription start, showed hypersensitivity to DNase I (K. Chapman, personal communication). It would be interesting to compare factors binding from different tissues to the 11ß-HSD1 promoter, particularly those in which 11ß-HSD1 transcription is initiated from the same promoter as used in liver e.g. in hippocampus, and it will also be of interest to determine whether the different promoters used in kidney utilise the same factors to regulate gene expression.

The promoter of the rat 11ß-HSD1 gene appears to be predominantly regulated by C/EBP, at least between -812 and +47, with 12 of the 14 footprints attributable to members of the C/EBP family of transcription factors (Chapter 4, Section 4.2.1). This, together with the presence of a C/EBP binding site at the transcription start suggests that C/EBP plays a uniquely important role in the regulation of this gene in liver. Most other C/EBP regulated genes contain only a single or a few C/EBP sites interspersed with other transcription factor binding sites in their promoter regions. It is tempting to speculate on the importance of the 11ß-HSD1 enzyme in providing the 'cross-talk' between the hormonal signals transduced by C/EBP and the glucocorticoid signalling pathways. Glucocorticoids regulate a wide range of genes

with diverse functions, but they also regulate 'groups' of genes performing similar functions e.g. genes involved in glucose metabolism (Cryer, 1992), and stress or infection (Baumann & Gauldie, 1994). It is interesting to note that additional factors which regulate these functions also regulate C/EBP. For example, in blood glucose control, the other major hormonal regulators insulin and glucagon (acting via cAMP) regulate C/EBP (Bosch et al., 1995; Matsuno et al., 1996), and in the acute phase response cytokines regulate C/EBP expression. These factors all alter the activity of C/EBP members, either by phosphorylation, or by altering gene expression, hence, C/EBP may be a mechanism for the organism to communicate the nutrient or 'stressed' status of the whole organism to the transcription apparatus. The regulation of 11ß-HSD1 by C/EBP provides a mechanism by which C/EBP is able to regulate the availability of glucocorticoid ligand to its receptor, amplifying the local effects of glucocorticoid. Thus when C/EBP activity is low, 11ß-HSD1 activity will be low and glucocorticoid will not have as large an effect on promoting glucose availability. When C/EBP activity increases, then 11ß-HSD1 activity increases and glucocorticoid availability and thus active GR is increased. It is also possible that different members of the C/EBP-family have different effects on 11ß-HSD1 activity e.g. C/EBPα is associated with regulating energy metabolism (Darlington et al., 1995) and C/EBPß and/or C/EBPδ have been primarily implicated in regulating genes involved in the acute phase response (Wedel & Ziegler-Heitbrock, 1995). So far, the major role of C/EBPα that has been investigated is that concerned with energy homeostasis (Darlington et al., 1995) and it is important now to investigate the effects C/EBPα may have in inducing the acute phase response. It would be interesting to explore the effects of C/EBPß and C/EBPδ upon 11ß-HSD1 promoter activity in transfected cells, and to examine whether these C/EBP-related factors bind to the same sites as C/EBPα in DNase I footprinting experiments. An indication of the major role C/EBP may play in regulating 11ß-HSD1 expression would be obtained by examining 11ß-HSD1 mRNA levels in C/EBPα and C/EBPß 'knock-out' mice. It would be predicted that 11ß-HSD1 expression would be reduced in these animals. The 11ß-HSD1 'knock-out' mice will be crucial for testing the role of 11ß-HSD1 in actions of glucocorticoids in liver and elsewhere, and it is possible that C/EBP expression is increased in these animals to compensate for the lack of active glucocorticoids.

The predominant role of C/EBP in liver raises questions as to what is regulating 11ß-HSD1 elsewhere, both in tissues that also express C/EBP and those that don't. Adipose tissue contains C/EBP (MacDougald & Lane 1995), and some preliminary data showed the prevalence of C/EBP in binding to the 11ß-HSD1 promoter between -88 and +47 and probably also to the transcription start. It will be interesting to compare DNase I footprints generated by liver and adipose tissue, especially at the -660 hypersensitive site. Transfection of 3T3-F442A cells has proved difficult (K. Chapman, personal communication), so it has not been possible to show a role for C/EBP in transfected 3T3-F442A cells. The function of 11ß-HSD1 in adipose tissue is even more speculative than the postulated role in liver. However, evidence from this laboratory has demonstrated 11ß-HSD1 expression in both 3T3-L1 and 3T3-F442A cells when differentiated into adipocytes with little or no expression of 11ß-HSD1 in the undifferentiated fibroblast-like state. Adipose tissue is the biggest energy store in the body, and it is therefore likely that 11ß-HSD1 regulates glucocorticoid mediated metabolism of lipids. The probable regulation of 11ß-HSD1 in adipocytes by C/EBPα suggests that the regulation of the 11ß-HSD1 promoter in adipose tissue is similar to that in liver.

One of the intriguing aspects of the work I have described in Chapter 6 is the possible involvement of members of the nuclear receptor superfamily in the regulation of 11ß-HSD1. 4 sequences from the 11ß-HSD1 gene were chosen for further analysis and 3 proved to bind factors from nuclear extracts made from bovine adrenal cortex, possibly SF-1, and all 4 bound SF-1 produced *in vitro*. Furthermore, 2 of these sites (both containing ER half sites AGGTCA) were also capable of binding COUP-TF *in vitro*. An AGGTCA sequence is an integral part of the recognition sequence of a number of receptors, including PPARγ, a crucial differentiation factor required to differentiate 3T3-F442A cells into adipocytes (MacDougald & Lane, 1995), raising the possibility that these sequences may play a role in the expression of 11ß-HSD1 in those tissues which express particular members of the nuclear receptor/steroid hormone receptor superfamily. It will be important to further explore the possible roles of these nuclear factor binding sites in expression of 11ß-HSD1 using transfections, mutagenesis and footprinting experiments. In addition, it will be informative to investigate the factors which bind to the 11ß-HSD1 promoter in extracts made from other tissues which express SF1 e.g. the gonads.

All of the work in this thesis has been carried out using *in vitro* techniques which give an indication of the factors that are capable of binding to the 11ß-HSD1 gene. It would be extremely interesting to carry out *in vivo* experiments to determine where factors in different tissues binding using *in vivo* footprinting which would provide more 'biological relevance' to the findings obtained by the *in vitro* techniques described in this thesis.

The gene for 11ß-HSD1 is expressed in a tissue specific manner and is regulated by specific transcription factors, particularly C/EBP, binding to the promoter to modulate 11ß-HSD1 expression. The work described in this thesis has provided an important start towards elucidating the mechanisms that govern expression of 11ß-HSD1 in a tissue-specific manner and has provided a testable hypothesis of a mechanism of 'cross-talk' between the glucocorticoid signalling pathway and an important group of transcription factors, the C/EBP family.

References

Aden DP, Fogel A, Plotkin S, Damjanov I & Knowles BB. (1979). Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature* **282:** 615-616.

Affolter M, Percival-Smith A, Muller M, Leupin W & Gehring WJ. (1990). DNA binding properties of the purified *Antennapedia* homeodomain. *Proc. Natl. Acad. Sci. USA* **87:** 4093-4097.

Agarwal AK, Monder C, Eckstein B & White PC. (1989). Cloning and expression of rat cDNA encoding corticosteroid 11ß-dehydrogenase. *J. Biol. Chem.* **264:** 18939-18943.

Agarwal AK, Mune T, Monder C & White, PC. (1994). NAD^+ dependent isoform of 11ß-hydroxysteroid dehydrogenase: cloning and characterization of cDNA from sheep kidney. *J. Biol. Chem.* **269:** 25959-25962.

Agarwal AK, Rogerson FM, Mune T & White PC. (1995). Gene structure and chromosomal localization of the human *HSD11K* gene encoding the kidney (type 2) isozyme of 11ß-hydroxysteroid dehydrogenase. *Genomics* **29:** 195-199.

Akira S, Isshiki H, Sugita T, Tanabe O, Kinoshita S, Nishio Y, Nakajima T, Hirano T & Kishimoto T. (1990). A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J.* **9:** 1897-1906.

Alam T, An MR & Papaconstantinou J. (1992). Differential expression of three C/EBP isoforms in multiple tissues during the acute phase response. *J. Biol. Chem.* **267:** 5021-5024.

Albiston AL, Obeyesekere VR, Smith RE & Krozowski ZS. (1994). Cloning and tissue distribution of the human 11ß-hydroxysteroid dehydrogenase type 2 enzyme. *Mol. Cell. Endocrinol.* **105:** R11-R17.

Amelung D, Hubener HJ, Roka L & Mayerheim G. (1953). Conversion of cortisone to compound F. *J. Clin. Endocrinol. Metab.* **13:** 1125-1126.

Amero SA, Kretsinger RH, Moncreif ND, Yamamoto KR & Pearson WR. (1992). The origin of nuclear receptor proteins: A single precurser distinct from other transcription factors. *Mol. Endocrinol.* **6:** 3-7.

Anderson WF, Ohlendorf DH, Takeda Y & Matthews BW. (1981). Structure of the cro repressor from bacteriophage λ and its interaction with DNA. *Nature* **290:** 754-758.

Antoni FA. (1993). Vasopressinergic control of pituitary adrenocorticotropin secretion comes of age. *Frontiers Neuroendocrinol.* **14:** 76-122.

Antonson P & Xanthopoulos KG. (1995). Molecular cloning, sequence, and expression patterns of the human gene encoding CCAAT/Enhancer Binding Protein α (C/EBPα). *Biochem. Biophys. Res. Comm.* **205:** 106-113.

Apfel R, Benbrook D, Lernhardt E, Ortiz MA, Salbert G & Pfahl M. (1994). A novel orphan receptor specific for a subset of thyroid hormone responsive elements and its interaction with the retinoid/thyroid hormone-receptor superfamily. *Mol. Cell. Biol.* **14:** 7025-7035.

Arnold J. (1866). Ein beitrag zu der feineren structur und dem chemismus der nebennieren. *Arch. Pathol. Anat. Physiol. Klin. Med.* **35:** 64-107.

Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE & Evans RM. (1987). Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science* **237:** 268-275.

Baes M, Gulick T, Choi HS, Martinoli MG, Simha D & Moore DD. (1994). A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. *Mol. Cell. Biol.* **14:** 1544-1552.

Baggia S, Albrecht ED & Pepe GJ. (1990). Regulation of 11ß-hydroxysteroid dehydrogenase activity in the baboon placenta by estrogen. *Endocrinology* **126:** 2742-2748.

Bakker O & Parker MG. (1991). CAAT/enhancer binding protein is able to bind to ATF/CRE elements. *Nucleic Acids Res.* **19:** 1213-1217.

Ballard PL. (1987). Glucocorticoid regulation of lung maturation. *Mead Johnson Symp. Perinat. Dev. Med* **30:** 22-27.

Bambino TH & Hseuh AJW. (1981). Direct inhibitory effects of glucocorticoids upon testicular leutinizing hormone receptor and steroidogenesis *in vivo* and *in vitro*. *Endocrinology* **108:** 2142-2148.

Baniahmed A & Tsai MJ. (1993). Mechanisms of transcriptional activation by steroid hormone receptors. *J. Cell. Biochem.* **51:** 151-156

Baumann H & Gauldie J. (1990). Regulation of hepatic acute phase plasma protein genes by hepatocyte stimulating factors and other mediators of inflammation. *Mol. Biol. Med.* **7:** 147-159.

Baumann H, Paulsen K, Kovacs H, Berglund H, Wright APH, Gustafsson JA & Hard T. (1993). Refined solution structure of the glucocorticoid receptor DNA-binding domain. *Biochemistry* **32:** 13463-13417.

Baumann H & Gauldie J. (1994). The acute phase response. *Immunol. Today* **15:** 74-80.

Bayer HS, Matta SG & Sharp BM. (1988). Regulation of the messenger ribonucleic acid for corticotrophin-releasing factor in the paraventricular nucleus and other brain sites of the rat. *Endocrinology* **123:** 2117-2123.

Beato M. (1987). Induction of transcription by steroid hormones. *Biochim. Biophys. Acta* **910:** 95-102.

Beato M. (1989). Gene regulation by steroid hormones. *Cell* **56:** 335-344.

Beato M, Chavez S & Truss M. (1996). Transcriptional regulation by steroid hormones. *Steroids* **61:** 240-251.

Becker DM, Fikes JD & Guarente L. (1991). A cDNA encoding a human CCAAT-binding protein cloned by functional complementation in yeast. *Proc. Natl. Acad. Sci. USA* **88:** 1968-1972.

Beitens IZ, Bayard F, Ances IG, Kowarski A & Migeon CJ. (1973). The metabolic clearance rate, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy near term. *Pediatr. Res.* **7:** 509-519.

Benediktsson R, Yau JLW, Low S, Brett LP, Cooke BE, Edwards CRW & Seckl JR. (1992). 11ß-hydroxysteroid dehydrogenase in the rat ovary: high expression in the oocyte. *J. Endocrinol.* **135:** 53-58.

Benediktsson R, Lindsay R, Noble J, Seckl JR & Edwards CRW. (1993). Glucocorticoid exposure in utero: a new model for adult hypertension. *Lancet* **341:** 339-341.

Ben-Or S & Okret, S. (1993). Involvement of a C/EBP-like protein in the aquisition of responsiveness to glucocorticoid hormones during chick neuronal retina development. *Mol. Cell. Biol.* **13:** 331-340.

Bird AP. (1992). The essentials of DNA methylation. *Cell* **70:** 5-8.

Bird AP. (1986). CpG-rich islands and the function of DNA methylation. *Nature* **321:** 209-213.

Bird AP. (1995). Gene number, noise reduction and biological complexity. *Trends in Genetics* **11:** 94-100.

Birkenmeier EH, Gwynn B, Howard S, Jerry J, Gordon JI, Landschulz WH & McKnight SL. (1989). Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. *Genes Dev.* **3:** 1146-1156.

Bodwell JE, Hu JM, Orti E & Munck A. (1995). Hormone induced hyperphosphorylation of specific phosphorylated sites in the mouse glucocorticoid receptor. *J. Steroid Biochem. Mol. Biol.* **52:** 135-140.

Bosch F, Sabater J & Valera A. (1995). Insulin inhibits liver expression of the CCAAT/enhancer-binding protein ß. *Diabetes* **44:** 267-71.

Bowers WJ & Ruddel A. (1992). α1/EBP: a leucine zipper protein that binds CCAAT/enhancer elements in the avain leukosis virus long terminal repeat enhancer. *J. Virol.* **66:** 6578-6586.

Boyes J & Bird A. (1992). Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein. *EMBO J.* **11:** 327-333.

Bradford M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254.

Braunstein M, Rose AB, Holmes SG, Allis CD & Broach JR. (1993). Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. *Genes Dev.* **7:** 592-604.

Breg JN, van Opheusden JHJ, Burgering MJM, Boelens R & Kaptein R. (1990). Structure of Arc repressor in solution: evidence for a family of ß-sheet DNA-binding proteins. *Nature* **346:** 586-589.

Brent GA, Harney JW, Chen Y, Warne RL, Moore DD & Larsen PR. (1989). Mutations of the rat growth hormone promoter which increase and decrease response to thyroid hormone define a consensus thyroid hormone response element. *Mol. Endocrinol.* **3:** 1996-2004.

Bretz JD, Williams SC, Baer M, Johnson PF & Schwartz RC. (1994). C/EBP-related protein 2 confers lipopolysaccharide-inducible expression of interleukin 6 and monocyte chemoattractant protein 1 to a lymphoblastic cell line. *Proc. Nat. Acad. Sci. USA* **91:** 7306-7310.

Brown RW, Chapman KE, Edwards CRW & Seckl JR. (1993). Human placental 11ß-hydroxysteroid dehydrogenase: evidence for and partial purification of a distinct NAD-dependent isoform. *Endocrinology* **132:** 2614-2621.

Brown RW, Chapman KE, Kotelevtsev Y, Yau JL, Lindsay RS, Brett L, Leckie C, Murad P, Lyons V, Mullins JJ, Edwards CRW & Seckl JR. (1996a). Cloning and production of antisera to human placental 11ß-hydroxysteroid dehydrogenase type 2. *Biochem. J.* **313:** 1007-1017.

Brown RW, Diaz R, Robson AC, Kotelevtsev YV, Mullins JJ, Kaufman MH & Seckl JR. (1996b). The ontogeny of 11ß-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development. *Endocrinology* **137:** 794-797.

Brownell JE, Zhou J, Ranalli T, Kobayashi R, Edmondson DG, Roth SY & Allis CD. (1996). Tetrahymena histone acetyltransferase A: a homolog to yeast Gin5P linking histone acetylation to gene activation.*Cell* **84:** 843-851.

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

Burgess-Beusse B & Darlington G. (1996). The study of the acute phase response in mice lacking C/EBPα. *FASEB J.* **10:** P2460 (Abstract).

Burke PA, Drotar M, Luo M, Yaffe M & Forse RA. (1994). Rapid modulation of liver-specific transcription factors after injury. *Surgery* **116:** 285-293.

Burley SK. (1994). DNA-binding motifs from eukaryotic transcription factors. *Curr. Opin. Struct. Biol.* **4:** 3-11.

Burt D, Creedy AM, Bird IM, Nicol M, Walker SW, Edwards CRW & Williams BC. (1991). Metabolism of cortisol by bovine zona fasciculata/reticularis cells-differential effects of acute stimulation on 18-hydroxylase and 11ß-hydroxysteroid dehydrogenase. *J. Endocrinol.* **129:** (suppl.) P57 (Abstract).

Calkhoven CF, Bouwman PRJ, Snippe L & Ab G. (1994). Translation start site multiplicity of the CCAAT/enhancer binding protein α mRNA is dictated by a small 5' open reading frame. *Nucleic Acids Res.* **22:** 5540-5547.

Cao Z, Umek RM & McKnight SL. (1991). Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.* **5:** 1538-1552.

Cappelletti M, Alonzi T, Fattori E, Libert C & Poli V. (1996). C/EBPß is required for the late phases of acute phase genes induction in the liver and for tumor-necrosis-factor-α, but not interleukin-6, regulation. *Cell Death and Differentiation* **3:** 29-35.

Carlson SG, Fawcett TW, Bartlett JD, Bernier M & Holbrook NJ. (1993). Regulation of the C/EBP-related gene *gadd153* by glucose deprivation. *Mol. Cell. Biol.* **13:** 4736-4744.

Carlstedt-Duke J, Okret S, O W & Gustafsson JA. (1982). Immunochemical analysis of the glucocorticoid receptor: identification of a third domain separate from the steroid-binding and DNA-binding domains. *Proc. Natl. Acad. Sci. USA* **79:** 4260-4264.

Cavailles V, Dauvois S, L'Horset F, Lopez G, Hoare S, Kushner PJ & Parker MG. (1995). Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. *EMBO J.* **14:** 3741-3751.

Cereghini S, Blumenfeld M & Yaniv M. (1988). A liver specific factor essential for albumin transcription differs between differentiated and dedifferentiated rat hepatoma cells. *Genes Dev.* **2:** 957-974.

Cereghini S. (1996). Liver-enriched transcription factors and hepatocyte differentiation. *FASEB J.* **10:** 267-282.

Chang CJ, Chen TT, Lei HY, Chen DS & Lee SC. (1990). Molecular cloning of a transcription factor, AGP/EBP, that belongs to members of the C/EBP family. *Mol. Cell. Biol.* **10:** 6642-6653.

Chen JD & Evans RM. (1995). A transcriptional repressor that interacts with nuclear hormone receptors. *Nature* **377:** 454-457.

Chen W, Manova K, Weinstein DC, Duncan SA, Plumb AS, Prezioso VR, Bachvarova RF & Darnell JE. (1994). Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. *Genes Dev.* **8:**2466-2477.

Choy B & Green MR. (1993). Eukaryotic activators function during multiple steps of preinitiation complex assembly. *Nature* **366:** 531-536.

Christy RJ, Yang VW, Ntambi JM, Geiman DE, Landschulz WH, Friedman AD, Nakabeppu Y, Kelly TJ & Lane MD. (1989). Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. *Genes Dev.* **3:** 1323-1335.

Clarkson R, Chen CM, Harrison S, Wells C, Muscat G & Waters MJ. (1995). Early responses of *trans*-activating factors to growth hormone in preadipocytes: differential regulation of CCAAT enhancer-binding protein-ß (C/EBPß) and C/EBPδ. *Mol. Endocrinol.* **9:** 108-120.

Cole TJ, Blendy JA, Monaghan P, Krieglstein K, Schmid W, Aguzzi A, Fantuzzi G, Hummler E, Unsicker K & Schutz G. (1995). Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Genes Dev.* **9:** 1608-1621.

Cole TJ. (1995). Cloning of the mouse 11ß-hydroxysteroid dehydrogenase type 2 gene: tissue specific expression and localization in distal convoluted tubules and collecting ducts of the kidney. *Endocrinology* **136:** 4693-4696.

Conaway RC & Conaway JW. (1993). General initiation factors for RNA polymerase II. *Ann. Rev. Biochem.* **62:** 161-190.

Connelly S & Manley JL. (1989). A CCAAT box sequence in the adenovirus major late promoter functions as part of an RNA polymerase II termination signal. *Cell* **57:** 561-571.

Cooper C, Johnson D, Roman C, Avitahl N, Tucker P & Calame K. (1992). The C/EBP family of transcriptional activators is functionally important for Ig V_H promoter activity *in vivo* and *in vitro. J. Immunol.* **149:** 3225-3231.

Costa RH, Grayson DR & Darnell JE. (1989). Multiple hepatocyte-enriched nuclear factors function in the regulation of transthyretin and α_1-antitrypsin genes. *Mol. Cell. Biol.* **9:** 1415-1425.

Courtios G, Morgan JG, Campbell LA, Fourel G & Crabtree GR. (1987). Interaction of a liver-specific nuclear factor with the fibrinogen and α_1-antitrypsin promoters. *Science* **238:** 688-692.

Crossley M & Brownlee GG. (1990). Disruption of a C/EBP binding site in the factor IX promoter is associated with haemophilia B. *Nature* **345:** 444-446.

Cryer PE. (1992). Glucose homeostasis and hypoglycemia. *Williams Textbook of Endocrinology* (eds Wilson JD & Foster DW., 8th Edition). 1223-1255.*WD Saunders Company, Philedelphia PA..*

Cupps TR, Gerrard TL, Falkoff RJM, Whalen G & Fauci AS. (1985). Effects of *in vitro* corticosteroids on B cell activation, proliferation and differentiation. *J. Clin. Invest.* **75:** 754-761.

Dalman FC, Sanchez ER, Lin ALY, Perini F & Pratt WB. (1988). Localisation of phosphorylation sites with respect to functional domains of the mouse L Cell glucocorticoid receptor. *J. Biol. Chem.* **263:** 12259-12267.

Dalman FC, Koenig RJ, Perdew GH, Massa E & Pratt WB. (1990). In contrast to the glucocorticoid receptor, the thyroid hormone receptor is translated in the DNA binding state and is not associated with hsp90. *J. Biol. Chem.* **265:** 3615-3618.

Dalman FC, Sturzenbecker LC, Levin AA, Lucas DA, Perdew GH, Petkovitch M, Chambon P, Grippo JF & Pratt WB. (1991). Retinoic acid receptor belongs to a subclass of nuclear receptors that do not form "docking" complexes with hsp90. *Biochemistry* **30:** 5605-5608.

Darlington GJ, Wang N & Hanson RW. (1995). C/EBPα: a critical regulator of genes governing integrative metabolic processes. *Curr. Opin. Genet. Dev.* **5:** 565-570.

Davis LG, Arentzen R, Reid JM, Manning RW, Wolfson B, Lawrence KL & Baldino F. (1986). Glucocorticoid sensitvity of vasopressin mRNA levels in the paraventricular nucleus of the rat. *Proc. Natl. Acad. Sci. USA* **83:** 1145-1149.

Davydov IV, Bohmann D, Krammer PH & Li-Weber M. (1995). Cloning of the cDNA encoding human C/EBPγ, a protein binding to the *PRE-I* enhancer element of the human interleukin-4 promoter. *Gene* **161:** 271-275.

Defranco D, Madan AP, Tang YT, Chandran UR, Xaio NT & Yang J. (1995). Nucleocytoplasmic shuttling of steroid receptors.*Vitamins and Hormones* **51:** 315-338.

Derman E, Krauter K, Walling L, Weinberger C, Ray M & Darnell JE. (1981). Transcriptional control in the production of liver-specific mRNA. *Cell* **23:** 731-739.

Deschatrette J, Moore EE, M. D & Weiss MC. (1980). Dedifferentiated varients of a rat hepatoma: reversion analysis. *Cell* **19:** 1043-1051.

Descombes P, Chojkier M, Lichtsteiner S, Falvey E & Schibler U. (1990). LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. *Genes Dev.* **4:** 1541-1551.

Descombes P & Schibler U. (1991). A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell* **67:** 569-579.

d'Escobar GM, Calvo R, Delrey FE & Obregon MJ. (1993). Differential effects of thyroid hormones on growth and thyrotropic hormones in rat fetuses near term. *Endocrinology* **132:** 2056-2064.

Diehl AM, Michaelson P & Yang SQ. (1994a). Selective induction of CCAAT/enhancer binding protein isoforms occurs during rat liver development. *Gastroenterology* **106:** 1625-1637.

Diehl JA & Hannink M. (1994b). Identification of a C/EBP-Rel complex in avian lymphoid cells. *Mol. Cell. Biol.* **14:** 6635-6646.

Dignam JD, Lebovitz RM & Roeder RG. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian cells. *Nucleic Acids Res.* **11:** 1475-1489.

Domalik LJ, Chaplin DD, Kirkman MS, Wu RC, Liu WW, Howard TA, Seldin MF & Parker KL. (1991). Different isozymes of mouse 11ß-hydroxylase produce mineralocorticoids and glucocorticoids. *Mol. Endocrinol.* **5:** 1853-1861.

Drapkin R, Reardon JT, Ansari A, Huang JC, Zawel L, Ahn K, Sancar A & Reinberg D. (1994). Dual role of TFIIH in DNA excision repair and in transcription by RNA polymerase II. *Nature* **368:** 769-772.

Du H, Roy AL & Roeder RG. (1993). Human transcription factor USF stimulates transcription through initiator elements of the HIV-1 and the Ad-ML promoters. *EMBO J.* **12:** 501-511.

Dunn JF, Nisula BC & Robard D. (1981). Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid binding globulin in human plasma. *J. Clin. Endocrinol. Metab.* **53:** 58-68.

Duperrex H, Kenouch S, Gaeggeler HP, Seckl JR, Edwards CRW, Farman N & Rossier BC. (1993). Rat liver 11ß-hydroxysteroid dehydrogenase complementary deoxyribonucleic acid encodes oxoreductase activity in a mineralocorticoid-responsive toad bladder cell line. *Endocrinology* **132:** 612-619.

Dynlacht BD, Hoey T & Tjian R. (1991). Isolation of coactivators associated with the TATA-binding protein that mediate transcriptional activation. *Cell* **66:** 563-576.

Ellenberger TE, Brandl CJ, Struhl K & Harrison SC. (1992). The GCN4 basic region leucine zipper binds DNA as a dimer of uninterupted α helices: crystal structure of the protein DNA complex. *Cell* **71:** 1223-1237.

Emili A, Greenblatt J & Ingles CJ. (1994). Species specific interaction of the glutamine rich activation domain with the TATA-box binding protein, TBP. *Mol. Cell. Biol.* **14:** 1582-1593.

Eriksson P & Wrange O. (1990). Protein-protein contacts in the glucocorticoid receptor homodimer influence its DNA binding properties. *J. Biol. Chem.* **265:** 3535-3542.

Evans RM. (1988). The steroid and thyroid hormone receptor superfamily. *Science* **240:** 889-895.

Exton JH. (1979). Regulation of gluconeogenesis by glucocorticoids. *In Glucocorticoid Hormone Action* (eds Baxter JD & Rousseau GG) pp 449-465. Springer-Verlag, NY.

Fain JH. (1979). Inhibition of glucose transport in fat cells and activation of lipolysis by glucocorticoids. In *Glucocorticoid Hormone Action* (eds Baxter JD & Rousseau GG) pp547-560.Springer Verlag, NY.

Federoff HJ, Grabczyk E & Fishman MC. (1988). Dual regulation of GAP-43 gene expression by nerve growth factor and glucocorticoids. *J. Biol. Chem.* **263:** 19290-19295.

Ferre-D'Amare AR, Prendergast GC, Ziff EB & Burley SK. (1993). Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain. *Nature* **363:** 38-45.

Flodby P, Antonson P, Barlow C, Blanck A, Porsch HI & Xanthopoulos KG. (1993). Differential patterns of expression of three C/EBP isoforms, HNF-1, and HNF-4 after partial hepatectomy in rats. *Exp. Cell Res.* **208:** 248-256.

Forman BM, Tontonoz P, Chen J, Brun P, Spiegelman BM & Evans RM. (1995). 15 deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. *Cell* **83:** 803-812.

Fornace AJ, Nebert DW, Hollander MC, Luethy JD, Papathanasiou M, Fargnoli J & Holbrook NJ. (1989). Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. *Mol. Cell. Biol.* **9:** 4196-4203.

Freedman LP, Luisi BF, Korszun ZR, Basavappa R, Sigler PB & Yamamoto KR. (1988). The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature* **334:** 543-546.

Freedman LP & Luisi BF. (1993). On the mechanism of DNA binding by nuclear hormone receptors: a structural and functional perspective. *J. Cell. Biochem.* **51:** 140-150.

Freytag SO & Geddes TJ. (1992). Reciprocal regulation of adipogenesis by Myc and C/EBPα. *Science* **256:** 379-382.

Friedman JM, Chung EY & Darnell JE. (1984). Gene expression during liver regeneration. *J. Mol. Biol.* **179:** 37-53.

Friedman AD, Landschulz WH & Mcknight SL. (1989). CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. *Genes Dev.* **3:** 1314-1322.

Galas DJ & Schmitz A. (1978). DNase footprinting: a simple method for the detection of protein-DNA binding specificity. *Nucleic Acids Res.* **5:** 3157-3170.

Gao HB, Shan LX, Monder C & Hardy MP. (1996). Suppression of endogenous corticosterone levels *in vivo* increases the steroidogenic capacity of purified rat leydig cells *in vitro*. *Endocrinology* 1714-1718.

Garcia-Blanco MA, Clerc RG & Sharp PA. (1989). The DNA-binding homeodomain of the Oct-2 protein. *Genes Dev.* **3:** 739-745.

Garcia-Ramirez M, Rocchini C & Ausio J. (1995). Modulation of chromatin folding by histone acetylation. *J. Biol. Chem.* **270:** 17923-17928.

Garland RC. (1986). Induction of glucose 6-phosphatase in cultured hepatoma cells by dexamethasone. *Biochem. Biophys. Res. Comm.* **139:** 1130-1134.

Ge H & Roeder RG. (1994). Purifiaction, cloning and characterisation of a human coactivator, PC4, that mediates transcriptional activation of class II genes. *Cell* **78:** 513-523.

Gearing KL, Gottlicher M, Teboul M, Widmark E & Gustafsson JA. (1993). Interaction of the peroxisome-proliferator activated receptor and retinoid X receptor. *Proc. Natl. Acad. Sci. USA* **90:** 1440-1444.

Gehring U & Arndt H. (1985). Heteromeric nature of glucocorticoid receptors. *FEBS Lett.* **179:** 138-142.

Gibbons GH, Dzau VG, Farhl ER & Barger AC. (1980). Interaction of signals influencing renin release. *Ann. Rev. Physiol.* **46:** 291-308.

Gillis S, Crabtree GR & Smith KA. (1979). Glucocorticoid-induced inhibition of T cell growth factor production. I. The effect on mitogen-induced lymphocyte proliferation. *J. Immunol.* **123:** 1624-1631.

Glass CK, DiRenzo J, Kurokawa R & Han ZH. (1991). Regulation of gene expression by retinoic acid receptors. *DNA and Cell Biol.* **10:** 623-638.

Glass CK. (1994). Differential recognition of target genes by nuclear receptor monomers, dimers and heterodimers. *Endocrine Rev.* **15:** 391-407

Godowski PJ, Rusconi S, Miesfeld R & Yamamoto KR. (1987). Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. *Nature* **325:** 365-368.

Goodfriend TL, Gibbons GH, Dzau VJ, Farhl ER & Barger AC. (1980). Interaction of signals influencing renin release. *Ann. Rev. Physiol.* **46:** 291-308.

Goodrich JA & Tjian R. (1994a). TBP-TAF complexes: selectivity factors for eukaryotic transcritpion. *Curr. Op. Cell Biol.* **6:** 403-409.

Goodrich JA & Tjian R. (1994b). Transcription factors IIE and IIH and ATP hydrolysis direct promoter clearance by RNA polymerase II. *Cell* **77:** 145-156.

Graham BS & Tucker WS. (1984). Opportunistic infections in endogenous Cushing's syndrome. *Ann. Intern. Med.* **101:** 334-338.

Granner DK, Andreone T, Sasaki K & Beale E. (1983). Inhibition of transcription of the phosphoenolpyruvate carboxykinase gene by insulin. *Nature* **305:** 549-551.

Graves BJ, Johnson PF & Mcknight SL. (1986). Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. *Cell* **44:** 565-576.

Green S, Kumar V, Theulaz I, Whali W & Chambon P. (1988). The N-terminal DNA-binding 'zinc finger' of the oestrogen and glucocorticoid receptors determines target gene specificity. *EMBO J.* **7:** 3037-3044.

Green H & Kehinde O. (1974). Sublines of mouse 3T3 cells that accumulate lipid. *Cell* **1:** 113-116.

Green H & Kehinde O. (1975). An established preadipose cell line and its differentiation in culture. *Cell* **5:** 19-27.

Guerra C, Benito M & Fernandez M. (1994). IGF-I induces the uncoupling protein gene expression in fetal rat brown adipocyte primary cultures: role of C/EBP transcription factors. *Biochem. Biophys. Res. Comm.* **201:** 813-819.

Gurney AL, Park EA, Giralt M, Liu J & Hanson RW. (1992). Opposing actions of Fos and Jun on transcription of the phosphoenolpyruvate carboxykinase (GTP) gene. *J. Biol. Chem.* **267:** 18133-18139.

Guyton AC. (1986). Metabolism and temperature regulation. *In 'Textbook of Medical Physiology' 7th Edition.* pp808-871. WB Saunders Co. Philadelphia.

Ha I, Roberts S, Maldonado E, Sun X, Kim LU, Green M & Reinberg D. (1993). Multiple functional domains of human transcription factor IIB: distinct interactions with two general transcription factors and RNA-polymerase II. *Genes Dev.* **7:** 1021-1032.

Hahn TJ, Halstead LR, Teitelbaum SL & Hahn BH. (1979). Altered mineral metabolism in glucocorticoid-induced osteopenia. Effect of 25-hydroxyvitamin D administration. *J. Clin. Invest.* **64:** 655-665.

Hall RK, Scott DK, Noisin EL, Lucas PC & Granner DK. (1992). Activation of phosphoenolpyruvate carboxykinse gene retinoic acid response is dependent on a retinoic acid receptor/coregulator complex. *Mol. Cell. Biol.* **12:** 5527-5535.

Hamada H & Kakunaga T. (1982). Potential Z-DNA forming sequences are highly distributed in the human geneome. *Nature* **298:** 296-398.

Hammami MM & Siiteri PK. (1991). Regulation of 11ß-hydroxysteroid dehydrogenase activity in human skin fibroblasts: enzymatic modulation of glucocorticoid action. *J. Clin. Endocrinol. Metab.* **73:** 326-334.

Hard T, Kellenbach E, Boelens R, Maler BA, Dahlman K, Freedman LP, Carlstedt-Duke J, Yamamoto KR & Gustafsson JA. (1990). Solution structure of the glucocorticoid receptor DNA-binding domain. *Science* **249:** 157-160.

Hardon E, Frain M, Paonessa G & Cortese R. (1988). Two distinct factors interact with the promoter regions of several liver-specific genes. *EMBO J.* **7:** 1711-1719.

Haas NB, Cantwell CA, Johnson PF & Burch JB. (1995). DNA-binding specificity of the PAR basic leucine zipper protein VBP partially overlaps those of the C/EBP and CREB/ATF families and is influenced by domains that flank the core basic region. *Mol. Cell. Biol.* **15:** 1923-1932.

Hebbes TR, Clayton AL, Thorne AW & Crane-Robinson C. (1994). A direct link between core histone acetylation and transcriptionally active chromatin. *EMBO J.* **7:** 1395-1402.

Hernandez N. (1993). TBP, a universal eukaryotic transcription factor. *Genes Dev.* **7:** 1291-1308.

Hochschild A & Ptashne M. (1986). Cooperative binding of λ repressors to sites separated by integral turns of the DNA helix. *Cell* **44:** 681-687.

Hoey T, Weinzierl ROJ, Gill G, Chen JK, Dynlacht BD & Tjian R. (1993). Molecular cloning and functional analysis of Drosophila TAF_{110} reveal properties expected of coactivators. *Cell* **72:** 247-260.

Hoffmann A, Chiang CM, Oelgeschlager T, Xie XL, Burley SK, Nakatani Y & Roeder RG. (1996). A histone octamer-like structure within TFIID. *Nature* **380:** 356-359.

Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, Thompson EB, Rosenfeld MG & Evans RM. (1985). Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature* **318:** 635-641.

Hong SC & Levine L. (1976). Inhibition of arachadonic acid release from cells as the biochemical action of anti-inflammatory corticosteroids. *Proc. Natl. Acad. Sci. USA* **73:** 1730-1734.

Horlein AJ, Naar AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Soderstrom M, Glass CK & Rosenfeld MG. (1995). Ligand-independent response by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature* **377:** 397-404.

Hsu W, Kerppola TK, Chen PL, Curran T & Chen-kiang S. (1994). Fos and jun repress transcription activation by NF-IL6 through association at the basic zipper region. *Mol. Cell. Biol.* **14:** 268-276.

Hu JM, Bodwell JE & Munck A. (1994). Cell cycle-dependent glucocorticoid receptor phosphorylation and activity. *Mol. Endocrinol.* **8:** 1709-1713.

Iami I, Stromstedt P, Quinn PG, Carlstedt-Duke J, Gustafsson JA & Granner DK. (1990). Characterisation of a complex glucocorticoid response unit in the phosphoenolpyruvate carboxykinase gene. *Mol. Cell. Biol.* **10:** 4712-4719.

Ingles CJ, Shales M, Cress WD, Triezenberg SJ & Greenblatt J. (1991). Reduced binding of TFIID to transcriptionally compromised mutants of VP16. *Nature* **351:** 588-590.

Inostroza J, Flores O & Reinberg D. (1991). Factors involved in specific transcription by mammalian RNA polymerase II. *J. Biol. Chem.* **266:** 9304-9308.

Isseman I & Green S. (1990). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* **347:** 645-650.

Jamieson PM, Chapman KE, Edwards CRW & Seckl JR. (1995). 11ß-hydroxysteroid dehydrogenase is an exclusive 11ß- reductase in primary cultures of rat hepatocytes: effect of physiochemical and hormonal manipulations. *Endocrinology* **136:** 4754-4761.

Jamieson PM, Chapman KE, Walker BR & Seckl JR. (1996). 11ß-Hydroxysteroid dehydrogenase type 1 and the control of liver specific glucocorticoid-inducible genes. *10th International Congress of Endocrinology*: P1-232 (Abstract).

Jeffcoate WJ, Silverstone JT, Edwards CRW & Besser GM. (1979). Psychiatric manifestations of Cushings syndrome: response to lowering of plasma cortisol. *Q. J. Med.* **48:** 465-472.

Jensen EV, Suzuki T, Kawashima T, Stumpf WE, Jungblut PW & DeSombre ER. (1968). A two step mechanism for the interaction of estradiol with rat uterus. *Proc. Natl. Acad. Sci. USA* **59:** 632-638.

Jensen EV. (1990). Molecular mechanisms of steroid hormone action in the uterus. In 'Uterine function: molecular and cellular aspects' (eds Carlson ME & Miller JD) Plenum Publishing Corp., NY.

Jenster G, Trapman J & Brinkmann AO. (1993). Nuclear import of the human androgen receptor. *Biochem. J.* **293:** 761-768.

Jewell CM, Webster JC, Burnstein KL, Sar M, Bodwell JE & Cidlowski JA. (1995). Immunocytochemical analysis of hormone mediated nuclear translocation of wild type and mutant glucocorticoid receptors. *J. Steroid Biochem. Mol. Biol.* **55:** 135-146.

Johansson S & Andersson G. (1990). Similar induction of the hepatic EGF receptor *in vivo* by EGF and partial hepatectomy. *Biochem. Biophys. Res. Comm.* **166:** 661-666.

Johnson PF, Landschulz WH, Graves BJ & McKnight SL. (1987). Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. *Genes Dev.* **1:** 133-146.

Kaestner KH, Christy RJ, McLenithan JC, Braiterman LT, Cornelius P, Pekala PH & Lane MD. (1989). Sequence, tissue distribution, and differential expression of mRNA for a putative insulin-responsive glucose transporter in mouse 3T3-LI adipocytes. *Proc. Natl. Acad. Sci. USA* **86:** 3150-3154.

Kaestner KH, Christy RJ & Lane MD. (1990). Mouse insulin-responsive glucose transporter gene: characterisation of the gene and *trans*-activation by the CCAAT enhancer binding protein. *Proc. Natl. Acad. Sci. USA* **87:** 251-255.

Kageyama R, Sasai Y & Nakanishi S. (1991). Molecular characterisation of transcription factors that bind to the cAMP responsive region of the substance P precurser gene: cDNA cloning of a novel C/EBP related factor. *J. Biol. Chem.* **266:** 15525-15531.

Katz S, Kowenz-Leutz E, Muller C, Meese K, Ness SA & Leutz A. (1993). The NF-M transcription factor is related to C/EBPß and plays a role in signal transduction, differentiation and leukemogenesis of avian myelomonocytic cells. *EMBO J.* **12:** 1321-1332.

Kaufmann J & Smale ST. (1994). Direct recognition of initiator elements by a component of the transcription factor IID complex. *Genes Dev.* **8:** 821-829.

Kerr LD, Ransone LJ, Wamsley P, Schmitt MJ, Boyer TG, Zhou Q, Berk AJ & Verma IM. (1993). Association between proto-oncoprotein Rel and TATA-binding protein mediates transcriptoinal activation by NF-κB. *Nature* **365:** 412-419.

Kinoshita S, Akira S & Kishimoto T. (1992). A member of the C/EBP family, NF-IL6ß, forms a heterodimer and transcriptionally synergizes with NF-IL6. *Proc. Natl. Acad. Sci. USA* **89:** 1473-1476.

Kissinger CR, Liu BS, Martin-Blanco E, Kornberg TB & Pabo CO. (1990). Crystal structure of an engrailed homeodomain-DNA complex at 2.8Å resolution: A framework for understanding homeodomain-DNA interactions. *Cell* **63:** 579-590.

Klein C & Struhl K. (1994). Increased recruitment of TATA-binding protein to the promoter by transcriptional activation domains *in vivo*. *Science* **266:** 280-282.

Klemm JD, Rould MA, Aurora R, Herr W & Pabo CO. (1994). Crystal structure of the Oct-1 POU domain bound to an octamer site: DNA recognition with tethered DNA-binding modules. *Cell* **77:** 21-32.

Kliewer SA, Umesono K, Mangelsdorf DJ & Evans RM. (1992a). Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D_3 signalling. *Nature* **355:** 446-449.

Kliewer SA, Umensono K, Noonan DJ, Heymann RA & Evans RM. (1992b). Convergence of 9-*cis* retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation and their receptors. *Nature* **358:** 771-774.

Klug A & Schwabe JWR. (1995). Zinc fingers. *FASAB J.* **9:** 597-604.

Knegtel RM, Katahira M, Schilthuis JG, Bonvin AM, Boelens R, Eib D, van der Saag PT & Kaptein R. (1993). The solution structure of the human retinoic acid receptorß DNA-binding domain. *J. Biomol. NMR* **3:** 1-17.

Knowles BB, Howe CC & Aden DP. (1980). Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science* **209:** 497-499.

Kobayashi N, Boyer TG & Berk AJ. (1995). A class of activation domains interacts directly with TFIIA and stimulates TFIIA-TFIID-promoter complex assembly. *Mol. Cell. Biol.* **15:** 6465-6473.

Koelle MR, Talbot WS, Segraves WA, Bender MT, Cherbas P & Hogness DS. (1991). The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* **67:** 59-77.

Koerner DR & Hellman L. (1964). Effect of thyroxine adminstration on the 11ß-hydroxysteroid dehydrogenases in rat liver and kidney. *Endocrinology* **75:** 592-601.

Koleske AJ & Young RA. (1994). An RNA polymerase II holoenzyme responsive to activators. *Nature* **368:** 466-469.

Konig P & Richmond TJ. (1993). The X-ray structure of the GCN4 bzip bound to ATF/CREB site DNA shows the complex depends on DNA flexability. *J. Mol. Biol.* **233:** 139-154.

Kornberg RD & Lorch Y. (1995). Interplay between chromatin structure and transcription. *Curr. Opin. Cell Biol.* **7:** 371-375.

Kotelevtsev YV, Jamieson PM, Best R, Stewart F, Edwards CRW, Seckl JR & Mullins JJ. (1996a). Inactivation of 11ß-hydroxysteroid dehydrogenase by gene targeting in mice. *Seventh Conference on the Adrenal Cortex* . P40 (Abstract).

Kotelevtsev YV, Jamieson PM, Edwards CRW, Seckl JR & Mullins JJ. (1996b). Gene targeting of 11ß-hydroxysteroid dehydrogenase. *10th International Congress of Endocrinology* OR52-1 (Abstract).

Kovesdi I, Reichel R & Nevins J. (1986). Identification of a cellular transcription factor involved in E1A *trans*-activation. *Cell* **45:** 219-228.

Kowenz-Leutz E, Twamley G, Ansieau S, Leutz A. (1994). Novel mechanism of C/EBPß (NF-M) transcriptional control: activation through derepression. *Genes Dev.* **8:** 2781-2791.

Krakoff L, Nicolis G & Amsel B. (1975). Pathogenesis of hypertension in Cushing's Syndrome. *Am. J. Med.* **58:** 216-220.

Kralli A, Bohen SP & Yamamoto KR. (1995). LEM, an ATP-binding-cassette transporter, selectively modulates the biological potency of steroid hormones. *Proc. Natl. Acad. Sci. USA* **92:** 4701-4705.

Krozowski Z, Stuchbery S, White P, Monder C & Funder JW. (1990). Characterization of 11ß-hydroxysteroid dehydrogenase gene expression: identification of multiple unique forms of messenger ribonucleic acid in the rat kidney. *Endocrinology* **127:** 3009-3013.

Krust A, Green S, Argos P, Kumar V, Walter P, Bornert JM & Chambon P. (1986). The chicken oestrogen receptor sequence: homology with *v-erbA* and the human oestrogen and glucocorticoid receptors. *EMBO J.* **5:** 891-897.

Krust A, Kastner P, Petkovich M, Zelnet A & Chambon P. (1989). A third human retinoic acid receptor, hRAR-γ. *Proc. Acad. Natl. Sci. USA* **86:** 5310-5314.

Kuo CK, Xanthopoulos KG & Darnell JE. (1990). Fetal and adult localization of C/EBP: Evidence for combinatorial action of transcription factors in cell-specific gene expression. *Development* **109:** 473-481.

Kurokawa R, Soderstrom M, Horlein A, Halachmi S, Brown M, Rosenfeld MG & Glass CK. (1995). Polarity-specific activities of retinolic acid receptors determined by a co-repressor. *Nature* **377:** 451-454.

Lai E, Prezioso VR, Tao WF, Chen WS & Darnell JE. (1991). Hepatocyte nuclear factor 3α belongs to a gene family in mammals that is homologous to the *Drosophila* homeotic gene *fork head. Genes Dev.* **5:** 416-427.

Lakshmi V & Monder C. (1988). Purification and characterization of the corticosteroid 11ß-dehydrogenase component of the rat liver 11ß-hydroxysteroid dehydrogenase complex. *Endocrinology* **123:** 2390-2398.

Lala DS, Rice DA & Parker KL. (1992). Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of *fushi tarazu* factor I. *Mol. Endocrinol.* **6:** 1249-1258.

Landschultz WH, Johnson PF, Adashi EY, Graves BJ & McKnight SL. (1988). Isolation of a recombinant copy of the gene encoding C/EBP. *Genes Dev.* **2:** 786-800.

Landschulz WH, Johnson PF & McKnight SL. (1989). The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. *Science* **243:** 1681-1688.

Laudet V, Hanni C, Coll J, Catzelis F & Stehlin D. (1992). Evolution of the nuclear receptor gene superfamily. *EMBO J.* **11:** 1003-1013.

Laudet V & Adelmant G. (1995). Nuclear receptors-lonesome orphans. *Curr. Biol.* **5:** 124-127.

Lax ER, Ghraf R & Schriefers H. (1978). The hormonal regulation of hepatic microsomal 11ß-hydroxysteroid dehyrogenase activity in the rat. *Acta Endocrinol.* **89:** 352-357.

Leckie CM & Seckl JR. (1996). Rat testicular 11ß-hydroxysteroid dehydrogenase and Leydig cell function. *10th International Congress of Endocrinology* P2-541 (Abstract).

Le Dovarin B, Zechel C/EBP, Garnier JM, Lutz Y, Tora L, Pierrat B, Heery D, Gronemeyer H, Chambon P & Losson, R. (1995). The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein TI8. *EMBO J.* **14:** 2020-2033.

Lee MS, Kliewer SA, Provencal J, Wright PE & Evans RM. (1993). Structure of the retinoid X receptor α DNA binding domain: a helix required for homodimeric DNA binding. *Science* **260:** 1117-1121.

Lee S & Hahn S. (1995). Model for binding of transcipriton factor TFIIB to the TBP-DNA complex. *Nature* **376:** 609-612.

Lee WS, Kao CC, Bryant GO, Liu X & Berk AJ. (1991). Adenovirus E1A activation domain binds the basic repeat in the TATA box transcirption factor. *Cell* **67:** 365-376.

Lee YH, Yano M, Liu SY, Matsunaga E, Johnson PF & Gonzalez FJ. (1994). A novel *cis*-acting element controlling the rat *CYP2D5* gene and requiring cooperativity between C/EBPß and an Sp1 factor. *Mol. Cell. Biol.* **14:** 1383-1394.

Leid M, Kastner P, Lyons R, Nakshatri H, Saunders M, Zacharewski T, Chen YY, Staub A, Garnier JM, Mader S & Chambon P. (1992). Purification, cloning and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* **68:** 377-395.

Lemaigre FP & Rousseau GG. (1994). Transcriptional control of genes that regulate glycolysis and gluconeogenesis in adult liver. *Biochem. J.* **303:** 1-14.

Li KXZ, Smith RE, Ferrari P, Funder JW & Krozowski ZS. (1996). Rat 11ß-hydroxysteroid dehydrogenase type-2 enzyme is expressed at low levels in the placenta and is modulated by adrenal steroids in the kidney. *Mol. Cell. Endocrinol.* 67-75.

Liao SM, Zhang J, Jeffery DA, Koleske AJ, Thompson CM, Chao DM, Viljoen M, van Vuuren HJ & Young RA. (1995). A kinase-cyclin pair in RNA polymerase II holoenzyme. *Nature* **374:** 193-196.

Lieberman PM & Berk AJ. (1991). The Zta *trans*-activator protein stabilizes TFIID association with promoter DNA by direct protein-protein interactions. *Genes Dev.* **5:** 2441-2254.

Lieberman PM & Berk AJ. (1994). A mechanism for TAFs in transcriptional activation: activation domain enhancement of TFIID-TFIIA-promoter DNA complex formation. *Genes Dev.* **8:** 995-1006.

Lin FT & Lane MD. (1992). Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes. *Genes Dev.* **6:** 533-544.

Lin FT, MacDougald OA, Diehl AM & Lane MD. (1993). A 30-kDa alternative translation product of the CCAAT/enhancer binding protein α message: transcriptional activator lacking antimitotic activity. *Proc. Natl. Acad. Sci. USA* **90:** 9606-9610.

Lin YS & Green MR. (1991). Mechanisms of action of an acidic transcriptional activator *in vitro*. *Cell* **64:** 971-981.

Lin YS, Ha I, Maldonado E, Reinberg D & Green MR. (1991). Binding of general transcription factor TFIIB to an acidic activating region. *Nature* **353:** 569-571.

Locker, J. (1993). Transcription controls: cis-elements and trans-factors. In 'Gene transcription. A practical approach' (Eds Hames BD & Higgins SJ)**:** A3

Low SC, Assaad SN, Rajan V, Chapman KE, Edwards CRW & Seckl JR. (1993). Regulation of 11ß-hydroxysteroid dehydrogenase by sex steroids *in vivo*: further evidence for the existence of a second dehydrogenase in rat kidney. *J. Endocrinol.* **139:** 27-35.

Low SC, Chapman KE, Edwards CRW & Seckl JR. (1994a). 'Liver-type' 11ß-hydroxysteroid dehydrogenase cDNA encodes reductase but not dehydrogenase activity in intact mammalian COS-7 cells. *J. Mol. Endocrinol.* **13:** 167-174.

Low SC, Chapman KE, Edwards CRW, Wells T, Robinson ICAF & Seckl JR. (1994b). Sexual dimorphism of hepatic 11ß-hydroxysteroid dehydrogenase in the rat: the role of growth-hormone patterns. *J. Endocrinol.* **143:** 541-548.

Low SC, Moisan MP, Noble JM, Edwards CRW & Seckl JR. (1994c). Glucocorticoids regulate hippocampal 11ß-hydroxysteroid dehydrogenase activity and gene expression *in vivo* in the rat. *J. Neuroendocrinol.* **6:** 285-290.

Lu H, Zawel L, Fischer L, Egly JM & Reinberg D. (1992). Human general transcription factor IIH phosphorylates the C-teminal domain of RNA polymerase II. *Nature* **358:** 641-645.

Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR & Sigler PB. (1991). Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* **352:** 497-505.

Lundbland JR & Roberts JL. (1988). Regulation of the proopiomelanocortin gene expression in pituitary. *Endocrine Rev* **9:** 135-158.

Lundgren M, Larsson C, Femino A, Xu M, Stavnezer J & Severinson E. (1994). Activation of the Ig germ-line γ1 promoter: Involvement of C/enhancer- binding protein transcription factors and their possible interaction with an NF-IL-4 site. *J. Immunol.* **153:** 2983-2995.

Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Shyamala G, Conneely OM & O'Malley BW. (1995). Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev.* **9:** 2266-2278.

MacDougald OA, Cornelius P, Lin FT, Chen SS & Lane MD. (1994). Glucocorticoids reciprocally regulate expression of the CCAAT/enhancer-binding protein α and δ genes in 3T3-L1 adipocytes and white adipose tissue. *J. Biol. Chem.* **269:** 19041-19047.

MacDougald OA, Cornelius P, Liu R & Lane MD. (1995). Insulin regulates transcription of the CCAAT/enhancer binding-protein (C/EBP) α, ß, and δ genes in fully-differentiate 3T3-L1 adipocytes. *J. Biol. Chem.* **270:** 647-654.

MacDougald OA & Lane MD. (1995). Transcriptional regulation of gene expression during adipocyte differentiation. *Ann. Rev. Biochem.* **64:** 345-373.

Magnuson MA, Quinn PG & Granner DK. (1987). Multihormonal regulation of phosphoenolpyruvate carboxykinase-chloramphenicol acetyltransferase fusion genes. *J. Biol. Chem.* **262:** 14917-14920.

Mahoney CW, Shuman J, Mcknight SL, Chen HC & Huang KP. (1992). Phosphorylation of CCAAT-enhancer binding protein by protein kinase C attenuates site-selective DNA binding. *J. Biol. Chem.* **267:** 19396-19403.

Maity SN, Goulmbek PT, Karsenty G & de Crombrugghe B. (1988). Selective activation of transcription by a novel CCAAT factor. *Science* **241:** 582-585.

Malawista SE & Weiss MC. (1974). Expression of differentiated functions in hepatoma cell hybrids: high frequency of induction of mouse albumin production in rat hepatoma-mouse lymphoblast hybrids. *Proc. Natl. Acad. Sci. USA* **71:** 927-931.

Mantero F, Tedde R, Opocher G, Fulgheri PD, Arnaldi G & Ulick S. (1994). Apparent mineralocorticoid excess Type II. *Steroids* **59:** 80-83.

Marks MS, Hallenbeck PL, Nagata T, Segars JH, Appela E, Nikodem VM & Ozato K. (1992). H-2RIIBP (RXRß) heterodimerisation provides a mechanism for combinational diversity in the regulation of retinoic acid and thyroid-hormone responsive genes. *EMBO J.* **11:** 1419-1435.

Matsuno F, Chowdhury S, Gotog T, Iwase K, Matsuzaki H, Takatsuki K, Mori M & Takiguchi M. (1996a). Induction of the C/EBPß gene by dexamethasone and glucagon in primary-cultured rat hepatocytes. *J. Biochem.* **119:** 524-532.

Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto T & Akira S. (1993). Transcription factors NF-IL6 and NF-κB synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc. Natl. Acad. Sci. USA* **90:** 10193-10197.

Maurer RA & Notides AC. (1987). Identification of an estrogen-responsive element from the 5'-flanking region of the rat prolactin gene. *Mol. Cell. Biol.* **7:** 4247-4254.

McEwen BS, Lambdin LT, Rainbow TC & De Nicola AF. (1986). Aldosterone effects on salt appetite in adrenalectomised rats. *Neuroendocrinol.* **43:** 38-43.

McKeon C & Pham T. (1991). Transactivation of the human insulin receptor gene by the CAAT enhancer binding protein. *Biochem. Biophys. Res. Comm.* **174:** 721-728.

McKnight SL, Lane MD & Gluecksohn-Waelsch S. (1989). Is CCAAT/enhancer-binding protein a central regulator of energy metabolism? *Genes Dev.* **3:** 2021-2024.

McKnight SL. (1993). Discovery of a transcription factor that catalyses terminal cell differentiation. *The Harvey Lectures.* **87:** 57-68.

Means AL & Farnham PJ. (1990). Transcription initiation from the dihydrofolate reductase promoter is positioned by HIP1 binding at the initiation site. *Mol. Cell. Biol.* **10:** 653-661.

Metzger S, Halaas JL, Breslow JL & Sladek FM. (1993). Orphan receptor HNF-4 and bZip protein C/EBPα bind to overlapping regions of the apolipoprotein B gene promoter and synergistically activate transcription. *J. Biol. Chem.* **268:** 16831-16838.

Michael AE, Pester LA, Curtis P, Shaw RW, Edwards CRW & Cooke BA. (1993a). Direct inhibition of ovarian steroidogenesis by cortisol and the modulatory role of 11ß-hydroxysteroid dehydrogenase. *Clin. Endocrinol.* **38:** 641-644.

Michael AE, Gregory L, Walker SM, Antoniw JW, Shaw RW, Edwards CRW & Cooke BA. (1993b). Ovarian 11ß-hydroxysteroid dehydrogenase: potential predictor of conception by in-vitro fertilization and embryo transfer. *Lancet* **342:** 711-712.

Michalopoulos GK. (1990). Liver regeneration: molecular mechanisms of growth control. *FASEB J.* **4:** 176-187.

Miesfeld R, Godowski PJ, Maler BA & Yamamoto KR. (1987). Glucocorticoid receptor mutants that define a small region sufficient for enhancer activation. *Science* **236:** 423-427.

Miller FD, Dixon GH, Rattner JB & van de Sande JH. (1985). Assembly and characterisation of nucleosomal cores on B- vs. Z-form DNA. *Biochemisrty* **24:** 102-109.

Miller J, McLachlan AD & Klug A. (1985). Repetative zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. *EMBO J.* **4:** 1609-1614.

Mischoulon D, Rana B, Bucher NL & Farmer SR. (1992). Growth-dependent inhibition of CCAAT enhancer-binding protein (C/EBPα) gene expression during hepatocyte proliferation in the regenerating liver and in culture. *Mol. Cell. Biol.* **12:** 2553-2560.

Moisan MP, Seckl JR & Edwards CRW. (1990a). 11ß-hydroxysteroid dehydrogenase bioactivity and messenger-RNA expression in rat forebrain: localization in hypothalamus, hippocampus, and cortex. *Endocrinology* **127:** 1450-1455.

Moisan MP, Seckl JR & Edwards CRW. (1990b). Effect of adrenalectomy and dexamethasone administration on 11ß-hydroxysteroid dehydrogenase *in vitro* activity in rat brain and kidney. *J. Physiol.* **434:** 87P.

Moisan MP, Edwards CRW & Seckl JR. (1992). Differential promoter usage by the rat 11ß-hydroxysteroid dehydrogenase gene. *Mol. Endocrinol.* **6:** 1082-1087.

Monder C & Lakshmi V. (1989). Evidence for kinetically distinct forms of corticosteroid 11ß-dehydrogenase in rat liver microsomes. *J. Steroid Biochem.* **12:** 77-83.

Monder C, Lakshmi V & Miroff Y. (1991). Kinetic studies on rat liver 11ß-hydroxysteroid dehydrogenase. *Biochi. Biophys. Acta* **1115:** 23-29.

Monder C. (1991). Corticosteroids, receptors, and organ-specific functions of 11ß-hydroxysteroid dehydrogenase. *FASEB J.* **5:** 3047-3054.

Monder C & White PC. (1993). 11ß-hydroxysteroid dehydrogenase. *Vit. Horm.. Advances in Res. Applications* **47:** 187-271.

Montell DJ, Rorth P & Spradling AC. (1992). Slow border cells, a locus required for a development regulated cell migration during oogenesis, encodes Drosophila C/EBP. *Cell* **71:** 51-62.

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

Moore CCD, Mellon SH, Murai J, Siiteri PK & Miller WL. (1993). Structure and function of the hepatic form of 11ß-hydroxysteroid dehydrogenase in the squirrel monkey, an animal-model of glucocorticoid resistance. *Endocrinology* **133:** 368-375.

Moreno M, Berry MJ, Horst C, Goglia F, Harney JW, Larsen PR & Visser TJ. (1994). Activation and inactivation of thyroid hormone by type 1 iodothyronine deiodenase. *FEBS Lett.* **344:** 143-146.

Mu D, Park CH, Matsunaga T, Hsu D, Reardon JT & Sancar A. (1995). Reconstitution of human DNA repair excision nuclease in a highly defined system. *J. Biol. Chem.* **270:** 2415-2418.

Mueller CR, Maire P & Schibler U. (1990). DBP, a liver-enriched transcriptional activator, is expressed late in ontogeny and its tissue specificity is determined posttranscriptionally. *Cell* **61:** 279-291.

Munck A. (1962). Studies on the mode of action of glucocorticoids in rats. II. The effects of *in vivo* and *in vitro* on net glucose uptake by isolated adipose tissue. *Biochi. Biophys. Acta.* **57:** 318-326.

Mune T, Rogerson FM, Nikkila H, Agarwal AK & White PC. (1995). Human hypertension caused by mutations in the kidney isozyme of 11ß-hydroxysteroid dehydrogenase. *Nature Genet.* **10:** 394-399.

Naar AM, Boutin JM, Lipkin SM, Yu VC, Holloway JM, Glass CK & Rosenfeld MG. (1991). The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. *Cell* **65:** 1267-1279.

Nakajima T, Kinoshita S, Sasagawa T, Sasaki K, Naruto M, Kishimoto T & Akira S. (1993). Phosphorylation at threonine-235 by a *ras*-dependent mitogen-activated protein-kinase cascade is essential for transcription factor NF-IL6. *Proc. Natl. Acad. Sci. USA* **90:** 2207-2211.

Naray-Feyes-Toth A & Feyes-Toth G. (1995). Expression cloning of the aldosterone target cell specific 11ß-hydroxysteroid dehydrogenase from rabbit collecting duct cells. *Endocrinology* **136:** 2579-2586.

Natsuka S, Akira S, Nishio Y, Hashimoto S, Sugita T, Isshiki H & Kishimoto T. (1992). Macrophage differentiation-specific expression of NF-IL6, a transcription factor for interleukin-6. *Blood* **79:** 460-466.

Nechushtan H, Benvenisty N, Brandeis R & Reshef L. (1987). Glucocorticoids control phosphoenolpyruvate carboxykinase gene expression in a tissue specific manner. *Nucleic Acids Res.* **15:** 6405-6417.

Nerlov C & Ziff EB. (1995). CCAAT/enhancer binding protein-α amino acid motifs with dual TBP and TFIIB binding ability co-operate to activate transcription in both yeast and mammaliancells. *EMBO J.* **14:** 4318-4328.

Ness SA, Kowenz-Leutz E, Casini T, Graf T & Leutz A. (1993). Myb and NF-M: combinatorial activators of myeloid genes in heterologous cell types. *Genes Dev.* **7:** 749-759.

Nikolov DB, Chen H, Halay ED, Usheva AA, Hisatake K, Lee DK, Roeder RG & Burley SK. (1995). Crystal structure of a TFIIB-TBP-TATA-element ternary complex. *Nature* **377:** 119-128.

Nishio Y, Isshiki H, Kishimoto T & Akira S. (1993). A nuclear factor for interleukin-6 expression (NF-IL6) and the glucocorticoid receptor synergistically activate transcription of the rat α_1-acid glycoprotein gene via direct protein-protein interaction. *Mol. Cell. Biol.* **13:** 1854-1862.

Nishizawa M & Nagata S. (1992). cDNA clones encoding leucine zipper proteins whcih interact with G-CSF gene promoter element 1-binding protein. *Febs. Lett.* **299:** 36-38.

Nye JA & Graves BJ. (1990). Alkylation interference identifies essential DNA contacts for sequence-specific binding of the eukaryotic transcription factor C/EBP. *Proc. Natl. Acad. Sci. USA* **87:** 3992-3996.

O'Brian RM, Lucas PC, Forest CD, Magnuson MA & Granner DK. (1990). Identification of a sequence in the PEPCK gene that mediates a negative effect of insulin on transcription. *Science* **249:** 533-537.

O'Malley BW. (1990). The steroid receptor superfamily: More excitement predicted for the future. *Mol. Endocrinol.* **4:** 363-369.

O'Shea EK, Rutkowski R & Kim PS. (1989). Evidence that the leucine zipper is a coiled coil. *Science* **243:** 538-542.

O'Shea EK, Klemm JD, Kim PS & Alber T. (1991). X-ray structure of the GCN4 leucine zipper a two-stranded, parallel coiled coil. *Science* **254:** 539-544.

Ohkuma Y & Roeder RG. (1994). Regulation of TFIIH ATPase and kinase activities by TFIIE during active initiation complex formation. *Nature* **368:** 160-163.

Ohlendorf DH, Anderson WF, Fisher RG, Takeda Y & Matthews BW. (1982). The molecular basis of DNA-protein recognition inferred from the structure of cro repressor. *Nature* **298:** 718-723.

Orth DN, Kovacs WJ & Debold CR. (1992). The adrenal cortex. *Williams Textbook of Endocrinology* (eds Wilson JD & Foster, DW, 8th Edition). 489-620. WD Saunders Company, Philedelphia PA.

Orti E, Bodwell JE & Munck A. (1992). Phosphorylation of steroid hormone receptors. *Endocr. Rev.* **13:** 105-128.

Ossipow V, Descombes P & Schibler U. (1993). CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. *Proc. Natl. Acad. Sci. USA* **90:** 8219-8223.

Ossipow V, Tassan JP, Nigg EA & Schibler U. (1995). A mammalian RNA polymerase II holoenzyme containing all components required for promoter-specific transcripiton initiation. *Cell* **83:** 137-146.

Pardridge WM. (1981). Transport of protein bound hormones into tissues *in vitro*. *Endocrine Rev.* **2:**103-123.

Park EA, Roesler WJ, Liu J, Klemm DJ, Gurney AL, Thatcher JD, Shuman J, Friedman A & Hanson RW. (1990). The role of the CCAAT/enhancer-binding protein in the transcriptional regulation of the gene for phosphoenolpyruvate carboxykinase (GTP). *Mol. Cell. Biol.* **10:** 6264-72.

Park EA, Gurney AL, Nizielski SE, Hakimi P, Cao Z, Moorman A & Hanson RW. (1993). Relative roles of CCAAT/enhancer-binding protein ß and cAMP regulatory element-binding protein in controlling transcription of the gene for phosphoenolpyruvate carboxykinase (GTP). *J. Biol. Chem.* **268:** 613-619.

Park EA, Jerden DC & Bahouth SW. (1995). Regulation of phosphoenolpyruvate carboxykinase gene transcription by thyroid hormone involves two distinct binding sites in the promoter. *Biochem. J.* **309:** 913-919.

Parker KL & Schimmer BP. (1993). Transcriptional regulation of the adrenal steroidogenic enzymes. *Trends Endocrinol. Metab.* **4:** 46-50.

Pasquarette MM, Stewart PM, Ricketts ML, Imaishi K & Mason JI. (1996). Regulation of 11ß-hydroxysteroid dehydrogenase type 2 activity and mRNA in human choriocarcinoma cells. *J. Mol. Endocrinol.* **16:** 269-275.

Pavletich NP & Pabo CO. (1991). Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1Å. *Science* **252:** 809-817.

Perlmann T & Vennstrom B. (1995). The sound of silence. *Nature* **377:** 387-388.

Phillips MD, Lakshmi V & Monder C. (1989). Corticosteroid 11ß-dehydrogenase in rat testis. *Endocrinology* **125:** 209-216.

Phillips SEV. (1991). Specific ß-sheet interactions. *Curr. Opin. Struc. Biol.* **1:** 89-98.

Plotz PA, Knowlton AI & Ragan C. (1952). The natural history of Cushing's syndrome. *Am. J. Med.* **13:** 597-614.

Poli V, Mancini FP & Cortese R. (1990). IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. *Cell* **63:** 643-653.

Pontoglio M, Barra J, Hadchovel M, Doyen A, Kress C, Bach JP, Babinet C & Yaniv M. (1996). Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonurea, and renal Fanconi Syndrome. *Cell* **84:** 575-587.

Pope RM, Leutz A & Ness SA. (1994). C/EBPß regulation of the tumour necrosis factor α gene. *J. Clin. Invest.* **194:** 1449-1445.

Potter JJ, Yang VW & Mezey E. (1993). Regulation of the rat class I alcohol dehydrogenase gene by growth hormone. *Biochem. Biophys. Res. Comm.* **191:** 1040-1045.

Ptashne M & Gann AF. (1990). Activators and targets. *Nature* **346:** 329-331.

Purnell BA & Gilmore DS. (1993). Contribution of sequences downstream of the TATA element to a protein-DNA complex containing the TATA-binding protein. *Mol. Cell. Biol.* **13:** 2593-2603.

Qian YQ, Billeter M, Otting G, Muller M, Gehring WJ & Wuthrich K. (1989). The structure of the *Antennapedia* homeodomain determined by NMR spectroscopy in solution: comparison with prokaryotic repressors. *Cell* **59:** 573-580.

Quinn PG, Wong TW, Magnuson MA, Shabb JB & Granner DK. (1988). Identification of basal and cyclic AMP regulatory elements in the promoter of the phosphoenolpyruvate carboxykinase gene. *Mol. Cell. Biol.* **8:** 3467-3475.

Quinn SJ & Williams GH. (1988). Regulation of aldosterone secretion. *Ann. Rev. Physiol.* **50:** 409-426.

Quirk SJ, Slattery JA & Funder JW. (1990). Epithelial and adipose cells isolated from mammary glands of pregnant and lactating rats differ in 11ß-hydroxysteroid dehydrogenase activity. *J. Steroid Biochem. Mol. Biol.* **37:** 529-534.

Rajan V, Chapman KE, Lyons V, Jamieson P, Mullins JJ, Edwards CRW & Seckl JR. (1995). Cloning, sequencing and tissue-distribution of mouse 11ß-hydroxysteroid dehydrogenase-1 cDNA. *J. Steroid Biochem. Mol. Biol.* **52:** 141-147.

Rajan V, Edwards CRW & Seckl JR. (1996). 11ß-hydroxysteroid dehydrogenase in cultured hippocampal cells reactivates inert 11-dehydrocorticosterone, potentiating neurotoxicity. *J. Neurosci.* **16:** 65-70.

Raught B, Liao WSL & Rosen JM. (1995). Developmentally and hormonally regulated CCAAT/enhancer-binding protein isoforms influence ß-casein gene expression. *Mol. Endocrinol.* **9:** 1223-1232.

Raumann BE, Brown BM & Sauer RT. (1994). Major groove DNA recognition by ß-sheets: the ribbon-helix-helix family of gene regulatory proteins. *Curr. Opin. Struct. Biol.* **4:** 36-43.

Ray A & Ray BK. (1994). Serum amyloid A gene expression under acute-phase conditions involves participation of inducible C/EBP-ß and C/EBP-δ and their activation by phosphorylation. *Mol. Cell. Biol.* **14:** 4324-4332.

Ray A, Hannink M & Ray BK. (1995). Concerted participation of NF-κB and C/EBP heteromer in lipopolysaccharide induction of serum amyloid A gene expression in liver. *J. Biol. Chem.* **270:** 7365-7374.

Rehberger P, Rexin M & Gehring U. (1992). Heterotetrameric structure of the human progesterone receptor. *Proc. Natl. Acad. Sci. USA* **89:** 8001-8005.

Reinehart JJ, Wuest D & Ackerman GA. (1982). Corticosteroid alteration of human monocyte to macrophage differentiation. *J. Immunol.* **129:** 1436-1440.

Reinisch JM, Simon NG, Karwo WG & Gandelman R. (1978). Prenatal exposure to prednisone in humans and animals retards intrauterine growth. *Science* **202:** 436-438.

Rexin M, Busch W, Segnitz B & Gehring U. (1992). Structure of the glucocortioid receptor in intact cells in the absence of hormone. *J. Biol. Chem.* **267:** 9619-9621.

Rice DA, Mouw AR, Bogerd AM & Parker KL. (1991). A shared promoter element regulates the expression of three steroidogenic enzymes. *Mol. Endocrinol.* **5:** 1552-1561.

Riggs KJ, Merrell KT, Wilson G & Calame K. (1991). Common factor 1 is a transcriptional activator which binds in the *c-myc* promoter, the skeletal α-actin promoter, and the immunoglobulin heavy-chain enhancer. *Mol. Cell. Biol.* **11:** 1765-1769.

Roberts SGE, Ha I, Maldonado E, Reinberg D & Green MR. (1993). Interaction between an acidic activator and transcription factor TFIIB is required for transcriptional activation. *Nature* **363:** 741-744.

Roeder, RG. (1991). The complexities of eukaryotic transcription initiation: regulation of preinitiation complex assembly. *Trends Biochem. Sci.* **16:** 402-407.

Roesler WJ, Vandenbark GR & Hanson RW. (1989). Identification of multiple protien binding domains in the promoter-regulatory region of the phosphoenolpyruvate carboxykinase (GTP) gene. *J. Biol. Chem.* **264:** 9657-9664.

Roesler WJ, McFie PJ & Dauvin C. (1992). The liver-enriched transcription factor D-site-binding protein activates the promoter of the phosphoenolpyruvate carboxykinase gene in hepatoma cells. *J. Biol. Chem.* **267:** 21235-21243.

Roesler WJ, Mcfie PJ & Puttick DM. (1993). Evidence for the involvement of a least two distinct transcription factors, one of which is liver-enriched, for the activation of the phosphoenolpyruvate carboxykinase gene promoter by cAMP. *J. Biol. Chem.* **268:** 3791-3796.

Roland BL, Li KXZ & Funder JW. (1995). Hybridisation, histochemical localisation of 11ß-hydroxysteroid dehydrogenase Type 2 in rat brain. *Endocrinology* **136:** 4697-4696.

Roland BL & Funder JW. (1996). Localization of 11ß-hydroxysteroid dehydrogenase type 2 in rat tissues: *in situ* studies. *Endocrinology* 1123-1128.

Roman C, Platero JS, Shuman J & Calame K. (1990). Ig/EBP-1: a ubiquitously expressed immunoglobulin enhancer binding protein that is similar to C/EBP and heterodimerizes with C/EBP. *Genes Dev.* **4:** 1404-1415.

Ron D, Brasier AR, Mcgehee RE & Habener JF. (1992). Tumor necrosis factor-induced reversal of adipocytic phenotype of 3T3-LI cells is preceded by a loss of nuclear CCAAT/enhancer binding protein (C/EBP). *J. Clin. Invest.* **89:** 223-233.

Ron D & Habener JF. (1992). CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev.* **6:** 439-453.

Roy AL, Meisterernst M, Pognonec P & Roeder RG. (1991). Cooperative interaction of an initiator-binding transcription initiation factor and the helix-loop-helix activator USF. *Nature* **354:** 245-248.

Roy R, Schaeffer L, Humbert S, Vermeulen W, Weeda G & Egly JM. (1994). The DNA dependent ATPase activity associated with the class II basic transcription factor BTF2/TFIIH. *J. Biol. Chem.* **269:** 9826-9832.

Ruppert S, Boshart M, Bosch FX, Schmid W, Fournier REK & Schutz G. (1990). Two genetically defined *trans*-acting loci coordinately regulate overlapping sets of liver-specific genes. *Cell* **61:** 895-904.

Rusconi S & Yamomoto KR. (1987). Functional dissection of the hormone and DNA binding activities of the glucocorticoid receptor. *EMBO J.* **6:** 1309-1315.

Rusvai E & Naray-Fejes-Toth A. (1993). A new isoform of 11ß-hydroxysteroid dehydrogenase in aldosterone target cells. *J. Biol. Chem.* **268:** 10717-10720.

Sadovsky Y, Crawford PA, Woodson KG, Polish JA, Clements MA, Tourlettotte LM, Simburger K & Milbrandt J. (1995). Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chaincleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. *Proc. Natl. Acad. Sci. USA* **92:** 10939-10943.

Saez JM, Morera AM, Haour F & Evain D. (1977). Effects of *in vivo* administration of dexamethasone, corticotropin and human chronic gonadotropin on steroidogenesis and protein and DNA synthesis of testicular interstitial cells in prepubertal rats. *Endocrinology* **101:** 1256-1263.

Sambrook J, Fritsch E & Maniatis T. (1989). Molecular cloning: a laboratory manual. *2nd edition Cold Spring harbour Laboratory Press, Cold Spring Harbour, NY*

Saruta T, Suzuki H, Handa M, Igarashi Y, Kondo K & Senba S. (1986). Multiple factors contribute to the pathogenesis of hypertension in Cushing's syndrome. *J. Clin. Endocrinol. Metab.* **62:** 275-279.

Sasaki K, Cripe TP, Koch SR, Andreone TL, Petersen DD, Beale EG & Granner DK. (1984). Multihormonal regulation of phosphoenolpyruvate carboxykinase gene transcription. *J. Biol. Chem.* **259:** 15242-15251.

Sauer RT, Smith DL & Johnson AD. (1988). Flexibility of the yeast α2 repressor enables it to occupy the ends of its operator, leaving the centre free. *Genes Dev.* **2:** 807-816.

Sauer F, Hansen SK & Tjian R. (1995). Multiple TAF_{II}s directing synergistic activaion of transcription. *Science* **270:** 1783-1788.

Sawadogo M & Roeder RG. (1985). Factors involved in specific transcription by human RNA polymerase II: Analysis by a rapid and quantitative *in vitro* assay. *Proc. Natl. Acad. Sci. USA* **82:** 4394-4398.

Schaeffer L, Moncollin V, Roy R, Staub A, Mezzina M, Sarasin A, Weeda G, Hoeijmakers I & Egly JM. (1994). The ERCC2/DNA repair protein is associated with the class II BTF2/TFIIH transcription factor. *EMBO J.* **13:** 2388-2392.

Schreiber JR, Nakamura K & Erickson GF. (1982). Rat ovary glucocorticoid receptor: identification and characterisation. *Steroids* **139:** 569-584.

Schwabe JWR, Neuhaus D & Rhodes D. (1990). Solution structure of the DNA-binding domain of the oestrogen receptor. *Nature* **348:** 458-461.

Schweizer-Groyer G, Groyer A, Cadepond F, Grange T, Baulieu EE & Pictet R. (1994). Expression from the tyrosine aminotransferase promoter (nt -350 to +1) is liver-specific and dependent on the binding of both liver enriched and ubiquitous *trans*-acting factors. *Nucleic Acids Res.* **22:** 1583-1592.

Screpanti I, Romani L, Musiani P, Modesti A, Fattori E, Lazzaro D, Sellitto C, Scarpa S, Bellavia D, Lattanzio G, Istoni F, Frati L, Cortese R, Gulino A, Ciliberto G, Costantini F & V. P. (1995). Lymphoproliferative disorder and imbalanced T-helper response in C/EBPß-deficient mice. *EMBO J.* **14:** 1932-1941.

Seckl JR. (1993). 11ß-hydroxysteroid dehydrogenase isoforms and their implications for blood pressure regulation. *European J. Clin. Invest.* **23:** 589-601.

Seckl JR, Benediktsson R, Lindsay RS & Brown RW. (1995). Placental 11ß-hydroxysteroid dehydrogenase and the programming of hypertension. *J. Steroid Biochem. Molec. Biol.* **55:** 447-455.

Segnitz B & Gehring U. (1995). Subunit structure of the nonactivated human estrogen receptor. *Proc. Natl. Acad. Sci. USA* **92:** 2179-2183.

Serizawa H, Conway RC & Conway JW. (1992). A carboxyl-terminal-domain kinase associated with RNA polymerase II transcription factor δ from rat liver. *Proc. Natl. Acad. Sci. USA* **89:** 7476-7480.

Serizawa H, Makela TP, Conaway JW, Conaway RC, Weinberg RA & Young RA. (1995). Association of Cdk-activating kinase subunits with transcription factor TFIIH. *Nature* **374:** 280-282.

Seto E, Shi Y & Shenk T. (1991). YY1 is an initiator sequence-binding protein that directs and activates transcription *in vitro*. *Nature* **354:** 241-245.

Shiekhatter R, Mermelstein F, Fisher RP, Drapkin R, Dynlacht B, Wessling HC, Morgan DO & Reinberg D. (1995). Cdk-activating kinase complex is a component of human transcription factor TFIIH. *Nature* **374:** 283-287.

Shimojo M, Condon J, Whorwood CB & Stewart PM. (1996). Adrenal 11ß-hydroxysteroid dehydrogenase. *Seventh Conference on the Adrenal Cortex.* P39 (Abstract).

Short JM, Wynshow-Boris A, Short HP & Hanson RW. (1986). Characterisation of the phosphoenolpyruvate carboxykinase (GTP) promoter-regulatory region. *J. Biol. Chem.* **261:** 9721-9726.

Simpson ER & Waterman MR. (1983). Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. *Ann. Rev. Physiol.* **50:** 427-440.

Singh H, Sen R, Baltimore D & Sharp PA. (1986). A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobin genes. *Nature* **319:** 154-158.

Smale ST & Baltimore D. (1989). The "Initiator" as a transcription control element. *Cell* **57:** 103-113.

Smith LI, Mendel DB, Bodwell JE & Munck A. (1989). Phosphorylated sites within the functional domain of the ~100kDa steroid-binding subunit of glucocorticoid receptors. *Biochemistry* **28:** 4490-4498.

Starkman MN, Schteingart DE & Schork MA. (1986). Cushings syndrome after treatment: changes in cortisol and ACTH levels, and amelioration of the depressive syndrome. *Psychiatry Res.* **19:** 177-188.

Stein B & Yang MX. (1995). Repression of the interleukin-6 promoter by estrogen-receptor is mediated by NF-κB and C/EBPß. *Mol. Cell. Biol.* **15:** 4971-4979.

Stewart PM, Corrie JET, Shackleton CHL & Edwards CRW. (1988). Syndrome of apparent mineralocorticoid excess: a defect in the cortisol-cortisone shuttle. *J. Clin. Invest.* **82:** 340-349.

Stringer KF, Ingles CJ & Greenblatt J. (1990). Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. *Nature* **345:** 783-786.

Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods in Enzymology* **185:** 60-89.

Suzuki M. (1990). The heptad repeat in the largest subunit of RNA polymerase II binds by intercalating into DNA. *Nature* **344:** 562-565.

Sylvester SL, ap Rhys CMJ, Luethy-Martindale JD & Holbrook NJ. (1994). Induction of *GADD153*, a CCAAT/enhancer-binding protein (C/EBP)-related gene, during the acute phase response in rats. Evidence for the involvement of C/EBPs in regulating its expression [published erratum appears in J. Biol. Chem. (1995), **270**:14842]. *J. Biol. Chem.* **269:** 20119-20125.

Tae HJ, Luo X & Kim KH. (1994). Roles of CCAAT/enhancer-binding protein and its binding site on repression and derepression of acetyl-CoA carboxylase gene. *J. Biol. Chem.* **269:** 10475-10484.

Tae HJ, Zhang SY & Kim KH. (1995). cAMP activation of CAAT enhancer-binding protein-ß gene expression and promoter I of acetyl-CoA carboxylase. *J. Biol. Chem.* **270:** 21487-21494.

Tanaka T, Akira S, Yoshida K, Umemoto M, Yoneda Y, Shirafuji N, Fujiwara H, Suematsu S, Yoshida N & Kishimoto T. (1995). Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. *Cell* **80:** 353-361.

Tanese N, Pugh BF & Tjian R. (1991). Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex. *Genes Dev.* **5:** 2212-2224.

Tannin GM, Agarwal AK, Monder C, New MI & White PC. (1991). The human gene for 11ß-hydroxysteroid dehydrogenase: structure, tissue distribution, and chromosomal localization. *J. Biol. Chem.* **266:** 16653-16658.

Tate P, Skarnes W & Bird A. (1996). The methyl-CpG bindin gprotein MeCP2 is essential for embryonic development in the mouse. *Nature Genetics* **12:** 205-208.

Theisen M, Behringer RR, Cadd GG, Brinster RL & McKnight GS (1993) A C/EBP-binding site in the transferrin promoter is essential for expression in the liver but not the brain of transgenic mice. *Mol. Cell. Biol.* **13:** 7666-7676.

Thomassin H, Hamel D, Bernier D, Guertin M & Belanger L. (1992). Molecular cloning of two C/EBP-related proteins that bind to the promoter and the enhancer of the alpha1-fetoprotein gene. Further analysis of C/EBPbeta and C/EBP. *Nucleic Acids Res.* **20:** 3091-3098.

Timchenko N, Wilson DR, Taylor LR, Abdelsayed S, Wilde M, Sawadogo M & Darlington GJ. (1995). Autoregulation of the human C/EBPα gene by stimulation of upstream stimulatory factor binding. *Mol. Cell. Biol.* **15:** 1192-1202.

Tjian R & Maniatis T. (1994). Transcriptional activation: a complex puzzle with few easy pieces. *Cell* **77:** 5-8.

Ubeda M, Wang XZ, Zinszner H, Wu I, Habener JF & Ron D. (1996). Stress-induced binding of the transcription factor CHOP to a novel DNA control element. *Mol. Cell. Biol.* **16:** 1479-1489.

Ueda H, Sun GC, Murata T & Hirose S. (1992). A novel DNA-binding motif abuts the zinc finger domain of insect nuclear hormone receptor FTZ-F1 and mouse embryonal long terminal repeat-binding domain. *Mol. Cell. Biol.* **12:** 5667-5672.

Ulick S, Levine LS, Gunczler P, Giovanni Z, Ramirez LC, Rauh W, Rosler A, Bradlow HL & New MI. (1979). A syndrome of apparent mineralocorticoid excess associated with defects in the peripheral metabolism of cortisol. *J. Clin. Endocrinol. Metab.* **49:** 757-764.

Umesono K, Murakami KK, Thompson CC & Evans RM. (1991). Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3 receptors. *Cell* **65:** 1255-1266.

Usheva A, Maldonado E, Goldring A, Lu H, Houbavi C, Reinberg D & Aloni Y. (1992). Specific interaction between the nonphosphorylated form of RNA polymerase II and the TATA binding protein. *Cell* **69:** 871-881.

Vallejo M, Gosse ME, Beckman W & Habener JF. (1995). Impaired cyclic AMP-dependent phosphorylation renders CREB a repressor of C/EBP-induced transcription of the somatostatin gene in an insulinoma cell line. *Mol. Cell. Biol.* **15:** 415-424.

Van den Hoff MJB, Vermeulen JLM, De Boer PAJ, Lamers WH & Moorman AFM. (1994). Developmental changes in the expression of the liver-enriched transcription factors LF-B1, C/EBP, DBP and LAP/LIP in relation to the expression of albumin, α-fetoprotein, carbamoylphosphate synthase and lactase mRNA. *Histochemical Journal* **26:** 20-31.

van Holde K & Zlatanova J. (1993). Unusual DNA structures, chromatin and transcription. *Bioessays* **16:** 59-68.

van Vuuren AJ, Vermeulen W, Ma L, Weeda G, Appeldorn E, Jaspers NGJ, van der Eb AJ, Bootsma D, Hoeijmakers JHJ, Humbert S, Schaeffer L & Egly JM. (1994). Correction of xeroderma pigmentosum repair defect by basal transcription factor BTF2 (TFIIH). *EMBO J.* **13:** 1645-1653.

Vasseur-Cognet M & Lane MD. (1993). CCAAT/enhancer binding protein alpha (C/EBPα) undifferentiated protein: A developmentally regulated nuclear protein that binds to the C/EBPα gene promoter. *Proc. Natl. Acad. Sci. USA* **90:** 7312-7316.

Vaulont S, Puzenat N, Kahn A & Raymondjean M. (1989). Analysis by cell-free transcription of the liver-specific pyruvate kinase gene promoter. *Mol. Cell. Biol.* **9:** 4409-4415.

Voice MW, Seckl JR, Edwards CRW & Chapman KE. (1996). 11ß-Hydroxystroid dehydrogenase type 1 expression in 2S FAZA hepatoma cells is hormonally regulated: a model system for the study of hepatic glucocorticoid metabolism. *Biochem. J.* **317:** 621-625.

vom Baur E, Zechel C, Heery D, Heine MJS, Garnier JM, Vivat V, Le Dovarin B, Gronemeyer H, Chambon & Losson R. (1996). Differential ligand dependent interactions between the AF-2 activation domains of nuclear receptors and the putative transcriptional intermediary factors mSUG1 and TIF1. *EMBO J.* 15: 110-124.

Walker BR, Campbell JC, Fraser R, Stewart PM & Edwards CRW. (1992). Mineralocorticoid excess and inhibition of 11ß-hydroxysteroid dehydrogenase in patients with ectopic ACTH syndrome. *Clin. Endocrinol.* **37:** 483-492.

Walker BR, Connacher AA, Lindsay RM, Webb DJ & Edwards CRW. (1995). Carbenoxolone increases hepatic insulin sensitivity in man: a novel role for 11-oxosteroid reductase in enhancing glucocorticoid receptor activation. *J. Clin. Endocrinol. Metab.* **80:** 3155-3159.

Walters MR. (1985). Steroid hormone receptors and the nucleus. *Endocrine Rev.* **6:** 512-543.

Wang ND, Finegold MJ, Bradley A, Ou CN, Abdelsayed SV, Wilde MD, Taylor LR, Wilson DR & Darlington GJ. (1995). Impaired energy homeostasis in C/EBPα knockout mice. *Science* **269:** 1108-1112.

Wang W, Carey M & Gralla JD. (1992a). Polymerase II promtoer activation: closed comlex fomation and ATP-driven start site opening. *Science* **255:** 450-453.

Wang W, Gralla JD & Carey M. (1992b). The acidic activator GAL-AH can stimulate polymerase II transcription by promoting assembly of a closed complex requiring TFIID and TFIIA. *Genes Dev.* **6:** 1716-1727.

Waterman MR & Simpson ER. (1989). Regulation of steroid hydroxylase gene expression is multifactorial in nature. *Recent Prog. Horm. Res.* **45:** 533-566.

Wedel A & Ziegler-Heitbrock WL. (1995). The C/EBP family of transcription factors. *Immunobiol.* **193:** 171-185.

Wegner M, Cao Z & Rosenfeld MG. (1992). Calcium-regulated phosphorylation within the leucine zipper of C/EBPß. *Science* **256:** 370-373.

Welsh JR, Bambino TH & Hseuh AJW. (1982). Mechanism of glucocortiocid-induced suppression of testicular androgen biosynthesis *in vitro*. *Biol. Repro.* **27:** 1138-1146.

Wharton RP, Brown EL & Ptashne M. (1984). Substituting an α-helix switches the sequence-specific DNA interactions of a repressor. *Cell* **38:** 361-369.

White PC, Curnow KM & Pascoe L. (1994). Disorders of steroid 11ß-hydroxylase enzymes. *Endocr. Rev.* **15:** 421-438.

Whorwood CB, Franklyn JA, Sheppard MC & Stewart PM. (1992). Tissue localization of 11ß-hydroxysteroid dehydrogenase and its relationship to the glucocorticoid receptor. *J. Steroid Biochem. Mol. Biol.* **41:** 21-28.

Whorwood CB, Sheppard MC & Stewart PM. (1993). Tissue-specific effects of thyroid hormone on 11ß-hydroxysteroid dehydrogenase gene expression. *J. Steroid Biochem. Mol. Biol.* **46:** 539-547.

Williams AC, Walker BR, Edwards CRW, Burt D, Williams BC & Walker SW. (1992). Differential effects of acute and chronic stimulation by ACTH on the secretion of cortisol, cortisone and 18-hydroxycortisol from bovine zona fasciculata cells in primary culture. *J. Endocrinol* **132:** (Suppl)P111 (Abstract).

Williams SC, Cantwell CA & Johnson PF. (1991). A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers *in vitro*. *Genes Dev.* **5:** 1553-1567.

Willy PJ, Umesono K, Ong ES, Evans RM, Heyman RA & Manglesdorf DJ. (1995). LXR, a nuclear receptor that defines a distinct retinoid response pathway. *Genes Dev.* **9:** 1033-1045.

Wilson CJ, Chao DM, Imbalzano AN, Schnitzler GR, Kingston RE & Young RA. (1996). RNA polymerase holoenzyme contains SWI/SNF regulators involved in chromatin remodelling. *Cell* **84:** 235-244.

Wilson RC, Krozowski ZS, Li K, Obeyesekere VR, Razzaghyazar M, Harbison MD, Wei JQ, Shackleton CHL, Funder JW & New MI. (1995a). A mutation in the HSD11ß2 gene in a family with apparent mineralocorticoid excess. *J. Clin. Endocrinol. Metab.* **80:** 2263-2266.

Wilson RC, Harbison MD, Krozowski ZS, Funder JW, Shackleton CHL, Hanauskeabel HM, Wei JQ, Hertecant J, Moran A, Neiberger RE, Balfe JW, Fattah A, Daneman D, Licholai T & New MI. (1995b). Several homozygous mutations in the gene for 11ß-hydroxysteroid dehydrogenase type 2 in patients with apparent mineralocorticoid excess. *J. Clin. Endocrinol. Metab.* **80:** 3145-3150.

Wilson TE, Fahrner TJ & Milbrandt J. (1993). The orphan receptors NGFI-B and Steroidogenic Factor-1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. *Mol. Cell. Biol.* **13:** 5794-5804.

Wu KJ, Wilson DR, Shih C & Darlington GJ. (1994). The transcription factor HNF1 acts with C/EBPα to synergistically activate the human albumin promoter through a novel domain. *J. Biol. Chem.* **269:** 1177-1182.

Xiao H, Pearson A, Coulombe B, Truant R, Zhang S, Regier JL, Triezenberg SJ, Reinberg D, Flores O, Ingles CJ & Greenblatt J. (1994). Binding of basal transcirpiton factor TFIIH to the acidic activation domains of VP16 and p53. *Mol. Cell. Biol.* **14:** 7013-7024.

Xie XL, Kokubo T, Cohen SL, Mirza UA, Hoffmann A, Chait BT, Roeder RG, Nakatani Y & Burley SK. (1996). Structural similarity between TAFS and the heterotetrameric core of the histone octamer. *Nature* **380:** 316-322.

Yang K, Smith CL, Dales D, Hammond GL & Challis JRG. (1992). Cloning of an ovine 11ß-hydroxysteroid dehydrogenase complementary deoxyribonucleic acid: tissue and temporal distribution of its messenger-ribonucleic-acid during fetal and neonatal development. *Endocrinology* **131:** 2120-2126.

Yang K & Matthews SG. (1995). Cellular-localization of 11ß-hydroxysteroid dehydrogenase 2 gene expression in the ovine adrenal gland. *Mol. Cell. Endocrinol.* **111:** R19-R23.

Yanuka-Kashles O, Cohen H, Trus M, Aran A, Benvenisty N & Reshef L. (1994). Transcriptional regulation of the phosphoenolpyruvate carboxykinase gene by cooperation between hepatic nuclear factors. *Mol. Cell. Biol.* **14:** 7124-7133.

Yeh WC & Mcknight. (1995). Regulation of adipose tissue maturation and energy homeostasis. *Curr. Opin. Cell Biol.* **7.** 885-890

Yeh WC, Cao ZD, Classon M & McKnight SL. (1995). Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes Dev.* **9:** 168-181.

Ylikomi T, Bocquel MT, Berry M, Gronemeyer H & Chambon P. (1993). Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. *EMBO J.* **11:** 3681-3694.

Yokomori K, Admon A, Goodrich JA, Chen JL & Tjian R. (1993). Drosohila TFIIA-L is processed into two subunits that are associated with the TBP/TAF complex. *Genes Dev.* **7:** 2235-2245.

Yoshinga SK, Peterson CL, Herskowitz I & Yamamoto KR. (1992). Roles of SW1, SW2, and SW3 proteins for transcriptional enhancement by steroid receptors. *Science* **258:** 1598-1604.

Yu VC, Delsert C, Andersen B, Holloway JM, Devary OV, Naar AM, Kim SY, Boutin JM, Glass CK & Rosenfeld MG. (1991). RXRß: a coregulator that enhances binding of retinoic acid, thyroid hormone and vitamin D receptors to their cognate response elements. *Cell* **67:** 1251-1266.

Zhang X-K, Wills KN, Graupner G, Tzukerman M, Hermann T & Pfahl M. (1991). Ligand binding domain of thyroid hormone receptors modulates DNA binding and determines their bifunctional roles. *New Biologist* **3:** 169-181.

Zhang XK, Hoffman B, Tran PBV, Graupner G & Pfahl M. (1992). Retinoid X receptor is an auxilliary protein for thyroid hormone and retinoic acid receptors. *Nature* **355:** 441-446.

Zhou Q, Lieberman PM, Boyer TG & Berk AJ. (1992). Holo-TFIID supports transcriptional stimulation by diverse activators and from TATA-less promoter. *Genes Dev.* **6:** 1964-1974.

Zhou Q, Boyer TG & Berk AJ. (1993). Factors (TAF's) required for activated transcirption interact with TATA-box binding protein conserved core domain. *Genes Dev.* **7:** 180-187.

Zhou MY, Gomezsanchez EP, Cox DL, Cosby D & Gomezsanchez CE. (1995) Cloning, expression, and tissue distribution of the rat nicotinamide adenine dinucleotide-dependent 11ß-hydroxysteroid dehydrogenase. *Endocrinology* **136:** 3729-3734.

Zweifach BW, Schorr E & Black MM. (1953). The influenc eof the adrenal cortex on the terminal vascular bed. *Ann. NY Acad. Sci.* **56:** 626-633.