HUMAN ALDOSTERONE SYNTHASE AND 11β-HYDROXYLASE. STUDIES ON THE RELATIONSHIP OF STRUCTURE AND FUNCTION AND THEIR CLINICAL IMPLICATIONS.

By

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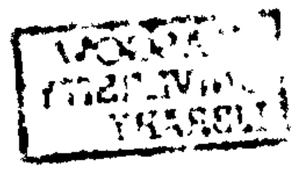

Abstract

In the human adrenal cortex, the genes CYP11B1 and CYP11B2 lie in tandem on chromosome 8 and encode the steroidogenic enzymes, 11β-hydroxylase and aldosterone synthase, respectively. Their steroidogenic capacities are markedly different despite their high degree of homology (93%). 11β-Hydroxylase mainly catalyses the conversion of 11-deoxycorticosterone (DOC) to corticosterone (B) and 11-deoxycortisol (S) to cortisol (F) while aldosterone synthase catalyses the 11β-hydroxylation, 18-hydroxylation and 18-oxidation necessary to convert DOC to aldosterone.

Abnormalities in adrenal steroid production have been implicated in certain forms of hypertension. Mutations in the CYP11B1 gene which result in complete loss of 11β-hydroxylase function cause 11β-hydroxylase deficiency and hypertension due to abnormally high levels of the mineralocorticoid, DOC. Mutations have been identified which destroy aldosterone synthase 18-hydroxylase activity or 18-oxidase activity or both, resulting in lack of aldosterone. Structure-function studies have identified aldosterone synthase residues specifically involved in 18-hydroxylation and 18-oxidation. In the DahI rat model of hypertension, mutations have been identified in the CYP11B2 and CYP11B1 genes which result in increased aldosterone and 18-OHDOC production respectively. Analogous mutations in the human CYP11B2 gene in exons 3 and 4 which result in amino acid substitutions, E136D and K251R have been shown to increase aldosterone production. In essential hypertension adrenal steroids have been implicated as a contributing factor in some cases and it is possible that mutations in aldosterone synthase and 11β-hydroxylase may be responsible in part for abnormalities in steroid production. The studies reported in this thesis have investigated some of the residues which may be responsible for the special properties of these enzymes and also the effects of potential inhibitors on enzyme steroid production *in vitro*.

In chapter 3 an attempt was made to identify which of the 7 % of residues that differ contribute to the unique enzymatic activities of aldosterone synthase and 11β-

hydroxylase. Several residues in the I-helix, which lies in close proximity to the haem and forms part of the active site, are known to contribute to the 18-hydroxylase and 18-oxidase activities of aldosterone synthase and cluster in regions encoded by exons 6,7 and 8. This does not provide information on the importance of residues distal to the active site and encoded by the other exons. Residues D147 and I248 in exons 3 and 4 of aldosterone synthase which lie close to residues E136 and K251 were replaced with the 11β-hydroxylase equivalent, E147 and T248. Substitutions of aldosterone synthase-specific residues, Q43 and T493 in exons 1 and 9, were also performed. Replacement of aldosterone synthase-specific residues Q43, I248 and T493 had no effect on DOC conversion to aldosterone. Substitution of D147 of aldosterone synthase to E147 caused a dramatic increase in corticosterone (B) production with a small concomitant increase in aldosterone production but had no effect on 11-deoxycortisol conversion to cortisol.

The converse mutation was also constructed where 11β-hydroxylase-specific residue E147 was replaced with the aldosterone synthase equivalent. This mutation of E147D in 11β-hydroxylase dramatically reduced corticosterone production but did not affect 11-deoxycortisol conversion to cortisol. Km values for DOC conversion to B for aldosterone synthase mutant B2-D147E and wild type aldosterone synthase were 1.4μmol/L and 5μmol/L respectively showing that the mutant had a higher affinity for DOC. Km values for the 11β-hydroxylase mutant B1-E147D and wild-type 11β-hydroxylase for B production from DOC were 7.5μmol/L and 2.5μmol/L respectively showing that mutant B1-E147D had a much lower affinity for DOC. This shows that residue E147 of 11β-hydroxylase contributes to the greater efficiency of 11β-hydroxylation by 11β-hydroxylase and is crucial for effective 11β-hydroxylation of DOC.

In chapter 4, an attempt was made to assign residues to a precise location in the protein molecule. The crystal structure of aldosterone synthase and 11β-hydroxylase is not known but those of closely related bacterial cytochrome P450 enzymes have been determined. These enzymes, although sharing only a low degree of homology, have a similar overall structure. It is therefore possible to model aldosterone synthase and 11β-hydroxylase on these simple enzymes. Using this approach, residue 147 in

both enzymes lies immediately flanking helix-G which forms part of the main framework surrounding the haem and the active site. It is possible that residue E147 of 11β-hydroxylase contributes to the positioning, of the helix holding it in a specific orientation which is highly favourable for DOC conversion to B.

In chapter 5, screening of DNA from a small group of hypertensive patients and normal subjects for nucleotide changes within the CYP11B1 and CYP11B2 genes revealed a number of mutations which resulted in residue changes. *In vitro* mutation analysis was performed for aldosterone synthase mutant B2-K357N and 11β-hydroxylase mutants B1-H107Y and B1-L186V. On the basis of modelling (chapter 4), residue 357 may form part of β sheet 3 or 4 and may possibly be involved in substrate interaction /adrenodoxin interaction. Residue 107 lies near a region shown previously to be important for hydroxylation efficiency. Residue 186 flanks a helix, the functional role of which is not known. These mutants did not affect enzyme activity *in vitro*. Other mutations which are polymorphic have been shown, by other authors, to be associated with hypertension and may find use as susceptibility markers for the disease. Association of these new mutations/polymorphisms with hypertension and their frequency within the hypertensive population were not assessed.

A third gene exists in the rare, autosomal dominant disorder, glucocorticoid suppressible hyperaldosteronism (GSH), which comprises the 5' regulatory region of CYP11B1 fused to the 3' coding regions of CYP11B2. Consequently, aldosterone synthase activity is expressed ectopically in the adrenal zona fasciculata where it is subject to ACTH control. In addition to producing aldosterone, the chimeric enzyme also catalyses the metabolism of cortisol (F), which is unique to the zona fasciculata, to 18-hydroxycortisol (18-OHF) and 18-oxocortisol (18-OXOF). These are therefore secreted in large quantities and, it had been suggested, may be the cause of the impaired 11β-hydroxylation observed in this condition. This study (chapter 6) has shown that high concentrations of 18-OXOF and 18-OHF do not affect 11β-hydroxylation *in vitro* and are therefore unlikely to do so *in vivo*. It is possible that these steroids may be further metabolised and that the metabolites act as inhibitors *in vivo*. This may explain the *in vitro/in vivo* differences. However, another closely

related steroid, 18-OHDOC, did reduce both 11-deoxycortisol conversion to cortisol and DOC conversion to B of both 11β-hydroxylase and aldosterone synthase *in vitro*. Another important finding was that aldosterone synthase, and to a much lesser extent 11β-hydroxylase, can use 18-OHDOC as an alternative substrate. The possibility therefore arose that 18-OHDOC conversion might contribute to the excessive aldosterone levels observed in GSH. Lineweaver-Burke analysis for DOC and 18-OHDOC conversion to aldosterone by aldosterone synthase produced Km values of 1.73μmol/L and 9.09μmol/L respectively. That is, aldosterone synthase has a much lower affinity for 18-OHDOC than for its preferred substrate, DOC. Thus, *in vivo*, 18-OHDOC is unlikely to be a substrate of significant importance.

In summary, this thesis presents new studies on the relationship between structure and function of aldosterone synthase and 11β-hydroxylase. Artificially induced changes, some relatively conservative and distant from centres of known functional importance, have been shown to alter activity significantly. A number of variations from consensus sequences of these enzymes have been identified in subjects with essential hypertension; whether these affect enzyme activity in such a way as to explain the clinical observations of mild 11β-hydroxylase deficiency or suppressed renin or whether they might be used as diagnostic markers remains to be evaluated.

Declaration

I declare that unless specified otherwise in the text the work presented in this thesis is my own.

Angela Fisher.

Dedication

I would like to dedicate this thesis to my parents and brother Stuart for their love, support and encouragement and also to the memory of my grandparents and Aunty Teen.

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Abbreviations

18-OHF	18-hydroxycortisol
18-OHB	18-hydroxycorticosterone
18-OHDOC	18-hydroxydeoxycorticosterone
18-OXOF	18-oxocortisol
ANG-I	angiotensin I
ANG-II	angiotensin II
ACE	angiotensin-I converting enzyme
ACTH	adrenocorticotropin
cAMP	adenosine 3', 5'-cyclic monophosphate
B	corticosterone
CaM	Calmodulin
CREB	cAMP response element binding protein
CYP11B1	gene encoding cytochrome P450 11β-hydroxylase
CYP11B2	gene encoding cytochrome P450 aldosterone synthase
DNA	deoxyribonucleic acid
DOC	11-deoxycorticosterone
DTT	dithiothreitol
EDTA	ethylene diamine tetracetate
F	cortisol
GSH	glucocorticoid-suppressible hyperaldosteronism
PCR	polymerase chain reaction
RNA	ribonucleic acid
S	11-deoxycortisol
SDS	sodium dodecyl sulphate
SF-1	Steroidogenic factor-1
StAR	Steroidogenic acute regulatory protein
SSCP	single-stranded conformational polymorphism
TE	Tris-HCl/EDTA buffer
TEMED	N,N,N,N'-tetramethylethylenediamine

CHAPTER 1

1. Introduction

In developed societies, the principal causes of morbidity and mortality are cardiovascular and cerebrovascular disease. These in turn are frequently the result of the so-called triple syndrome which is associated with obesity, glucose intolerance and hypertension. While it is clear that a major proportion of the risk of developing these conditions is due to aspects of life style such as diet, smoking and exercise, genetic predisposition is also important. Thus, offspring of hypertensive parents have a significantly enhanced risk of themselves becoming hypertensive (Watt et al. 1992). Understanding the mechanism of this inherited predisposition is a pre-requisite of developing strategies of prevention and treatment.

Hormones are key factors in the control of metabolism and blood pressure; many have been studied in connection with the aetiology of primary or essential hypertension. It is, however, striking that most of the rare monogenic hypertensive syndromes relate to aberrations of corticosteroid biosynthesis and metabolism and, moreover, that corticosteroids are key factors in the control of glucose metabolism and of blood pressure. Whether or how they contribute to the genetic component of essential hypertension has not, however, been established. To obtain a fuller understanding, the structure of the genes involved in corticosteroid synthesis must be understood and, in particular, how small changes in this structure alter biosynthesis and therefore risk of disease. In this introductory review, the structure and function of the adrenal cortex are described with particular attention to the genes, enzymes and biochemical processes controlling the secretion of corticosteroids in normal subjects and in adrenocortical disorders in hypertensive animal and human patients.

1.1. The adrenal gland

An adrenal gland can be found at the superior pole of each kidney in mammals. The glands differ in shape between species, being for example, roughly triangular in cross section in man and round in the rat. They comprise the adrenal medulla, which secretes catecholamines such as adrenaline, surrounded by the cortex, the source of a number of steroid hormones (see below). The cortex consists of distinct zones with

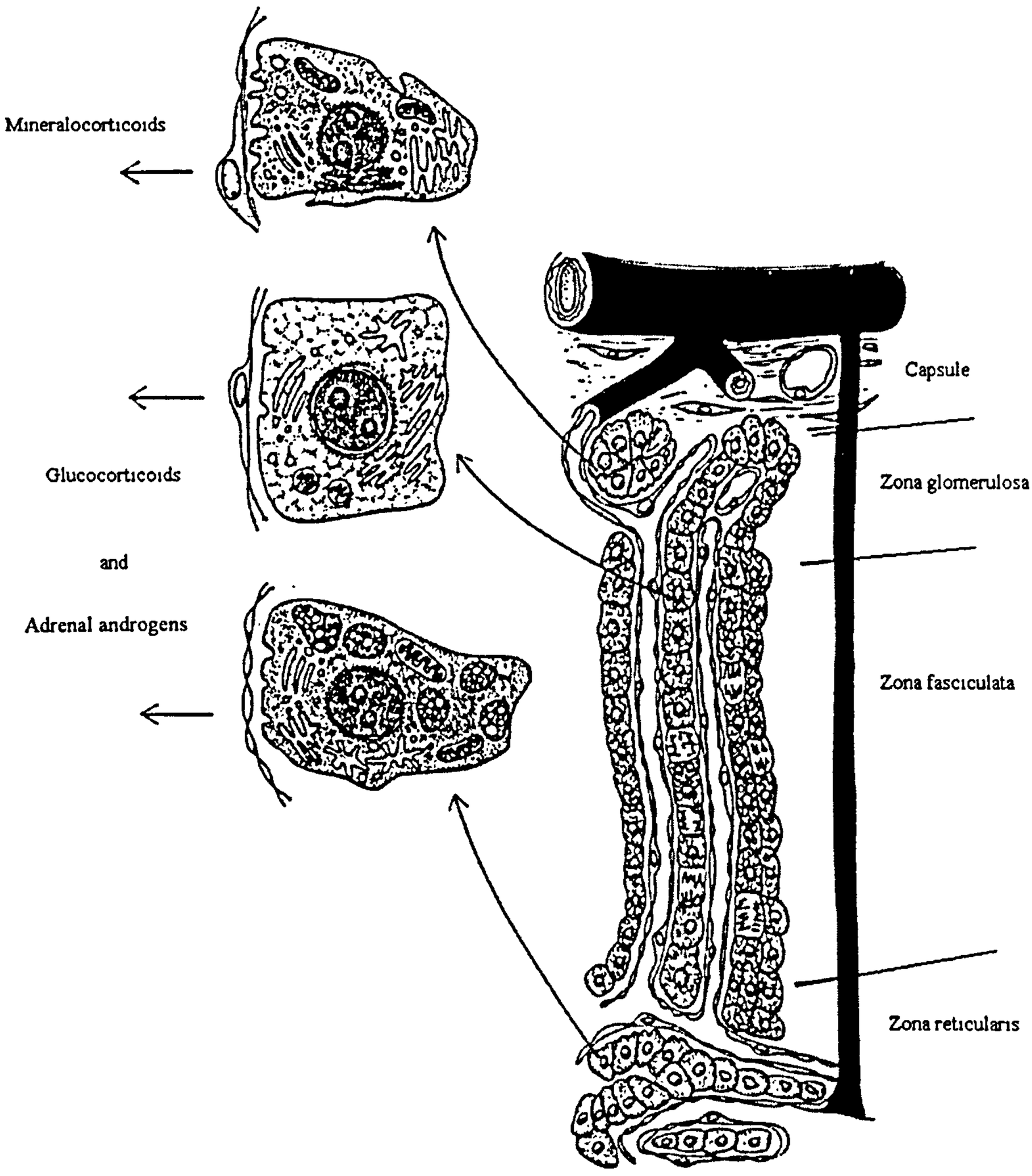


Figure 1.1a Microscopic anatomy of the adrenal cortex.

After Gray's Anatomy, 36 Ed.

different steroidogenic capability and capacity (see **figure 1.1a.**). Adjacent to the medulla is the zona reticularis. The middle zone, the zona fasciculata is usually the widest and consists of large lipid-laden cells with mitochondria which have tubo-vesicular critsae. The zona glomerulosa is thinner and often incomplete. Its cells are smaller and more compact with no lipid deposits and fewer mitochondria. The gland is enclosed in an outer capsule composed of loose fibrous connective tissues.

The adrenal cortex is highly vascularised (Flint 1990). Arterial blood enters the gland via a series of small arteries originating from the dorsal aorta with additional supplies from branches of the renal and the inferior phrenic arteries. An extensive network of arterioles from these arteries pervade the capsule. Venous blood drains via the left adrenal vein into the renal vein and via the right adrenal vein into the vena cava.

A dense network of nerves supplies the gland. Cholinergic innervation and also vasoactive intestinal peptide (VIP) immunoreactive nerve fibres have been identified (Kleitman et al. 1985, Holzwarth 1984). In addition to interacting with adrenocytes, these nerves may assist in the control of adrenal blood flow, a factor important in hormone secretion rate (L'age et al. 1970).

1.2. Products and function of the adrenal cortex.

The adrenal cortex synthesises and secretes a greater variety of steroid hormones than any other steroidogenic organ. The steroid hormones are derived from cholesterol which is progressively modified by a series of locus- and orientation-specific enzyme-catalysed reactions which progressively shorten the side chain and add substituents such as hydroxyl groups (see below). They may be classified as C19 compounds, the adrenal androgens, and C21 compounds, the corticosteroids.

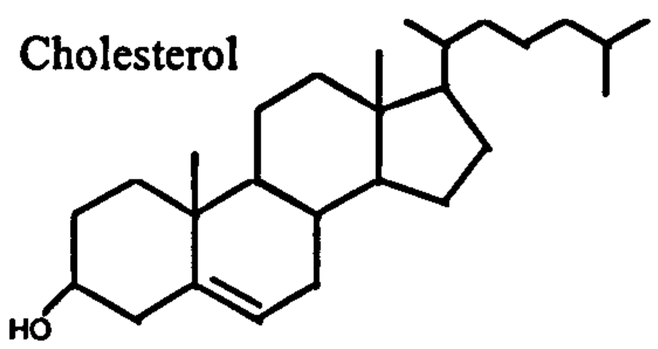
1.2.1. Androgens The principal adrenal androgens are androstenedione and dehydroepiandrosterone (DHEA) and its sulphate (DHEAS). They are synthesised in the zona fasciculata and reticularis (**Figure 1.1a**). Their function in normal subjects is unclear but they may contribute to virilisation in some forms of adrenal disease and in post-menopausal women. Reduced levels of DHEA and DHEAS are associated with the aging process (Vermeulen 1995). Increased levels of DHEA are also associated

with reduced cancer rates. Studies in mice showed that direct injection of DHEAS into the brain improved long term memory (Roberts et al. 1987). A recent study has shown an association of cognitive impairment in the elderly with a high ratio of cortisol (see below) over DHEAS (Kalmijn et al. 1998). This suggests that high cortisol and low DHEAS levels are associated with the aging process. The subject remains controversial.

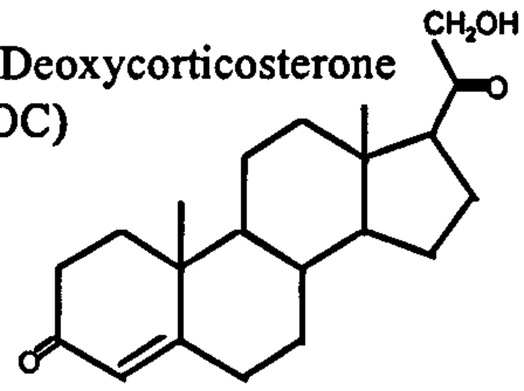
1.2.2.Corticosteroids In man, the functionally most important C21 products are cortisol and aldosterone **(Figure 1.2a)**. Cortisol is synthesised mainly in the zona fasciculata while aldosterone is a product of zona glomerulosa and exists in solution mainly in the hemiacetal form. (The rat adrenal cortex is unable to synthesise 17α-hydroxylated steroids; its principal zona fasciculata product is corticosterone). In addition, a large number of other corticosteroids have been isolated and characterised **(Figure 1.2a, Table 1.2)**. These are frequently intermediates in the biosynthesis of the corticosteroid end products and their importance will become evident later in this review.

The corticosteroids may be subdivided on the basis of their metabolic effects. Aldosterone acts on the distal nephron to promote sodium reabsorption in exchange for potassium and hydrogen ions. The large intestine and the salivary glands are also target organs as is the amphibian bladder. Aldosterone is thus a **mineralocorticoid**. DOC has much lower potency but may become important in some adrenal diseases (see section 1.8). Mineralocorticoids influence blood pressure partly by modulating intravascular volume and partly because extracellular sodium (and potassium) concentrations determine vascular smooth muscle sensitivity to pressor agonists such as angiotensin II. Recently, it has been shown that aldosterone may affect the function of tissues not usually associated with electrolyte transport e.g in the heart where it is thought to be associated with cardiac fibrosis and arrhythmias (Rahman 1992) and also in the central nervous system and vascular smooth muscle where it may affect blood pressure (Gomez-Sanchez. 1997, Takeda et al. 1995). Thus, high levels of aldosterone promote cardiac remodelling and stimulate collagen synthesis in cardiac fibrocytes, resulting in cardiac fibrosis (Brilla et al. 1992). The aldosterone antagonist, spironolactone, inhibits this process (Brilla et al. 1993). Aldosterone at very

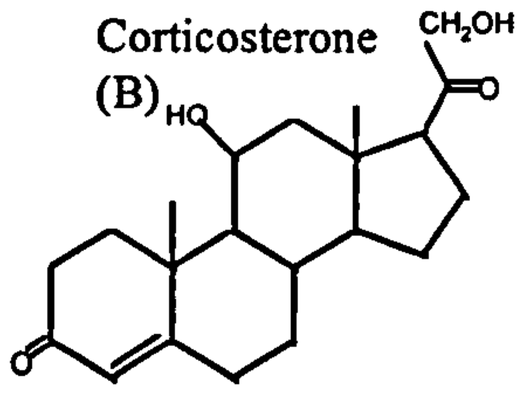
Cholesterol



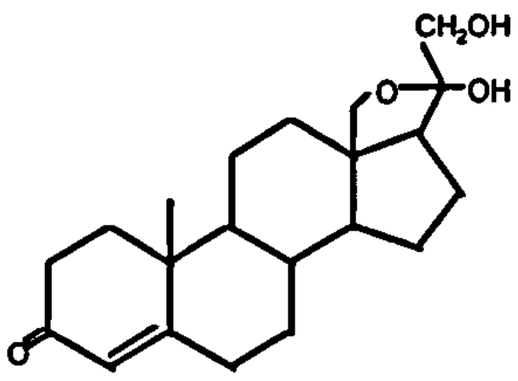
11-Deoxycorticosterone
(DOC)



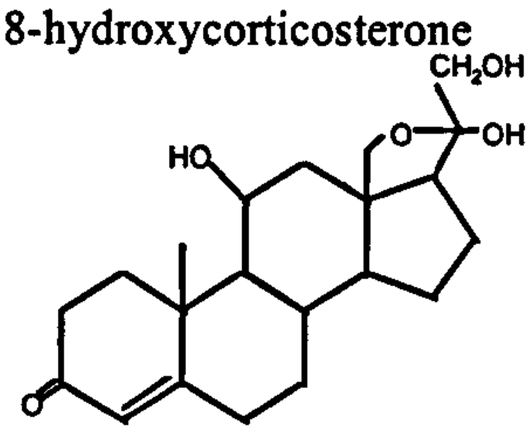
Corticosterone
(B)



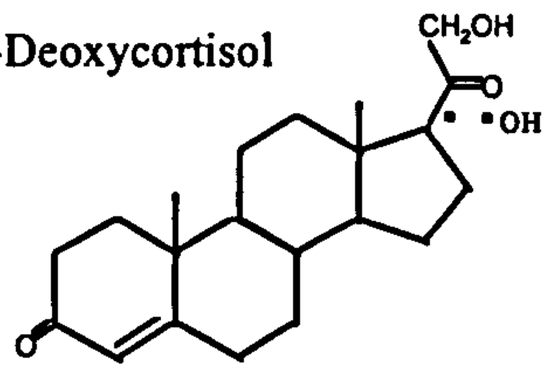
18-hydroxydeoxycorticosterone
(18-OHDOC)



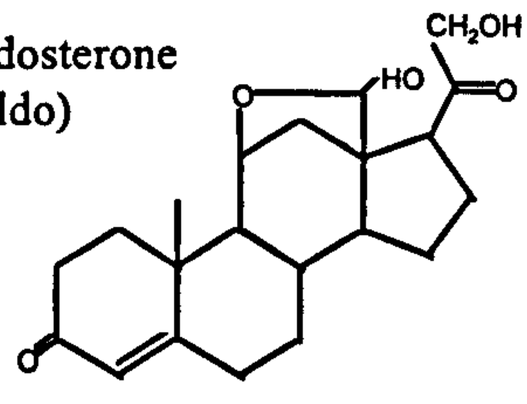
18-hydroxycorticosterone



11-Deoxycortisol
(S)



Aldosterone
(Aldo)



Cortisol
(F)

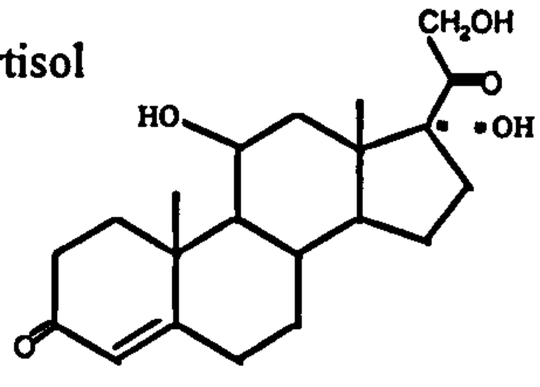


Figure1.2a. Steroid structures and common names

low concentrations also stimulates Na+/H+ exchange in leucocytes (Wehling.1997), possibly by activating a novel membrane receptor (see below).

Cortisol (corticosterone in the rat) is defined as a **glucocorticoid**. It has profound effects on intermediary metabolism, stimulating gluconeogenesis, enhancing protein catabolism and, in excess, increasing fat deposition of characteristic central distribution. Glucocorticoids are important as inhibitory factors of the immune response and inflammatory processes and are used as immunosuppressants, for example, in organ transplantation. However, it is important to emphasise that glucocorticoids also have profound effects on blood pressure. Although the precise mechanism of these effects is not understood, glucocorticoids affect renal function (GFR, tubular flow, Na+/H+ exchange) and also control the rate of synthesis of many other hormones and factors which alter vascular and peripheral nerve function (see review Fraser and Blackhurst in press). A much used and potent synthetic glucocorticoid is dexamethasone **(Figure 1.2a, Table 1.2)**

1.3 Mechanism of action

Steroid hormones are lipophilic and thus are readily able to penetrate the cell membrane. In common with thyroid hormones and retinoids, they act by binding to intracellular receptors (in the case of corticosteroids, in the cytoplasm) to form a steroid-receptor complex which binds to chromatin and induces the synthesis of new proteins. For this reason, in contrast to hormones acting on cell membrane receptors, there is significant delay - 'latent period' - between steroid binding and hormone effects. The glucocorticoid (type2; GR) and mineralocorticoid (type1; MR) receptors belong to this large nuclear receptor super family and are distinguished by their different ligand affinities. However, their basic mechanisms of action are similar. The cDNAs for all the major receptors have been cloned and much is known about their cytoplasmic state, alteration on binding to the ligand and the processes which subsequently lead to altered rates of gene transcription . While the GR has been more intensively studied, it is likely that the MR acts in a similar manner.

COMMON NAME	SYSTEMATIC NAME
11-Deoxycortisol (S)	17,21-dihydroxy-pregn-4-ene-3,20-dione
Cortisol (F)	11β,17α, 21-trihydroxy-pregn-4ene-3, 20-dione
11-Deoxycorticosterone (DOC)	21-hydroxypregn-4-ene-3,20-dione
Corticosterone (B)	11β, 21-dihydroxy-4-ene-3,20-dione
18-Hydroxycorticosterone (18-OHB)	11β,18,21-trihydroxy-4-ene-3,20-dione
Aldosterone (Aldo)	11β, 21-dihydroxy-pregn-4-en-18al-3, 20-dione
18-OHDOC	18,21-dihydroxy-4-ene-3,20-dione
Dexamethasone (DEX)	9α-fluoro-16α-methyl, 1-dehydrocortisol

Table 1.2 Common and sytematic names of adrenal steroids and Dexamethasone.

In the ligand-free state , the GR exists as a 310kda complex. It was first cloned and expressed in 1985 (Hollenberg et al. 1985). Two isoforms have been identified GRα and GRβ. The latter form does not bind active glucocorticoids but is involved in ligand independent negative regulation of glucocorticoid action (Bamberger et al. 1995). The activated receptor is much smaller (90KDa) indicating a dissociation of a multicomponent complex (**Figure 1.3a**). Further research has shown that the free receptor complex consists of a single receptor molecule associated with two 90KDa heat shock protein 90 (Hsp90) molecules as well as one each of Hsp56 and Hsp70 (for review see Panarelli and Fraser 1994). Another smaller peptide constituent, p53, has also been identified. Finally, several heat stable molecules such as polyunsaturated fatty acids and aminoether phosphoglycerides have been isolated from the complex. Hsp90 is a crucial component of the complex, acting as a structure -stabilising chaperone protein. Its loss by heat inactivation or mutation decreases ligand binding affinity to about 1% and increases turnover rate (Werner et

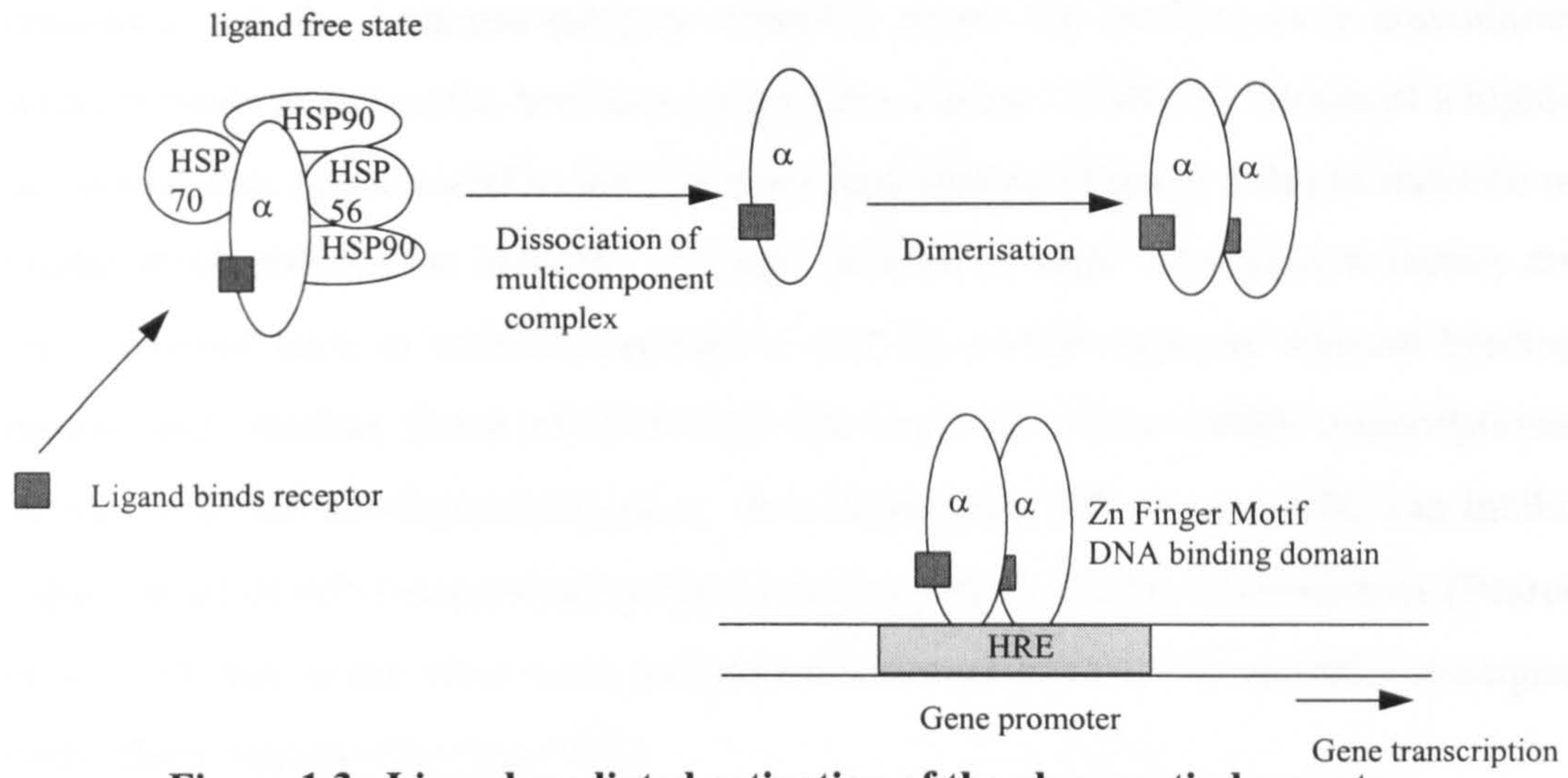


Figure 1.3a Ligand mediated activation of the glucocortiod receptor Adapted from (DeRijk et al. 1997)

1992). It is thought to protect the DNA-binding domain (see below) of the ligand -free receptor. The receptor protein consists of domains responsible for the specific functions (**Figure 1.3b**), principally a DNA-binding domian and C-terminal ligand-binding domain. The immunogenic region occupies the N-terminus.

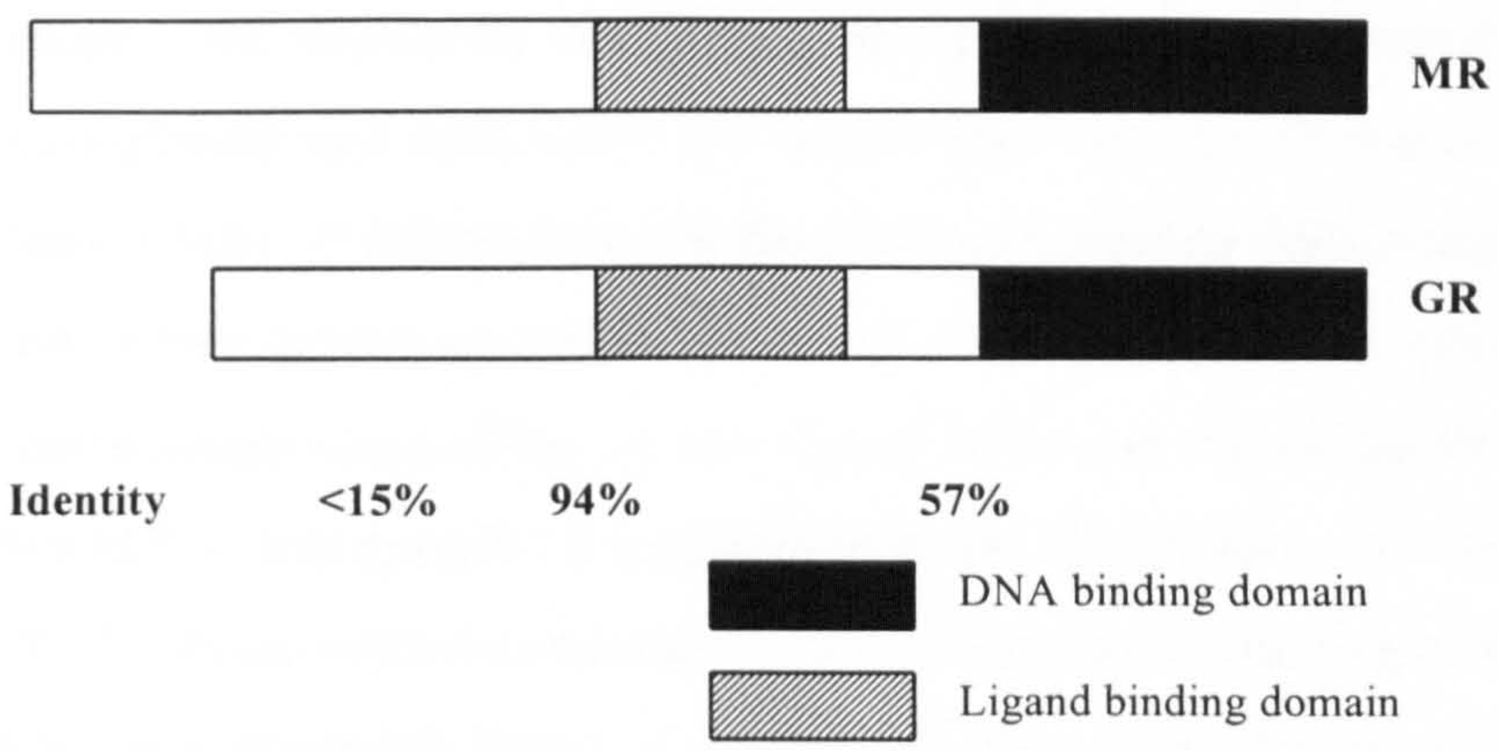


Figure 1.3 b. Diagram of human mineralocorticoid receptor (MR) and glucocorticoid receptor (GR).

Binding of the ligand decreases the receptor affinity for the chaperones proteins which dissociate and the hormone-receptor complex enters the nucleus as a homodimer where it binds to a specific, hormone -responsive element (HRE) by means of a highly conserved zinc finger motif in the DNA-binding domain (**Figure 1.3a**) to institute or inhibit RNA-polymerase activity. A large number of other transcription factors are also involved such as activation-protein 1 (AP-1), cAMP-response element binding protein and nuclear factor κB (NF-κB); GR binds NF-κB to inhibit transcriptional activation of NF-κB-dependent genes. It is known that GR, but not MR, can inhibit induction of of AP-1-dependent genes by interaction of fos/jun heterodimers (Pearce et al. 1993) but on the other hand, jun/jun homodimers and the GR mutually synergise each others activity (Teurich 1995).

The GR binds cortisol, corticosterone and dexamethasone. It is widely, almost ubiquitously expressed in tissues and has great importance in the liver, brain, vascular tissue, kidney and the immune system. Glucocortiocoids, for example, modulate the activities of several neurotransmitter systems such as the adrenergic and cholinergic-muscarinic systems (Torres et al. 1991). Moreover, they exert an important influence on vascular tone, assisting in the control of sensitivity to pressor agents such as ANGII and noradrenaline (Kornel 1993, Scott et al. 1987). Independently of this, they affect the transcription of the genes encoding angiotensinogen, renin, the AT-1 receptor, the endothelin (ET-1) receptor, endothelin, the metabolism of noradrenalin, prostaglandin and nitric oxide (for review see Fraser and Blackhurst , In press).

Their effects on intermediary metabolism have already been mentioned. The GR can have either inhibitory action, as is the case in the immune sytem where it has an immuno-supressant effect, or stimulatory effects as seen in the liver.

The MR is less specific. It binds cortisol and aldosterone with equal affinity (Funder 1997). Since cortisol circulates in normal subjects at concentrations up to 1000 times those of aldosterone, it should (a) prevent aldosterone binding to the MR and (b) exert potent mineralocorticoid effects itself. That it does not is due to the action of 11β-hydroxysteroid dehydrogenase type 2, the 'cortisol-cortisone shuttle' (Stewart et al. 1991). This microsomal enzyme is present in mineralocorticoid-responsive tissues and oxidises cortisol to cortisone which does not bind to the MR.

The major site of aldosterone action in the nephron of the kidney is in the distal convoluted tubule and the cortical collecting tubules where there is a dense population of MR (Schwartz et al. 1978, Horisberger et al. 1983). However, these receptors are also expressed in the liver, hippocampus pituitary, peripheral blood leucocytes , colon and the salivary glands (Orth et al. 1992, deKloet et al. 1998). In the basolateral membrane of the luminal cells of the tubules, Mg^{2+}- dependent, $Na^{+}K^{+}$ -dependent ATPases mediate sodium:potassium exchange and maintain the electrochemical gradient across the cell membrane. When aldosterone is administered chronically, these cells undergo changes in structure and the basolateral membrane surface area is increased with higher expression of the genes encoding the α- and β- subunits of the $Na^{+}K^{+}$ATPase (Komesaroff et al. 1994). The epithelial Amiloride-sensitive channel also found in the distal nephron (Rossier 1994) is also regulated by aldosterone and helps to maintain Na^{+} balance, extracellular volume and blood pressure by controlling Na^{+} entry into the cells of the distal nephron (Canessa et al. 1994).

Aldosterone -sensitive sodium reabsorption by the distal nephron constitutes only 5% of filtered sodium; the remaining 95% has already been absorbed prior to arriving at these sites. Never the less, the remaining 5% constitutes about 80 grams per day and therefore the regulation of its absorption by aldosterone is crucial in the control of extracellular electrolytes, volume and blood pressure. Hydrogen ion secretion is also regulated by the kidney by the intercalated cells of the collecting tubule. Aldosterone acts on the ATP-dependent apical hydrogen ion pump combined with regulation of the basolateral membrane Cl-/HCO3 exchanger (Hays 1992). The net result of these effects on sodium, potassium and hydrogen ions is to increase the extracellular volume and increase blood pressure. Aldosterone excess results in hypokalaemia, metabolic alkalosis, raised exchangeable sodium content and a low body potassium (Ferriss et al. 1983, Kremer et al. 1977).

Recently, studies have been published which suggest that aldosterone may affect cell functions other than electrolyte transport. A specific example is that aldosterone mediated via a non-epithelial non-protected MR can induce experimental cardiac hypertrophy and fibrosis (Brilla et al. 1992). The receptors described above are cytosolic. However, a membrane receptor has been reported (Wehling et al. 1997).

This receptor is found on the red blood cell membrane, has a very high affinity for aldosterone and a very rapid response. In white blood cells, it increases Na+/H- and in cardiac fibrocytes it is associated with increased collagen synthesis.

1.4 Biosynthesis

Cholesterol is the principal building block of steroid hormone biosynthesis. Steroid hormone biosynthesis occurs mainly in the mitochondria of steroidogenic tissues where a series of enzymes, the majority of which belong to the haem-containing cytochrome P450 superfamily, convert cholesterol to endogenous steroids.

1.4.1 Steroidogenesis

The biosynthetic pathways within the adrenal gland will be discussed in some detail later in this section. Firstly however, cholesterol must be delivered to the mitochondria. It can be synthesised in the adrenal cortex de novo from acetate or mobilised from cholesterol esters in intracellular pools. Approximately 80% of cholesterol is derived from circulatory lipoproteins, predominantly low density lipoprotein (LDL). Acting at a cell surface receptor (Gwynne and Strauss 1992), the LDL particle is internalised by receptor-mediated endocytosis, releasing the cholesterol esters within the cell for steroid biosynthesis (Kovanen 1979, Brown 1979). Adrenocortical cells, particularly those of the zona fasciculata (ZF), are characterised by abundant fat vacuoles packed with stores of esterified cholesterol which are mobilised for steroidogenesis. The rate limiting step of steroidogenesis was originally thought to be cholesterol conversion to pregnenolone by the cytochrome P450 side chain cleavage enzyme (P450scc) (see below) but it is now well-established that the mobilisation and delivery of cholesterol to this enzyme within the mitochondria is the real rate-limiting factor. The enzyme, cholesterol ester desmolase, hydrolyses cholesterol esters, releasing free cholesterol available for steroidogenesis in response to hormonal stimulation (see section 1.5). Once mobilised, the cholesterol must cross the outer and inner mitochondrial membrane. A number of cholesterol carrier/translocation systems have been identified.

1.4.2 Cholesterol translocation

Sterol carrier protein, SCP2 is a well-characterised carrier of sterols and is found in the liver. It also enhances the formation of pregnenolone from cholesterol in adrenal mitochondria. Involvement of this protein in adrenal steroidogenesis has been documented (Vahouny et al. 1985). Following hydrolysis of cholesterol esters, the sterol is transferred to the inner mitochondrial membrane, a process facilitated by a SCP2-like protein.

More recently, a cholesterol carrier protein localised to the mitochondria and induced in steroidogenic cells by ACTH (see section 1.5) has been identified (see review Stocco 1998) and is known as steroidogenic acute regulatory protein (StAR). As levels of cholesterol increase within the mitochondria, so too do levels of StAR (Strauss abstr. 1998). StAR acts directly at the mitochondrial surface, possibly with a surface protein, and forms an import core through which cholesterol flows down a gradient from the outer to the inner membrane. When cholesterol moves into the mitochondrial matrix, StAR activity stops (Strauss abstr. 1998). Missense mutations in the StAR gene have been detected in patients with Congenital lipoid hyperplasia. Patients have a total inability to make steroids and the adrenal cortex is packed full of the steroid building block cholesterol. The resulting defective StAR protein is unable to carry cholesterol to the mitochondrial enzymes.

In the last ten years, a novel cholesterol transport mechanism distinct from those described above has been identified. It is based on the peripheral-type benzodiazepine receptor PBR (Papadopoulos 1993) and occurs in response to acute hormonal stimulation (see section 1.5). Benzodiazepines are a class of anticonvulsant drugs which act in the CNS at $GABA_A$ receptors (Haefly et al. 1975, Costa et al. 1979). However, a different receptor from that in the CNS has been identified in the periphery and localised specifically to the outer mitochondrial membrane (Anholt et al. 1986). It is associated with a voltage-dependent anion channel protein (VDAC) and an inner mitochondrial membrane adenine nucleotide carrier (McEnry et al. 1992). This suggests that the PBR is a multimeric complex. The VDAC protein is a

large diameter ion channel which is located in outer membrane of mitochodria at junctions or contact sites with the inner mitochondrial membrane (Levitt et al. 1990). VDAC, as well as anions, allows small molecules to be translocated from the cytoplasm to the inner mitochondrial membrane (Levitt et al. 1990). PBR molecules are found in clusters and are associated with these VDAC channels. Their arrangement on the outer mitochondrial membrane at contact sites enables them to transport free cholesterol from the outer to the inner mitochondrial membrane. The mechanism by which the translocation of cholesterol is controlled will be discussed in section 1.7.

After translocation into the mitochondria, cholesterol undergoes complex modification. A series of hydroxysteroid dehydrogenases and mixed function oxidases catalyse the biosynthesis of adrenal steroid hormones. The oxidases belong to a superfamily of haem-containing mono-oxygenases which also includes liver enzymes such as those involved in drug detoxification (Williams 1973). Adrenal enzymes are either microsomal or mitochondrial. Side chain cleavage enzyme, 11β-hydroxylase and Aldosterone synthase are mitochondrial. They require a coupled coenzyme system which transfers electrons to the P450 enzyme as the reducing equivalents necessary for the hydroxylation reaction (see **figure 1.4a**)

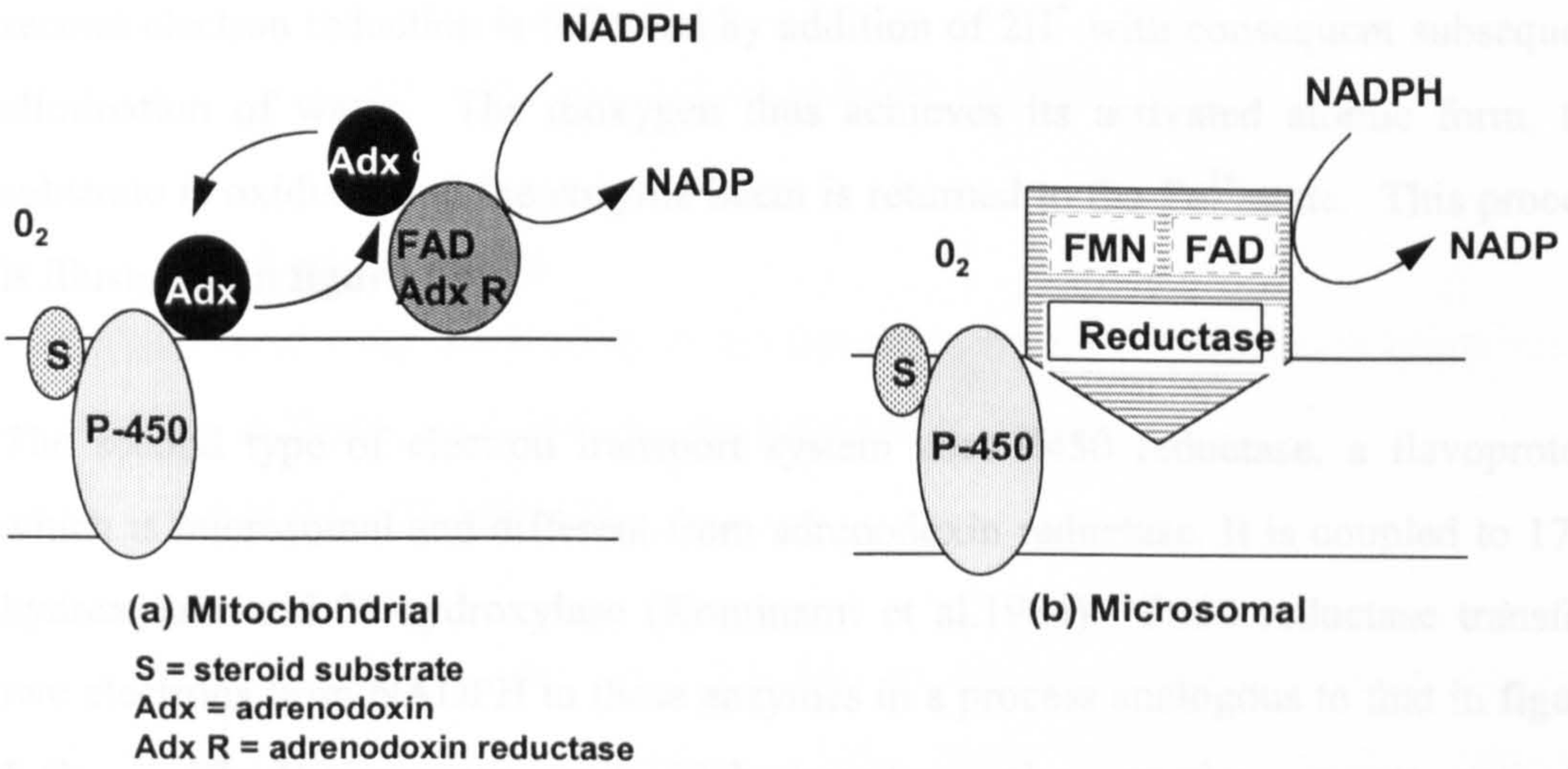


Figure 1.4a. Steroidogenic electron transfer system in (a) mitochondria and (b) microsomes. Adapted form Kominani et al (1984).

1.4.3. Electron sources for hydroxylation

Two such electron transport systems are found in man. The adrenodoxin/ adrenodoxin reductase system is utilised by side chain cleavage enzyme, 11β-hydroxylase and aldosterone synthase which are mitochondrial (Kimura and Suzuki. 1967). Adrenodoxin, a non-haem iron protein, is linked to the flavoprotein adrenodoxin reductase which accepts electrons from NADPH supplied mainly from the citric acid cycle. In man, adrenodoxin and adrenodoxin reductase are each encoded by single genes; the reductase has two isoforms due to alternative DNA splicing (Solish et al. 1988). There are also two adrenodoxin pseudogenes. The adrenodoxin/adrenodoxin reductase "electron shuttle" system is illustrated in **figure 1.4a**.

By providing electrons, NADPH transforms adrenodoxin reductase into its reduced state which in turn reduces adrenodoxin. This then transfers reducing equivalents to the P450 enzyme. Its active site has a single iron protoporphyrin prosthetic group where dioxygen binds, is reduced and cleaved. The steroid substrate binds the low spin Fe^{3+} P450 enzyme to generate a high spin ferric complex. An electron from NADPH converts Fe^{3+} to Fe^{2+} which is then able to bind molecular oxygen. This second electron reduction is followed by addition of $2H^{+}$ with consequent subsequent elimination of water. The dioxygen thus achieves its activated atomic form, the substrate is oxidised and the enzyme haem is returned to the Fe^{3+} state. This process is illustrated in **figure 1.4b**.

The second type of electron transport system uses P450 reductase, a flavoprotein which is microsomal and different from adrenodoxin reductase. It is coupled to 17α-hydroxylase and 21-hydroxylase (Kominami et al.1984). P450 reductase transfers two electrons from NADPH to these enzymes in a process analogous to that in **figure 1.4b**. 17α-Hydroxylase and 21-hydroxylase also employ cytochrome b5 (Yanagibashi and Hall, 1986).

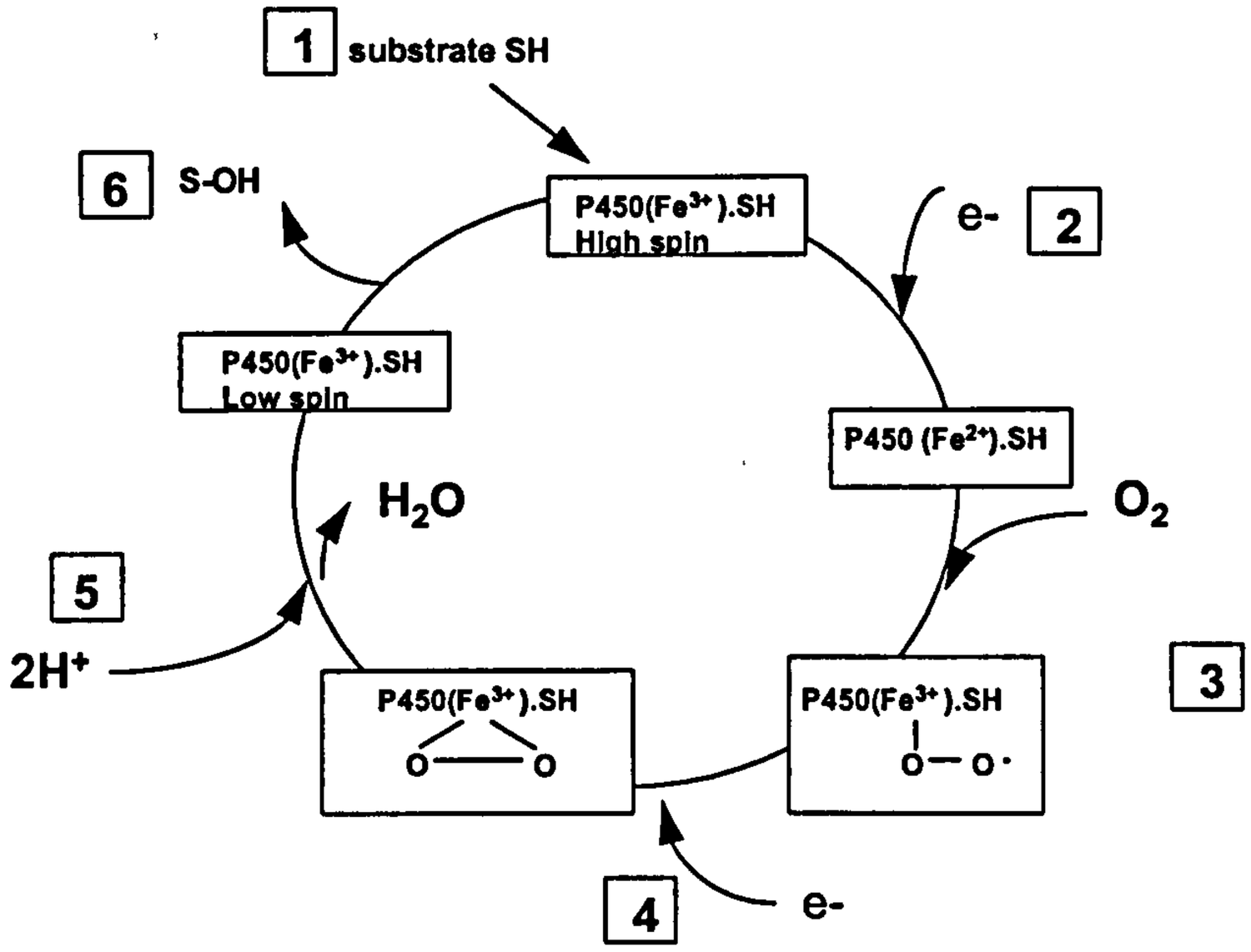


Figure 1.4b. The sequence of reactions in P450 catalysed hydroxylation. Adapted from Dawson (1988).

Both electron transport mechanisms enable the mono-oxygenases to insert one atom of oxygen into the substrate (Hall et al. 1985, Gwynne and Mahaffee 1986); the other atom of molecular oxygen combines with H^+ to form water. The overall reaction is as follows:

$$\text{Substrate-H} + \text{NADPH} + H^+ + O_2 = \text{Substrate-OH} + \text{NADP+} + H_2O$$

1.4.4 Biosynthetic pathways

The sequence of reactions leading to the formation of the corticosteroid hormones is summarised in **figure 1.4c.** Briefly, the steps are as follows.

(a) Cortisol and Corticosterone

1. Cholesterol enters the inner mitochondrial membrane where side chain cleavage enzyme converts it to pregnenolone. Side chain cleavage enzyme catalyses hydroxylations at the 22R and 22S positions. The side chain is cleaved as isocaproic acid leaving the C21 steroid, pregnenolone (Strott et al. 1990). This enzyme is

encoded by a single gene (CYP11A) on chromosome 15 and is expressed in all steroidogenic tissues (Chung et al. 1986a).

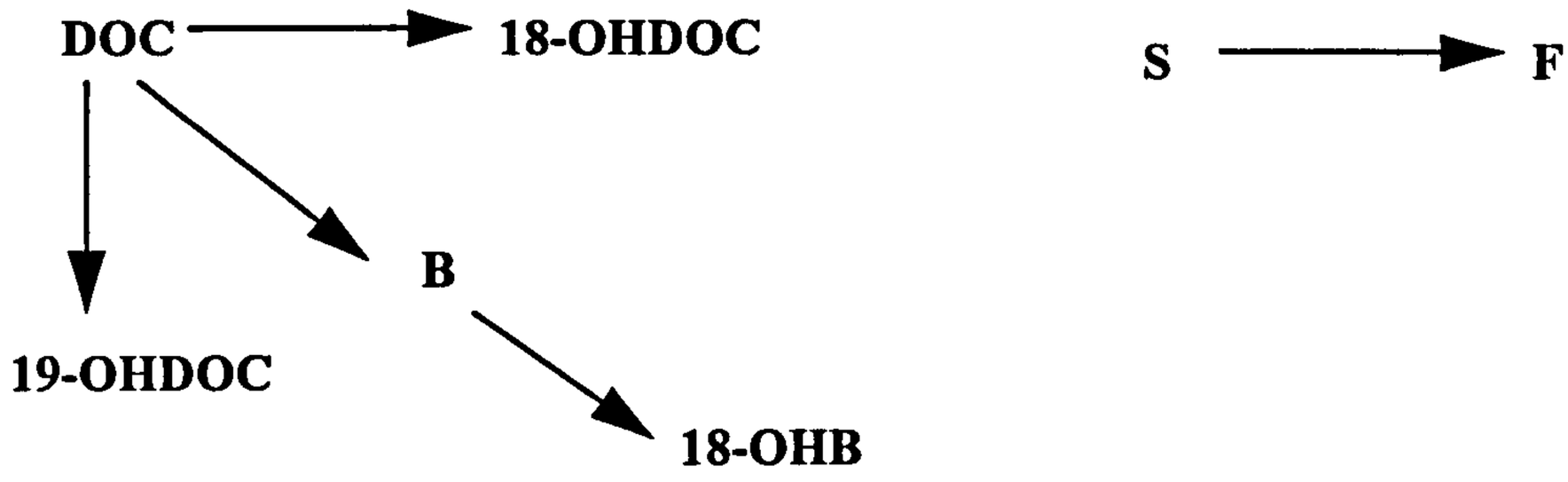
2. Pregnenolone is converted to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD). It is also able to catalyse the conversion of 17α-hydroxypregnenolone to 17α-hydroxyprogesterone (see below). This is not a cytochrome P450 enzyme. It is encoded by a single gene on chromosome 1 (Berube et al. 1989). The gene exhibits tissue-specific expression and a number of isoforms have been described (Lachance et al. 1991).

3. Pregnenolone leaves the mitochondria, passing to the smooth endoplasmic reticulum where it is converted to the 17α-hydroxyderivative by 17α-hydroxylase. This enzyme is encoded by a single gene, CYP17A, on chromosome 10 (Matteson et al. 1986). 17α-Hydroxypregnenolone is converted to 17α-hydroxyprogesterone as described in 2. Progesterone may also be 17α-hydroxylated to produce 17α-hydroxyprogesterone. These 17α-hydroxysteroids are required for cortisol biosynthesis. The enzyme possesses an additional 17-20 lyase activity which converts 17α-hydroxylated C21 steroids into C19 steroids, the adrenal androgens (dehydroepiandrosterone (DHEA), androstenedione and testosterone (Orth et al. 1992). There is no 17α-hydroxylase activity in the ZG. Moreover, the rat adrenal gland contains no 17α-hydroxylase.

4. 17α-Hydroxyprogesterone is then converted to 11-deoxycortisol in the ZF by the enzyme 21-hydroxylase, also in the smooth endoplasmic reticulum. Progesterone from **2.** can also be converted to 11-deoxycorticosterone (DOC) by P450C21. This enzyme is encoded by the CYP21A gene which lies on chromosome 6. A highly homologous inactive pseudogene, CYP21P, lies in tandem with CYP21A within the Major Histocompatibity Complex region.

5. 11-Deoxycortisol (S) is shuttled back to the inner mitochondrial membrane where 11β-hydroxylase converts it to cortisol (F), the major human glucocorticoid. This enzyme is encoded by CYP11B1 which resides on the long arm of chromosome 8

(8q22) (Mornet et al. 1989). As well as cortisol formation, this enzyme can 11β-hydroxylate 11-deoxycorticosterone (DOC) to produce corticosterone (B), and 18- and 19-hydroxylate to produce 18-hydroxydeoxycorticosterone (18-OHDOC), 18-hydroxycorticosterone (B) and 19-hydroxy-11-deoxycorticosterone (19-OHDOC) respectively, also in the ZF.



(b) Aldosterone

Conversion of cholesterol to DOC in the ZG is identical to steps 1-2 and step 5 converts progesterone to pregnenolone above. Production of aldosterone, the major human mineralocorticoid, from DOC is catalysed by the enzyme aldosterone synthase and involves three enzymatic reactions, 11β-hydroxylation, 18-hydroxylation and 18-oxidation. This enzyme is encoded by the CYP11B2 gene and also resides on chromosome 8 situated in tandem approximately 40kb upstream of CYP11B1 (see section 1.6). Aldosterone synthase is exclusively expressed in the ZG and it is in this zone that the three terminal stages of aldosterone biosynthesis occurs.

1. DOC is the preferred substrate of aldosterone synthase.
The 11β-hydroxylase activity of this enzyme converts DOC to corticosterone (B) by adding a hydroxyl group at C11.

2. Corticosterone (B) is then converted to 18-hydroxycorticosterone (18OHB) by 18-hydroxylation and addition of a hydroxyl group at position C18.

3. 18-OHB is finally converted to aldosterone by the final 18-oxidation step which converts the hydroxyl group at C18 to an aldehyde with subsequent removal of water.

DOC ⟶ **B** ⟶ **18-OHB** ⟶ **Aldosterone**

There has been some controversy over the existence of an 18-oxo product in the interconversion between 18-OHB and aldosterone and an 18-hydroxysteroid dehydrogenase activity of aldosterone synthase between 18-OHB and aldosterone has been described. 18-OHB has a hydroxyl group at C18 and aldosterone has an aldehyde group at this position. This suggests that an 18-hydroxysteroid dehydrogenase catalyses the interconversion. However, when 18-OHB is incubated with aldosterone synthase, a small amount of aldosterone is produced (Vinson and Whitehouse 1970). It is therefore the consensus opinion that aldosterone arises through a second hydroxylation at C18 with subsequent water loss to produce an aldehyde and hence aldosterone. The absolute requirement for reduced NADP is strong evidence of this (Fraser and Lantos 1978).

In vitro studies using cloned human aldosterone synthase showed that B was less efficient at producing 18-OHB and aldosterone than DOC (Denner et al. 1995). These findings show that DOC is the prefered substrate and that it probably stays bound to the active site throughout the three stages of conversion releasing B and 18-OHB only as by-products. Aldosterone is the major, most potent mineralocorticoid in human subjects and is produced in small amounts (100-500pM). It is this aspect which delayed its discovery.

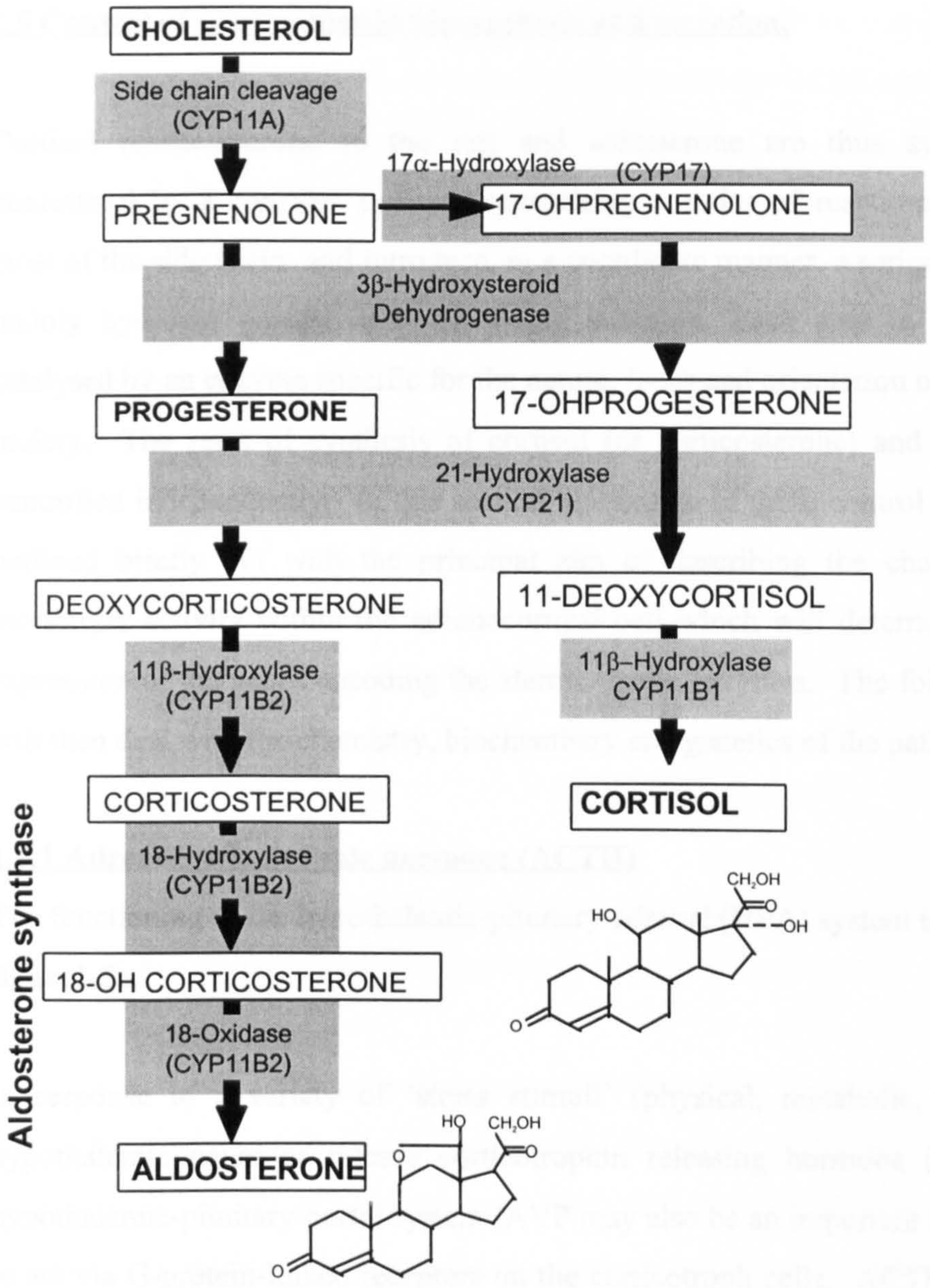


Figure 1.4c Biosynthetic pathway

1.5 Control of Corticosteroid biosynthesis and secretion.

Cortisol (corticosterone in the rat) and aldosterone are thus synthesised from cholesterol by a complex pathway comprising a series of reactions which remove most of the side chain and introduce, in a coordinate manner, a series of substituents, mainly hydroxyl groups, into the 4-ring structure. Each step in the pathway is catalysed by an enzyme specific for the nature, locus and orientation of the substituent moiety. The rates of synthesis of cortisol (or corticosterone) and aldosterone are controlled independently. In this section, the nature of these control systems will be outlined briefly but with the principal aim of describing the changes in second messenger activity within the adrenocortical cell which will determine the rates of expression of the genes encoding the steroidogenic enzymes. The following sections will then deal with the chemistry, biochemistry and genetics of the pathway.

1.5.1 Adrenocorticotrophic hormone (ACTH)

The functioning of the hypothalamic-pituitary-adrenal (HPA) system is summarised in **figure1.5a.**

In response to a variety of 'stress stimuli' (physical, metabolic, psychological), hypothalamic neurones release corticotrophin releasing hormone (CRH) into the hypothalamic-pituitary-portal system (AVP may also be an important releasing factor) to act via G-protein-linked receptors on the corticotroph cells. ACTH is synthesised as part of a precursor molecule, pro-opiomelanocorticotropin (POMC), which also contains β-endorphin, melanocyte-stimulating hormone and a number of other peptides of uncertain function. ACTH is a 39 amino acid peptide, the first 24 residues of which are highly conserved. Biological activity resides in the N-terminal region (1-18); the C-terminal region is immunogenic. ACTH is released from POMC during post-translational modification by enzymatic cleavage. Its secretion follows a circadian rhythm, highest early in the morning and lowest late at night. ACTH stimulates, almost immediately, the secretion of cortisol and corticosterone from the zona fasciculata/reticularis. It also stimulates adrenal blood flow and, chronically, promotes hypertrophy of the zona fasciculata. Acutely and *in vitro,* aldosterone

secretion from the zona glomerulosa is also enhanced. However, continued ACTH use *in vivo* leads to inhibition of secretion and eventual atrophy of the zone.

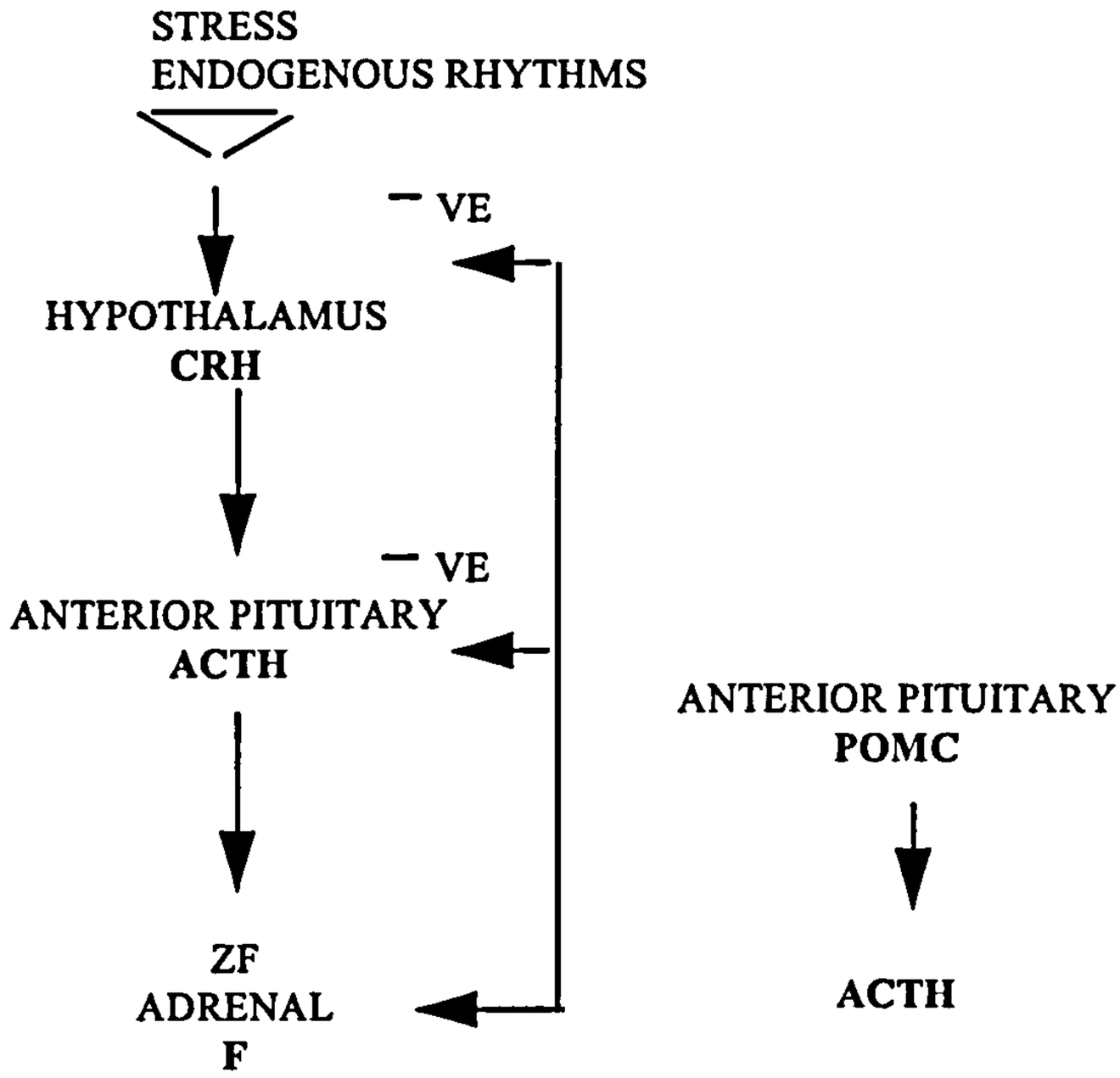


Figure 1.5a. The hypothalamic-pituitary-adrenal (HPA) system

ACTH's primary action is to increase cortisol secretion by activating its synthesis; intra-adrenal storage of cortisol is minimal (Dickerman et al. 1984, Hall 1985). At the cellular level, ACTH acts on the zona fasciculata cells by binding to high-affinity specific cell surface receptors on the plasma membrane, initiating a series of intracellular second messenger systems. The adrenocortical cell membrane is said to possess approximately 3600 ACTH-binding sites (Orth et al. 1992). Only a small receptor occupancy is required to obtain a maximal steroidogenic response. ACTH binding stimulates the guanine nucleotide binding protein, Gs, which stimulates adenylate cyclase and the production of cAMP (**Figure 1.5b**). This intracellular cyclic nucleotide then phosphorylates a number of proteins and transcription factors which leads to the transcription of enzymes required for steroid hormone biosynthesis.

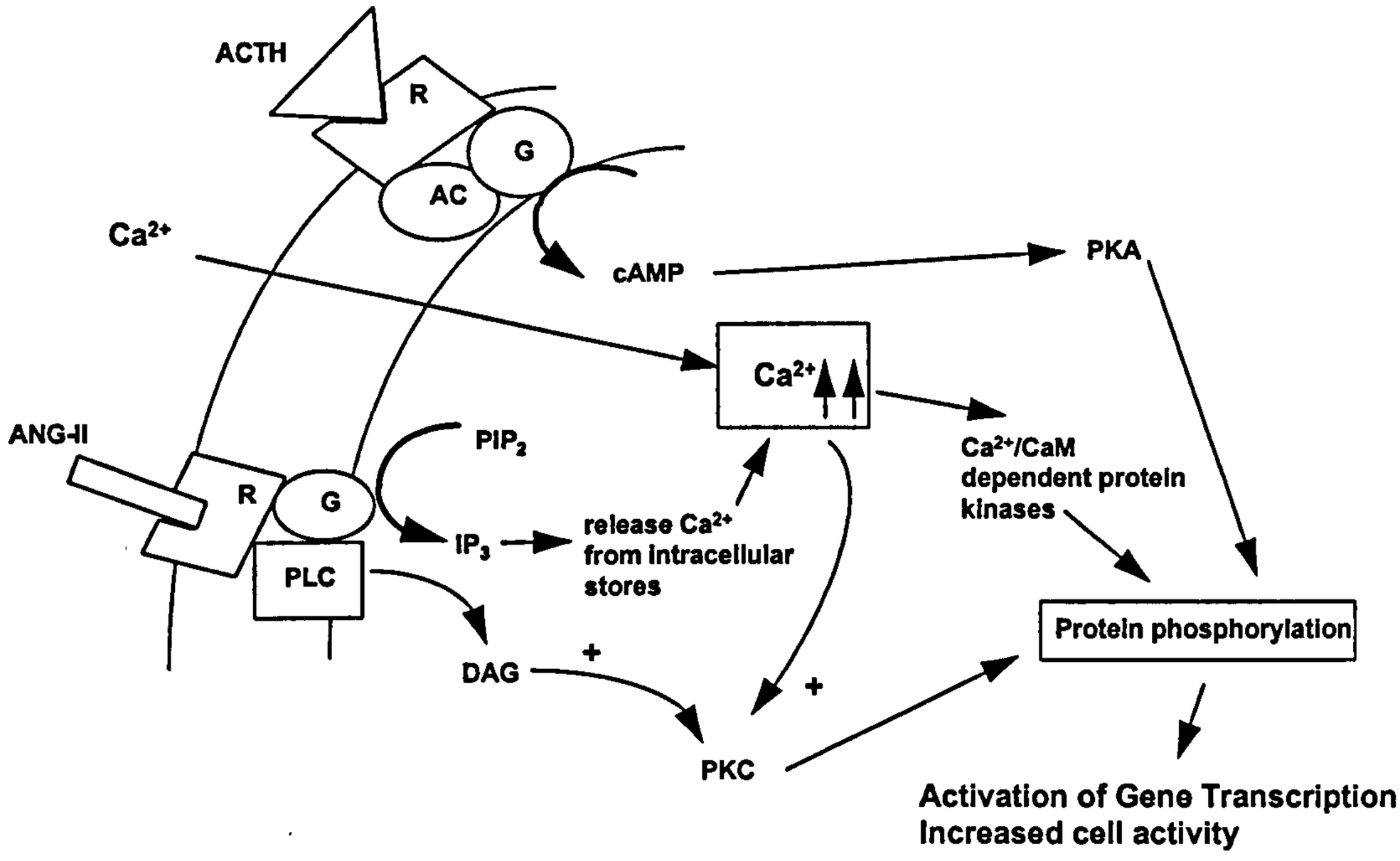


Figure 1.5b. Diagram of ACTH and ANG-II receptors and intracellular mechanisms.

Adapted from The Adrenal Cortex. Ed. Vinson, Whitehouse and Hinson (1992).

For example, cholesterol ester desmolase is phosphorylated directly and converts cholesterol esters to free cholesterol. From these fatty stores, cholesterol is then transported to the site of steroid hormone biosynthesis, the inner mitochondria membrane (see section 1.4). ACTH increases transcription and expression of side chain cleavage enzyme, 11β-hydroxylase, 21-hydroxylase, 17α-hydroxylase and adrenodoxin genes *in vitro* in bovine adrenocortical cells and similarly, cAMP also demonstrates these effects (Simpson and Waterman 1988). The mechanism by which ACTH-dependent cAMP increases transcription is discussed in depth later in this section.

Chronic effects of ACTH include increased synthesis of the steroidogenic enzymes (excluding aldosterone synthase) as well as actions on cell growth (Hall 1985, Simpson and Waterman 1988). Prolonged absence of ACTH causes levels of steroidogenic enzymes and RNA to fall in association with atrophy of the adrenal

cortex. However, these effects are reversed over a period of a few days when ACTH is re-administered (Orth et al. 1992).

ACTH may also affect other second messenger systems such as intracellular calcium $[Ca^{2+}]_i$. Extracellular Ca^{2+} is required to promote hormone-receptor interaction and to maintain receptor occupancy (Haksar and Peron 1973, Cheitlin et al. 1985). The generation of cAMP by ACTH stimulation requires an influx of extracellular Ca^{2+} (Kojima et al. 1985) which inturn causes a rapid transient increase in $[Ca^{2+}]_i$ (Kojima et al. 1986). The effects of these intracellular mechanisms are discussed later in this section.

1.5.2 Angiotensin

Angiotensin II (ANG-II) is a pressor octapeptide and is the product of the renin angiotensin system (RAS) (**Figure 1.5.c**). Briefly, the enzyme renin is secreted from epitheloid cells of the juxtaglomerular apparatus of the afferent glomerular arterioles in response to a decrease in intravascular volume or sodium levels. In the circulation, it cleaves an inactive decapeptide, ANG-I, from an α2-globulin, angiotensinogen, which is of hepatic origin. Plasma and tissue angiotensin converting enzyme (ACE) hydrolytically removes two further amino acid residues to release ANG-II. This has a short half-life and is rapidly degraded by angiotensinases. Recent studies have shown that complete RAS systems also exist in the brain, vasculature, adrenal cortex and other tissues (Samani 1994).

ANG-II acts on target cells via specific membrane receptors. Two have been identified, AT1 and AT2. Most biological responses are mediated through AT1 receptors. ZG cells respond via AT1 receptors which, on binding ligand, stimulate phospholipase C (PLC) hydrolysis of phosphatidyl inositol biphosphate (PIP2), generating diacyl glycerol (DAG) and inositol triphosphate (IP3) which releases bound Ca^{2+} from intracellular stores, thus increasing $[Ca^{2+}]_i$. Studies using the technique of patch clamping or following the rate of K+ flow across the cell membrane have shown that ANG-II also rapidly depolarises the ZG cell membrane.

As a result, voltage-dependent-Ca^{2+} channels open. Since $[Ca^{2+}]_e$ massively exceeds $[Ca^{2+}]_i$, there is a rapid influx which also contributes to the increase in$[Ca^{2+}]_i$(Connor et al. 1987, Shepherd 1989).

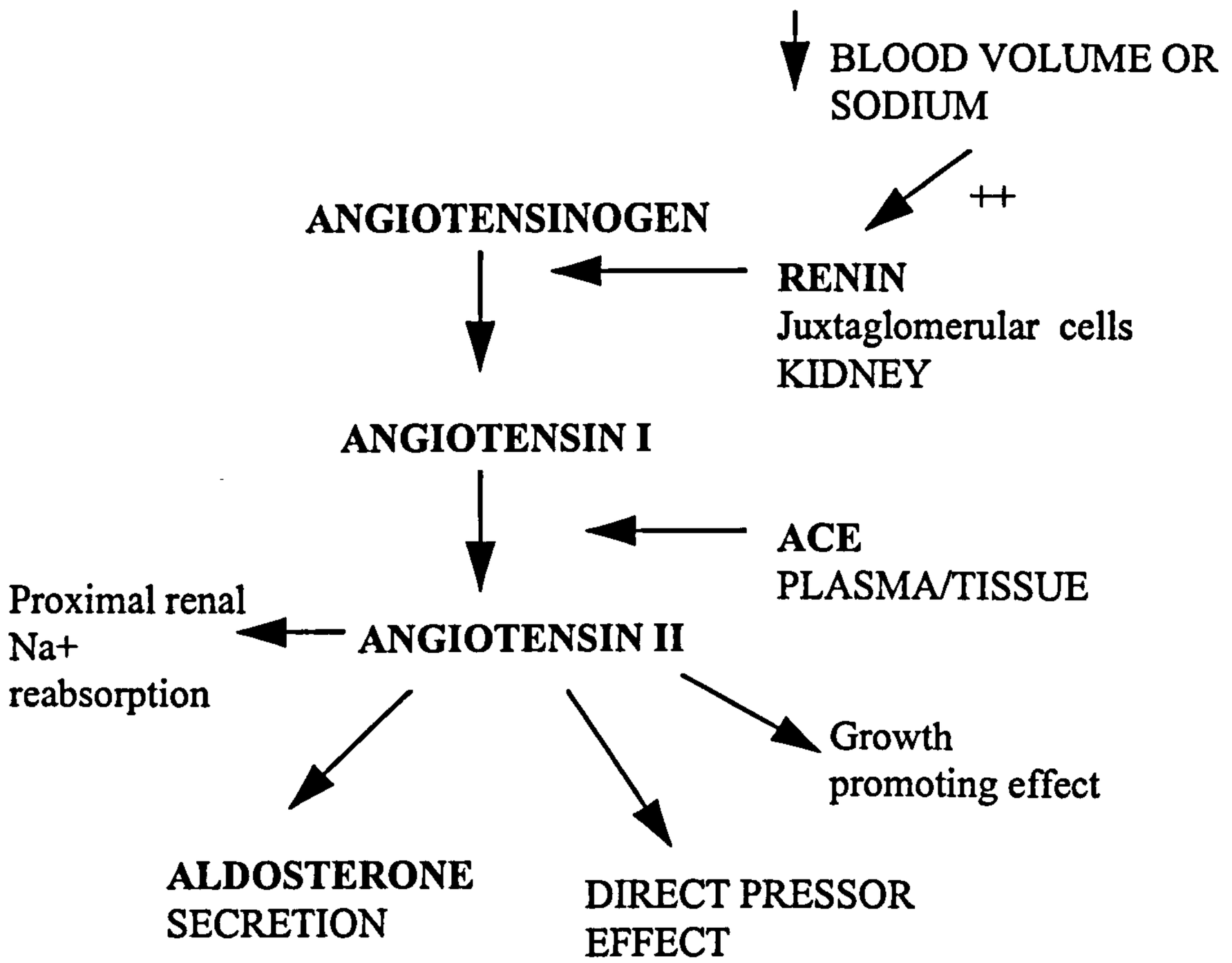


Fig 1.5c. The Renin-angiotensin system

ANG-II is a key regulator of sodium status and extracellular volume. It is perhaps not surprising, therefore, that sodium status itself modulates the quantitative relationship between ACTH, ANG-II or K+ with aldosterone. Thus infusion of ANG-II into sodium deplete subjects produces higher increases of aldosterone than the same amount of ANG-II than in sodium loaded subjects (Oelkers et al. 1974, Gordon et al. 1980). The opposite is true for pressor sensitivty to ANG-II. The mechanism of this modulation has been the subject of much research but has yet to be satisfactorily resolved. The ANG-II receptor is up-regulated in the kidney in response to Na+ but this is not the case in the adrenal (Aguilera and Catt 1978a).

1.5.3 Potassium

Cells of the ZG are extremely sensitive to changes in extracellular K^+. Increased extracellular K^+ levels increase aldosterone secretion (Muller 1987) which in turn facilitates renal potassium excretion thus, controlling body potasssium balance. Potassium, like sodium, can modify response to other agonists. High dietary potassium intake enhances responsiveness, while low potassium reduces the response (Dluhy et al. 1972). In the rat, Northern blotting showed that adrenal aldosterone synthase mRNA levels increased some 5 to 6 fold following dietary potassium supplementation and sodium restriction (Tremblay et al. 1992). Rat 11β-hydroxylase mRNA levels were not altered by these dietary changes which is consistent with unchanged cortisol levels. Captopril, an inhibitor of angiotensin converting enzyme (ACE), significantly decreased the level of aldosterone synthase message induced by these monovalent cation changes, indicating that these effects require ANG-II production (Tremblay et al. 1992).

1.5.4 Potassium activates Calcium channels

In rat ZG cells *in vitro*, an increase in extracellular K^+ of as little as 0.5 mM produces an increase in cytoplasmic $[Ca^{2+}]_i$ (Pralong et al. 1992) underlining the extreme sensitivity to K+. This increase in $[Ca^{2+}]_i$ occurs by the activation of voltage-dependent Ca2+ channels (VDCC's) (Cohen et al. 1988, Durroux et al. 1988). VDDC's in these cells are mostly of the T-type (Varnai et al. 1995) but there have also been reports of L-type channels (Durroux et al. 1988, Varnai et al. 1995). T-type channels are low activation threshold (-69mV) , transient, rapid activation channels whereas L-type are high threshold (-40mV) with larger Ca^{2+} conductance (Varnai et al. 1995). The T-type channel in rat ZG cells is activated even by raising extracellular $[K^+]$ by an increment not exceeding the physiological range. Thus it is possible that these T-channels or a sub-type do not require membrane depolarisation for activation which suggests the occurrence of a non-voltage -operated Ca^{2+} influx (Varnai et al. 1995). Further channel subtypes are receptor-operated and second-messenger operated. ZG cells have a K^+ activated, Ca^{2+} permeable channel possessing a K^+

binding site (Pardo et al. 1992). Variations in extracellular $[K^+]$ therefore modify the rate of influx of Ca^{2+} through a variety of channels in order to alter $[Ca^{2+}]_i$ (see **figure 1.5d**).

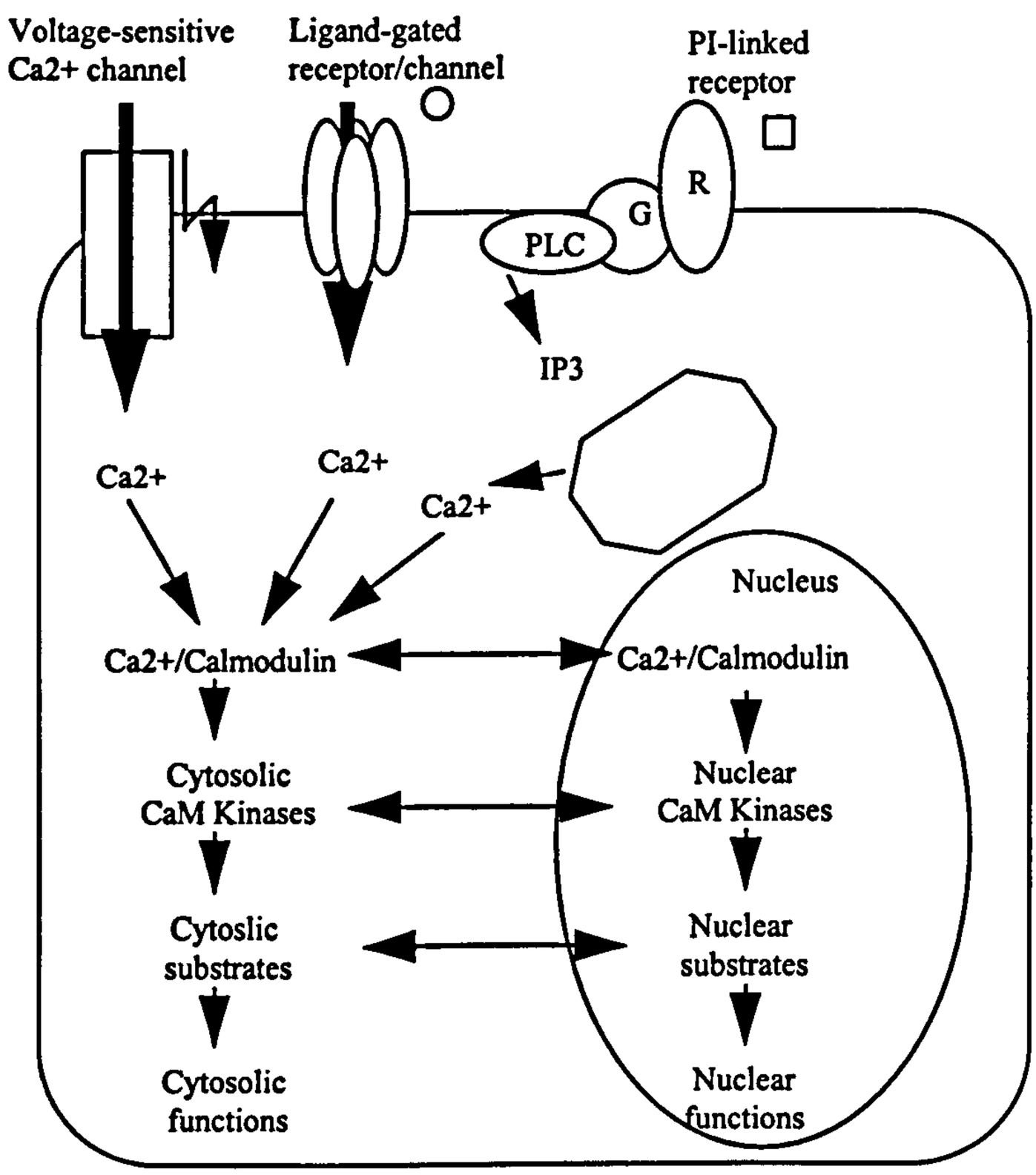


Figure 1.5d. Calcium channels and routes of entry

Adapted from Heist and Schulman (1998).

1.5.5 Other factors

More and more endogenous ligands are joining the list of control factors exerting effects on aldosterone secretion. A few of the well-known ones are shown in **table 1.5**

CONTROL FACTORS	EFFECT	RECEPTOR/ MECHANISM	REFERENCE
Atrial natriuretic peptide	Inhibitory ↓Aldosterone	ANP Receptor ↓pregnenolone	Goodfriend et al 1984
Adrenaline, Noradrenaline	Stimulatory ↑Aldosterone	β-adrenergic receptors	De Lean et al.1984
Acetylcholine	Stimulatory ↑Aldosterone	Muscarinic receptors	Hadjan. 1981
Vasoactive intestinal peptide	Stimulatory ↑Aldosterone	Synergises with ACTH	Holzwarth et al.1984
Dopamine	Inhibitory ↓Aldosterone	Tonic inhibition via DA receptor	Norbiato et al .1977, Dunn et al.1981, Connell et al 1987
Adrenomedullin	Inhibitory ↓Aldosterone	Receptor mediated	Mozzochi et al. 1996

Table 1.5. Table showing effects of several control factors on aldosterone secretion.

Thus, agonists of the adrenal cortex have certain common effects on intracellular second messenger systems. From the point of view of eventual translation of these changes into altered enzyme activity, it is now necessary to consider what changes each of these messengers evoke on gene transcription. It would be expected that the second messenger would interact directly or indirectly within specific control sites on the gene promoter. These are discussed in the following sections.

1.5.6 Effects of cAMP

ACTH, but not ANG-II or potassium, stimulates levels of cAMP. As a second messenger, cAMP results in the phosphorylation of a variety of proteins required for transcription of genes involved in adrenal steroidogenesis. It activates protein kinase A (PKA) which uses ATP to phosphorylate a number of proteins and increases nuclear mRNA production of a number of ACTH-dependent genes (Daniel et al. 1998). Phosphoprotein phosphatases (PP1 and/or PP2) are also required to maintain steroidogenic responses to ACTH and cAMP in rat ZG cells (Sayed et al. 1997).

These enzymes dephosphorylate phosphoproteins and recent evidence suggests that they may be involved in the regulation of the activity of a phosphoprotein which acts before PKA activation (Sayed et al. 1997). Activation of the PKA pathway in H295R cells (a human adrenal cell line) using an exogenous activator of this pathway, dibutyryl cAMP or forskolin, preferentially increases mRNA levels of CYP11B1 which suggests that ACTH may act through this pathway to increase CYP11B1 transcription (Denner et al. 1996).

Levels of StAR (see section 1.4) mRNA and StAR protein are increased by cAMP through increases in the rate of transcription also mediated by PKA phosphorylation (Clark et al. 1995, Kiriakidou et al. 1996). More recently, northern and western blotting showed that administration of ACTH to hamsters *in vivo* caused a 2- to 3-fold increase in StAR mRNA and a 1.5-fold increase in StAR protein respectively, 1 hour post-treatment (Fleury et al. 1998). This small mammal is a good model for studying the *in vivo* effects of ACTH because, like man, cortisol is the major glucocorticoid (LeHoux et al. 1992).

cAMP regulates the expression of specific genes by mediating the PKA-dependent phosphorylation of cAMP-responsive element binding protein (CREB) transcription factor (Gonzalez et al. 1989) see **figure 1.5e**. It interacts directly with CREB in the cytosol of adrenocortical cells. This protein forms part of a cAMP -dependent protein kinase. CREB then binds CPB (CREB binding protein) and this complex then interacts directly with genes such as the CYP11B1 and CYP11B2 which have a CRE (cAMP responsive element) (see section 1.6) in their control regions. Recently, ANG-II and K^+ have been shown to regulate human CYP11B2 transcription in H295R cells through common steroidogenic factor-1 (SF-1) and CRE-like cis-elements, thus providing a plausible molecular mechanism by which these physiological regulators may combine to control aldosterone production (Clyne et al. 1997). cAMP activation of PKA preferentially activates gene transcription of CYP11B1 over that of CYP11B2 *in vitro* (Denner et al. 1996).

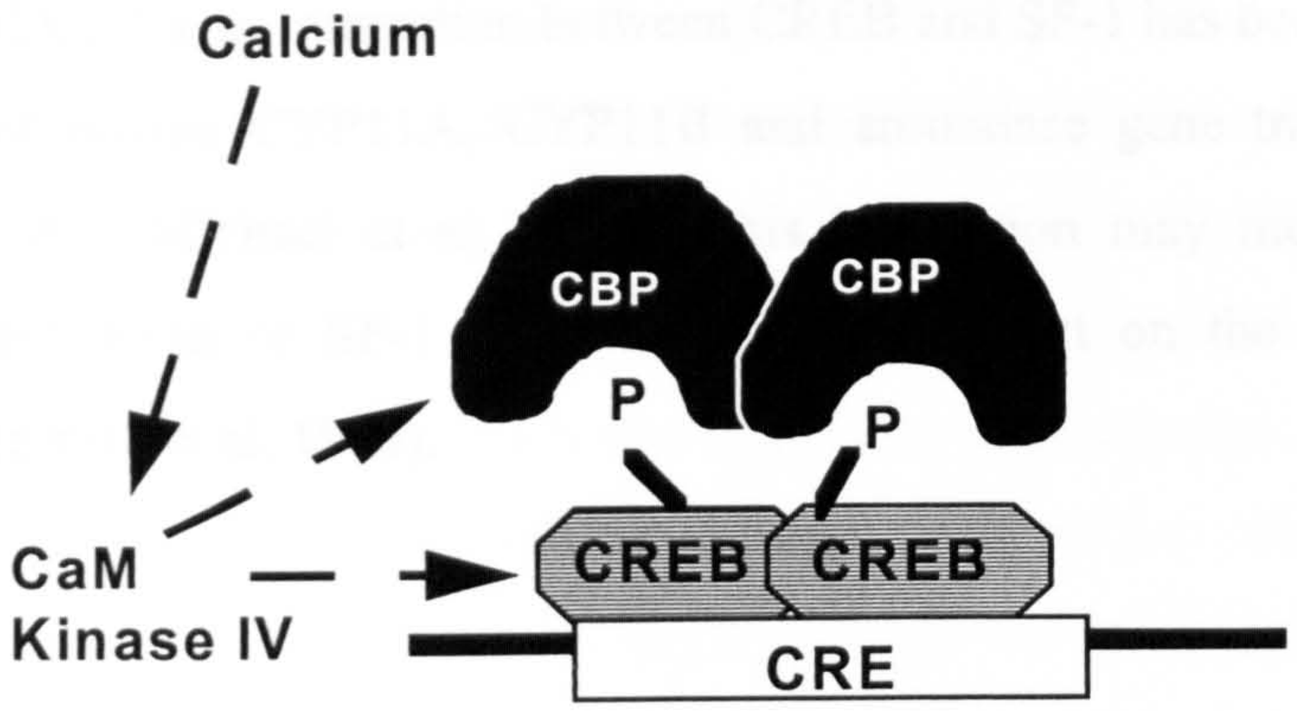


Figure 1.5e Transcriptional activation of CRE-dependent genes by CREB Adapted from Hardingham et al (1998)

Steroidogenic factor 1 (SF-1), also known as adrenal 4-binding protein (Ad4BP), is a major regulator of tissue specific expression of the cytochrome P450 steroid hydroxylase enzymes of the adrenal cortex and the gonads (Lala et al. 1992, Morohashi et al. 1992). It is thought to be activated by PKA phosphorylation (Pon et al. 1986). SF-1 is a nuclear hormone receptor and SF-1 cDNAs from human, bovine, rat and mouse share conserved regions of known functional importance with other members of this superfamily (Evans 1988). SF-1 cDNA is highly homologous with known transcription factors: embryonal long terminal repeat-binding protein (ELP) from mouse embryonal carcinoma cells (Tsukiyama et al. 1992) and fushi tarazu factor-1 (FTZ F1), a Drosophila orphan nuclear receptor (Ueda et al. 1990, Lavorgna et al. 1991) which are involved in retroviral expression and developmental regulation respectively. These functional domains consist of two zinc fingers which mediate DNA binding, an AF-2 transactivation domain at the carboxy terminus, a proline -rich domain which is thought to mediate transactivation and also a consensus site for PKA phosphorylation in response to cAMP. The mechanism by which SF-1 exerts its regulatory effects on gene transcription is not known. However, in a recent study, SF-1 has been shown to mediate cAMP and phorbol ester upregulation of some steroidogenic enzymes, such as bovine P45017 and human 3β-HSD directly(Bakke & Lund 1995, Leers-Suchetat et al 1997). The efficiency with which SF-1 activates transcription is increased upon binding 25-OH-cholesterol (Lala et al. 1997).

Recently an interaction between CREB and SF-1 has been demonstrated in the control of bovine CYP11A, CYP11B and aromatase gene transcription (Morohashi et al. 1993, Michael et al. 1997). This interaction may mediate cAMP-dependent gene activation or SF-1 may exert a direct effect on the CREB-binding protein itself (Smith et al. 1996).

1.5.7 Effects of Calcium as an intracellular messenger

The routes by which Ca^{2+} enters the cell are shown in **figure 1.5d.**

ANG-II and extracellular K^+ induce aldosterone secretion by increasing $[Ca^{2+}]_i$ via signal transduction mechanisms (Spät et al. 1991, Ganguly et al. 1994). A recent study has shown that a sustained increase $[Ca^{2+}]_i$ increases the levels of mitochondrial NADPH, ATP and GTP in ZG cells stimulated with ANG-II or K^+. This may contribute to increased hormone production (Rochas et al. 1997) by increasing the rate of steroid hydroxylation (Rochas et al. 1997). As well as ANG-II and K^+ stimulation of aldosterone production, Ca^{2+} has been implicated in the mechanism of ACTH stimulation of cortisol synthesis (Davies et al. 1985).

There is increasing evidence that the calcium-signalling pathway can directly utilize CREs to increase transcription via the calcium/calmodulin-dependent protein kinase pathway by phosphorylation of CREB (Schwaninger et al. 1993, Eckert et al. 1993, Gonzalez. et al. 1989, Lee et al. 1990, Sun. et al. 1996). Ca^{2+} binds to calmodulin (CaM). Stimulation of adrenocortical cells with ACTH increases levels of CaM in ZF nucleii (Harper et al. 1980), a change associated with the phosphorylation of CREB (Deisseroth et al. 1998). The Ca2+/CaM complex then activates a series of Ca^{2+}/calmodulin-dependent protein kinases (I, II or IV) which mediate the action of many other agonists which also elevate $[Ca^{2+}]_i$ (Hanson et al. 1992, Braun et al. 1995, Schulman et al. 1998). These kinases activate a number of nuclear proteins such as transcription factors and other DNA-binding proteins (Heist and Schulman 1998).

CaMkinases I and IV phosphorylate CREB at serine 133 (Sun et al. 1996) which increases transcriptional activation by CREB of genes with CREs (Krebs and Beavo

1979) CaMkinase II phosphorylates CREB serines at positions 133 and 142 causing inactivation (Sun et al. 1994, Sun and Maurer 1995). CaMkinase IV, once activated by Ca^{2+}/CaM complexes, can phosphorylate CREB with similar kinetics to phosphorylation by PKA (Enslen et al.1995). The CaM kinase cascade activates CBP which then binds CREB and associates as a complex with CRE elements on gene promoters to activate CRE-dependent transcription. The CaM kinases are expressed in adrenocortical cells and appear to be involved in the acute stimulation of aldosterone production (Papadopolous et al 1990, Fern et al.1995, Clyne et al 1995, Pezzi et al.1996) (see **figure 1.5e**).

Evidence for possible involvement of this pathway in ACTH-induced StAR expression has been obtained from studies of cultured adrenal ZF cells (Nishikawa et al 1997). It is known that cAMP and protein kinase C -dependent processes play a crucial role in the regulation of expression of StAR protein when bovine cells are stimulated by ACTH. Also in bovine ZF cells, ACTH increases cytosolic [Ca^{2+}] and activates StAR expression with a resultant increase in cortisol production (see section 1.3). Pre-treatment with a specific inhibitor of CaM kinase II, KN-93, inhibited the stimulatory effects of ACTH on both StAR and cortisol production. This suggests that ACTH can enhance StAR expression and cortisol not only through cAMP mechanisms but also via the calcium/calmodulin dependent pathway (Nishikawa et al. 1997).

Activated CaM has a variety of other functions such as binding and activation of proteins involved in RNA-processing or splicing, RNA binding proteins and nuclear ribosomal proteins (Agell et al. 1998). These findings further suggest the existance of cross-talk mechanisms between intracellular messenger pathways.

1.5.8 Transcription factors and zone-specific expression

It is well known that CYP11B1 expression in the adrenal cortex is restricted largely to the ZF and CYP11B2 is expressed exclusively in the ZG cells. The reason for this zone-specific expression and tight regulation within the adrenal cortex remains

controversial. Some recent data have described the involvement of activated protein (AP-1) transcription factor in the zone-specific expression of CYP11B1.

The immediate early genes such as c-fos can be activated by Ca^{2+}, cAMP, DAG and other second messengers (Morgan et al.1991, Ghosh et al. 1995). Their promoters have CRE sequences, the regulation of which involves Ca^{2+} activation of CREB (Sheng et al. 1990). The products of the immediate early genes Fos and Jun produce jun (c-jun, junB, junD) and fos (c-fos, fosB, Fra-1, Fra-2) family proteins. Homodimer or heterodimer complexes between jun and fos members which then function as a transcription factor known as activated protein (AP-1) transcription factor. AP-1 transcription factor binds a regulatory element AP-1 in the promoter region of target genes. The human CYP11B1 gene promoter has two sequence elements similar to the consensus sequence for binding AP-1 (Kawamoto et al 1990). The exact role of these elements in human CYP11B1 transcription is not yet known.

A recent study in ZF cells from rat adrenal cortex showed that an AP-1 transcription factor is found only in ZF cells of the adrenal cortex (Mukai et al. 1995). It binds to a regulatory element of the rat CYP11B1 promoter, an AP-1 binding site responsible for transcriptional activation of CYP11B1 in response to ACTH or cAMP (Mukai et al. 1995).

The combination of AP-1 dimers in response to ACTH and cAMP induction has been studied in rat ZF cells (Mukai et al. 1998). These cells constitutively express basal levels of jun and fos family members: c-jun, JunB and Fra-2. ACTH or cAMP induction increases c-jun to 3-times the basal level and also cause a rapid transient increase in junB and c-fos (Mukai et al. 1998). Heterodimer protein complex combinations all bind to the AP-1 element with similar strengths (Mukai et al. 1998).

To determine which heterodimers mimicked the transcriptional activation of CYP11B1 by ACTH and cAMP, genes encoding the various early gene proteins were cotransfected into cells (Mukai et al. 1998). Combinations of c-jun and c-fos genes dramatically increased transactivation of the CYP11B1 gene and worked synergistically. In non-stimulated cells, heterodimer AP-1 complexes are different from complexes seen in cells exposed to ACTH or cAMP (Mukai et al. 1998). This suggests that ACTH or cAMP regulate CYP11B1 transcription by inducing a

particular subset of these proteins that mediate zone-specific expression of CYP11B1 in ZF cells in the rat. The activation of these proteins may involve the cAMP/PKA pathway (Mukai et al. 1998). Whether ANG-II regulates ZG cells in a similar manner is not known but recent data show that ANG-II in ZF cells, *in vitro*, caused a different pattern of stimulation of fos and jun complexes compared to ACTH (Viard et al. 1992). Thus, in normal ZF cells ACTH and ANG-II regulate these compnonents differently which may account for differences in action of ACTH and ANG-II on differentiated adrenal cell function (Viard et al. 1992). Whether ANG-II induces a particular subset of transcription factors exclusive to the ZG which regulates zone specific expression of CYP11B2 is not known . Moreover, whether finding in animals are applicable to man is uncertain. However, the human CYP11B1 promoter does have two AP-1 binding sites which may be involved in transcription of this gene in the ZF (see section 1.4)

1.5.9 Summary

In summary, ACTH, ANG-II and K^+ stimulate cAMP and Ca^{2+} levels respectively within the cell. These second messengers activate a series of intracellular pathways which ultimately switch on gene transcription and hence control cortisol and aldosterone production.

1.6 Gene structure and control of expression

The previous section discussed the mechanisms by which ACTH, ANG-II and K^+ control cortisol and aldosterone secretion respectively and the intracellular mechanisms which lead to transcription of those genes required for steroid hormone biosynthesis. In this section, the structure of the genes encoding 11β-hydroxylase and aldosterone synthase will be discussed.

11β-Hydroxylase and aldosterone synthase are encoded by the CYP11B1 and CYP11B2 genes respectively and lie in tandem on chromosome 8q21-22 in man (Chua et al 1987, Mornet et al 1989, Wagner et al 1991). Studies of large restriction fragments separated by pulse field electrophoresis suggest that the two genes are only approximately 40kb apart (Lifton et al. 1992a, Pascoe et al. 1992a). This arrangement is also known to exist in the mouse where CYP11B2 lies on the left if the genes are shown being transcribed right to left (Domalik et al. 1991). In man, no genomic clones have been identified which have the genes in this arrangement but studies of patients with congenital hypoaldosteronism due to aldosterone synthase deficiency (corticosterone methyloxidase II deficiency) (Pascoe et al. 1992b) or with glucocorticoid-suppressible hyperaldosteronism (see section 1.8) suggest that a similar arrangement exists. Each gene spans approximately 7Kb of genomic DNA and is composed of 9 exons and 8 introns (see **figure 1.6 a**)

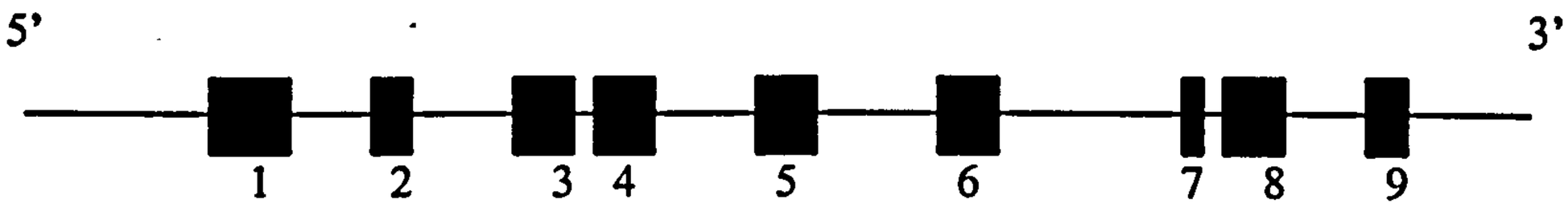


Figure 1.6a Exonic-intronic arrangement of CYP11B1/B2 genes (not to scale) >8000bp

The coding region nucleotide sequence of their exons is 95% identical and their intronic regions share 90% identity. The putative proteins encoded are composed of 503 amino acids including a 23 amino-acid signal peptide (Kawamoto et al. 1992). Their amino acid sequences share 93% identity. Due to sequence similarities, these

genes have been assigned to a specific sub-group of the cytochrome P450 gene superfamily (Nelson et al. 1993). CYP11A, the gene encoding side chain cleavage enzyme has a similar arrangement of exons and introns and the protein shares 36% identity with aldosterone synthase and 11β-hydroxylase (Morohashi et al. 1987).

The 5' UTR (promoter) regions of the CYP11B1 and B2 genes share least homology, presumably due to differences in transcriptional regulation of these genes. In both genes, the TATA box (GATAAAA) lies at position -35 to -29 upstream of the transcription initiation site. Binding sites for transcription factors such as CREB, SF-1 and AP-1 (see section 1.5) which switch on gene transcription have been identified. The cAMP-responsive element (CRE) is a palindromic sequence (TGACGTA) upstream of the TATA box which binds cAMP-responsive element binding protein (CREB). The promoter regions of the CYP11B1 and B2 genes are shown in **(Figure 1.6 b)**.

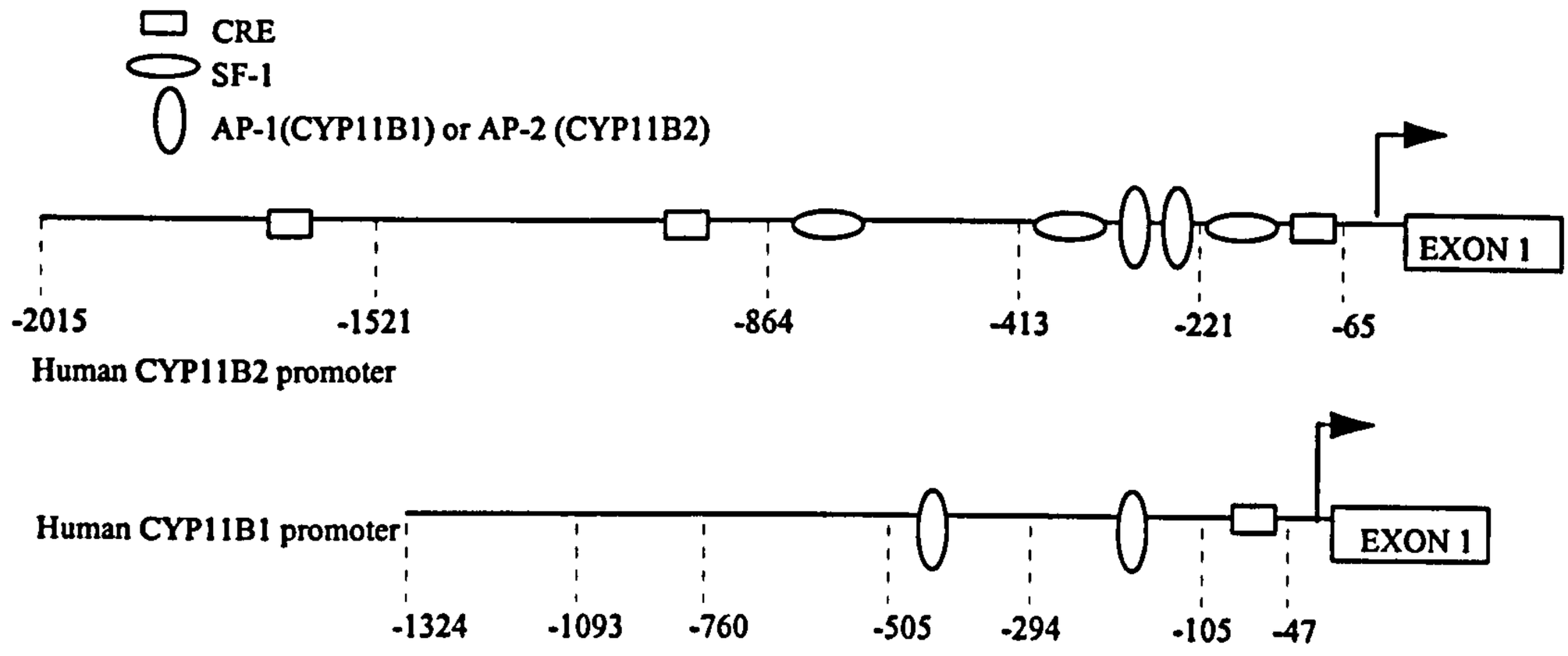


Figure 1.6b promoter regions of human CYP11B1 and CYP11B2 genes

1.6.1 CYP11B1 promoter

Sequence analysis of the CYP11B1 promoter revealed a CRE at position -71 to -64 and two AP-1 elements at -383 to -377 and -139 to -133 (Kawamoto et al. 1990).

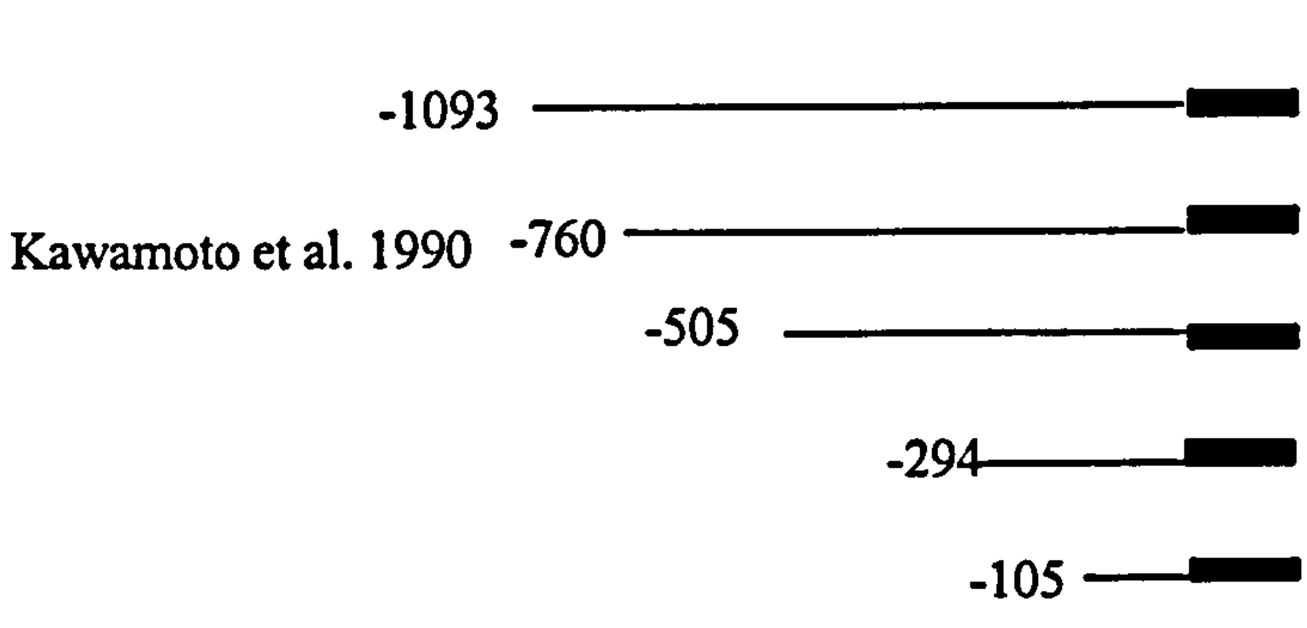


Figure 1.6c CYP11B1 promoter constructs

In vitro expression studies of the CYP11B1 promoter where successive deletions were made have demonstrated that cAMP regulates transcription through the CRE (Kawamoto et al. 1990) (**Figure 1.6c**). Promoter constructs fused to a CAT reporter gene were expressed in Y-1 cells, a mouse adrenal cortex tumour cell-line, and tested for their response to 8-bromo-cAMP, a cAMP analogue. The 5' flanking region of CYP11B1 up to -1324 had promoter activity but deletion up to -1094 increased activity 4-fold. This suggests that a negative cis element suppressing transcription is present in the region deleted. Constructs deleted further to -761 caused a marked decrease in activity, suggesting that the region between -1094 and -761 is required for full promoter activity. Deletion to -506, -295, -105 and -47 progressively decreased activity further (Kawamoto et al.1990). These results suggest that there are several positive cis acting elements in the region from -1094 to -47 necessary for full promoter activity (see **figure 1.6c**). From these experiments, the CRE and the two AP-1 binding sites identified are essential for CYP11B1 promoter activity and other positive regulatory elements present in the region -1093 to -506 are also required. Recent evidence has shown transcriptional activation of CYP11B1 by AP-1 transcription factors in response to ACTH and cAMP in rat, ovine and bovine ZF cells. These are thought to mediate zone-specific expression (see section 1.5).

1.6.2 CYP11B2 Promoter

CYP11B1 and CYP11B2 differ in the control of their expression and consequently differ most at the structural level at their control regions.

A long palindromic sequence from -1734 to -1001 is present in CYP11B2 but not CYP11B1 (Kawamoto et al. 1992). According to Kawamoto et al (1992), 8-bromo cAMP-responsive promoter ability differs between the two genes when regions of the respective promoter are deleted. The CAT expression system was used to measure promoter activty *in vitro* in Y-1 cells. Expression of a CAT construct containing up to -2015 of the 5' flanking region of CYP11B2 did not respond to 8-bromo cAMP. Progressive deletion to -1490, -654 or -373 also did not respond but deletion up to -64 increased expression by producing a small basal promoter response to 8-bromo cAMP. This suggested that the region -2015 to -65 inhibits the promoter activity of CYP11B2 (Kawamoto et al. 1992) whereas, in CYP11B1, a large section -1093 to -47 contains elements which increase promoter activity (Kawamoto et al, 1990)(see **figure 1.6d)**

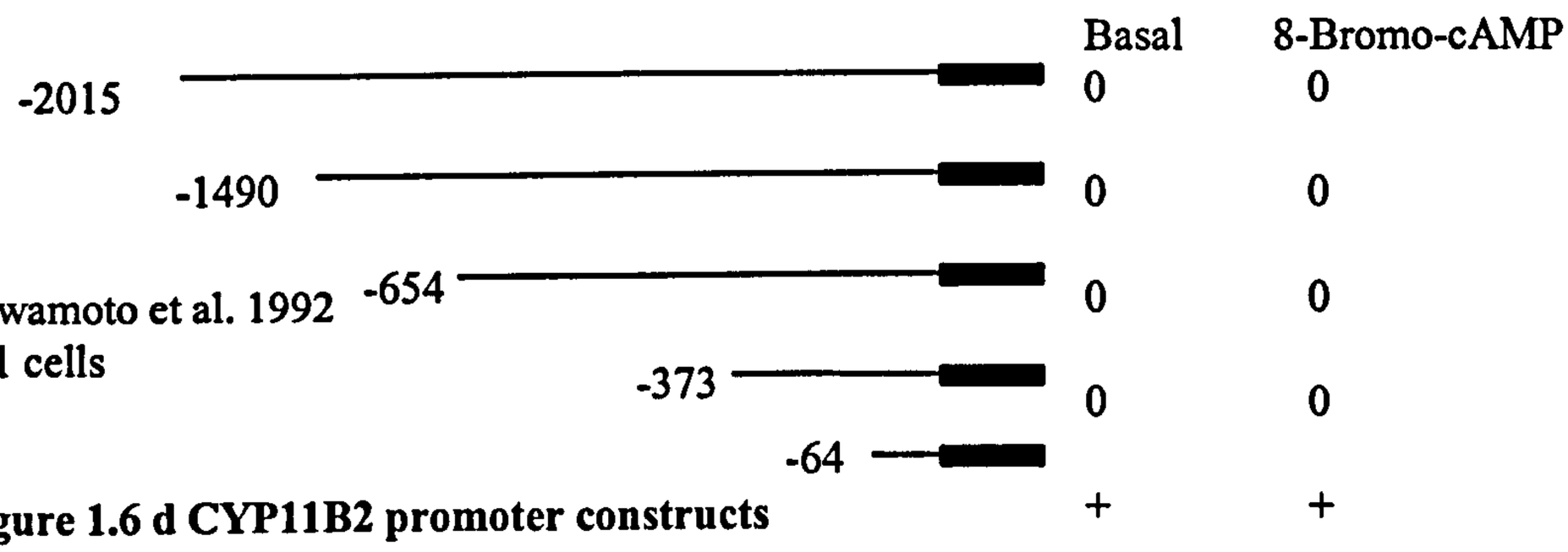


Figure 1.6 d CYP11B2 promoter constructs

However, results conflicting with those of Kawamoto have recently been reported although the conflict may have been due to the different cell lines used. In this second study, human CYP11B2 expression was stimulated by ANG-II and K^+ through common cis-elements (Clyne et al. 1997). Concentrations of K^+ used in this study were much higher than the physiological range and therefore one must consider that the effects observed by this stimulus *in vitro* are not representative of the *in vivo* situtation. Analysis of the 5' flanking region identified a CRE at position -71 to -64 (TGACGTGA) and an SF-1 binding site at -129 to -114 (CTCCAGCCT). Electrophoretic mobility shift assays demonstrated that the -129 to -114 element could

bind SF-1. Promoter deletion constructs showed that the first 2015 bp of 5' flanking region was sufficient to direct basal reporter gene transcription when expressed in H295R cells (human adrenal tumour cell-line). Progressive deletions, -1521, -864, -413 to -221 retained similar basal activity but deletion to -65 reduced promoter activity to 15%. These results indicate that there are DNA sequences between -221 and -65 which are essential for basal CYP11B2 gene expression (Clyne et al.1997) (see **figure 1.6e**).

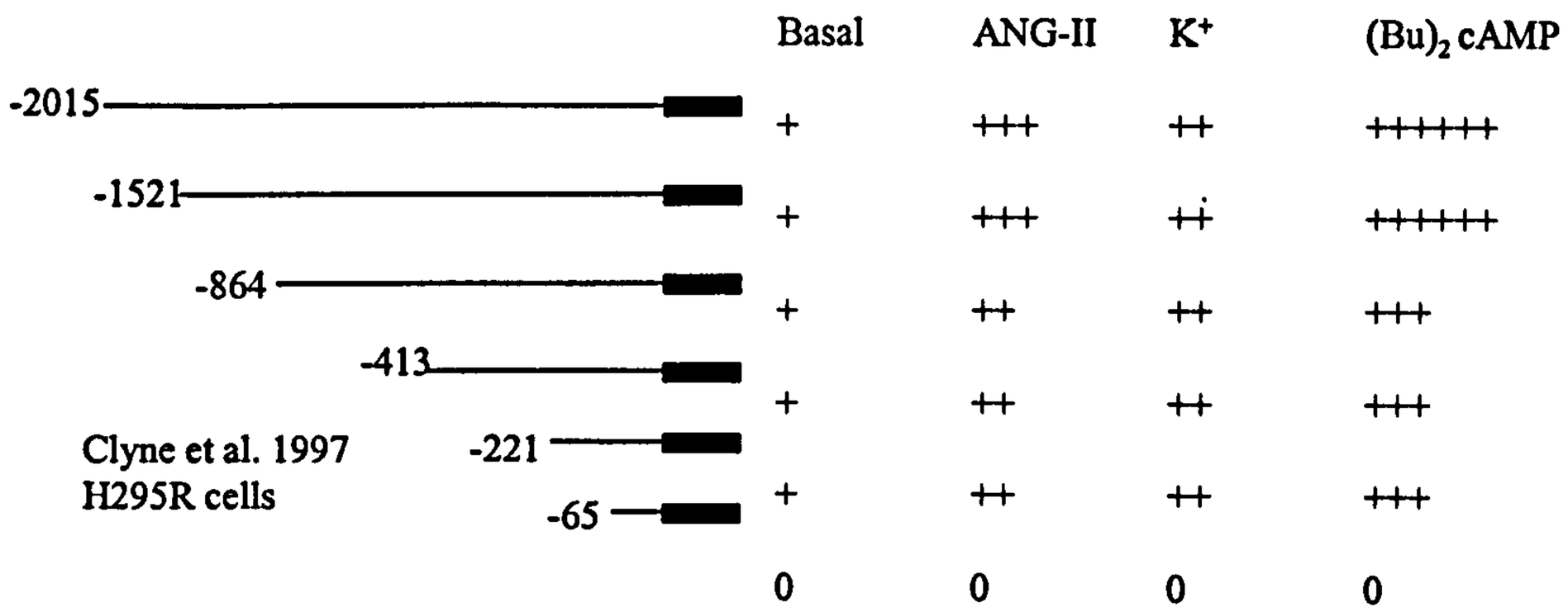


Figure 1.6 e CYP11B2 promoter constructs

When cells transfected with the construct containing the 2015bp 5'flanking region of CYP11B2 were treated with ANG-II or K^+, the basal activity was increased 5.5 and 4.5 fold respectively. Deletion mutants up to -221 retained this augmented response to ANG-II and K^+ but deletion up to -65 completely abolished ANG-II or K^+ induction (Clyne et al. 1997).

Another analogue of cAMP, (Bu)2cAMP, was used to test the efficiency of promoter constructs. The full promoter construct activity increased by 13 times basal level whereas deletion to -864 reduced the response by 50-60% and deletion to -65 completely abolished the response (Clyne et al 1997). This suggests that there are CRE's in the region between -2015 to -864 and between -864 and -221 which are required for full promoter activity in response to cAMP (Clyne et al 1997). These findings are also in conflict with those of Kawamoto et al (1992) who suggested that the region -2015 to -65 repressed promoter activity but this may again be due to the different cell lines in which they were expressed. It is possible that cAMP stimulates

induces different sub-sets of regulatory proteins in the two cell-lines which have very different consequences. These findings collectively show that the -221 to -65 region of the CYP11B2 promoter is essential for basal and cAMP- and Ca^{2+} -induced transcription.

DNaseI footprinting analysis was performed using H295R cell nuclear extracts to identify protein binding sites within the promoter. The -129/-114 element binds SF-1 and is required for full basal and agonist-induced reporter activity and the -71/-64 element binds CREB and is necesssary for induction by increased $[Ca^{2+}]_i$ (Clyne et al. 1997). Mutation of the CRE -71/-64 reduced basal activity to 50% and drastically decreased induction by ANG-II, K^+ and cAMP. Thus, this CRE is required not only for response to cAMP but also to increased $[Ca^{2+}]_i$. This element itself, however, is not sufficient for full basal activity as deletion of the SF-1-129/-114 element further reduced this activty. Therefore, it follows that CYP11B2 promoter activty requires the interaction of the CRE and SF-1 binding sites for full transcriptional activity (Clyne et al. 1997). These results once again contradict the findings of Kawamoto et al (1992); these differences may be due to the differing cell-lines utilised. From alignment studies of sequences from other species, another SF-1 site at position -351/-343 has been identified in the human CYP11B2 promoter. Deletion studies have, however, ruled out a functional role for this site even though it binds SF-1 tightly (Clyne et al. 1997). SF-1 sites also bind another factor called chicken ovalbumin upstream promoter transcription factor (COUP-TF). Like SF-1, this factor is an orphan nuclear receptor. Two additional SF-1 sites in the CYP11B2 promoter which bind COUP-TF in H295R cells were identified (Clyne et al. 1997). In the bovine CYP17 promoter, COUP-TF competes with SF-1 for the binding site. Possibly COUP-TF functions in this way to repress transcription (Lund et al. 1995). Whether COUP-TF is a repressor or activator of human CYP11B2 transcription is not known.

In summary, CYP11B1 and B2 consist of a 9 exon structural component and a promoter region which contains specific sites for interaction with known transcription factors which are essential for both basal and agonist-stimulated activity.

1.6.3 Polymorphisms in control regions

Two polymorphisms have been identified in the human CYP11B2 gene. The first is a polymorphism in the consensus sequence for the SF-1 binding site at position -351/-343 and the second is a gene conversion in intron 2. The SF-1 binding site polymorphism involves a -344C/T substitution in the putative SF-1 binding site (White et al. 1995). In functional studies, the -344C allele binds SF-1 4 times more strongly than the -344T allele (White et al 1995). Deletion constructs of the CYP11B2 promoter show that this SF-1 site has no apparent functional importance in promoter activity (Clyne et al 1997). It is therefore unlikely that the -344C allele causes increases in transcription of CYP11B2 in subjects carrying this allele. The intronic conversion, also identified in the human CYP11B2 gene, has almost all of intron 2 replaced with that of CYP11B1 (White et al. 1995). The -344T and the intronic conversion are in linkage disequilibrium.

Studies of these two biallelic polymorphisms have been performed in hypertensive and control populations (Brand et al. 1998, Benetos et al. 1997, Davies et al. in press). There was a higher frequency of the intronic conversion in the hypertensive population (Davies et al. in press). There was no change in frequency observed between the two groups in studies by Benetos (1997) and Brand (1998). There was a significant association of the -344T allele of the SF-1 site with essential hypertension (Brand et al 1998, Davies et al. in press). Benetos et al (1997) found an association with the -344C allele and increased plasma aldosterone levels. In 486 subjects from the North Glasgow MONICA normotensive population, subjects with the SF-1 -344 TT or TC genotype had significantly higher excretion rates of tetrahydroaldosterone (the excretory product of aldosterone found in urine) (Davies et al. in press). In agreement with the other studies, the intronic conversion showed no association with hypertension or urinary excretion rate of aldosterone (Davies et al. in press). Thus a possible intermediate phenotype has been identified on the basis of higher urinary aldosterone excretion in subjects bearing the allele which is overrepresented in patients with essential hypertension. Aldosterone rate, together with TT/CT genotype, may thus be linked to raised blood pressure. In a different study, the -344C

allele was shown to be associated with higher plasma aldosterone (Pojoga et al. 1998, Benetos et al. 1997). The -344C allele has also been shown to be a predictor of increased left ventricular mass (Kupari et al. 1998).

From these studies, it is apparent that there is considerable disagreement over which allele is important and is associated with hypertension. One would expect the same allele to be associated with increases in blood pressure, increased plasma aldosterone or increased urinary aldosterone as well as increased left ventricular mass but this is not the case. It is possible that, due to the small number of studies, that the observations are a consequence of type II statistical error. In a recent linkage study, the CYP11B2 was not significantly associated with hypertension. Similar studies from other populations are required to establish whether this locus is a useful or reliable marker for hypertension. Further studies are also required to define the precise nature of the intermediate phenotype and the physiological implications of this association.

1.7 Protein structure

The exact structure of mammalian cytochrome P450 enzymes are unknown but simpler bacterial cytochrome P450 enzymes have been crystallised and their structures determined. These provide the basic and core structure on which modelling of human and other cytochrome P450s can be performed. Specific enzyme architecture determines individual activities and substrate specificity. The following sections describe the protein structure and how its features account for the catalytic properties.

1.7.1 Bacterial/microsomal P450 structure

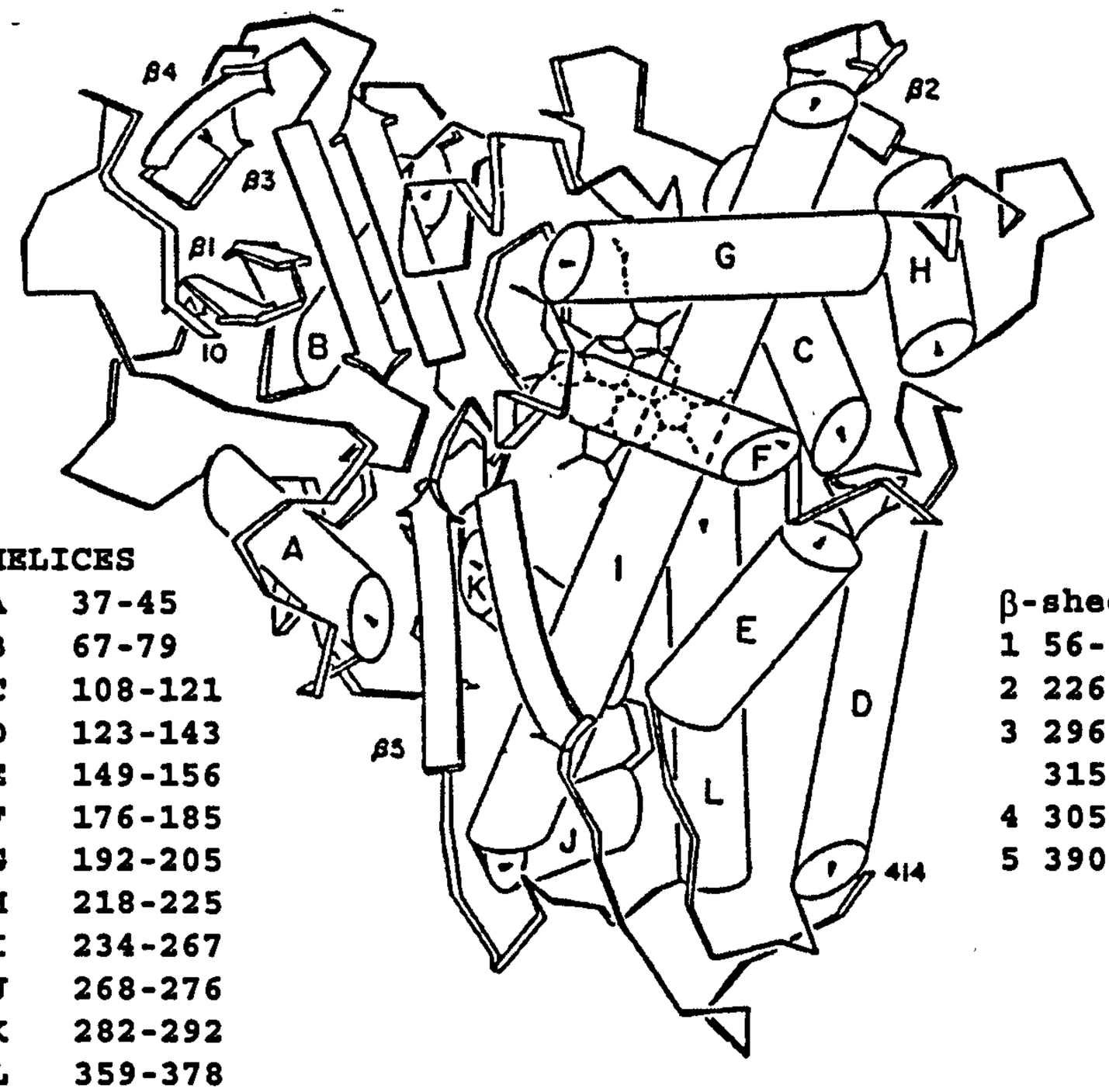
The high resolution crystal structure of camphor mono-oxygenase ($P450_{cam}$) from Pseudomonas putida was determined in 1987 (Poulos et al. 1987). In depth biochemical and biophysical studies followed and it is this structure which has become the model for the study of all other cytochrome P450s. Various techniques were used to investigate the relation of structure to function including studies of various inhibitor/substrate complexes and recombinant expression utilising site-directed mutagenesis. Both techniques have identified domains and key residues of structural and functional importance. Residues essential for and characteristic of cytochrome P450 structure have been shown by sequence comparison to be absolutely conserved in all cytochrome P450s and will be discussed in detail. More recently, other prokaryotic P450 enzyme crystal structures have been determined: microsomal $P450_{BM3}$ (Ravichandrin et al. 1993), $P450_{terp}$ (Hasemann et al. 1994) and most recently $P450_{eryF}$ (Cupp-Vickery et al. 1994). Comparison of these structures has identified similar secondary and tertiary structure and key functional regions (Hasemann et al. 1995). These are discussed below.

1.7.2 Protein topology: appearance.

Despite low sequence identity (less than 20%), the overall folding patterns and topology of cytochrome P450s are very similar. From this information, it is probable that all P450 enzymes, including membrane-bound enzymes such as human 11β-hydroxylase and aldosterone synthase, have a conserved tertiary structure. The polypeptide chain is composed of an α-helical (A-L) rich region (α-domain) towards

the C-terminal end and a β-sheet rich region (β-domain) towards the N-terminal end. The long α-helices and β-sheets lie in a plane parallel to the plane of the haem, thus producing a flat, triangular shaped molecule. The C-terminal portion forms the inner core of the molecule with helices I and L bracketing either side of the haem; any β-structure in the C-terminal portion is towards the surface of the molecule (**Figure 1.7** of $P450_{cam}$).

Figure 1.7 Diagram of P450cam



HELICES

A	37-45
B	67-79
C	108-121
D	123-143
E	149-156
F	176-185
G	192-205
H	218-225
I	234-267
J	268-276
K	282-292
L	359-378

β-sheets

1	56-62
2	226-233
3	296-301
	315-320
4	305-312
5	390-400

Taken from FEBS (Suppl.) Cytochrome P450 systems: from structure to application. Bernhardt 1998: L13.

1.7.3 Regions of conservation

Comparison of $P450_{cam}$, $P450_{BM3}$ and $P450_{terp}$ has revealed regions important for their structure and function.

(a). Haem-binding region

P450 enzymes have haem groups and use iron-oxo intermediates for oxidative reactions (see section 1.4). Helices are arranged around the haem in an orientation that leaves a cavity enabling the substrate to bind to the oxyferryl centre in the heart of the molecule. The haem containing domain and the region surrounding it are well conserved and include the central I-helix, the cys pocket (see below), the L-helix and the J-helix (Hasemann et al. 1995). The haem is maintained in a hydrophobic environment due to the conservation of no-polar resiues in this region.

The cys pocket.

A cysteine residue at position 357 ($P450_{cam}$ numbering) which is absolutely conserved in all P450 enzymes forms the 5th ligand to the haem prosthetic group and is found at the N-terminus of the L-helix. The haem iron is covalently bound to the cysteine through the sulphur atom in the cys pocket, loop or β–bulge. In $P450_{cam,}$ residues 350-400 form the cys ligand loop. Lying closest to the haem, this is the most highly conserved region between all P450 enzymes. The β–bulge, which forms part of the loop, creates a hydrophobic environment or pocket around the cys ligand. This region is hydrophobic which is required to maintain the anionic form of the sulphur ligand in the cys pocket on which the redox potential of the haem irn and substrate binding depend (Kassner et al. 1973).

Propionate co-ordination

The haem has propionate groups (see **table 1.7b)** which interact with residues of the protein holding the haem in a specific orientation (termed propionate co-ordination) which influences the redox potential of the haem iron (Gunner 1991, Mathews 1985). These residues are polar or charged **(see table 1.7b)** (Hasemann et al 1995). In $P450_{terp}$, two residues are found at the amino terminal end of the C-helix, another in

β1–4 and another in the cys pocket (Hasemann et al. 1995). These residues are His110, Arg114, Arg319 and His375 respectively. In $P450_{BM3,}$ residue 319 is a lysine but this residue is chemically similar to arginine and therefore still maintains the environment required to stabilise the haem A-ring propionate (Hasemann et al. 1995). Other amino acids occupy this position in other P450 enzymes but their properties must be such as to still maintain the polar environment. However, there may be important exceptions. For example, the residues which interact with propionates in $P450_{scc}$ have been identified as Arg 357 and Arg 421 (Vijayakamur and Salerno 1992). Only two residues are involved, each forming two bonds. From the secondary structure prediction, 357 lies in β-sheet 3 and presumably 421 is within the cys pocket (Vijayakamur and Salerno 1992) which suggests that residues forming propionate bonds are not located in exactly the same regions in all P450 enzymes. This information is relevant to 11β-hydroxylase and aldosterone synthase as arginine residue (R448) in these enzymes is thought to be involved in haem binding (White et al. 1991) (see section 1.8).

The Meander and ERR-triad

The meander is a region of the K-helix and the cys pocket which has neither α-helices nor β-sheet configuration. The ERR-triad is three residues, Glu-Arg-Arg, which are conserved. The first two residues of the triad are invariant and found in the K-helix of all P450s whereas the third residue can be Arg, His or Asn (Hasemann et al. 1995). The ERR triad forms a set of salt bridges. As a consequence the third residue of the triad can form H-bonds with carnohyl oxygens. These bonds produce a highly conserved 3-D structure, establishing a folding motif which holds the cys pocket firmly in place and provides a stable association between haem and protein. This network of H-bonds connects the haem to the K-helix. The location of the meander suggests that it may be involved in redox-partner interaction, for example with adrenodoxin (see later).

(b). Substrate binding region

The substrate binding regions exibit most diversity. Gotoh et al (1992) proposed, from analysis of CYP2 family proteins, that all eukaryotic P450 enzymes have six regions

involved in substrate recognition. These regions or substrate recognition sequences (SRS) are described below:

SRS1	B' helix
SRS2	Carboxy terminal of F helix
SRS3	Amino terminal of G helix
SRS4	Central I helix
SRS5	β6-1 and β1-4 (β6-1 is present in some but not all P450 structures)
SRS6	β4

SRS1 varies most between P450 enzymes, probably due to amino acid insertions at both ends. Residues which alter substrate specificity have been identified in the turn between B'-C helices in a variety of enzymes (Matsunga et al. 1990, Lindberg et al. 1989, Kronbach et al. 1989, Halpert et al. 1993). Several amino acids of the F-helix (SRS2) have been identified as substrate contact points in a number of enzymes (Lindberg et al. 1989, Iwasaki et al. 1993, Juoven et al. 1991). Where the loop between helices F and G, is very large this may alter the position of the G-helix (SRS3) so that it does not have any contact with substrate (Hasemann et al. 1995). In most eukaryotic P450 enzymes the loop is large. Therefore, this region is unlikely to act as an SRS in the eukaryotic enzymes. The B' helix and F-G regions are thought to form part of the substrate access channel. We would expect these areas to be most variable to allow selective access of different substrates to the active site. SRS4 is the central I-helix and has the least inter-cytochrome P450 variabilty. It is involved in the proton delivery essential for catalysis (see section below). Between $P450_{cam}$, $P450_{BM3}$ and $P450_{terp}$, this region is the most conserved (Hasemann et al. 1995). The I-helix is associated with both substrate and haem binding. A threonine or an aspartate at position 252, found at the local widening of this helix in $P450_{cam}$, plays a crucial role in enzyme catalysis by activating molecular oxygen. It is conserved in all cytochrome P450 enzymes (Imai et al. 1989, Martinis et al. 1989, Raag et al. 1991). This key catalytic group is discussed in depth later. In 11β-hydroxylase and aldosterone synthase , residues 288, 301, 302 and 320 in the I-helix have been shown to affect substrate specifity and enzyme activity (Bottner et al. 1996, Curnow et al.1997) (see section 1.8.)

SRS5, the β6-1 and β1-4 regions, spans part of the haem binding domain and contains several residues which have been shown to influence catalytic activity in several enzymes (Lindberg et al. 1989, Halpert et al. 1993, Hsu et al. 1993, He et al. 1992). SRS6 has some variability between $P450_{cam}$, $P450_{BM3}$ and $P450_{terp}$ in terms of length and composition.

The above sections have described conserved structural regions between cytochrome P450 enzymes. It is possible to align these regions with eukaryotic P450 enzymes such as 11β-hydroxylase and aldosterone synthase to predict where SRS regions lie in these enzymes. The sections below describe how these structures correspond to their catalytic function.

(c). Binding and cleavage of molecular oxygen and proton delivery

The residue Thr252, found at the local widening of the I helix in $P450_{cam}$, is a key catalytic group involved in the cleavage of molecular oxygen, a process requiring protonation. It is in a stretch of residues directly adjacent to the oxygen binding site. This region, bewteen residues 248 to 253, acts as a pocket to accomodate the bound oxygen (Poulos et al. 1987). The threonine residue 252 interacts by H-bonding to the carbonyl O of Gly248 and directly donates a proton to molecular oxygen. When threonine is replaced by other residues, there is uncoupling of electron transfer and no substrate hydroxylation (Imai et al. 1989, Furuya et al. 1989, Imai et al. 1989, Martinis et al. 1989). In human 11β-hydroxylase and aldosterone synthase, the corresponding threonine is at position 318. Mutation of this residue to a methionine, T318M, completely abolishes enzyme activity *in vitro* (Curnow et al. 1993) (see section 1.7). This threonine residue may also exert steric control over the way in which oxygen binds. Other residues may shuttle the protons required for hydroxylation into the active site for O-O bond cleavage; an example is Asp251, another highly conserved residue, which ion pairs with Lys178 and Arg186 ($P450_{cam}$ numbering) (Ortiz de Montellano 1992). It is likely that a similar system for generating protons exists in eukaryotic enzymes such as human 11β-hydroxylase and aldosterone synthase.

(d). Redox partner interaction

Splitting of molecular oxygen depends on the binding of the enzyme to its redox partner e.g. adrenodoxin. The redox-partner enzyme association permits delivery of electrons to the haem which causes a shift in redox potential of the haem iron. The redox potential of the haem influences activity but this is also dependent on the spin state of the iron and binding of the substrate (see section 1.4). In the substrate-free structures of $P450_{cam}$, $P450_{BM3}$ and $P450_{terp}$, the active sites are hydrated, have haem low spin state iron and a low redox potential (Hasemann et al. 1995). In $P450_{terp}$, when substrate binds, all the solvent moves out of the active site (Hasemann et al. 1994) with an accompanying increase in redox potential; the iron is then in high spin state (see section 1.4). The electron donor (e.g. adrenodoxin) has negatively charged amino acids which associate with positive amino acid charges on the enzyme redox-partner docking site on the cytochrome P450 surface (Coughlan et al. 1991, Geren et al. 1984, Wada and Waterman 1992). A tyrosine at position 82 on adrenodoxin has been shown to be important for binding to 11β-hydroxylase (Beckert et al. 1994). The positive charges are centred around the cys pocket but are assymmetrically distributed, thus producing a molecular dipole which stabilises the redox partner interaction by pulling the partner close to the protein surface. Mutations of CYP11A, which encodes side chain cleavage enzyme, within the K-helix at positions K377 and K381 dramatically decrease binding of adrenodoxin to side chain cleavage enzyme (Wada and Waterman 1992), showing that these residues are involved in redox-partner interaction. In 11β-hydroxylase and aldosterone synthase , residue R374 is thought to be involved in adrenodoxin interaction (Curnow et al. 1993, see section 1.7).

1.7.4. Modelling based on crystallised structures

The above sections have described briefly the core structure using information based on the crystalline structures of $P450_{cam}$, $P450_{BM3}$ and $P450_{terp}$. This information has been used to model eukaryotic P450 enzymes such as bovine side chain cleavage enzyme (Vijayakamur and Salerno 1992). The first problem of this approach is that, unlike bacterial structures, eukaryotic P450 proteins possess an N-terminal membrane

anchor. Alignment of side chain cleavage enzyme to $P450_{cam}$ has been performed beginning after this anchor sequence so that the N-terminal sequence is excluded. Structures common to P450s start after this region. In a separate study, the first 44 residues of human cytochrome P450s could not be aligned to the core structure of $P450_{cam}$ (Zvelebil et al. 1991). Sequence analysis suggests that residues 1-26 form a single membrane spanning segment (Zvelebil et al. 1991). The tables below show **(1.7a)** the secondary structure prediction of side chain cleavage enzyme based on $P450_{cam}$ and **(1.7b)** the haem propionate bonds (Vijayakamur and Salerno 1992). The key catalytic residue Thr252 in P450cam is found at position 291 in side chain cleavage enzyme and the cysteine haem ligand is found at position 423.

Secondary Structure	**$P450_{cam}$**	**bovine side chain cleavage enzyme**
α-helix A	37-46	56-65
α-helix B'	89-96	105-112
α-helix C	106-126	124-143
α-helix D	127-145	144-162
α-helix F	173-185	214-224
α-helix G	192-214	231-253
α-helix I	234-267	273-306
α-helix L	359-378	425-444
β3	295-301	353-359
	315-323	374-382
β4	305-312	363-370
β5	382-405	448-471
	146-150	163-167

Table 1.7.a Conserved secondary structure of side chain cleavage enzyme compared to $P450_{cam}$ (Vijayakamur and Salerno 1992).

$P450_{cam}$	bovine side chain cleavage enzyme
haem O1A Arg 299	Arg 357
haem O2A Arg 299	Arg 357
haem O2D His 355	Arg 421
haem O1D Arg 112	Arg 421

Table 1.7.b Haem-propionate bonds of cytochrome $P450_{cam}$ compared to side chain cleavage enzyme (Vijayakamur and Salerno 1992).

1.7.5 Summary

This alignment shows that there is indeed conservation of structure between bacterial and eukaryotic cytochrome P450 enzymes. A similar comparison can be used with confidence to predict the structure of 11β-hydroxylase and aldosterone synthase which are closely related to side chain cleavage enzyme. The basic structure is highly conserved between members of the cytochrome P450 superfamily but variations in substrate binding regions between more closely related enzymes such as 11β-hydroxylase and aldosterone synthase are due to changes in tertiary structure which tightly regulate substrate specificity and it is these regions which share least homology.

1.8 Molecular biology of adrenal diseases.

Previous studies have discussed the structure of the CYP11B1 and CYP11B2 genes (Chua et al. 1987, Mornet et al. 1989, Wagner et al. 1991) and described what is currently known about the primary , secondary and tertiary structure of the proteins they encode, 11β-hydroxylase and aldosterone synthase. These enzymes are structurally highly homologous but their catalytic properties are markedly different. What are the differences, in terms of amino acid sequence and 3-dimensional structure, which account for these distinct properties?

Although there is as yet no complete answer to this question, relevant information is available from two general sources, the effects of mutations in man and the rat, which have pathological consequences and more recent experiments in which site-specific mutations have been introduced and the biochemical consequences subsequently examined *in vitro*. The following sections deal with this in detail.

1.8.1 Disorders of corticosteroid biosynthesis.

Characteristic diseases are associated with both excess and deficiency of particular corticosteroids. Excess cortisol secretion, such as occurs in Cushing's syndrome where the excess may be due to a cortisol-secreting tumour in the adrenal cortex or an ACTH-secreting adenoma in the arterior pituitary, leads to hypertension and metabolic abnormalities such as glucose intolerance and altered bone metabolism among others.Conversely, inability to synthesise cortisol, for example, when the ACTH receptor is non-functional, results in neonatal death. Similarly, excess secretion of aldosterone by an adrenocortical adenoma (Conn's syndrome) causes sodium retention, hypokalaemia, a metabolic alkalosis and hypertension. In Addison's disease or in rare inherited diseases (see below), a deficiency of aldosterone results in sodium loss, hyperkalaemia, hypovolaemia and hypotension. With the possible exception of the adrenocortical tumour tissue (see below), these syndromes, which are not inherited, provide little insight into enzyme structure-function relationships.

The biosynthesis of cortisol and aldosterone from cholesterol is illustrated in **figure 1.4c**. Genetic defects have been described for each of the many enzyme-catalysed reactions in these pathways (New et al. 1998). While the biochemistry of these inborn errors of corticosteroid biosynthesis and its relationships to pathology have in most cases been understood for many years, the underlying explanation in terms of molecular biology is more recent. Since the concern of this thesis is the cytochrome P450-containing enzymes, 3β-hydroxysteroid dehydrogenase/isomerase deficiency will not be considered. Similarly, although originally thought to be due to cytochrome P450scc deficiency, congenital adrenal lipoid hyperplasia is now thought to be due to StAR protein deficiency and will not be discussed further. The following discussion will concentrate on inherited deficiences of 11β-hydroxylase and aldosterone synthase. Those of 17α-hydroxylase and 21-hydroxylase deficiencies will be mentioned where relevant. In addition, a discussion of glucocorticoid-suppressible hyperaldosteronism is relevant here.

1.8.2 Congenital adrenal hyperplasia (CAH)

Congenital adrenal hyperplasia (CAH) is due to decreased production of cortisol which is required to exert feedback inhibition on ACTH secretion. Deficiences of 17α-hydroxylase , 21-hydroxylase or 11β-hydroxylase all result in cortisol deficiency (in 21-hydroxylase deficiency, aldosterone may also be deficient). As a consequence, ACTH secretion is high and causes hyperplasia of the adrenal cortex and excessive production of steroid precursors and other products. These steroids, produced in inappropriately large amounts, cause undesirable effects such as hypertension and disturbance of secondary sex characteristics and reproduction. The steroid abnormalities can be treated and corrected by glucocorticoid replacement therapy.

1.8.3 11β-Hydroxylase deficiency

Deficiency of 11β-hydroxylase, an autosomal recessive disorder, again causes CAH. Most cases of CAH which are due to 21 hydroxylase deficiency, present with salt-wasting but a small proportion develop hypertension when cortisol synthesis is impaired (White et al.1994). In 11β-hydroxylase deficiency, which constitutes 5-8% of CAH cases (Zachmann et al. 1983), activity of this enzyme is impaired but

aldosterone biosynthesis, a process requiring 11β-hydroxylation but which is accomplished by aldosterone synthase, is unaffected. Plasma aldosterone levels are low but this effect is due to suppression of renin production (Bongiovanni and Eberlein 1967). The adrenal cortex fails to synthesise cortisol in the zona fasciculata because 11β-hydroxylase is required to convert 11-deoxycortisol (S) to cortisol (F). ACTH levels are elevated due lack of negative feedback inhibition resulting from low levels of cortisol. 11β-Hydroxylase catalyses the conversion of S to F and DOC to B. Abnormally high levels of 17α-hydroxy-21-deoxy compounds resulting from high ACTH drive are shunted into adrenal androgen biosynthesis causing masculinization of external genitalia in females and premature appearance of secondary sexual characteristics in males.

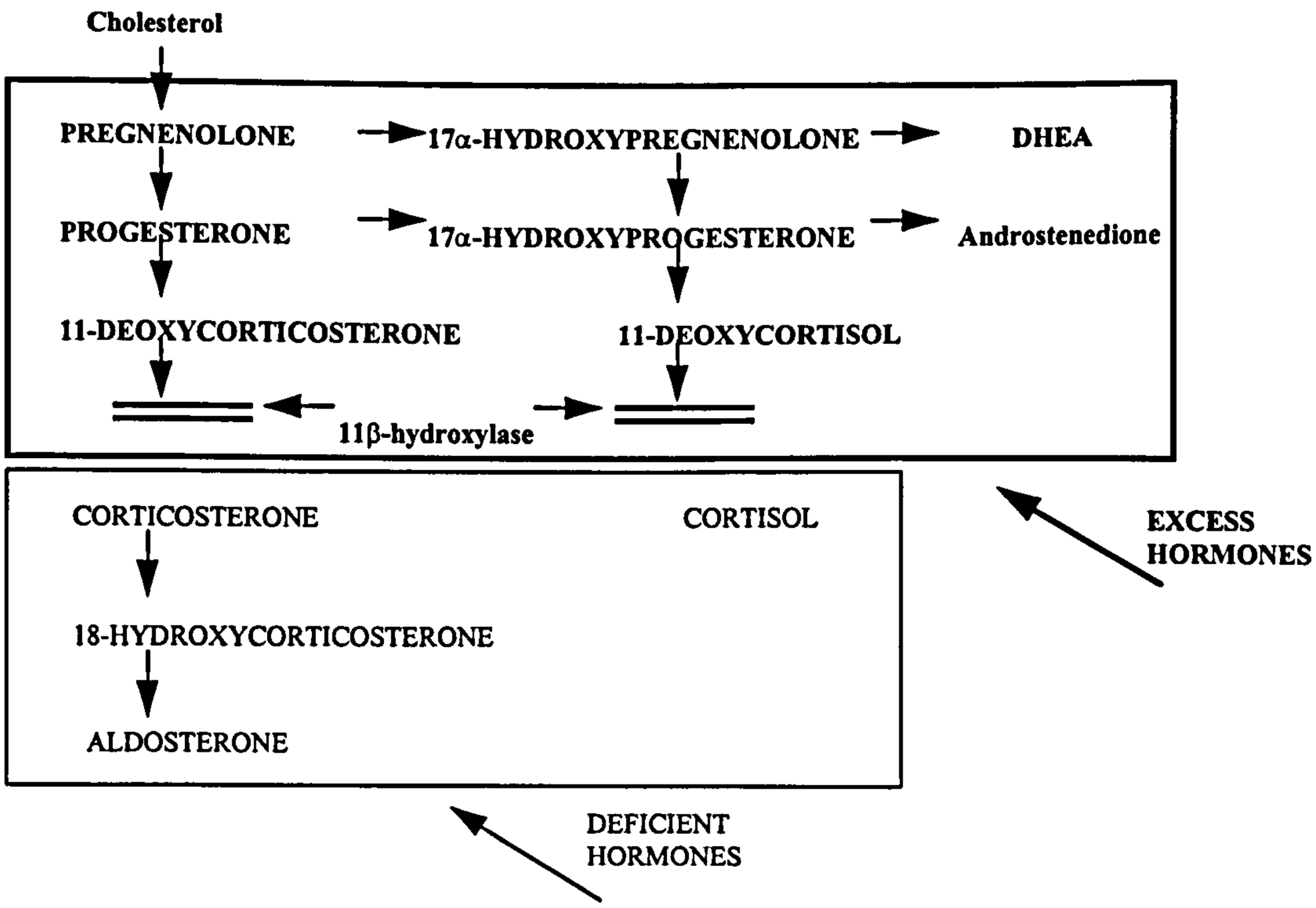


Figure 1.8a Deficiency of 11β-hydroxylase in man.

The elevated levels of DOC, which possesses mineralocorticoid activity, causes hypertension, suppression of the renin-angiotensin system and hypokalemia. If the patients are left untreated, approximately two-thirds become hypertensive and this usually occurs before adulthood (Rösler et al. 1988) (see **figure 1.8a**).

1.8.4 Mutations in the CYP11B1 gene causing 11β-hydroxylase deficiency

The 11β-hydroxylase enzyme is encoded by the CYP11B1 gene (see section 1.6). To date, 25 different mutations in the CYP11B1 causing 11β-hydroxylase deficiency have been identified which can be characterised as missense, nonsense, frameshift or insertions (**Table 1.8a and Table 1.8b**). For amino acid names, abbreviations and symbols see **appendix 1**. Missense mutations abolish *in vitro* 11β-hydroxylase activity (**Table 1.8c**). Amino acid names, symbols and abbreviations are shown in appendix 2. All other mutations result in a premature stop codon which would result in a non-functional truncated protein lacking haem and substrate binding domains. All mutations result in 11β-hydroxylase deficiency.

Missense mutation R448H in exon 8 of CYP11B1 changes an arginine residue to a histidine. Its frequency is 1/5000 births in a Jewish population of Moroccan origin (White et al. 1991). This residue lies within a conserved haem-binding region containing a cysteine residue (C450) which forms the fifth ligand to the iron atom of the heme prosthetic group. This arginine R448 residue is conserved among all known eukaryotic cytochrome P450 enzymes (Gotoh et al. 1989, Nebert et al. 1991) (see section 1.7). In Pseudomonas putida P450cam, however, the residue at the analogous position is a histidine (Gotoh et al. 1989). The R448H mutation abolishes *in vitro* 11β-hydroxylase activity when simulated in a transient transfection assay incorporating the eukaryotic expression vector pCMV4 and a non-steroidogenic COS-1 cell-line (Curnow et al. 1993). At this position, substituting a different residue, R448C, also abolishes *in vitro* activity (Geley et al. 1996).

Mutation R427H was found in combination with V384A and a 5bp insertion at codon 121 in an Asian patient of Kenyan origin (Skinner et al. 1994). The insertion itself would produce a truncated non-functional protein. R427 also lies within the haem-

binding region, is highly conserved (Nelson and Strobel 1987) and is thought to be involved in adrenodoxin interaction (Ravichandran et al. 1993).

Mutation	Exon	Type of mutation	Ethnic Group	Reference
ΔC32 + possible splice variant (htz)	1	Frameshift	White	Curnow et al. 1993
Δ28bpEx2 + V129M (compound htz)	2	Frameshift/ missense	Caucasian	Geley et al. 1996
W116X (hmz)	2	Nonsense	Japanese	Nakai et al. 1993
Ins 5bp121+ V348A, R427H (compound htz)	2,6,8	Frameshift/ missense	Asian Kenyan	Skinner et al. 1994
Ins 5bp121, R427H (hmz)	2,8	Frameshift/ missense	Indian Asian	Skinner et al. 1996
Δ28bpEx2 (htz)+ G267R (hmz)	2,5	Frameshift/ missense	Caucasian	Skinner et al. 1996
K174X +R384Q (htz)	3,7	Nonsense/ missense	White	Curnow et al. 1993
W247X (hmz)	4	Nonsense	Caucasian	Geley et al. 1996
W247X, R448H (htz)	4,8	Nonsense/ missense	Caucasian	Geley et al. 1996
W247X, E371G (htz)	4,6	Nonsense/ missense	Caucasian	Geley et al. 1996
Q338X (hmz)	6	Nonsense	Indian Sikh	Curnow et al. 1993
Q356X (hmz)	6	Nonsense	Afro-American & Nigerian-African	Curnow et al. 1993, Skinner et al. 1996
Ins2bp 394 (hmz)	7	Insertion/ Frameshift	Jewish	Helmberg et al. 1992
Ins3bp464 (hmz)	8	Insertion/ Frameshift	Caucasian	Geley et al. 1996

Table 1.8a Complex mutations in the CYP11B1 gene causing 11β-hydroxylase deficiency. hmz=homozygous, htz=heterozygous.

Mutation	Exon	Ethnic Group	Reference
G267D hmz or + possible splice variant(htz)	5	Caucasian	Geley et al. 1996
T318M (hmz)	5	Yemenite	Curnow et al. 1993
A331V (hmz)	6	Caucasian	Geley et al. 1996
R374Q (hmz)	7	Lebanese	Curnow et al. 1993
R384G (hmz)	7	Japanese	Yang et al. 1995
V441G (hmz)	8	White	Curnow et al. 1993
R448H, or R448C (hmz)	8	Morroccan & Caucasian/Iranian Jews	Curnow et al. 1993, Geley et al. 1996
C494F (hmz) or + possible splice variant(htz)	9	Indian(Asian) / Turkish	Skinner et al. 1996

Table 1.8b Missense mutations in the CYP11B1 gene causing 11β-hydroxylase deficiency. hmz=homozygous htz = heterozygous.

If this mutation or other mutations within this region, were found alone and were homozygous, they would probably influence enzyme activity (Skinner et al. 1994).

The substrate binding domain is a highly conserved area and spans residues 362-375 (White et al. 1987). Residues E371 and R374 lie within this region (Curnow et al. 1993). Two other residues within this region have been shown to be essential for binding of adrenodoxin, an electron transport protein, in a closely related enzyme (Wada and Waterman 1992) (see section 1.7.3d). Residues T318 and R384, where missense mutations have been identified, are highly conserved among members of the P450 family (Gotoh et al. 1989). R384 forms part of a β-sheet thought to be important in substrate binding (Curnow et al. 1993). T318 is specifically involved in the proton transfer which cleaves molecular oxygen (see section 1.7.3c). Residues at positions 331, 337 and 427 are arginines or histidines in all cytochrome P450 enzymes. They have structural importance and mutations at these sites which replace them with innappropriate residues will therefore exert detrimental effects on helices I,

K and L respectively and hence on the secondary structure of the protein (Geley et al. 1995).

Mutation CYP11B1	EXON	Activity *in vitro*	Functional significance	Reference
V129M	2	0	substrate binding	Geley et al. 1996
G267R	5	NA		Skinner et al. 1996
G267D	5	NA		as above
T318M	5	0	alters proton transfer	Curnow et al. 1993
A331V	6	0	disturbs I-helix	Geley et al. 1996
V348A	6	NA	Little effect conservative	Skinner et al. 1994
E371G	6	0	Adx interaction K-helix	Geley et al 1996
R384Q	7	0	β-strand/ substrate binding	Curnow et al. 1993
R384G	7	0	as above	Yang et al. 1995
R427H	8		Adx interaction/ haem binding	Skinner et al. 1994, Skinner et al. 1996
V441G	8	0	Alters secondary structure	Curnow et al. 1993
R448H	8	0	Haem binding	Curnow et al. 1993, Geley et al. 1996
R448C	8	0	as above	Geley et al. 1996
C494F	9	NA	Substrate/Adx interaction	Skinner et al. 1996

Table 1.8c Effects of missense mutations of CYP11B1 on 11β-hydroxylase activity *in vitro* .

Residue 337 is also thought to be important in adrenodoxin interaction (Geley et al. 1996). An insertion of a leucine at position 464 also disturbs L-helix structure (Geley

et al. 1996). The valine at residue 129 is also highly conserved but its exact function is unknown.

A recent *in vitro* study showed that I112, which lies close to V129, is important for hydroxylation potency and is perhaps involved in substrate binding (see section 1.9.2). The exact structural and functional role of the other residues affected by missense mutations is not known. However, it is clear from their clinical consequences that they are important for enzyme activity. Residues 267, 441 and 494 are not highly conserved between members of the cytochrome P450 family but, from this evidence, must be important.

An additional 10 mutations have been identified which result in premature termination of the coding sequence and a truncated, non-functional gene product. Mutations of this kind are nonsense mutations; K174X, Q338X, Q356X, W116X and W247X or frameshift mutations; ΔC32, 2bp insertion at codon 394, a 28bp deletion or a 5bp duplication in exon 2 (see **tables 1.8a to 1.8c** for references). The haem-binding and substrate binding domains are absent or incomplete which explains the lack of enzyme activity.

It is clear that all the mutations identified within CYP11B1 exert detrimental effects. However, they do not provide enough information to explain in detail the relation between gene/protein structure and enzyme activity.

1.8.5 Aldosterone synthase deficiency

Inborn errors of aldosterone biosynthesis were first described by Ulick and colleagues in 1976. It was originally thought that the final conversion steps in aldosterone biosynthesis involved two enzymes, corticosterone methyloxidase type I which generated 18-hydroxycorticosterone and corticosterone methyloxidase type II, responsible for the generation of aldosterone. It is this concept which led to the classification of the disorders as CMO-I and CMO-II deficiencies which are categorised by either deficient levels or elevated levels of 18-hydroxycorticosterone

(18-OHB) respectively (Ulick et al. 1992). In CMO-I deficiency, there is loss of the 18-hydroxylation capacity of aldosterone synthase which should be reflected in a build up of corticosterone although this may be difficult to detect. On the other hand, in CMO-II deficiency, the problem is directed towards the 18-oxidative capacity of aldosterone synthase and prevents the oxidation at the C18 position of 18-hydroxycorticosterone to aldosterone. In both cases, there is a lack of aldosterone and consequently disruption of the fine balance of fluid homeostasis which may result in marked sodium loss. The severity of this disorder becomes less as the kidney matures. Sodium reabsorption capacity, which is aldosterone-independent, improves and the symptoms of the disorder apparently become increasingly undetectable in adulthood, occurring only during sodium losing crises such as vomiting, diarrhoea, excessive sweating or menstruation (Rösler et al. 1984) (see **figure 1.8b**).

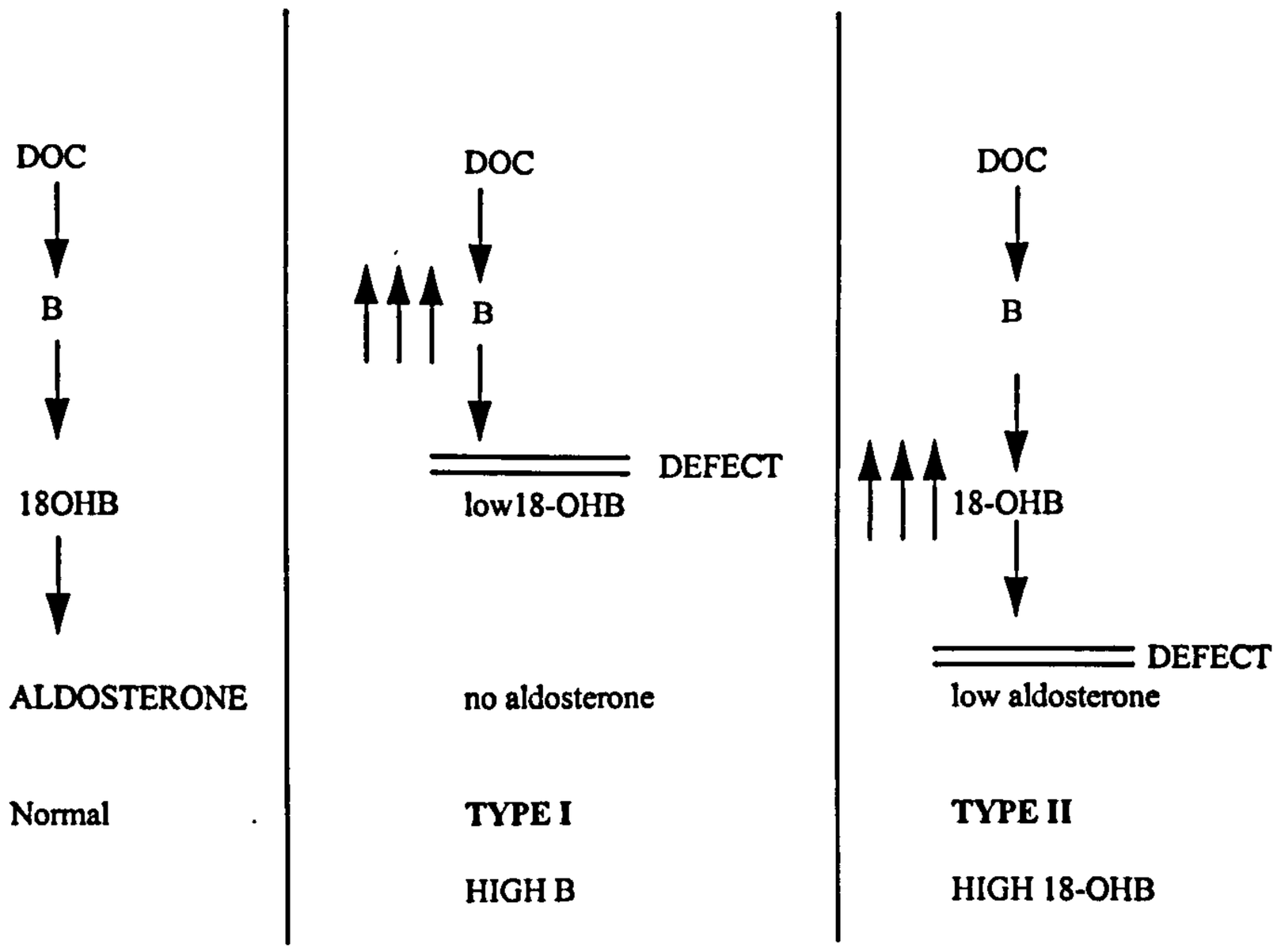


Figure 1.8b Aldosterone synthase deficiencies type I and II

CMO-I and II deficiencies are autosomal recessive inherited disorders due to a defect in the CYP11B2 gene. Mutations have been identified in coding regions of CYP11B2 in nearly all cases of aldosterone synthase deficiency and are shown in **table 1.8d.**

1.8.6 Mutations causing CMO-I deficiency

Mitsuuchi et al (1993) identified homozygous inheritance of a 5bp deletion in exon 1 of the CYP11B2 gene which results in a premature stop codon in CMO-I deficient patients. A premature stop codon in exon 4 (E255X) was identified (Peter et al. 1997) in a patient described by Visser and Cost (1964). The mutant enzyme was predicted to lack the 5 terminal exons that contain the haem-binding domain. Homozygous amino acid substitution R384P also causes CMO-I deficiency (Geley et al. 1995). A similar mutation has been identified CYP11B1 which completely completely abolishes enzyme activity (Curnow et al. 1993) (see section 1.8.4). This arginine is highly conserved in all mitochondrial cytochrome P450 enzymes and is involved in binding of the haem group (Geley et al. 1995). More recently, a homozygous point mutation, L461P, which lies in exon 8 and is involved in the putative haem-binding site, has been identified (Nomoto et al. 1997). Expression of missense mutations causing CMO-I deficiency *in vitro* have been shown that they cause complete loss of aldosterone synthase activity (**Table 1.8e**).

This is surprising as the biochemical phenotype, *in vivo*, suggests that only 18-hydroxylase activity is deficient. In a recent study, a patient with classical CMO-I deficiency was homozygous for three mutations (R173K, E198D and V386A) (Portrat-Doyen et al. 1998). R173K and V386A are polymorphic in the French population (Portrat-Doyen et al. 1998). *In vitro* studies have shown that R174K is no different from wild-type aldosterone synthase (Fardella et al. 1996b). V386A and E198D, when introduced singly, reduced aldosterone production only mildly but a construct with all three mutations (R173K, E198D and V386A) had reduced 11β-hydroxylase and some residual 18-hydroxylase activity but no 18-oxidase activity (Portrat-Doyen et al. 1998). The mutant construct suggests that the patient should have CMO-II deficiency and not CMO-I as there is still some 18-hydroxylase activity (Portrat-Doyen et al. 1998). Thus, the relationship between phenotype and genotype is not as clear as once thought.

CMO-I Mutation	Exon	Type of mutation	Ethnic Group	Reference
5bp deletionEx1 (hmz)	1	deletion	Amish North America	Mitsuuchi et al 1993 Nomoto et al 1997
E255X (hmz)	4	Nonsense	Netherlands	Peter et al 1997
R384P (hmz)	7	Missense	White	Geley et al.1995
L461P (hmz)	8	Missense	Turkish	Nomoto et al 1997
R173K, E198D, V386A (hmz)	3,7	Missense	French	Portrat-Doyen et al. 1998.
CMO-II Mutation				
R181W/ V386A hmz	3,7	Missense	Iranian jews	Pascoe et al 1992, Mitsuuchi et al 1992
ΔC372/R181W one allele + T318M/V386 A other allele (Compound htz)	7/3 + 5/7			Zhang et al 1995
Exon 3+4 conversion	3/4	Conversion	Scot/Irish/ Afro-American	Fardella et al 1996

Table 1.8d Complex mutations in the CYP11B2 gene causing aldosterone synthase deficiencies CMO-I and II. hmz=homozygous, htz=heterozygous

1.8.7 CMO-II deficiency.

Several mutations have been identified in the coding regions of the CYP11B2 gene in this disorder (**Table 1.8d**). However, it is possible that mutations in the promoter region or other non-coding regions, or mutations in genes other than CYP11B2 which exert control over CYP11B2 expression or enzyme function, may cause the syndrome. Of course, this may also apply to other inherited syndromes of corticosteroid excess. For example, a polymorphism in one of the SF-1 binding sites within the promoter region and also an intronic conversion in intron 2 of CYP11B2 has been identified (see section 1.6).

Two substitutions, R181W and V386A, have been identified in patients with CMO-II deficiency (Pascoe et al. 1992b). Individuals affected by the disorder were homozygous for both mutations but relatives who were homozygous for either R181W or V386A were unaffected (Pascoe et al. 1992b). Also, recently a T185I mutation, which lies close to R181, was identified in two cases of CMO-II deficiency (Peter et al. 1998a).

Expression studies *in vitro* demonstrated that a mutant enzyme carrying both substitutions resulted in loss of both 18-hydroxylase and 18-oxidase activities of aldosterone synthase (Pascoe et al. 1992b). When considered individually, the mutant enzyme with R181W had reduced 18-hydroxylase and a complete loss of 18-oxidase activity, whereas mutant V386A had only mildly impaired aldosterone synthase capacity (Pascoe et al. 1992b). In combination with the biochemical *in vivo* observations, this suggests that the R181W mutant still produces a very small amount of aldosterone *in vivo* and that, *in vitro*, this is undetectable (Pascoe et al. 1992b).

Several other mutations have been identified. However, again these occur in combination in CYP11B2 and are somewhat complex. In one patient, ΔC372 was found on one allele and two substitutions, T318M and V386A, on the other allele (Zhang et al.1995). We know that T318 is highly conserved and plays a crucial role in proton transfer and, from the previous study (Pascoe et al. 1992b), V386A results in slightly impaired loss of function of P450Aldo. However, we do not know the combined effect of these mutations *in vitro*. This suggests that, although one allele is

totally dysfunctional and carries ΔC372, the other less affected allele determines the biochemical phenotype (Zhang et al. 1995). Effects of missense mutations on aldosterone synthase *in vitro* are shown in **table 1.8e.**

Missense Mutation	**Exon**	**Activity *in vitro***	**Functional significance**	**Reference**
R173K	3	normal aldosterone	-	Fardella et al. 1996b, Portrat-Doyen et al. 1998
R181W	3	↓ 18-Hydroxylase no 18-oxidase	enzyme activity	Pascoe et al. 1992, Mitsuuchi et al. 1992
R181W, V386A	3,7	as above	enzyme activity conformational change	as above
E198D	3	slight reduced aldosterone	highly conserved-functional role	Portrat-Doyen et al. 1998
V386A	7	as above, no change	-	Mitsuuchi et al. 1992, Pascoe et al. 1992, Zhang et al. 1995, Portrat-Doyen et al. 1998.
R173K, E198D, V386A	3,7	↓11β-hydroxylase no 18-functions	conformational change in protein	Portrat-Doyen et al. 1998.
D147E	3	normal aldosterone	-	Fardella et al. 1996a
K151N	3	as above	-	as above
I248T	4	as above	-	as above
Ex3/4 conversion , D147E, K151N, I248T	3,4	normal aldosterone	-	as above
R384P	7		Sunstrate binding	Geley et al. 1995
L461P	8		enzyme activity	Nomoto et al. 1997

Table 1.8e Effects of missense mutations of CYP11B2 on aldosterone synthase activity *in vitro* .

A possible gene conversion event between CYP11B1 and CYP11B2 exons 3 and 4 has been identified in a patient with CMO-II deficiency (Fardella et al. 1996). This produced an aldosterone synthase enzyme containing all residues from exons 3 and 4 of 11β-hydroxylase. Aldosterone synthase and 11β-hydroxylase are highly homologous and between the regions encoded by exons 3 and 4, only 3 residues differ in the protein (at positions 147, 152 and 248). Seven constructs were prepared expressing each mutation singly, each of the three possible pairs of mutations and the triple mutant. *In vitro* expression studies only measured aldosterone production. None of the mutants affected aldosterone production. No attempt was made to identify which of the several functions of aldosterone synthase was affected in the patient. From these findings, it appears that this gene conversion event is associated with, but is not the cause of, the deficiency (Fardella et al. 1996a). Other factors affecting aldosterone biosynthesis may be responsible. Polymorphisms/ mutations of the CYP11B2 gene have not been restricted to aldosterone synthase deficiency. Recently a genetic variant of aldosterone synthase was identified in Chilean patients with low renin essential hypertension (Fardella et al. 1996b). In this disorder, the ratio of aldosterone concentration to plasma renin activity (PRA) is high, as aldosterone levels are normal but renin levels are low. It is possible that inappropriately increased aldosterone biosynthesis could be due to a CYP11B2 defect. The CYP11B2 genes were sequenced and found to possess two allelic variants which resulted in a aldosterone synthase with Arg173 or Lys173. *In vitro* expression studies revealed that both had similar Vmax and Km values. Although these variants apparently exert no detrimental effects on enzyme function, the Arg173 variant of CYP11B2 had a higher frequency in patients with low renin essential hypertension (Fardella et al. 1996b). Recently, homozygous deletion of R173 in aldosterone synthase has been identified in a patient with CMO-II deficiency (Peter et al. 1998b). Deletion of this residue which lies in helix-D may alter secondary structure (Peter et al. 1998b). This does suggets that this specific locus of aldosterone synthase is important.

1.8.8 Glucocorticoid-suppressible hyperaldosteronism (GSH)

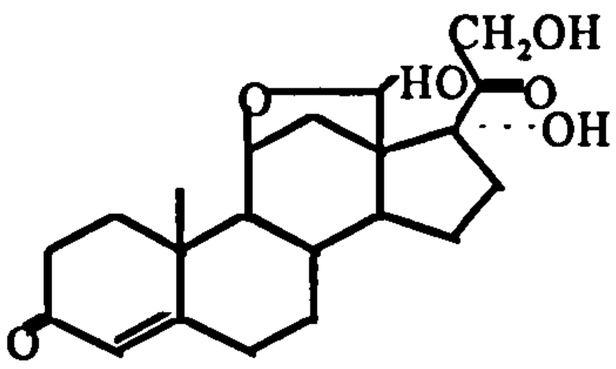
The previous sections have discussed mutations in the CYP11B1 and B2 genes. GSH is due to the presence of a chimeric gene formed between CYP11B1 and CYP11B2 and is thought to occur via unequal crossover at meiosis. To briefly summarise, CYP11B1 and B2 are found approximately 40kb apart on chromosome 8q22 in a similar arrangement to that of CYP21 and CYP21P (Pascoe et al. 1992, Lifton et al. 1992). CYP11B1 is regulated by ACTH, can convert DOC to B and S to F and is expressed in the ZF. CYP11B2 is expressed exclusively in the ZG, is regulated by ANGII *in vitro* and is required for the conversion of DOC to aldosterone. The biochemical phenotype of GSH arises from a chimeric gene possessing control regions of CYP11B1 fused to the coding regions of CYP11B2 and expressed ectopically in the ZG (Lifton et al. 1992a).

Hybrid genes of this description has been observed in several kindreds with this disorder (Pascoe et al. 1992, Lifton et al. 1992a, Lifton et al. 1992b). This rare autosomal dominantly inherited disorder is characterised by hypersecretion of aldosterone with suppressed plasma renin activity, hypertension and hypokalaemia. These abnormalities are successfully reversed by administration of small doses of the synthetic glucocorticoid, dexamethasone. Aldosterone levels are subject to control by ACTH and the diurnal variation in plasma cortisol levels correlate strongly with plasma aldosterone levels as do levels of DOC (Connell et al. 1986).

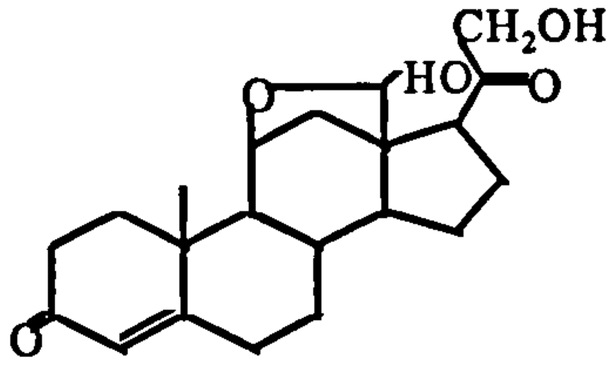
Steroid ratios of DOC:B and S: F are elevated, suggesting an additional defect in 11β-hydroxylation (Jamieson et al. 1996, Fallo et al. 1994). The chimeric enzyme further metabolises cortisol in the ZF to 18-hydroxy and 18-oxocortisol. In normal patients, small amounts of 18-hydroxycortisol are secreted (Corrie et al. 1985). In patients with GSH, urinary secretion rates of both 18-hydroxy and 18-oxocortisol are markedly raised (Ulick and Chu 1982, Gomez-Sanchez. 1984, Connell et al. 1986). Both 18-hydroxycortisol and 18-oxocortisol are structurally similar to 18-

hydroxycorticosterone and aldosterone (an alternative name for 18-hydroxycortisol is 17α-hydroxyaldosterone) (see **figure 1.8c**).

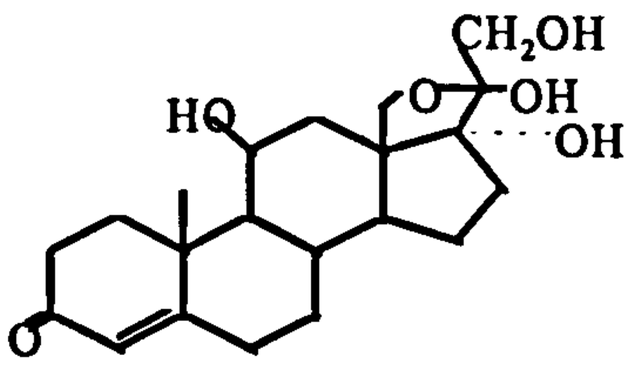
18-oxocortisol



Aldosterone



18-hydroxycortisol



18-hydroxycorticosterone

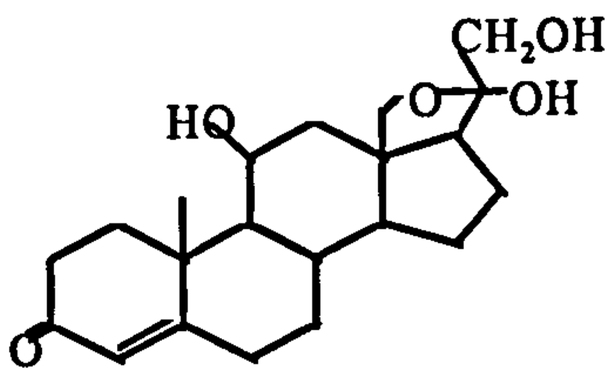


Figure 1.8c. 18-oxocortisol and 18-hydroxycortisol

18-Hydroxycortisol has extremely low affinity for both GR and MR (Gomez-Sanchez et al. 1984); 18-oxocortisol has higher affinity than 18-hydroxycortisol for both but still very low when compared to aldosterone (1%) and dexamethasone (3%) (Ulick et al .1983, Gomez-Sanchez et al. 1985). It is therefore unlikely that these steroids exert significant mineralocorticoid or glucocorticoid actions *in vivo*. As mentioned previously, 11β-hydroxylation is defective in patients with GSH. It has been suggested that 18-hydroxycortisol and 18-oxocortisol may act as inhibitors of enzyme activity (see chapter 6).

In summary, patients with GSH have excessive levels of aldosterone, 18-hydroxycortisol and 18-oxocortisol which is responsive to ACTH. This suggests that there is ACTH-responsive aldosterone synthase activity in the ZG and the ZF. These changes are associated with the hypertension, hypokalaemia and suppression of the renin-angiotensin system although affected subjects vary in severity of phenotype which is even absent in some subjects possessing the chimeric gene. The alternative

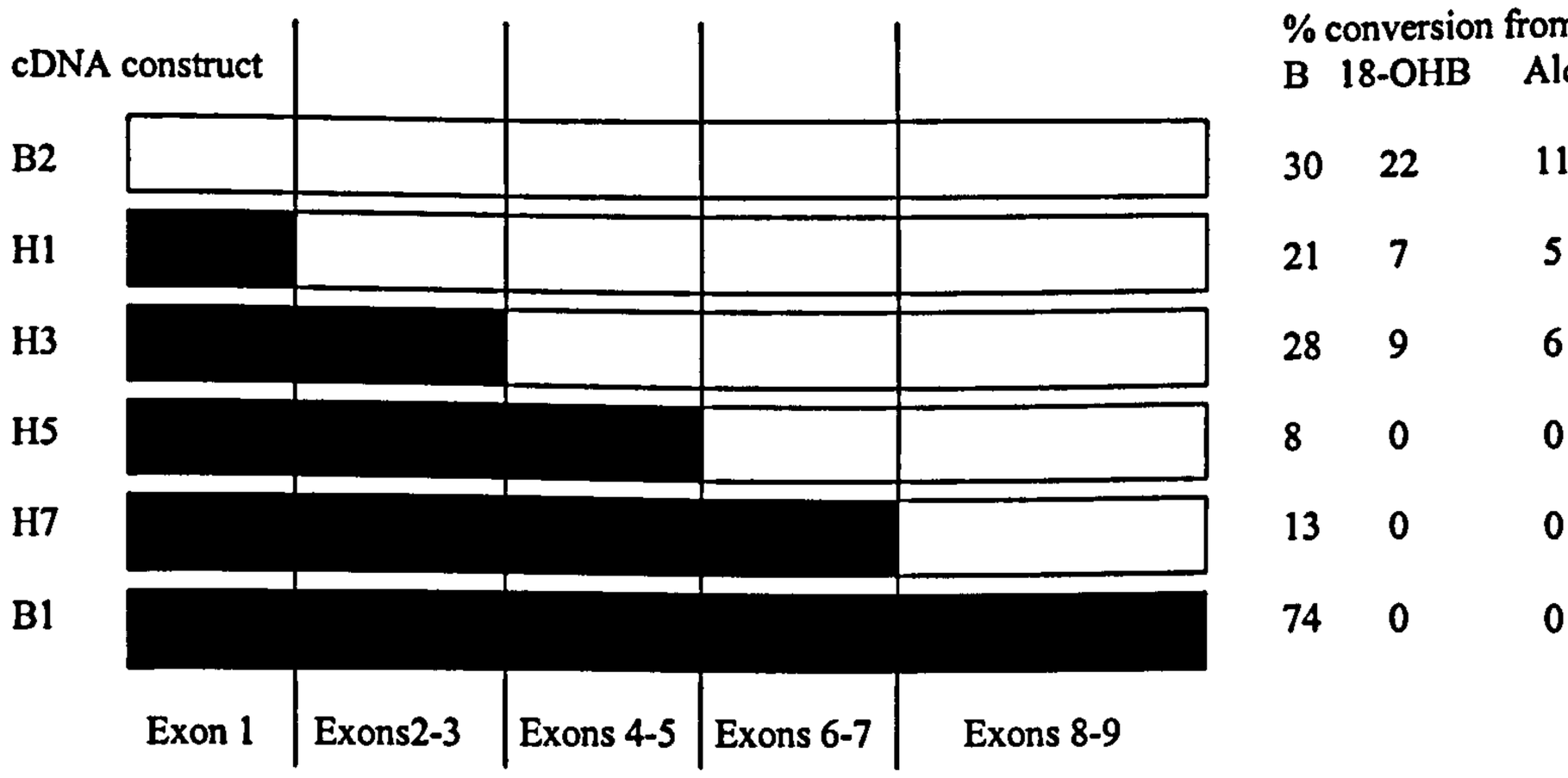
chimera which possesses the 5'regions of CYP11B2 fused to the 3' regions of CYP11B1, has not to date been found.

1.8.9 Molecular Genetics of GSH

By Southern blotting, the chimeric gene was identified in subjects from a single North American kindred (Lifton et al.1992). DNA from patients was digested with the restriction enzyme BamHI, and transferred to a nylon membrane. A radiolabelled probe containing exons 3-4 of CYP11B1 was used to probe the membrane. Individuals with GSH had three hybridising species, the two normal species 8.5kb and 4.5kb for CYP11B1 and CYP11B2 respectively, and an additional 6.3kb band representing the chimeric gene.

Linkage analysis of the kindred yielded a maximum lod score of 5.23 for complete linkage of the 6.3kb band with GSH. Using densitometry, the band intensities were analysed. The 6.3kb band showed intensity approximately 50% of that of the bands representing CYP11B1 and CYP11B2. This suggests that affected subjects have one copy of the additional chimeric gene in addition to their normal copies of the CYP11B1 and CYP11B2 genes. From these observations evolved the idea of the unequal crossover event. The resulting gene would possess the 5' regions of CYP11B1 fused to the coding regions of CYP11B2. In subjects from four unrelated kindreds, a similar gene has been identified, thus confirming Lifton's findings (Pascoe et al. 1992a). In this latter study, the chimeric genes were selectively amplified in all four subjects using the polymerase chain reaction (PCR). Primers were designed which were specific to CYP11B1 and B2 to locate the crossover site. Twelve kindreds of Celtic origin have been reported (Lifton et al. 1992). Eight out of the twelve had Irish ancestry (Lifton et al. 1992). In the United Kingdom, seven kindreds have been identified, all of which were of Scottish origin except one Irish kindred. Therefore, of the 21 kindreds studies so far affected with GSH, 15 have a Celtic background. A founder effect is unlikely as sequence analysis has shown that there are at least 7 different chimeric genes in these kindreds (Lifton et al. 1992, Jamieson 1995).

The sites of crossover have been determined (Pascoe et al. 1992b, Lifton et al. 1992a). In all cases, the site is between intron 2 and intron 4 and always before exon 5 of CYP11B2 (Lifton et al. 1992, Miyahara et al. 1992). This information alone indicates that the aldosterone synthesising capacity of human P450aldo is conferred by amino acids specific to this enzyme encoded by exons 5-9 of the CYP11B2 gene. All GSH kindreds studied so far have inherited a copy of the chimeric B1/B2 gene from either parent which includes exon 5 of CYP11B2. To investigate the effect of the exonic arrangement of these chimeric genes, *in vitro* studies of the steroidogenic activity of cDNA constructs with variable proportions of CYP11B1 and CYP11B2 exonic DNA have been performed (Pascoe et al. 1992a). Constructs and *in vitro* enzyme activity are shown in **figure 1.8d.**



B2 Exons 1-9 of CYP11B2 only
H1 Exon 1 from CYP11B1 - Exons 2-9 from CYP11B2
H3 Exons 1-3 from CYP11B1- Exons 4-9 from CYP11B2
H5 Exons 1-5 from CYP11B1- Exons 6-9 from CYP11B2
H7 Exons 1-7 from CYP11B1- Exons 8-9 from CYP11B2
B1 Exons 1-9 of CYP11B1 only

Figure 1.8d Aldosterone synthase activity of chimeric CYP11B1/CYP11B2 cDNA constructs *in vitro*.

Constructs possessing exons 1-3 of CYP11B1 fused to exons 4-9 of CYP11B2 have full aldosterone synthase activity. Constructs with exons 1-5 or more of CYP11B1

had no detectable aldosterone synthase activity. This confirms that exon 5 of CYP11B2 encodes residues important for aldosterone synthase activity.

In a recent study, crucial residues of CYP11B2 important for aldosterone synthase activities were identified in exons 4, 5 and 6 (Mulatero et al. 1998). At the corresponding positions in CYP11B1, the residues were replaced for the aldosterone synthase specific residues. *In vitro*, the mutant CYP11B1 construct had aldosterone synthesising capacity (Mulatero et al. 1998). If found *in vivo,* this gene would be regulated by ACTH but possess aldosterone synthase activity and would be the result of a conversion event between exons 4, 5 and 6 between the CYP11B1 and CYP11B2 genes and result in GSH. In all cases studied, no such a conversion was found (Mulatero et al. 1998).

In summary, GSH is caused by chimeric genes where the crossover is in the first 4 exons of CYP11B1and fused to exons 5-9 of CYP11B2. These exons contain the active site consisting of the haem-binding and substrate-binding domains, both essential for enzyme function. Specific residues within these exons of CYP11B2 confer aldosterone synthase activities of the chimeric gene product. The precise importance of these individual residues has been investigated (see section 1.9)

1.8.10 Animal models of hypertension

Steroidogenesis and adrenal cytochrome P450 enzymes in the DahI salt sensitive (S) and salt- resistant (R) rat models of salt-induced hypertension have provided useful insights into structure-function relationships. The pathophysiological mechanisms underlying blood pressure regulation in these strains have been studied extensively. Dahl et al (1962) selectively bred rats for their blood pressure response to high sodium dietary intake. Two strains were identified. The Dahl S rat is sensitive to the hypertensive effects of sodium whereas the DahI R rat is resistant. Differences in the structure of both CYP11B1 and B2 may be responsible. 18-Hydroxy-11-deoxycorticosterone (18-OHDOC) secretion by the adrenal glands of the DahI S rat was 2-fold higher than those of the DahI R rat (Rapp and Dahl 1972) suggesting that

the DahI R rat had a reduced capacity to synthesise 18-OHDOC due to mutations in CYP11B1.

RT-PCR and sequence analysis of the CYP11B1 from the DahI S and R models revealed six nucleotide differences five of which caused amino acid changes, R127C, V351A, V381L, I384L, V443M (Matsukawa et al. 1993, Cicila et al 1993). When the cDNA was expressed in COS-7 cells, 18-OHDOC synthesis was dramatically reduced compared to the DahI S cDNA (Matsukawa et al. 1993). Hybrids formed between DahI S and R cDNA were constructed to determine the effects of these diferences on steroid production. Replacement of R127 and V351 of DahI S with valine (V) and cysteine (C) of DahI R within the DahI S cDNA did not alter 18-OHDOC production of DahI S. Replacement of V381, I384 and V443 of DahI S with leucine (L), leucine (L) and methione (M) of DahI R within the DahI S cDNA markedly altered 18-OHDOC, making DahI S production more like that from the DahI R cDNA.

The reverse of these changes were also constructed. Substituting DahI R residues 381,384 and 443 with the DahI S equivalent in the DahI R cDNA made DahI R more like DahI S (Matsukawa et al 1993). Therefore, the alternative residues at 127 and 351 do not determine the rates of 11β and 18-hydroxylation in the DahI rat P45011β. Residues 381, 384 and 443 are within a region which affects 11β and 18-hydroxylation (Matsukawa et al. 1993). Residues 351, 381 and 384 are located near Ozol's region (Ozol 1989) which is important for orientation of the substrate and the haem in the active site (Nonaka et al. 1989). It has also been implicated in the electron transfer interaction between cytochrome P450s and adrenodoxin (Wada and Waterman 1992). Residues 381 and 384 reside in a region known to be a substrate recognition site (SRS-5) in this family of P450s (Gotoh et al 1992) (see section 1.7.3b). Although located near the haem binding region, residue 443 probably does not affect substrate orientation (Matsukawa et al. 1993).

From these structural observations, it is most likely that the alterations of amino acids at residues 381 and 384 in the DahI R rat account for the dramatic decrease in 18-OHDOC (Matsukawa et al. 1993). In the DahI S rat, high levels of 18-OHDOC may

be at least partly responsible for pressor sensitivity to dietary sodium. Cicila et al (1993) showed that in inbred DahI S and R rats, CYP11B1 polymorphisms cosegregate with both the capacity to synthesise 18-OHDOC and with blood pressure. This CYP11B1 locus has been implicated as a hypertension QTL (quantitative trait locus) in chromosome 7 (Garret et al. 1998).

Aldosterone synthase cDNA is another potential candidate for the chromosome 7 QTL (Cicila et al. 1997). The DahI R rat aldosterone synthase has amino acid substitutions, E136D and Q251R, compared to the DahI S rat enzyme (Cover et al 1995). cDNA's from both were expressed *in vitro*. When corticosterone (B) was used as substrate, the DahI R rat cDNA produced >1000 times more aldosterone than the DahI S cDNA (Cover et al. 1995). Kinetic experiments followed but 11-deoxycorticosterone (DOC) was used as substrate (the reason for this switch in substrate is not explained). This produces a DahI R enzyme with an increased V_{max} and reduced K_m compared to DahI S enzyme *in vitro* (Cover et al. 1995). The gross over- production of aldosterone *in vitro* by DahI R rat compared to the DahI S rat does not reflect the biochemical phenotype as there is only a 2-fold difference in plasma aldosterone levels reported between these strains (Rapp et al. 1978, Kusano et al. 1986). This suggests that other factors, in addition to inherent enzymatic properties, control aldosterone levels (Cover et al. 1995).

Mutations of human aldosterone synthase were prepared, at the analogous positions to those identified in the DahI R rat, to yield three constructs altering E136D, K251R or both (Fardella et al. 1995). Transfection studies revealed that the K251R substitution, in particular, produced 3 to 4 times more 18-OHB than wild-type aldosterone synthase as well as a 50% increase in aldosterone (Fardella et al. 1995). Substitutions of this kind which increase aldosterone production may be implicated in human hypertension (Fardella et al. 1995).

1.8.11 Summary

In summary, mutations have been identified in the CYP11B1 and B2 genes of the DahI R strain which have functional implications. In the case of CYP11B2, these

mutations produce an overactive aldosterone synthase. Similar mutations manufactured in the human CYP11B2 had lesser effects than the rat mutations but did increase human aldosterone synthase activity. A mutation of this kind found in human subjects would be likely to cause hypertension. The use of animal models provides a useful tool by which the mechanism of disease can be understood. This particular model has provided useful information concerning adrenal steroids and their involvement in hypertension.

1.9 Structure function studies- Site-directed mutagenesis

Disorders of the adrenal gland and its steroidogenic enzymes are often the consequence of rare mutations. For example, in 11β-hydroxylase deficiency, all of the mutations described to date completely abolish enzyme activity which explains the resulting clinical changes (see section 1.8.2). These mutations, therefore, do not provide much useful information about the residues which are structurally important or those which are essential for enzymatic activity and substrate specificity. Mutations on the other hand which increase activity or provide a new activity for the enzyme are much more interesting and are very likely to be in close proximity to the active site. As previously discussed, 11β-hydroxylase and aldosterone synthase are highly homologous isozymes. However, their enzymatic properties differ dramatically and this must be due ultimately to the residues which differ between the two isozymes. Therefore, it is of interest to identify those residues responsible for the aldosterone synthesising capacity and the lower 11β-hydroxylase activity in aldosterone synthase compared to 11β-hydroxylase. It is also important to identify those natural polymorphic residues which do not contribute to their structural and enzymatic differences. The amino acid differences between 11β-hydroxylase and aldosterone synthase are shown in **figure 1.9a.**

To date neither 11β-hydroxylase nor aldosterone synthase tertiary structure has been determined. Their association with the inner mitochondrial membrane has made preparation, purification and crystallisation difficult. Regions of structural importance have been deduced by close comparison with the crystallised bacterial P450 enzymes. Although displaying low homology with the bacterial P450 enzymes, all p450s in this family have highly conserved secondary and tertiary structure. This information along with a knowledge of the residues that differ between aldosterone synthase and 11β-hydroxylase, will enable precise identification of residues which are structurally important as well as those residues conferring specific enzymatic activities (see section 1.7).

CYP11B1		CYP11B2	Exon
Met	11	Val	
Val	13	Ala	
Gln	22	Arg	
Val	31	Ala	
Arg	43*	Gln	1
Arg	44	His	
Asp	63	His	
Val	68	Met	
Asp	82	Asn	
Ala	86	Pro	
Gly	87	Arg	2
His	109	Cys	
Ser	112	Ile	
Glu	147	Asp	
Asn	152	Lys	3
Lys	173*	Arg	
Thr	248	Ile	4
Ser	281	Asn	
Gln	285	His	
Ser	288	Gly	
Asn	296	Lys	5
Pro	301	Leu	
Asp	302	Glu	
Val	320	Ala	
Asn	335	Asp	
Ala	339	Ile	6
Ala	386*	Val	7
Arg	404	Gln	
Pro	414	Ala	8
Tyr	439	His	
Leu	471	Phe	
Gln	472	Leu	
Ser	492	Gly	
Met	493*	Thr	9
Phe	494	Ser	

Figure 1.9a. Differences in amino acids between human 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2). The diagram shows 35 residue differences with locations of exon junctions. Asterisks are naturally occurring polymorphisms which do not always differ.

The molecular biological explanation of GSH has been described (section 1.8). Many pedigrees have been studied. All chimeric genes have exons 1-4 of CYP11B1 and

exons 5-9 of CYP11B2 with the crossover site within intron 4. This indicates that residues in exons 5-9 of aldosterone synthase are essential for aldosterone synthase activity (Pascoe et al. 1992). Hybrid cDNA in vitro transfection studies have clearly shown that the crossover point must lie upstream of exon 5 for the resultant enzyme hybrid to possess efficient 18-hydroxylase and 18-oxidase activities (Pascoe et al. 1992). When the genes are fused after exon 3, aldosterone synthase activity is retained. However, if the genes are fused after exon 5 (i.e retaining exons 6-9) aldosterone synthase activity is lost. This suggests that exon 5 is essential for 18-hydroxylase and 18-oxidase activities (Pascoe et al. 1992). Similar hybrid cDNA construction has been performed between cloned rat CYP11B1 and CYP11B2 (Zhou et al. 1994). A hybrid containing the first five exons of CYP11B1 fused to the last 4 exons of CYP11B2, when expressed in COS-7 cells and incubated with DOC, possessed 11β-hydroxylase activity but had no 18-hydroxylase or 18-oxidase activity. That is, it synthesised corticosterone but not 18-hydroxycorticosterone or aldosterone. This suggests that residues upstream of exon 6 of aldosterone synthase are required for aldosterone synthesising capacity in the rat. The reverse of this construct, exons 1-5 of CYP11B2 fused to exons 6-9 of CYP11B1) was also studied. It was inactive. Thus residues present in the first 5 exons of CYP11B1 are essential for the 11β-hydroxylase activity in the rat (Zhou et al.1994).

1.9.1 Residues important for 11β-hydroxylation of 11-deoxycortisol

Exon 5 encodes amino acids essential for aldosterone synthase activity. This exon and part of exon 6, which encodes residues 299-388 of human aldosterone synthase and 11β-hydroxylase, were aligned to the I-helix region of P450cam, P450BM3 and P450terp which forms the active site domain in these simpler enzymes (Bottner et al. 1996). This careful alignment permitted the investigation of the different activities and substrate specificities in the two human enzymes (Bottner et al. 1996). As described above, hybrid cDNAs constructed between these two human genes demonstrated that the crucial breakpoint was amino acid 256 (exon 4) and that the C-terminal portion beyond this residue of aldosterone synthase seems to be important in aldosterone synthesising capacity (Pascoe et al. 1992). In P450cam, this I-helix

region contributes mainly to the active site. In an earlier study, Thr 252, which is highly conserved in all P450 proteins (Bernhardt et al. 1995), was reported to be required for oxygen activation, a key stage in P450 enzyme activity (Imai et al.1989, Martinis et al. 1989, Raag et al. 1991) (see section 1.7). From this information alone, it is probable that residues close to or within this domain play functional roles in enzyme activity. At positions 296, 301, 302, 320 and 355, aldosterone synthase-specific residues (K, L, E, A and D respectively) which align to this domain were replaced by the 11β-hydroxylase equivalents (N, P, D, V and N respectively). The mutant cDNAs were expressed in COS-1 cells and conversion of 11-deoxycortisol (S) and DOC measured by HPLC or radioimmunoassay (Bottner et al. 1996). With respect to ability to convert S to F, i.e. 11β-hydroxylase activity, mutants K296N and D335N showed a slight increase in activity compared to wild-type aldosterone synthase. However, greatly increased 11β-hydroxylation was observed with mutants L301P, E302D or A320V. When these single mutations were combined, a more pronounced positive effect on 11β-hydroxylation was seen. Double substitution of L301P and A320V increased 11β-hydroxylation by aldosterone synthase to 60% of wild-type 11β-hydroxylase (aldosterone synthase 11β-hydroxylase activity is normally only 10% of wild-type 11β-hydroxylase). Conversion of DOC to aldosterone was suppressed compared to wild-type aldosterone synthase, the triple mutant L301P/E302D/A320V losing 90% of its aldosterone synthesising capacity. The replacement of these residues demonstrated a switch in the substrate specificity of aldosterone synthase, increasing its ability to convert S, the preferred 11β-hydroxylase substrate, by replacing residues with those essential for 11β-hydroxylation by human 11β-hydroxylase.

1.9.2 Residues of aldosterone synthase which are important for aldosterone synthesising capacity.

A more recent careful alignment study of human aldosterone synthase and 11β-hydroxylase with the equivalent enzymes of other species, has identified two key residues which contribute to the 18-oxidase and 18-hydroxylase activities (i.e. aldosterone-synthesising capacity) of aldosterone synthase (Curnow et al. 1997).

Substitution within I-helix of Aldosterone synthase	Effect on S to F conversion
K296N	Slight ↑ compared to WT aldosterone synthase
L301P	↑↑↑
E302D	↑↑↑
A320V	↑↑↑
D355N	Slight ↑ compared to WT aldosterone synthase
L301P, A320V	↑↑↑↑↑ (60% of WT 11β-hydroxylase activity)
L301P,E302D, A320V	↑↑↑↑↑

Table 1.9a Effects of replacing aldosterone synthase specific residues with the 11β-hydroxylase equivalents on S to F conversion *in vitro (*Bottner et al. 1996).

Residues 248-340 (a region encoded by part of exon 4-6) of human aldosterone synthase were aligned to the corresponding region of aldosterone synthases from other species and also to human 11β-hydroxylase. Residues G288 and A320 were conserved in aldosterone synthase in all species. Interestingly, at the corresponding loci in human 11β-hydroxylase, there were a S288 and a V320 respectively (Curnow et al. 1997). Using in vitro site-directed mutagenesis, progressive C-terminal substitutions were performed. Resulting cDNA mutants were expressed in vitro in COS-7 cells and conversion of DOC to B, 18-OHB and ALDO measured by TLC with subsequent RIA. Results showed that efficient 18-hydroxylation and 18-oxidation were dependent on the nature of ten central residues located between 248 and 339. 18-Oxidase activity was markedly reduced when exon 6 residues of

aldosterone synthase (A320, D335, I339) were replaced by those of 11β-hydroxylase (V320, B335, A339). On the other hand, when the differing residues of exon 4 and 5 (248, 281,285,288,296, 301, 302) of 11β-hydroxylase were replaced by those of aldosterone synthase, this 11β-hydroxylase mutant was able to produce low levels of aldosterone (Curnow et al. 1997). Further substitution of the 11β-hydroxylase residues P301 and B302 with the corresponding aldosterone synthase residues L301 and Z302 did not influence this low level of aldosterone produced by this hybrid 11β-hydroxylase (Curnow et al. 1997). The most profound effect was seen when residues S288 and or V320 of 11β-hydroxylase were substituted for their aldosterone synthase equivalents G288 and A320. This alone was sufficient to confer 18-hydroxylase and 18-oxidase activities respectively upon 11β-hydroxylase, enabling aldosterone synthesis (Curnow et al. 1997). Thus, efficient 18-hydroxylation requires a glycine at position 288 and subsequent 18-oxidation requires an alanine at position 320. Such mutations, should they occur naturally, might have important implications in hypertension. Screening of normal and hypertensive populations for mutations and polymorphisms within these genes may reveal some interesting results.

Substitution within 11β-hydroxylase	**Activity**
S288G	Confers 18-hydroxylase activity
V320A	Confers 18-oxidase activity
S288G, V320A	Full aldosterone synthase activity

Table 1.9b Amino acid substitutions conferring aldosterone synthesising capacity on 11β-hydroxylase (Curnow et al. 1997).

A more recent study, also based on structural alignement, focussed on the regioselective role of isoleucine (I) at position 112 (exon 2) in human aldosterone synthase (Bechtel and Bernhardt 1998). Structural and sequence comparison with P450cam suggested that I112 of human aldosterone synthase may be of importance to the enzymes catalytic properties. Site-directed mutagenesis was used to alter this

residue to either a serine I112S (the equivalent residue found in human 11β-hydroxylase) or a proline I112P (residue found at corresponding position in mouse and rat 11β-hydroxylase). The resultant mutants were then expressed in COS-1 cells and the rate of conversion of DOC to aldosterone compared to wild-type aldosterone synthase. The rates of all three stages of aldosterone biosynthesis from DOC were increased by the I112P mutation but unaffected by the I112S mutation: 11β-hydroxylation was increased by 30%, 18-hydroxylation by 78% and 18-oxidation by 22%. This residue may be excluded from forming interactions with the substrate due to its distance from the active site but, from this study, it is clearly important for hydroxylation by aldosterone synthase (Betchel and Bernhardt 1998).

1.9.3 Summary

It is clear from these studies that several residues close to the active site play essential roles in the specific functions of these two enzymes. However, little is known about residues outwith this region and their role, if any, in enzymatic function with the exception of residue 112 in exon 2 whose role has been clearly defined (Betchel and Bernhardt 1998). It is unwise to class residues as functionally irrelevant solely on the basis of their locii. Although they may lie at some distance from the active site, they may still influence enzymatic function. Possible roles may be initial substrate contact points, binding of facilitatory proteins and protein folding all of which will contribute to activity and function.

1.9.4 Aims of this study

From the survey of the literature there is strong evidence that corticosteroid metabolism is frequently changed and may be involved in the aetology of essential hypertension. In order to establish this relationship two things are necessary. Firstly, a clearer understanding of the relationship of structure-function of alosterone synthase and 11β-hydroxylase not only at sites of known functional importance but throughout the molecule is required. Secondly, to determine whether mutations/polymorphisms occuring in essential hypertension change activity, bearing in mind that changes in the pattern of steroid synthesis, for example , the synthesis of abnormal steroids, may indirectly affect function.This problem has been addressed using techniques of molecular biology and established biochemical methods.

CHAPTER 2

2.1 Structure-function studies of the CYP11B1 (11β-hydroxylase) and CYP11B2 (aldosterone synthase) genes

2.1.1. Plasmids

The cDNA encoding human 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) were in the vector pCMV4; named pCMVB1 and pCMVB2 respectively (referred to as B1 or B2) and were kindly provided by Professor Perrin White (Curnow et al. 1991). The inserts constitute 1.5kb and the plasmid vector is 4.8kb. The cDNA encoding bovine adrenodoxin was contained in the plasmid pCD Adx (Okamura et al. 1985) and was a gift from Prof. J Ian Mason. As a reporter gene for transfection studies, the plasmid pSVβ-gal (Promega, Madison , USA) was used. All plasmids share a common general CMV-derived promoter. Plasmid DNA was prepared by CsCl/EtBr purification both small-scale and large-scale DNA preparation protocols (see below).

2.1.2 Preparation of competent cells

A stock of E.coli DH5 α in glycerol was used to inoculate 2ml L-broth supplemented with glucose (0.2% w/v) and $MgCl_2$ (10mM) and incubated overnight at 37°C in an orbital shaker (225rpm). 1ml of this fresh overnight culture was inoculated into 100ml of L-broth supplemented with glucose (0.2% w/v), $MgCl_2$ (10mM) and incubated at 37°C with orbital shaking (225rpm). When the optical density at 600nm was between 0.2 and 0.3, the culture was placed on ice/water for 10 minutes to cool to 4°C. The cells were then pelleted in pre-cooled sterile 250ml centrifuge bottles by centrifugation at 3000 rpm for 15 minutes at 4°C. The supernatant was decanted and the cells were resuspended by pipetting and gentle triturating in 10ml of ice-cold "Miller" transformation solution (see **appendix 3**). The resuspension was completed by gentle swirling. The suspension was divided into aliquots (100µl), frozen on "dry ice" and stored at -70°C until required. Transformation efficiency tests were performed on transformed cells.

2.1.3 Transformation of bacterial cells

Plasmid DNA (10-100ng) was added to a 100µl suspension of competent E.coli DH5α. in a 1.5ml Eppendorf tube. The mixture of cells and DNA was stored on ice for 30 minutes and then subjected to a heat shock of 30 seconds at 37°C. The cells

were allowed to recover on ice for 2 minutes before adding 500µl of L-broth supplemented with glucose (0.2% w/v), $MgCl_2$ (10mM) and incubating at 37°C for 45 minutes in an orbital shaker (225rpm). The cells were pelleted by microfuging for 30 seconds and all but 100µl of the supernatant was removed. The cell pellet was resuspended in this residual medium and spread onto L-agar plates (L-broth, 1.5% agar) containing 100µg/ml ampicillin. The plates were allowed to dry and then inverted and incubated overnight at 37°C. Colonies were visible after 16 hours.

2.1.4 Screening transformed colonies

L-agar ampicillin plates allow the selection of clones containing closed circular plasmid DNA with an intact, ampicillin-resistant gene. Analysis of clones was performed by restriction analysis and sequencing as necessary (see below)

2.1.5a Small scale plasmid purification by alkaline lysis method

A transformed colony was used to inoculate 2ml L-broth supplemented with 100µg/ml ampicillin and incubated overnight at 37°C with orbital shaking. 1.5ml of the resulting culture was pelleted in a microcentrifuge at 4000rpm for 5min and the supernatant was removed. The pellet was resuspended in 100µl of ice-cold solution 1, pH 8.0 (50mM glucose, 25mM Tris, 10mM EDTA), then 200µl of freshly prepared room temperature solution 2 was added (1% SDS, 0.2M NaOH). The mixture was inverted 5 times and stored on ice. 150µl of ice-cold solution 3, pH 4.8 (3M potassium acetate, 5M acetic acid) was added and the suspension was mixed by gentle vortexing in an inverted position and then stored on ice for 15 minutes. Cell debris was removed by centrifugation at 12000rpm for 5 minutes. The DNA in the supernatant was then precipitated with ethanol (see below) and resuspended in 50µl of TE buffer (10mM Tris.HCl pH 8.0, 1mM EDTA, pH 8.0) or sterile water, supplemented with DNase-free RNase at a final concentration of 20µg/ml.

2.1.6a Large scale DNA purification

A single colony was used to inoculate 2ml of L-broth supplemented with 100µg/ml ampicillin. It was incubated overnight at 37°C in an orbital shaker (225rpm). This overnight culture was used to inoculate 500ml of L-broth supplemented with 100µg/ml ampicillin in a 2L flask which was incubated for 16hours at 37°C with orbital shaking at 225rpm. Cells were harvested by centrifugation in a Sorval ultracentrifuge (Beckman J series rotor) at 5000rpm for 5minutes at 4°C in 250ml

centrifuge bottles. The pellet was resuspended in 18mls of ice cold solution 1 (see 2.1.5a). Freshly prepared lysozyme (2ml) (10mg/ml in 10mM Tris.HCl (pH8.0)) was then added. Freshly prepared solution 2 (40ml) was then added, the suspension mixed by inverting 10 times and then stored on ice for 5 minutes. Ice cold solution 3 (20ml) was then added, the suspension mixed gently by inverting 10 times and stored on ice for 15 minutes until a white flocculent precipitate appeared. The bacterial lysate was then centrifuged at 10000rpm for 15 minutes at 4°C. The centrifuge was allowed to decelerate gently without braking. The supernatant was filtered through four layers of sterile gauze into a fresh 250ml centrifuge bottle containing 48ml of isopropanol (0.6 x volume). This was mixed and then left at room temperature for 10 minutes. The resulting mixture was transferred to a 50ml Beckman ultracentrifuge tube and DNA was recovered by centrifugation at 12000rpm for 15 minutes at room temperature. The supernatant was decanted and the pellet was rinsed in 70% ethanol and allowed to dry. The pellet was then resuspended in 7ml H_2O or TE. Plasmid DNA was then purified by CsCl-EtBr gradient centrifugation.

2.1.6b Caesium chloride-ethidium bromide density gradient centrifugation

For every ml of DNA solution, 1g of solid caesium chloride (CsCl) was added. Ethidium bromide (EtBr, 10mg/ml in water), 0.8ml, was added for every 10ml of DNA/CsCl solution. The tubes (Beckman quick-seal centrifuge tubes) were balanced using mineral oil and centrifuged at 49000rpm for 16 hours at 20°C (with a Ti70 rotor in a Beckman centrifuge). The DNA was detected as fluorescent bands using a long wavelength UV light source and the lower band containing supercoiled plasmid DNA was collected using a syringe. The higher band is chromosomal DNA. EtBr was removed by successive extractions with an equal volume of water-saturated butanol. Water (3 x volume) was added followed by 2 volumes (DNA solution + 3 volumes water) of ethanol and the mixture placed on ice for 15 minutes. It was then centrifuged at 15000 rpm for 20minutes at 4°C. The supernatant was removed and the pellet was resupended in 1ml sterile dH_2O. CsCl was removed by dialysis against dH_2O or TE for 24 hours with approximately 6-8 changes of dH_2O or TE.

2.1.6c Ethanol precipitation

Nucleic acids were precipitated by adjusting the monovalent cation concentration to 0.3M using 3M sodium acetate (pH 5.7) stock solution, adding 2.5 x volumes of

ethanol and incubating at -20°C for 30 minutes. The precipitated nucleic acids were pelleted at 12000rpm in a centrifuge at 12 000rpm. The pellet was washed with 70% (v/v) ethanol and air-dried before resuspension in water or TE.

2.1.7 Determination of DNA/RNA concentration.

The concentration of each preparation was determined by measuring the optical density of samples at 260nm. Five microlitres of the DNA solution were added to a final volume of 1ml of H_2O in a quartz cuvette and the extinction measured at 260nm in a dual beam spectrophotometer with a deuterium lamp. An optical density OD260 of 1 corresponds to 50µg/ml of double stranded DNA. An OD260 of 1 corresponds to 40µg/ml of RNA. The optical density of pure preparations of DNA and RNA have an extinction 260/280 ratio of approximately 1.8 and 2.0 respectively (Sambrook et al. 1989).

DNA quality and identification of the individual plasmids was verified by sequencing, digestion by restriction enzymes and gel electrophoresis.

2.1.8 Restriction enzyme digestion of plasmid DNA.

Two micrograms of plasmid DNA was placed in a 1.5ml Eppendorf tube with 1µl of high concentration of the desired enzyme and 2µl of 10X buffer (specific for each enzyme, see **Appendix 4**). The enzyme SpeI for example was used to distinguish pCMV4-B1 from B2. Double digests, Not I/Bgl II and BamHI/HindIII, were used to digest pCD-Adx and pSV-β-gal respectively. Where required, 2µl of 10x BSA was added. H_2O was added to make the final reaction volume of 20µl. Digests were incubated at 37°C for at least 2hours or overnight. The completeness of each digestion and characteristic bands generated were identified by electrophoresis of the total reaction volume plus 5µl of loading buffer (see **Appendix 3**) on a 1.0% agarose gel.

2.1.9 Preparation of agarose gels for electrophoresis.

Agarose (1% w/v) was added to 100ml of TAE buffer (see **appendix 3**), mixed and heated in a 650W microwave oven for 120 seconds. Whilst cooling , 1µl of ethidium

bromide was added to the agarose, the agarose mixed and poured into gel moulds with teflon combs. The gel was allowed to set for 1hour before removing the combs. Gels were placed in standard electrophoresis tanks with 100ml of TAE buffer and connected to a constant power source at 60V for 1hour.

2.2. Direct DNA sequencing from PCR products.

The sequenase system.

Determination of nucleotide sequences to confirm mutations created by site directed mutagenesis and ensure that undesirable mutations were not incorporated was performed on PCR-amplified plasmid DNA by a modification of the chain termination method using a commercially available Sequenase kit (United States Biochemicals, Ohio, U.S.A). Dimethyl sulphoxide (DMSO) was included to enhance breakdown of DNA secondary structure. Buffer ingredients and details are in **appendix 3.**

The chain termination method involves the synthesis of a DNA strand by DNA polymerase in vitro using a single-stranded DNA template. Synthesis is initiated at the site of hybridisation of a target specific sequencing primer which anneals to the DNA of interest and a DNA strand is elongated until the reaction is terminated by the incorporation of a nucleotide analogue that does not permit further elongation. Such analogues include the 2',3'-deoxynucleoside 5'-triphosphates (ddNTP's) which lack the 3'OH group required for DNA chain elongation. Four such reactions are carried out in parallel using different ddNTP's mixed with the complementary dNTP's to provide complete sequence information A radioactively labelled nucleotide is included in the synthesis so that the labelled chains of varying length can be visualised by autoradiography after high resolution electrophoresis.

2.2.1 Sequencing protocol

Annealing

Each of the reactions listed below was carried out in a sterile Eppendorf tube:

a) 4 tubes containing 2.5 µl of each of the four ddNTP's in a termination mix were prepared.

b) A mixture was prepared containing the following:

DNA	6 µl (1 µg/ µl)

H_2O	1 µl
DMSO 25%	1 µl
Sequencing primer	2 µl (10 µM)
Total volume	10 µl

The mixture was placed on a heating block at 99°C for 5 minutes to denature DNA and then centrifuged briefly. The tube was then placed on a mixture of dry ice and 100% ethanol.

2.2.2 Preparation of the label reaction

c) In a sterlie Eppendorf tube:

DTT	1 µl
label mix	2 µl (1:5 diln)
^{35}S-ATP label	1 µl

All subsequent reactions were performed behind perspex to shield against radioactivity.

d) The plasmid mix from a) was gently thawed and centrifuged briefly. Immediately 2 µl of Sequenase buffer was added and mixed well. After 5 minutes at room temperature to allow complete denaturation, the sample was briefly spun and stored on ice.

Dilution of Sequenase enzyme

e) 1 µl Sequenase enzyme was diluted with 7 µl Sequenase dilution buffer to give a 1:8 dilution, mixed well, centrifuged briefly and transferred directly onto dry ice.

f) 2 µl of diluted enzyme from e) was added to the label reaction from c) mixed well and transferred directly onto ice.

g) 5.5 µl of this mixture from f) was added to the denatured plasmid DNA from step d) mixed well, spun briefly and stored on ice. This mixture was referred to as the 'total mix'.

h) The four ddNTP tubes from a) were placed on a pre-heated block at 37°C for 1 minute. Then 3.5 µl of total mix was added, the tubes capped to prevent evaporation and incubated for a further 5 minutes at 37°C. To each reaction were 4 µl of stop solution (see **appendix 3**) was added and stored at -20°C until run on a polyacrylamide gel.

2.2.3 Polyacrylamide gel preparation

Polyacrylamide gels were prepared using a modification of a standard protocol. Two glass plates were cleaned using isopropanol and then siliconised and left to dry in air with teflon spacers in place. A 6% polyacrylamide gel with urea was prepared:

Acrylamide:bisacrylamide (40%, 19:1)	22.5ml
10X TBE	15ml
Urea	63g

These were mixed and the volume adjusted to 150ml with H_2O. The acrylamide:urea mix was polymerised by addition of 150µl of 25% ammonium persulfate and 150µl TEMED, and poured. Combs were inserted into the top of the gel and the gel allowed to set for approximately 3 hours.

2.2.4 Polyacrylamide gel electrophoresis

The gel heated to 45°C. The lanes were cleared of bubbles by a fine-tipped Pastuer pipette and the loading combs replaced .

Samples were removed from -20°C storage and placed on a heating block at 75°C for 2 minutes before loading. For each lane of the gel, 3.5 µl of the terminated sequencing mix were placed in a well. Four reactions were run in parallel on the gel for each sequencing pimer in the order: GATC allowing the complete sequence of the DNA to be determined. Gels were run at 55-70 Watts, ensuring that the surface temperature of the gel did not rise above 50°C. Gels were run from 3 to 5 hours depending on the primer site in relation the region to be sequenced.

2.2.5 Preparation and autoradiography of sequencing gels

After electrophoresis, the gel was removed from the tank and the plates soaked in cold water in a flat tank for 30 minutes. The top plate was then carefully removed and gels were absorbed onto 3mm Whatman paper, covered in clingfilm and then dried for 60 minutes. The dried gels were subjected to autoradiography overnight and autoradiographs developed (Kodak X-Omat).

2.2.6 Determination of DNA sequence from polyacrylamide gels

Sequences were determined directly from the autoradiograph and comparison with the consensus sequence of the CYP11B1 and CYP11B2 genes.

2.2.7 Automated Cycle Sequencing

Automated sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Reaction Kit (PE Applied Biosystems, Foster City, CA, USA) using a Perkin Elmer ABI Prism 377XL DNA Sequencer by Dr Wai Kwong Lee and Nick Brain (Blood Pressure Group). The technique is an adaptation of the dideoxy termination method of sequencing (Sanger et al. 1977).

Sequencing reaction: 100-500ng of template DNA
3.2 pmol of primer
8µl Ready Reaction Mix (supplied in kit)
20µl Total

Reactions were overlaid with 40µl of mineral oil. The reaction was run over 25 cycles on a Perkin Elmer PCR block:

Protocol: 96°C for 30s
(Annealing Temp °C primer) for 25s
60°C for 4min

Prior to the gel run, sequencing products were precipitated in sodium acetate and ethanol (2.1.6c). To the pelleted DNA 6µl of 5:1 deionised formamide:25mM EDTA with 50mg/ml blue dextran dye, pH8.0 was added, the sample vortexed and denatured at 95°C for 2min. 1.5µl of each sample was loaded onto a 5% polyacrylamide gel (36 cm "well-to-read" distance, 0.2mm thick). Samples were electrpohoresed in afield of 1.68kV at 51°C in TBE buffer. At these settings the instrument resolves at 100bp/hour. The ready reaction mix contains 2',3' dideoxy terminators which are labelled with dichlor-rhodamine fluoresecent dyes. Each ddNTP flouresces at a particular wavelength between 532 and 620nm. As the labelled DNA fragments migarte down the gel, an argon-ion laser scans horizontally back and forth emmitting two wavelengths of 488nm (blue light) and 514.5nm (green light). This emmission excites the flourescent dye on each DNA fragmnent which flouresces, emmitting light at a particular wavelength. This is detected by the instrument and the data sent to a Power macintosh G3 computer and analysed using ABI Prism Sequencing analysis v3.0 (Perkin Elmer).

2.3. Screening of the CYP11B1 and CYP11B2 genes for mutations in normal and hypertensive populations

2.3.1 Blood samples

Blood samples were taken using Vacutainer needles and bottles (Becton Dickson, Europe, Meylan, Cedex-France).

2.3.2 Genomic DNA extraction from blood

Genomic DNA was extracted from leukocytes using a variation of the method of Sambrook (Sambrook et al. 1989). Ten millilitres of EDTA-preserved whole blood (10ml) was placed in a Universal tube and 40ml of cell lysis mix (**appendix 3**) added. Tubes were left on ice for 10 minutes before centrifugation at 2800 rpm. for 10 minutes at 4°C. The resulting pellet was resuspended in 3ml of nucleic lysis mix (**appendix 3**), 200µl 10% SDS and 100µl proteinase K (10mg/ml) were added.. After overnight incubation at 37°C incubation, 1 ml of 6M NaCl was added with vigorous shaking, then 5ml of phenol:chloroform:isoamyl alcohol (25:24:1), (pH aqueous phase >7.6) and the tubes centrifuged at 2800 rpm for 20 minutes at 4°C. The upper aqueous phase of the supernatant was then transferred to a fresh universal container and two volumes of ethanol added. DNA was then spooled out with a glass rod, washed in 70% ethanol, allowed to air dry and then suspended in 100µl TE buffer and stored at 4°C.

2.3.3 PCR amplification of genomic DNA

Preparation of synthetic oligonucleotides

Synthetic oligonucleotides which were HPLC-purified were designed from published sequences of CYP11B1 and CYP11B2 and obtained from a commercial source (Oswel DNA Service, Southampton). Primers were generally between 20-24 base pairs and had approximately 50% guanine and cytosine nucleotides (G/C).The melting temperature TM of primers was around 65°C. The primer was also designed not to contain any hairpin structure.

2.3.4 Polymerase chain reaction amplification

Amplification of genomic DNA exons for SSCP analysis was performed using the polymerase chain reaction (PCR). Genomic DNA extracted from the blood of individuals from both normotensive and hypertensive subjects were used as the reaction template. Reactions were carried out in sterile polypropylene Eppendorf

tubes. A standard protocol was used for amplification reactions of genomic DNA and oligonucleotide primers were altered according to the gene and exonic region. Before actual SSCP was performed, the PCR conditions were optimised using genomic DNA from 4 normal subjects.

A 1µl (approximately 50ng/µl) aliquot of the genomic DNA to be amplified was placed in a sterile Eppendorf tube and then placed on ice.

A reaction "pre-mix" was prepared for addition to the DNA prior to incubation:

dH_2O:	15.75µl
dNTP's (1mM each):	2.5µl of mixture of dATP, dCTP, dGTP, dTTP
$MgCl_2$ (25mM):	0.75µl
Sense primer:	1µl (10pmoles/µl)
Antisense primer:	1µl (10pmoles/µl)
10X Buffer:	2.5µl
Taq Polymerase:	0.5µl (5U/µl)

The total reaction volume was 25µl. Taq polymerase, a thermostable DNA polymerase, was used. Reaction mixtures were covered with 50 µl of mineral oil and placed in a thermal cycler. The heating block temperature was raised to 94°C for 3 minutes for the initial reaction to allow complete denaturation of the DNA template to occur prior to cycling.

2.3.5 A typical amplification protocol used was:

1.Denaturation:	94°C for 60seconds
2.Annealing:	65°C for 60seconds (dependent on primers)
3.Extension:	72°C for 120seconds

1 to 3 were repeated for 35 cycles and followed by a final extension step of 72°C for 7 minutes.

Different pairs of primers were used to amplify different exons of the two different genes. Details of these primers and exons are shown in **tables 3 and 4 of appendix 2.** For some exons it was not possible to obtain primers specific for each gene. When this was the case primers chosen amplified both CYP11B1 and B2 exons. It was then necessary to separate CYP11B1 from CYP11B2 by subcloning into T-vector to facilitate sequencing (detailed later).

Following completion of the PCR, samples were placed on ice until run on 1% agarose gels stained with ethidium bromide to verify the presence of the desired reaction products.

2.3.6 Single-stranded conformational polymorphism (SSCP)

Once the PCR reaction had been optimised, conditions for SSCP were set up. This technique is used to detect mutations in DNA sequences which have been amplified from genomic DNA by PCR. It uses a PCR product which, by incorporating a radioactively labelled substrate in the PCR reaction, becomes labelled itself. These PCR products are then run on a non-denaturing acrylamide gel. The electrophoretic mobility of single-stranded nucleic acids depends on size and sequence. Using single-stranded fragments of ≤ 250bp single base changes in the sequence can be detected as mobility shifts on the gel.

2.3.7 SSCP PCR

PCR conditions were the same as those detalied in section 2.3.5. PCR products greater than 250bp were digested to generate fragments of size suitable for SSCP (see 2.3.8). Details of restriction enzymes used are shown in the **table 3**. In each run of SSCP a total of 50 (1μl) genomic DNA samples were run simultaneously. This consisted of 10 samples chosen at random from normotensive participants in the Monica IV survey , a project surveying coronary risk in random samples of patients on the lists of Glasgow general practitioners and 40 samples from patients with essential hypertension. A reaction PCR mix was prepared adequate for 60 samples. To this mix, 2μl of radioactive dCTP was added and the mixture carefully mixed behind perpsex.

Aliquots (24µl) of the radioactive mix was then added to each DNA sample and mixed. Reactions were covered with mineral oil and placed in a thermal cycler as described in 2.3.6.

2.3.8 Digestion of PCR fragments greater than 250bp

In some cases the PCR product amplified required digestion in order to obtain fragment sizes of 250 bases or smaller. A non-denaturing SSCP gel is more sensitive in detection of band shifts in fragments of this size. Details of the enzymes used are shown in **table 3 appendix 2.**

An enzyme reaction mix was prepared and added directly to the completed PCR reaction:

Enzyme	0.5µl
Buffer	2.5µl
Total	5.0µl

Again a master mix sufficient for 60 reactions was prepared and 5µl aliquots pipetted into each completed PCR reaction, carefully mixed and incubated overnight at 37°C.

2.3.9 Stopping reactions

A 5µl aliquot of the completed SSCP PCR reaction containing digested or undigested fragments was transferred to fresh tubes and 10µl of stop blue (see **appendix 3**) was added and carefully mixed. 10µl of this mixture were loaded per well on a non-denaturing SSCP gel.

2.3.10 Preparation of the non-denaturing gel for SSCP

Gels for SSCP (30 x 40 cm) were prepared using a modification of a standard protocol. Two glass plates were cleaned using isopropanol and the top plate siliconised and left to dry in air with (0.2mm) teflon spacers in place. Unlike sequencing gels, there is no urea present in a non-denaturing gel. The mixture for a polyacrylamide 6% gel was prepared as follows:

Acrylamide:bisacrylamide (30%, 37.5:1)	30ml
10X TBE	15ml
Glycerol	7.5ml
0.5M pH 8.0 EDTA	0.3ml
dH_2O	97.2ml
Total	150ml

The resulting 6% polyacrylamide gel contained 5% glycerol. The acrylamide mix was polymerised by addition of 150 µl of 25% ammonium persulfate and 150 µl TEMED.

2.3.11 SSCP gel electrophoresis

Approximately 1L of 1 X TBE was added to the apparatus tank. The lane combs were removed and the gel equilibrated at room temperature.

Samples were removed from storage and placed on a heating block at 95°C for 5 minutes before loading to denature. For each lane of the gel, 5µl of the stop blue PCR mix were placed in a well. Gels were run for approximately 4 to 5 hours at 30 Watts (1875V, 16mA) at room temperature, ensuring that the surface temperature of the gel did not rise above 50°C.

2.3.12 Preparation and autoradiography of SSCP gels

After completion of the electrophoresis, the gel was removed, dried and exposed to photographic film as before.

2.3.13 Determination of band shifts from SSCP gels

The banding pattern of the individual samples were compared. Samples with a unique band shift were urther analysed by sequencing to detect base changes in the DNA sequence as described in 2.2 to 2.2.6.

2.4.14 Sub-cloning into T-vector

For some exons it was not possible to select primers which amplified selectively only CYP11B1 or CYP11B2. In these instances exonic regions were amplified using non-selective primers which produced CYP11B1 and CYP11B2 amplicons. The resulting PCR products were sub-cloned into T-vector (Promega) to facilitate sequencing.

2.4.15 T-vector ligation reaction

0.6 µl PCR product (≈ 25ng)

1.0 µl DNA ligase

1.0 µl ligase buffer

1 µl Vector

6.4 µl dH_2O

10 µl Total

Incubated at 4°C for 16 hours. The ligation reaction was then transformed into JM109 cells as described in the manufacturers' protocol and plated onto LB-amp plates (2.1.3). 20 colonies expressing ampicillin resistance were picked off and 2ml cultures inoculated. These were grown overnight at 37°C and shaken at 225rpm in an orbital shaker. DNA was extracted as described in (2.1.5a). The resulting DNA was ran on a 1% agarose gel to check quality and then sequenced directly following ethanol precipitation.

More colonies were screened as necessary to decide whether a subject was homozygous or heterozygous for a particular polymorphism.

2.4 Site-directed mutagenesis.

Mutations identified in either CYP11B1 or CYP11B2, detected by SSCP, which altered the amino acid sequence of either 11β-hydroxylase or aldosterone synthase were constructed in vitro using the cloned cDNAs for both genes: plasmids pCMV4-B1 or pCMV4B2. Other constructs were also prepared which resulted in aldosterone synthase-specific residues being altered to the 11β-hydroxylase equivalent in aldosterone synthase and in some cases vice versa in an attempt to identify residues which account for their differing activities.

Site-directed mutagenesis was performed using the Quick-Change site-directed mutagenesis kit (Stratagene Ltd., Cambridge, UK). Mutation of nucleotides which altered the amino acid sequence was carried out by PCR using sense and antisense primers incorporating the desired mutation. The primers used are shown in table 1 and 2 and were purified by HPLC (Oswell DNA Service, University of Southampton).

2.4.1 The PCR reaction contained:

5µl reaction buffer (10x)

2µl plasmid DNA (pCMV4-B1 or B2, 20ng/µl)

1µl (125ng) sense primer

1µl (125ng) antisense primer

1µl dNTP mix (10mM)

1µl pfu DNA polymerase (2.5U/µl)

39µl H_2O

50µl total.

2.4.2 The PCR protocol consisted of :

95°C for 30 secs initial denaturation step

1. 95°C for 30secs denaturation
2. 55°C for 1min annealing
3. 68°C for 14 min extension

1, 2 and 3 were repeated for 12 cycles.

2.4.3 Digestion of parental strand

Parental (non-mutated) supercoiled dsDNA template remaining in the PCR reaction was digested by addition of 1µl of the restriction enzyme Dpn I for 1hour at 37°C. Dpn I specifically digests DNA which is methylated and of bacterial origin. Therefore the newly PCR synthesised strand remains intact.

2.4.4 Transformation of bacterial cells with mutant plasmid

Epicurion Coli XL1-Blue supercompetent cells (50µl) (Promega Corp. Southampton UK.) were transformed with 1µl of the PCR reaction after DpnI digestion by heat shock at 42°C for 45 seconds and grown at 37°C in NZY+ broth for 1 hour. Details of medium ingredients are shown in **appendix 3**. The transformation reaction was plated onto LB/ ampicillin plates (100µg/ml) and incubated at 37°C for >16hours. Single white colonies were selected and innoculated into 2ml of LB/ ampicillin broth (100µg/ml). Plasmid DNA was prepared and the entire insert sequenced to ensure the

desired mutation had been incorporated as described in 2.2 to 2.2.6. Large scale plasmid DNA preparations were carried out by CsCl/Etbr purification centrifugation as described in 2.1.6a to 2.1.6c.

2.5 Transient transfection system

2.5.1 COS cell maintenance

The preparation of media and all cell handling procedures were performed in a type II vertical laminar flow tissue culture hood COS-7 cells (African Green Monkey kidney cells, European cell culture collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2mM L-glutamine. The culture medium was supplemented with 5% foetal calf serum, 100U/ml pennicillin G, 100µg/ml streptomycin and amphotericin B. The latter three reagents were supplemented as an antibiotic/antimycotic complex solution(Gibco BRL). Cells were either cultured in 175cm^2 culture flasks (containing 20ml medium) or 100mm dishes (containing 10ml medium) and placed in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

2.5.2 Sub-culturing

Cells were passaged upon reaching 90% confluence. Medium was aspirated and the cells washed twice with phosphate-buffered saline (PBS). Trypsin solution (0.25% trypsin and 0.02% EDTA in Dulbecco's buffered saline) was layered over the the cell monolayer and then aspirated off. Cells were incubated at 37°C for 5 minutes. Cells were suspended in fresh medium and pelleted at 1000rpm for 5 minutes to remove traces of trypsin solution and resuspended in the appropriate growth medium at the required density.

2.5.3 Storage and revival of frozen stocks

Cells grown to 90% confluence were trypsinised and pelleted as before and then resuspended in media containing 10% dimethyl sulfoxide (DMSO). Aliquots (1ml) were transferred to 1.5ml cryovials which were placed in Nalgene cryo 1°C freezing container. This was placed in a -70°C freezer for 24 hours to ensure gradual chilling of the cells (approximately 1°C/minute). These vials were then transferred to a liquid nitrogen freezer for long term storage. Cells were removed from liquid nitrogen source for use when required and quickly thawed by placing in a 37°C water bath. The cells were washed in DMEM, supplemented as before, to remove DMSO and

pelleted. The pellet was then resuspended in fresh growth medium and transferred to a small flask. Cells were incubated at 37°C, 5% CO_2 .

2.5.4 COS cell Transfection

COS-7 cells are non-steroidogenic apart for some basal 11β-HSD activity and are therefore suitable for transfection with aldosterone synthase and 11β-hydroxylase genes. Transfections were carried out using DOTAP Liposomal Transfection Reagent (Boehringer Mannheim, Germany). The reagent and DNA form cationic DNA-liposome complexes which fuse with the cell membrane. Approximately 5 x 10^7 cells at 80% confluence in 100mm dishes were pre-incubated for 3 hours with 8mls of Optimem I Reduced Serum Medium supplemented with 100 units /ml penicillin, 0.1mg/ml streptomycin and 0.025mg amphotericin-B (antibiotic-antimycotic solution from Sigma-Aldrich Company, Dorset, England, UK). A transfection mixture was then supplemented and the cells were exposed to this for 8 hours. The transfection mixture was prepared as follows sufficient for duplicate dishes:

VIAL A			VIAL B		
DNA (1μg/μl)			(μl)		
Adx	β-gal	Test	20mM HEPES	DOTAP	20mM HEPES
10	20	20	150	150	50

Vial A was mixed with that of vial B and incubated at room temperature for 15 minutes. DOTAP was supplemented accordingly so that the DOTAP:DNA ratio was 3:1. 200μl of the mixture was carefully poured over the cells per 100mm dish and evenly distributed.

The test plasmids were:

wild-type plasmids: pCMV4-B1 or pCMV4-B2

CYP11B2 mutants: B2-Q43R, B2-D147E, B2-I248T, B2-K357N or B2-T493M

CYP11B1 mutants: B1-H107Y, B1-E147D or B1-L186V.

Control transfections were also performed

1. 10μg of pSV-β-gal
2. 10μg pSV-β-gal plus 5μg of pCD-Adx
3. 10μg pSV-β-gal, 5μg of pCD-Adx plus 10μg plasmid vehicle.

For every μg of DNA transfected, 3 μl of DOTAP was used and the total volume was made up to 200 μl with 20mM HEPES buffer per dish. Control transfections were also performed in duplicate. 200 μl of transfection mixture was used per 100mm tissue culture dish. Also a negative control was also performed where cells were not exposed to any transfection mixture. After 24 hours, the cell medium was replaced with DMEM and incubated for 48 hours to allow protein expression.

2.5.5 Steroid Incubation

After 48 hours, cells were permeabilised with 10% DMSO in PBS for 2 minutes at room temperature and then washed twice with PBS. Transfected cells were then incubated with DMEM supplemented with 5μM 11-DOC plus [^{3}H] 11-DOC (10 000 cpm,36 Ci/mmol) or 5μM 11-deoxycortisol plus [^{3}H] 11-deoxycortisol (10 000 cpm, 50 Ci/mmol) for a further 48 hours. When more accurate quantification of steroids was required, non-tritiated steroids were incubated and the resultant steroid products analysed by radioimmonoassay. All transfections were performed in duplicate and repeated 4 times. Medium was retained for steroid analysis and cell extracts prepared for protein measurement and β-galactosidase activity. RNA was prepared for semi-quantative RT-PCR.

2.6 Measurement of transfection efficiency

Protein and β-galactosidase measurements

COS-7 cell extracts were prepared post-transfection for measurements of protein content (Biorad Laboratories Ltd., Hertfordshire) and β-galactosidase activity (Promega Corp. Southampton UK.) following the manufacturers' protocols. Transfection efficiency was determined using these data and steroid results corrected as necessary.

2.6.1 Preparation of cell lysates

Cell lysates were prepared using Reporter Lysis Buffer (Promega). Steroid-containing medium was removed and stored for analysis and then cells were washed twice with PBS and any residual traces of PBS removed. 1ml of 1x Reporter lysis buffer was added per 100mm dish to ensure complete coverage of the cell surface. Dishes were

incubated at room temperature for 15 minutes with gentle rocking of the dishes several times during the incubation. The cells were scraped off using a 'rubber policeman', transferred to a pre-chilled 1.5ml Eppendorf tube and placed on ice. Tubes were then vortexed for 10-15 seconds and centrifuged at full speed in a microcentrifuge for 2 minutes at 4°C. The supernatant was then assayed directly for β-galactosidase activity. For the protein assay, the whole cell lysate was used to determine the protein concentration and this was retained at -70°C until use.

2.6.2 Promega β-galactosidase assay

This proceedure was carried out according to the manufacturer's protocols. β-Galactosidase activity was determined in cell extracts transfected with the pSV-β-gal reporter gene. The standard assay was used where an equal volume of standards (150µl: 1-6 milli units purified β-galactosidase enzyme) in duplicate or appropriately diluted samples (150µl) were added to an equal volume of 2x assay buffer which contains the substrate ONPG (o-nitrophenyl-β-D-galactopyranoside). Samples were mixed by vortexing and incubated at 37°C for 30 minutes. During this time, a faint yellow colour appears as the β-galactosidase hydrolyses the colourless substrate to o-nitrophenol which is yellow. The reactions were terminated by addition of 500µl of 1M sodium bicarbonate and vortexed. The absorbance was read at 420nm in a spectrophotometer. β-Galactoisdase concentration was determined using the standard curve.

2.6.3 Biorad Protein Assay

Bovine serum albumin standard solutions (100µl: 200-1400µg/ml) were prepared in duplicate. Biorad reagent concentrate was diluted 1 in 5 and filtered through Whatman No 1 filter paper. Diluted dye reagent (5ml) was added to standards and 100µl of appropriately diluted samples to be analysed, gently vortexed and incubated at room temperature for 5-30 minutes. Absorption was measured at 595nm. Protein concentration was determined using the standard curve.

2.7. Semi-quantitative RT-PCR

A semi-quantitative PCR system was used to detect noticeable differences in the transcriptional levels of wild-type and mutant cDNAs. Using this method PCR amplification of the CYP11B1 and B2 cDNAs was carried out and and standardised

against the ubiquitously expressed GAPDH (Glyceraldehyde 3-phosphate dehydrogenase, a key enzyme in glycolysis) gene. In tissues or cells of the same origin the level of GAPDH transcription should be the same, providing similar levels of RNA are utilised. For this technique, RNA is isolated and any residual DNA is digested with Dnase. mRNA is then selected using oligo dT primers which select the poly adenylation site of the mRNA. The mRNA is reverse transcribed to produce cDNA and the desired region amplified by PCR. Primers were chosen which amplified a region of the GAPDH mRNA or which amplified a region of the test gene i.e B1, B2 or mutants.

2.7.1 RNA isolation

RNAzol B (Biogenesis, Poole, England, UK.) was used to isolate RNA. COS-7 cells were washed twice with PBS and then 1ml of RNAzol B added per flask/dish. Using a 'rubber policeman', the resulting mixture was transferred to a sterile, prechilled 1.5ml Eppendorf tube and placed on ice. 100 µl of chloroform were added per tube, vigorously shaken to mix and placed on ice for a further 5 minutes. The tubes were then spun at 4°C for 5 minutes at 3200rpm. The top layer was retained and transfered to a fresh tube containing an equal volume of isopropanol (500 µl). This was gently mixed and left on ice for 15 minutes to precipitate the RNA. The RNA pellet was obtained by microcentrifugation for 15 minutes at 4°C at 10000rpm. The supernatant was removed and the pellet was washed with 70% ethanol, gently resuspended and once again microcentrifuged for 15 minutes at 4°C at 10000rpm. The pellet was left to air-dry on ice and then resuspended by gentle triturating in 100 µl of cold (diethyl pyrocarbonate) DEPC H_2O, which prevents RNA degradation. The RNA was stored at -70°C.

2.7.2 Agarose Gel Electrophoresis of RNA

A 1% (w/v) agarose gel containing 2µl of ethidium bromide was prepared in 100mls 1x TAE. The gel was allowed to set for 1hour before removing the combs. Gels were placed in standard electrophoresis tanks with 100ml of TAE buffer and connected to a constant power source. A mixture of 2µl of RNA solution, 3µl of DEPC H_2O and 5µl of 6x loading dye was added to per well with 6µl of λ HaeIII digest as a marker in one well to ensure the gel was runing correctly. The gel was run at 5V/cm (~55V per

gel) for about 45 minutes and then visualised using a UV illuminator. Distinct rRNA bands were visualised.

Any contaminating DNA was digested with RNase free DNase for 1 hour at 37°C as follows.

$MgCl_2$ (25mmol/L)	20 µl
10x Mg^{2+} free PCR buffer	10 µl
RQ1 DNase (1U/µl)	20 µl
RNA (10µg)	50 µl

The reaction was terminated by addition of an equal volume of phenol/chloroform. The tube was then vortexed and spun at 14 000 rpm at 4°C for 5 mins. The aqueous top layer was removed and an equal volume of chloroform added. This was spun at 14 000 rpm at 4°C for 5 mins, the top layer removed and 3 volumes of 100% ethanol and 0.1 volumes of DEPC treated 3M sodium acetate were added. The RNA was precipitated at -20°C for 30 mins and spun at 14 000 rpm at 4°C for 30mins. The pellet was washed in 70% ethanol and air dried for 15 mins. The pellet was resuspended in 10ul of DEPC water and quantified.

RNA Quantification see 2.1.7

2.7.3 Reverse Transcription

The following components were placed in a Perkin-Elmer tube. All solutions were kept on ice throughout:

Components	Volume
RNA (1µg/µl)	1.0µl
$MgCl_2$ (25µM)	4.0µl
10X PCR Buffer II	2.0µl
dATP (10µM)	2.0µl
dCTP (10µM)	2.0µl
dGTP (10µM)	2.0µl

dTTP (10μM)	2.0μl
RNase inhibitor (20U/μl)	1.0μl
MuLV reverse transcriptase (50U/μl)	1.0μl
Oligo-$(dT)_{16}$ primers (25μM)	1.0μl
DEPC-treated water	2.0μl
Total	20.0μl

Control reactions were also constructed omitting either reverse transcriptase (R.T.) or RNA (water blank). In all the above cases, alterations in the various amounts of components that were added were compensated for by altering the volume of DEPC-H_2O, such that a final volume of 20 μl was achieved. Each reaction was overlayed with a drop of mineral oil.

The reactions were then incubated in a Perkin-Elmer thermal cycler as follows:

15mins	42°C
5mins	99°C
5mins	5°C

Samples were stored at -20°C until required for PCR.

2.7.4 PCR of first strand cDNA

The completed reaction product from 2.7.3 was prepared for PCR by adding the following components to Perkin Elmer tubes: Primers used amplified GAPDH or CYP11B1 or CYP11B2 (see **table 5 appendix 2).** The number of cycles carried out for CYP11B1/B2 and GAPDH was 30 and 34 respectively, which are in the linear part of the amplification curve (see **Figure 3.6a, chapter3**).

Components	Volume
R.T. reaction product	20.0μl
10X PCR buffer II	8.0μl
Primers (sense) (1.5pmoles/μl)	1.0μl
Primers (antisense) (1.5pmoles/μl)	1.0μl
$MgCl_2$ (25μM)	4.0μl

AmpliTaq DNA polymerase	0.5µl
Water	65.5µl
Total	100.0µl

The tubes were centrifuged briefly in a microcentrifuge and then placed in a Perkin Elmer cycle. The optimised programs were as follows:

CYP11B1/B2		**GAPDH**	
94°C for 3 min		94°C for 3 min	
94°C for 1min	30 cycles	94°C for 45 secs	34 cycles
56°C for 1min		60°C for 45 secs	
72°C for 1 min		72°C for 2 mins	
72°C for 7min		72°C for 7min	

PCR reactions were run on 1% agarose gel where 10µl of loading dye was added to each sample, then 30µl was loaded on the agarose gel. 6µl of HaeIII ØX174 digest and/or HindIII λ digest were added as markers. The gel was run at 80V for a length of time appropriate to the amplified fragment size and then visualised under UV light. The intensity of the GAPDH and CYP11B1/B2 bands were measured by phosphorimaging analysis. The ratio of the CYP11B1/B2: GAPDH was calculated for each RNA sample. Ratios for RNA samples from wild-type plasmid transfected cells were compared to mutants.

2.8 Enzyme activity and steroid production

This was assessed 48 hours post-transfection by measuring the conversion of the substrate ^{3}H-11-DOC to B, 18-OH-B, aldosterone or 18-OH-deoxycorticosterone (18-OH-DOC). In some experiments, the conversion of ^{3}H -11-deoxycortisol to cortisol was also measured. Alternatively, non-tritiated products were measured by radioimmunoassays.

2.8.1 Steroid extraction

Steroids were extracted from 1ml aliquots of culture medium with 5 volumes of freshly distilled methylene chloride. Phases were separated by centrifugation at 1500g at room temperature for 5 min and the aqueous layer aspirated and discarded. The organic phase was washed with dH_2O (1ml) to remove the residual medium.

2.8.2 Measurement of tritiated products

For measurement of tritiated products unlabelled 11-DOC, B, 18-OH-B and aldosterone (3μg) were added as carriers to each organic phase, which was evaporated to dryness under a stream of N_2 at 37°C. The residues were resuspended in chloroform:methanol (2:1, 20μl) and applied to glass-backed silica F254-coated TLC plates. Standards (3μg) were also applied. The plates were developed in methylene chloride-methanol-water (300:20:1). Steroids were located under UV light and ^3H content measured by liquid scintillation spectrometry. These results were used to construct ratios of product to substrate (e.g. B:DOC or F:S, 18-OHB:B, Aldo:18-OHB) which are indices of the individual enzyme activities 11β-hydroxylation, 18-hydroxylation or 18-oxidation respectively.

2.8.3 Radioimmunoassay.

Concentrations of steroids were measured by radioimmunoassay after extraction and partial purification by paper chromatography (Belkien et al. 1980, Fraser et al. 1975). 1ml samples to which ^3H-steroid standards had been added were extracted with freshly distilled dichloromethane and the extract evaporated to dryness under nitrogen at 30°C. The residues were chromatographed on paper (Whatman 2) using a volatile system **(appendix 3)** and the steroid regions located using isotope scanning and eluted in methanol. Aliquots of corticosterone and 18-hydroxycorticosterone were assayed using ^3H-steroids and antisera raised in rabbits to steroid-3-carboxymethyloxine conjugated to bovine serum albumin. Bound and free steroid were separated using the dextran-coated charcoal method (Fraser et al 1975). Measurement of aliquots of aldosterone were performed using a solid phase (coated tube) radioimmunoassay (Diagnostic products (UK) Ltd). The coefficient of variation is <10.4% and the limit of detection 1ng/dL. Cortisol measurements were also made using a commercially

available kit, using a coated tube radioimmunoassay (Diagnostic products (UK) Ltd). The coefficient of variation is <6.4% and the limit of detection is 0.2μg/dL.

2.9 Pseudosubstrate inhibition studies

2.9.1.Cell culture techniques

Cells used were parental V79 cells, stably transfected CYP11B1 or CYP11B2 V79 cells. These cells are Chinese hamster lung cell-lines which express the necessary electron transport proteins required by cytochrome P-450 enzymes.

2.9.2 Cell culture conditions

V79 cells were grown, handled and maintained in the same manner as COS-7 cells as described in 2.5 to 2.5.2.

2.9.3 Subculturing cell lines

A 175cm^2 tissue culture flask containing cells at confluence was submitted to the following protocol: the culture medium was removed and cells washed with 10ml phosphate buffered saline (PBS) as a prelude to washing with 5ml 1x trypsin-EDTA solution (Gibco, Life Technologies, Paisley, Scotland, UK). The cells were incubated at room temperature for 2min and then resuspended in 10ml of cell culture medium. The cell suspension was then transferred into a sterile universal and spun for 5min at 37°C at 1000rpm. The supernatant was removed and the pellet of cells resuspended in 90ml of cell culture medium. Of this volume, 20ml was transferred to a fresh large flask as the continuing flask (producing a splitting ratio of 1:4 approx). Of the remaining suspension, 4ml aliquots were transferred per well into 6-well plates. In order to provide adequate wells for a single experiment, 2 large flasks were required and treated in exactly the same way as described above. Following 24 hours culture, cells in the 6-well plates were used in inhibitor studies.

2.9.4 Steroid incubation in presence and absence of potential steroid inhibitors

Steriod Substrate incubation

A 1mg/ml working concentration of DOC, S or 18-OHDOC was prepared in pure ethanol. For experiments, concentrations ranging from 10^{-3}μM to 10μM were prepared in tissue culture medium.

Steroid effectors

1mg/ml working solutions in ethanol were prepared of the following potential inhibitors: 18-OHDOC, 18-OXOF and 18-OHF. As a starting point, 10μM of inhibitor was used. This was added to medium containing either a fixed concentration of 1μM substrate or substrate concentrations ranging from 10^{-3}μM to 1μM of substrates DOC or S. This was performed for both substrates used and concentration ranges were set up in duplicate. One set was prepared in the presence of inhibitor.

2.9.4 Steroid incubation

Each concentration point, in the presence and absence of inhibitor, was set up in quadruplicate using 4 wells of a 6-well plate. 4ml of each of the steroid medium mixtures were incubated per well for a period of 24 hours (determined by time-course experiments) and at the end of this time-point, medium was transferred to a 5ml tube, capped and stored at -20°C for steroid analysis. Cell lysates were prepared for protein determination.

2.9.5 Preparation of cell lysates

Following removal of steroid medium, cells were washed twice with PBS. The cells were then scraped off the surface of the well and resuspended in 1ml of PBS and transferred to pre-chilled 1.5ml Eppendorf tubes. The resultant cell lysate was stored on ice, vortexed at full speed for 10secs and then stored at -20°C until protein concentration was determined.

Determination of protein concentration

Protein concentration of cell lysates was determined using Biorad protein Assay kit as described in section 2.6.3.

2.9.6 Radioimmunoassay

Steroids were extracted, partially purified by paper chromatography and measured by radioimmunoassay as described in section 2.8.3. Steroid measurements were corrected for protein concentration.

CHAPTER 3

Chapter 3. Results

3.1 Structure-function studies of human 11β-hydroxylase and aldosterone synthase

In the zona fasciculata (ZF) of the adrenal cortex, 11β-hydroxylase is regulated by ACTH and catalyses the conversion of 11-deoxycorticosterone (DOC), a weak mineralocorticoid, to corticosterone (B), 18-OHB, 18-OH-DOC or 19-OH-DOC. It also catalyses the conversion of 11-deoxycortisol (S) to cortisol (F). In the zona glomerulosa (ZG), aldosterone synthase is controlled by angiotensin II and potassium (see section 1.5). It catalyses the initial 11β hydroxylation to convert DOC to B, and is then able to perform the necessary 18-hydroxylation and dehydration steps to produce 18-hydroxycorticosterone and aldosterone respectively. It is clear that the relative efficiency of 11β- and 18-hydroxylation will strongly influence the ratio of glucocorticoid: mineralocorticoid secreted by the adrenal cortex.

The genes encoding 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) lie in tandem on chromosome 8 in man. They share 93% nucleotide sequence identity but have quite different functional properties (see 1.6) which have been outlined above. To investigate the molecular basis of the functional differences between the enzymes and to determine the effects of small genetic changes on steroid production, aldosterone synthase-specific amino acids (Glutamine Q43, Aspartate D147, Isoleucine I248 and Threonine T493) were substituted for 11β-hydroxylase specific-residues (Arginine R43, Glutamate E147, Threonine T248 and Methionine M493) respectively within aldosterone synthase. Mutants which caused a significant alteration in DOC conversion were further investigated by constructing the analogous mutation i.e substitution of 11β-hydroxylase specific residues for the aldosterone synthase specific residues within 11β-hydroxylase (Glutamate E147 to Aspartate D147).

3.2 Methods (see section 2)

Mutants were prepared using site directed mutagenesis (section 2.4). Large scale preparations of mutant plasmids, wild-type plasmids $pCMV_4$-B1 and B2, pSV-β-gal

and pCD-Adx were made (section 2.1.6a.). These were analysed by restriction digests (section 2.1.8) and mutant plasmids were sequenced (section 2.2). Transient transfection in vitro was performed in COS-7 cells (section 2.5). 5μM ^{3}H or non-tritiated substrates were added to the medium of transfected cells and incubated for a period of 48 hours. (section 2.5.5). Steroid conversion rate was measured by liquid scintillation and production rate by radioimmunoassay (section 2.8). Cell lysates were prepared (section 2.6.1) and protein (section 2.6.3) and β-galactosidase assays performed (section 2.6.2) . RNA was prepared from cells for semi-quantitative RT-PCR (2.7). Where a mutation showed a significant alteration in DOC conversion rate, kinetic studies were performed. This was as described above except that varying concentrations of substrate, ranging from 0.01 to 15μM, were used.

3.3 RESULTS

3.3.1 Restriction digests of plasmids

Plasmid DNA was digested using restriction enzymes to identify and assess its quality. Spe I was used to digest both $pCMV_4$-B1 and B2 plasmids. This enzyme hydrolyses a unique site in $pCMV_4$-B1 but cuts $pCMV_4$-B2 at two sites, therefore clearly distinguishing the two plasmids. Double digests were used for pCD-Adx and pSV-β-gal which were Not I/ Bgl II and BamHI/HindIII respectively. Spe I digests are shown in **figure 3.3a**.

3.3.2. Confirmation of mutant sequences

The incorporation of the mutations was verified by sequencing. Codon 43 in exon 1 was altered from CAG to CGG, thus changing a glutamine to an arginine residue. Codon 147 in exon 3 was altered from GAT to a GAA, thus changing an aspartate to a glutamate residue. Codon 248 in exon 4 was altered from ATC to ACC, thus coding an isoleucine instead of threonine residue. Codon 493 in exon 9 was altered from ACG to ATG, thus coding a threonine instead of methionine. Codon 147 of CYP11B1 cDNA was altered from GAA to GAT thus changing a glutamate to an aspartate. Sequence data are shown in **figures 3.3b, 3.3c and 3.3d.**

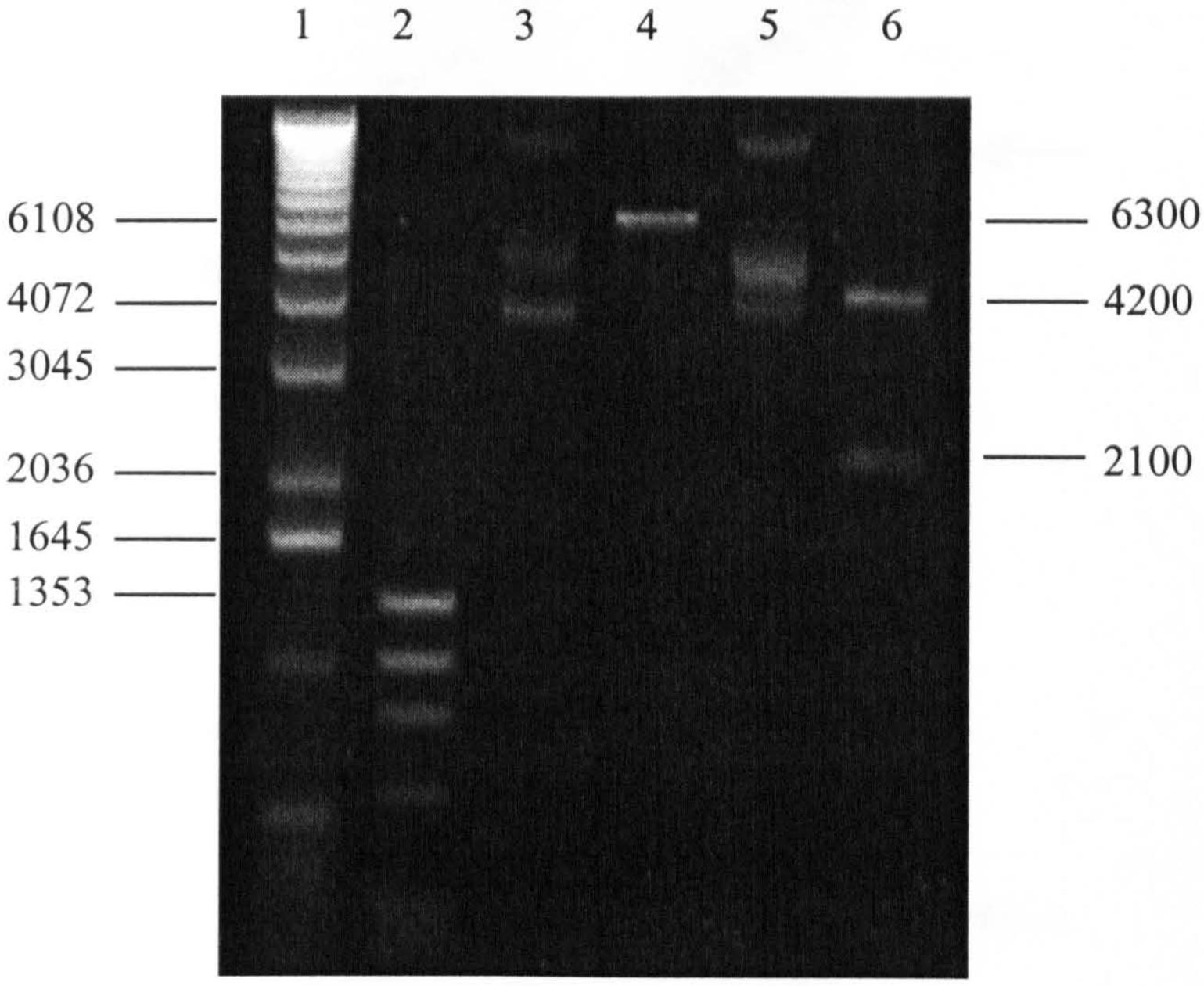


Figure 3.3a Spe I digests of wild-type plasmids pCMV4-B1 and pCMV4-B2

A 1% agarose gel showing Spe-I digests of pCMV4-B1 and pCMV4-B2. Uncut plasmids are shown in lanes 3 and 4 respectively with digests in lanes 4 and 5. Lane 1 shows λ DNA 1kb ladder and lane 2 shows λ DNA Hae III digest as a marker. Spe-I linearises pCMV4-B1 to generate a 6.3Kb band. Spe-I cuts pCMV4-B2 twice to generate two fragments of 4.2 and 2.1Kb approximately.

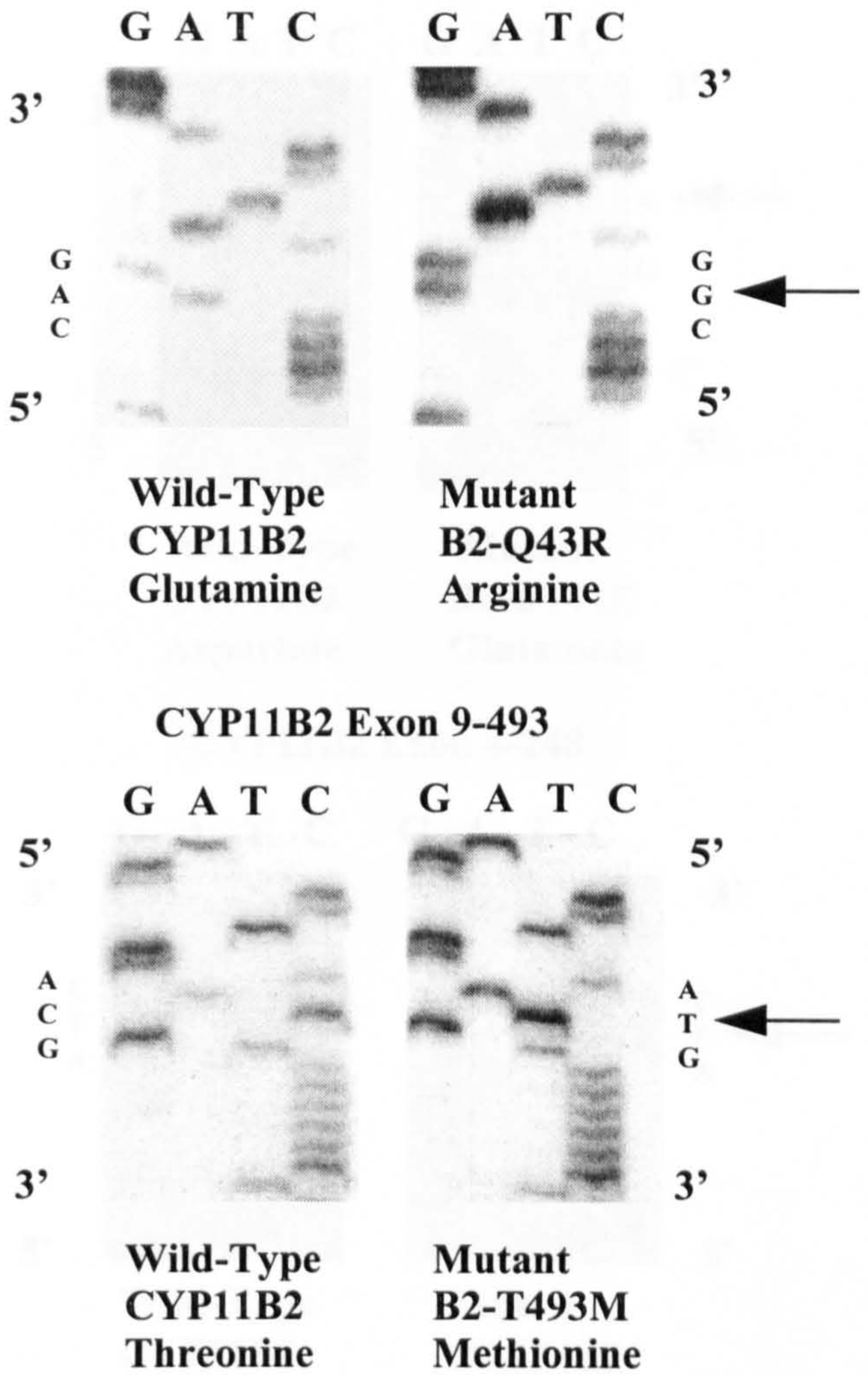


Figure 3.3b.

Sequence of wild-type plasmid pCMV4-B2 and mutant constructs B2-Q43R and B2-T493M

A portion of nucleotide sequence of exon 1 from wild-type plasmid pCMV4-B2 and from mutant B2-Q43R are shown in the top panel. In the panel directly above, a portion of nucleotide sequence of exon 9 from the wild-type plasmid pCMV4-B2 and from mutant B2-T493M are shown. Arrows indicate where the mutations have been incorporated at codons 43 and 493.

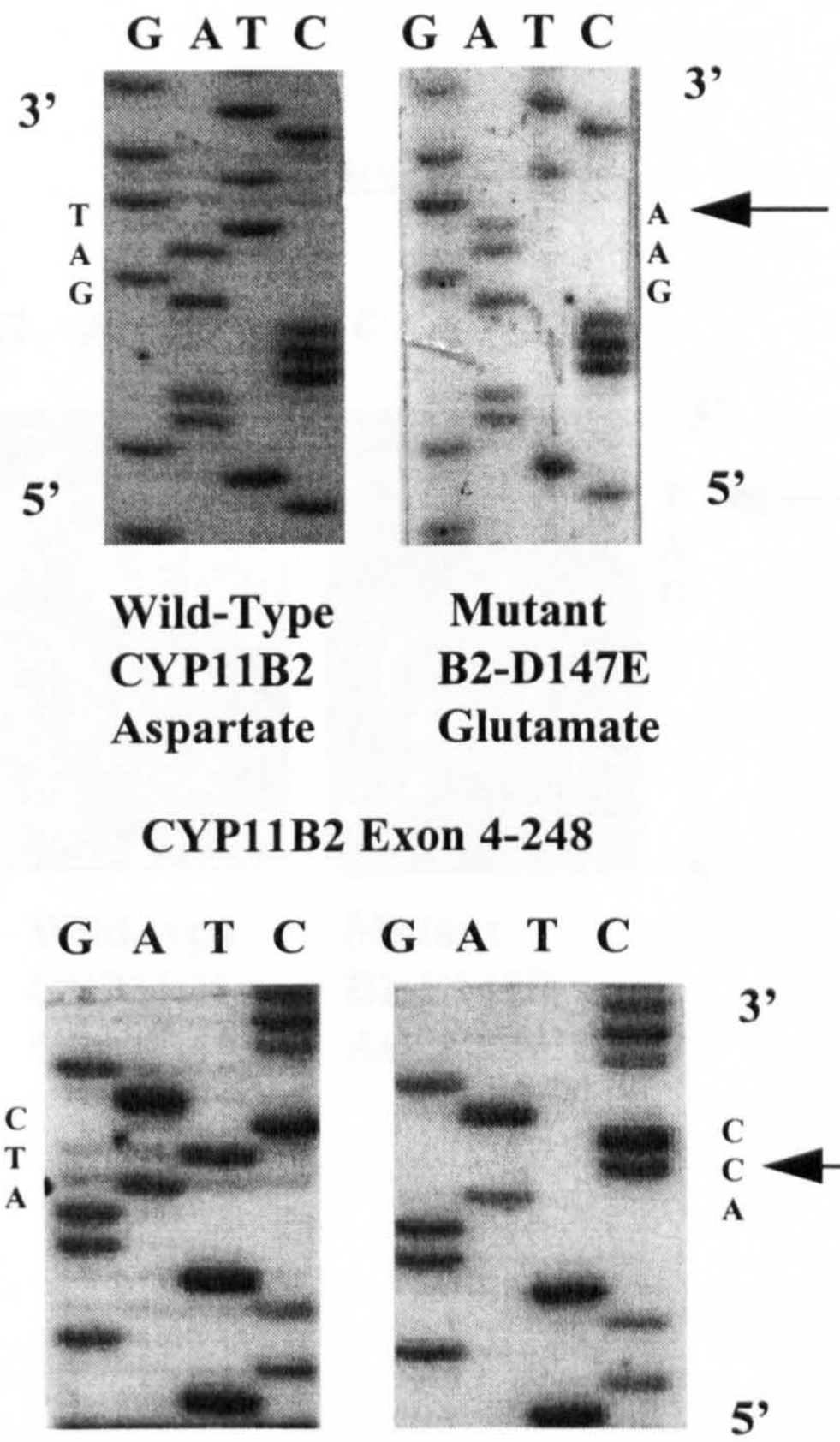


Figure 3.3c.
Sequence of wild-type plasmid pCMV4-B2 and mutant constructs B2-D147E and B2-I248T

A portion of nucleotide sequence of exon 3 from wild-type plasmid pCMV4-B2 and from mutant B2-D147E are shown in the top panel. In the panel directly above, a portion of nucleotide sequence of exon 4 from the wild-type plasmid pCMV4-B2 and from mutant B2-I248T are shown. Arrows indicate where the mutations have been incorporated at codons 147 and 248.

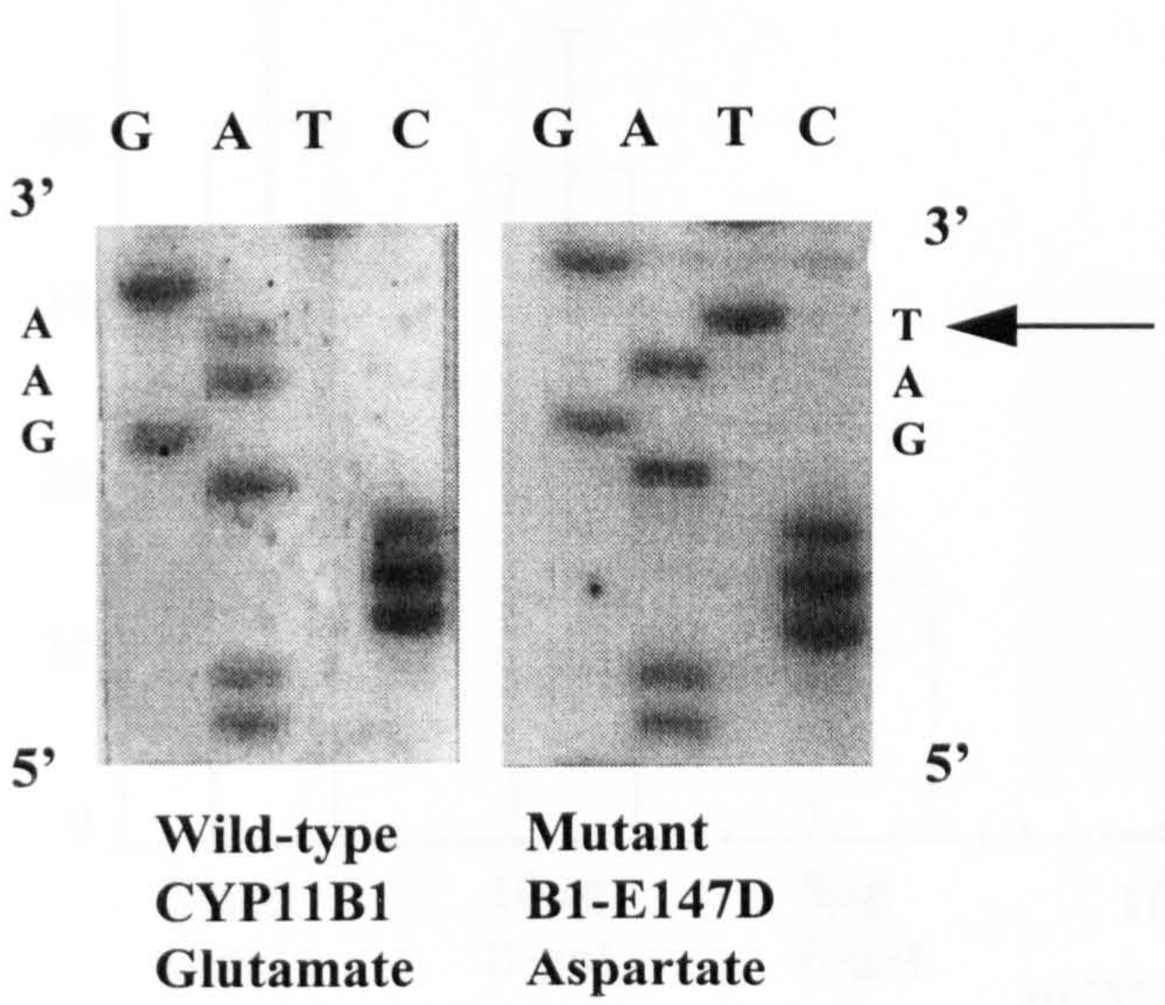


Figure 3.3d.
Sequence of wild-type plasmid pCMV4-B1 and mutant construct B1-E147D

A portion of nucleotide sequence of exon 3 from wild-type plasmid pCMV4-B1 and from mutant B1-E147D are shown. An arrow indicates where the mutation has been incorporated at codon 147.

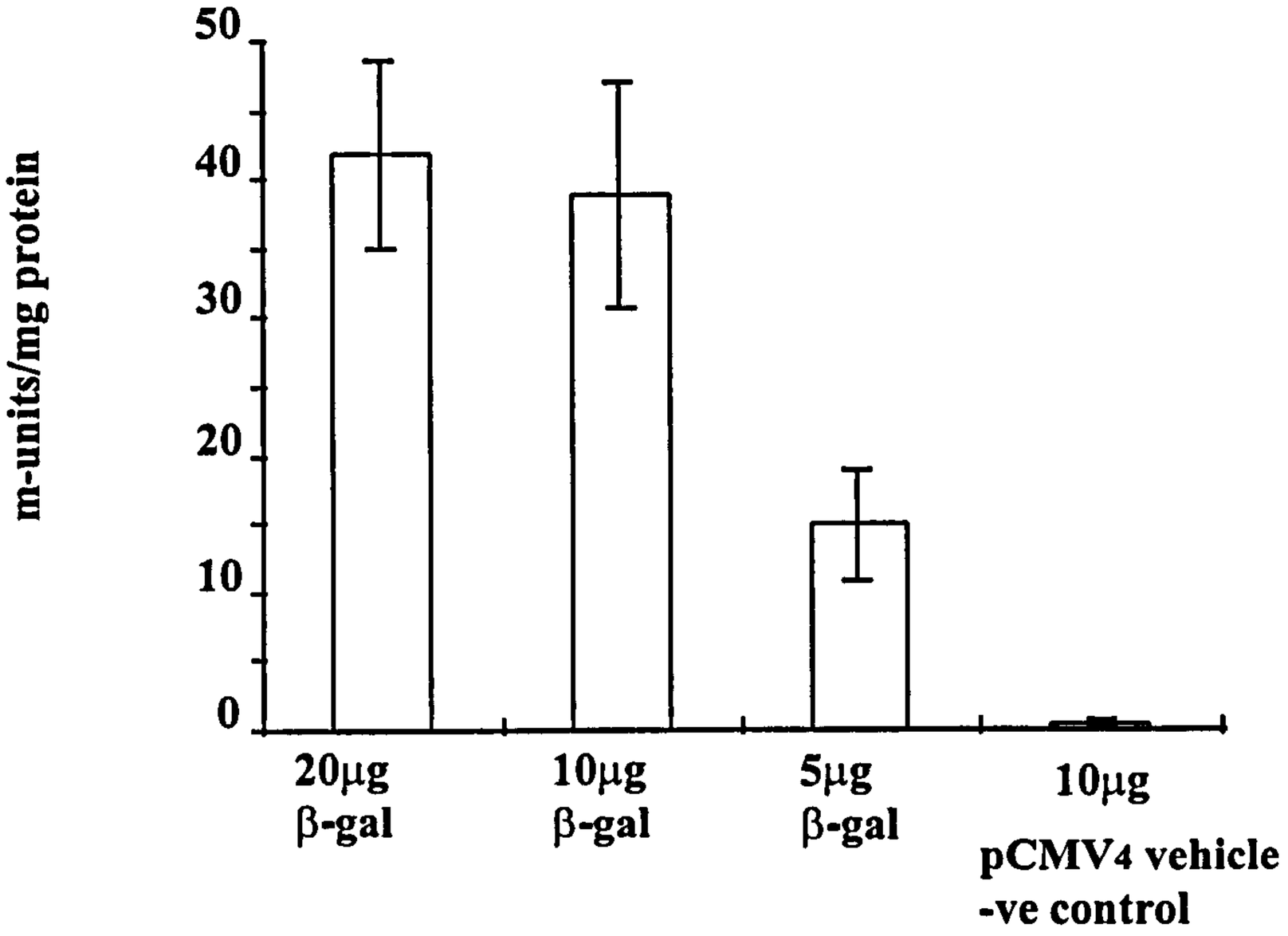


Figure 3.3e.
The effect of increasing transfected pSV-β-gal DNA on cell lysate β-galactosidase activity expressed as milli-units (m-units) per mg of protein. COS-7 cells were transfected with 10μg of pCMV4 vehicle or increasing doses of pSV-β-gal. β-galactosidase activity was performed on cell lysates.

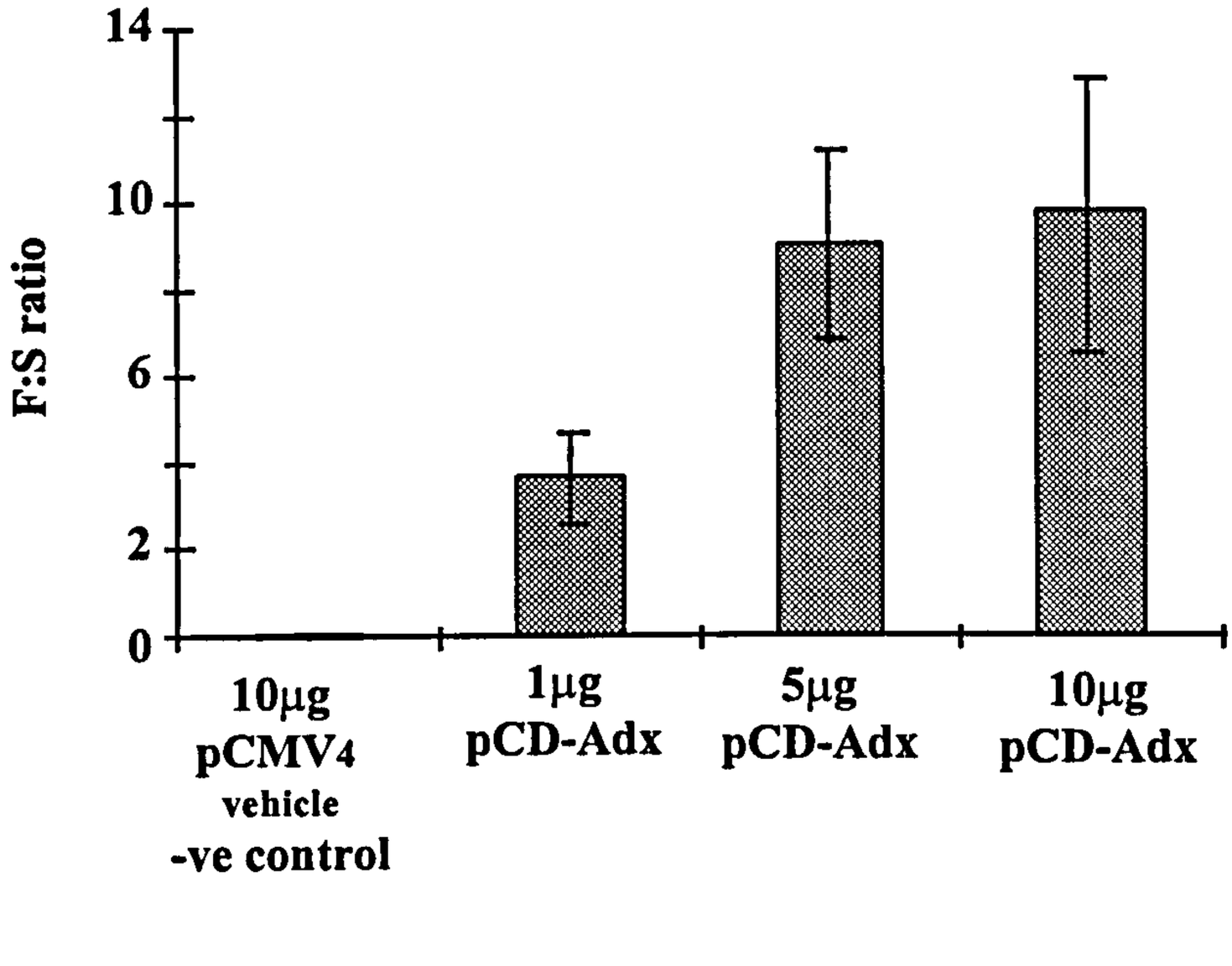


Figure 3.3f.
Effect of various pCD-Adx concentration in transfected cells on substrate conversion.
The effect on steroid conversion of 11-deoxycortisol (S) of varying concentrations of pCD-Adx in cells transfected with wild-type 11β-hydroxylase. COS-7 cells transfected with either 10µg of pCMV4 vehicle (-ve control) or increasing doses of pCD-Adx and 10µg of pSV-β-gal were incubated with 5µM ^{3}H S for 48 hours. ^{3}H-steroids from the medium were extracted and separated by TLC and analysed in duplicate by liquid scintillation counting. Results are expressed as ratio of product to substrate (cortisol) F:S which is an index of 11β-hydroxylase activity.

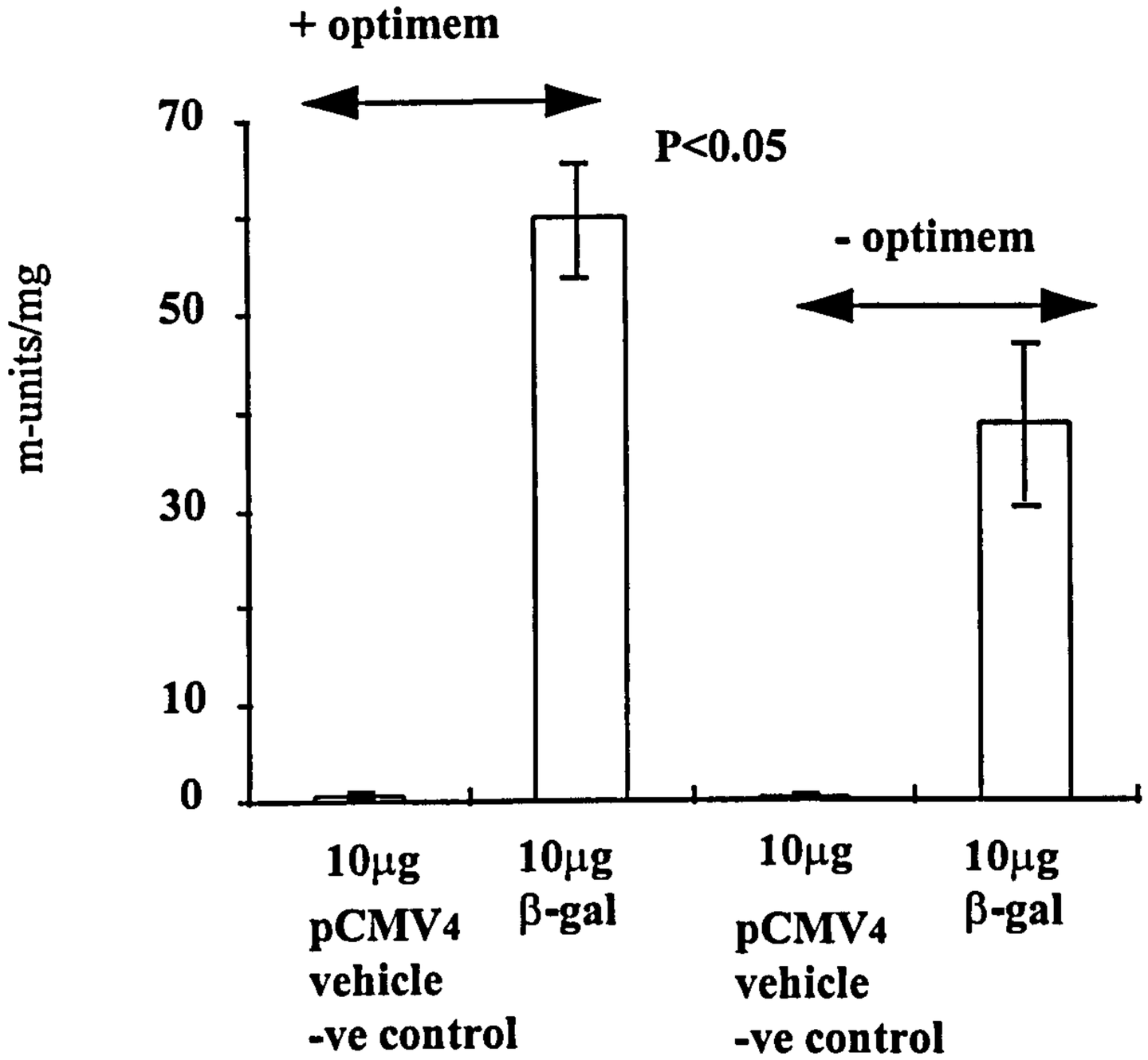


Figure 3.3g.
Effect of Optimem -I on transfection efficiency.
Incubation of COS-7 cells with Optimem-I prior to, and for the duration of transfection increases β-galactosidase activity in cell lysates. COS-7 cells were transfected with 10μg of pCMV4 vehicle or 10μg of pSV-β-gal. β-galactosidase activity was performed on cell lysates.

3.3.3 Transient transfection system

Optimal conditions for transfections were assessed. Varying doses of pSV-β-gal and pCD-Adx were transfected to determine the lowest dose which produced the maximum response (**Figures 3.3e and 3.3f**). Increasing the dose of pSV-β-gal transfected increased the cell lysate β-galactosidase activity. A dose of 10μg was chosen for all subsequent transfections as this was the lowest dose providing maximum activity. Increasing doses of pCD-Adx increased *in vitro* enzyme activity, as determined by steroid conversion, which reached a plateau at concentrations greater than 5μg. This dose was chosen as it produced the maximum steroid conversion at the lowest dose of pCD-Adx. The effect of incubating cells with Optimem I, a form of starvation medium which induces cells to enter simultaneously the same phase of the cell-cycle, prior to and for the duration of transfection on transfection efficiency of β-galactosidase was also assessed (**Figure 3.3g**). Transfection efficiency values were calculated by converting protein and β-galactosidase concentrations of cell lysates to milli-units (m-units) β-galactosidase per mg of protein. These values were then converted to percentages of the maximum pSV-β-gal control transfection. Use of Optimem I clearly increases transfection efficiency by 20%.

3.4.1 Steroid conversion ratios of aldosterone synthase mutants

The results from ^{3}H-DOC steroid incubations are expressed as ratios of product to substrate, which are indices of the individual enzymatic activities of aldosterone synthase. The B:DOC, 18-OH-B:B and aldosterone:18-OH-DOC ratios represent 11β-hydroxylase, 18-hydroxylase and 18-oxidase activities respectively. The aldosterone :B ratio is indicative of combined 18-hydroxylase and 18-oxidase activities. All steroid results were corrected for transfection efficiency as necessary. The ratios of product:substrate for the wild type aldosterone synthase and the four mutants are shown in **figures 3.4a. and 3.4b**. Compared to the wild-type aldosterone synthase, mutant B2-D147E significantly increased the B:DOC ratio from 0.5 ± 0.1 to 3 ± 0.4 ($p<0.001$, $n=8$), suggesting that 11β-hydroxylase function was increased. B2-D147E also caused a small but nonetheless significant decrease in the 18-OH-B:B ratio from 1 ± 0.1 to 0.7 ± 0.1 ($p<0.05$). There was no significant

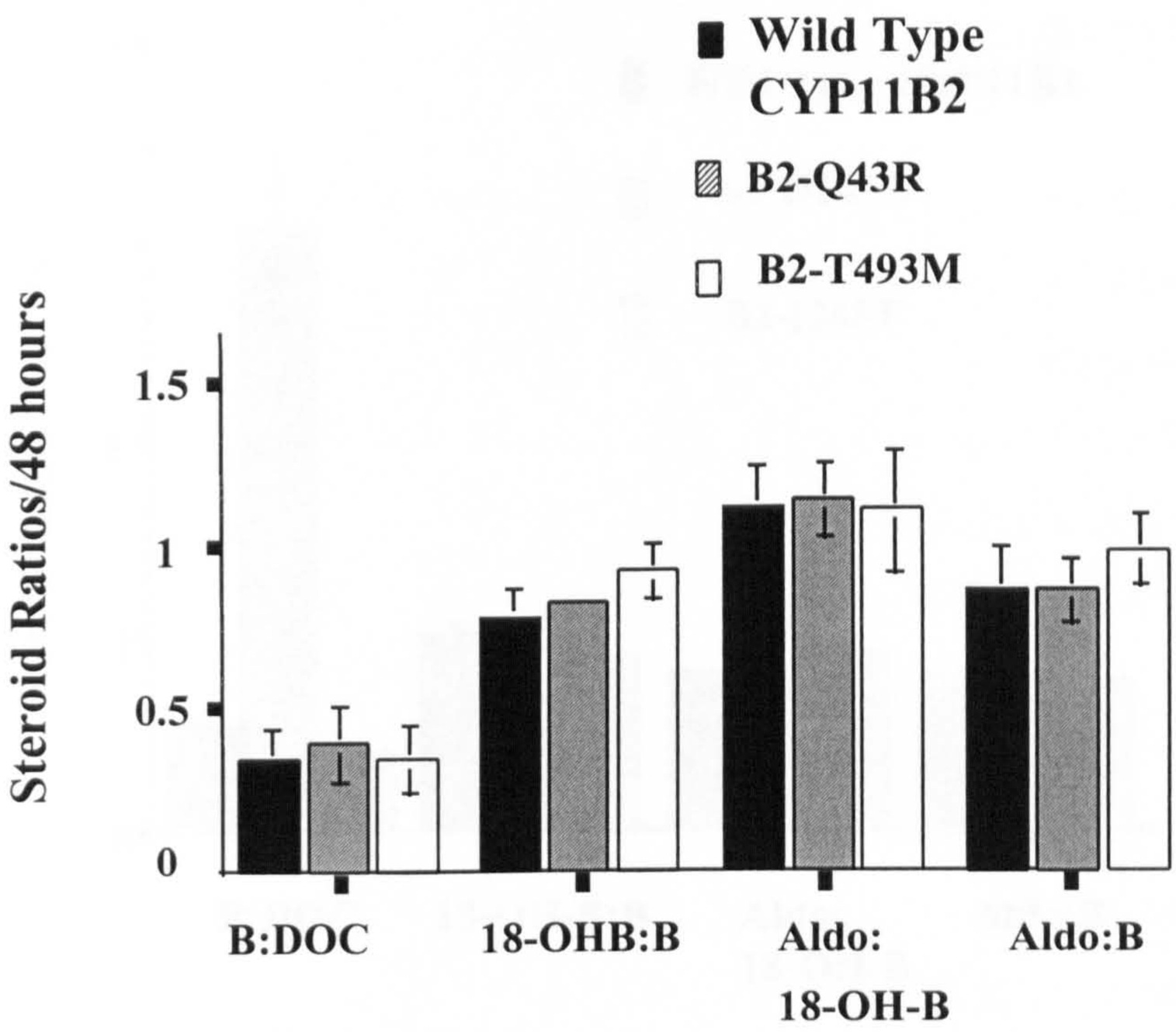


Figure 3.4a.
Steroid conversion ratios of aldosterone synthase mutants B2-Q43R and B2-T493M.

Mutants B2-Q43R and B2-T493M conversion ratios are shown compared to wild-type aldosterone synthase (CYP11B2). COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with 5μM ^{3}H DOC for 48 hours. ^{3}H steroids from the medium were extracted and separated by TLC and analysed in duplicate by liquid scintillation counting. Results are expressed as ratios of product to substrate B:DOC, 18-OHB:B, Aldo:18-OHB which are indices of 11β-hydroxylase , 18-hydroxylase and 18-oxidase activities. The Aldo:B ratio represents overall 18-function. Results are mean ± SEM from four separate transfections, each done in duplicate. Statistical analysis was done by the Mann-Whitney U test.

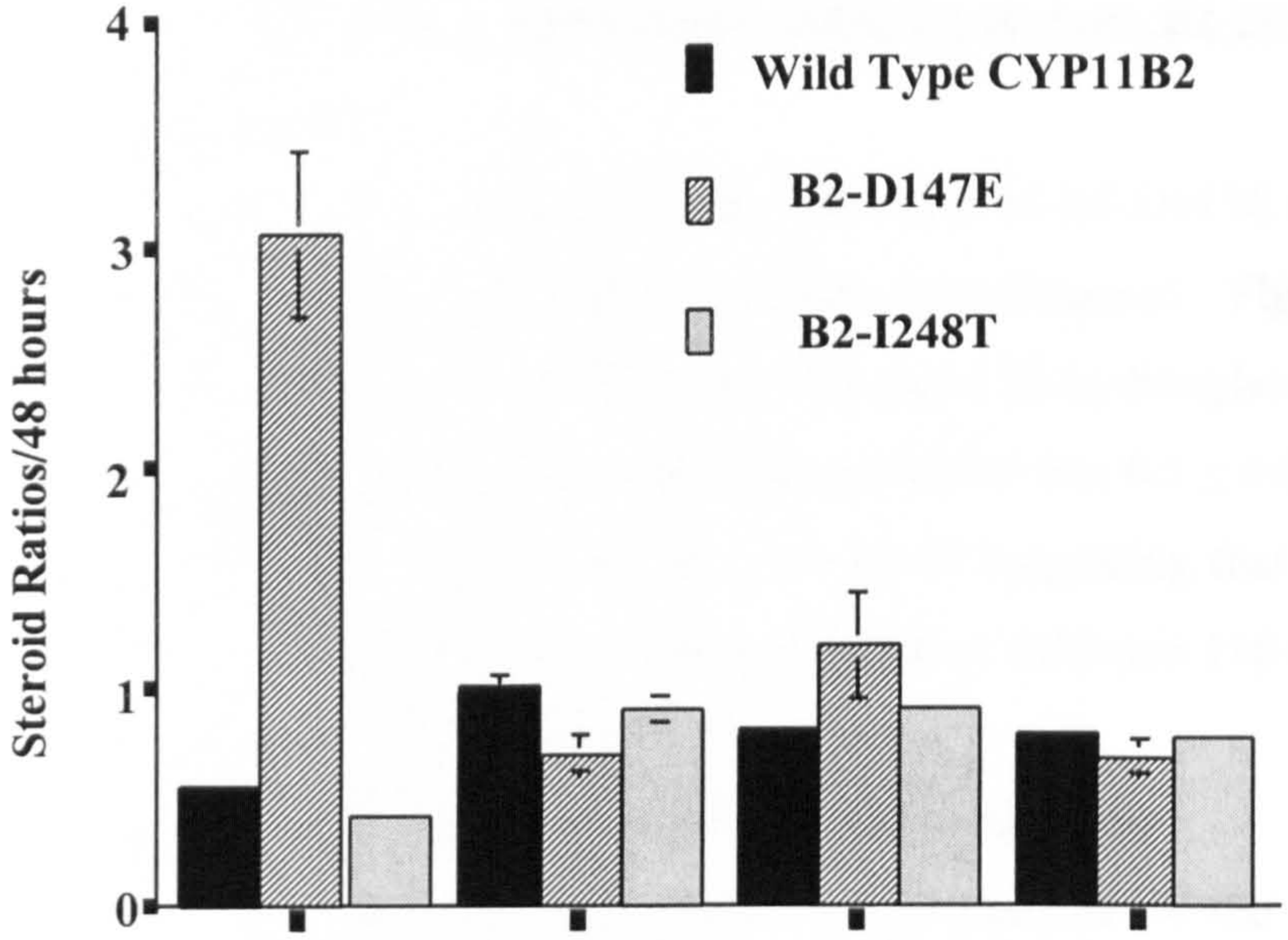


Figure 3.4b.
Steroid conversion ratios of aldosterone synthase mutants B2-D147E and B2-I248T.

Mutants B2-D147E and B2-I248T conversion ratios are shown compared to wild-type aldosterone synthase (CYP11B2). COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with 5μM ^{3}H DOC for 48 hours. ^{3}H steroids from the medium were extracted and separated by TLC and analysed in duplicate by liquid scintillation counting. Results are expressed as ratios of product to substrate B:DOC, 18-OHB:B, Aldo:18-OHB which are indices of 11β-hydroxylase, 18-hydroxylase and 18-oxidase activities. The Aldo:B ratio represents overall 18-function. Results are mean ± SEM from four separate transfections, each done in duplicate. Statistical analysis was done by the Mann-Whitney U test.

difference in the ratio of aldosterone:18-OH-B or aldosterone:B between the wild-type aldosterone synthase and B2-D147E, B2-Q43R, B2-I248T and B2-T493M had no significant effect on steroid production compared to wild-type aldosterone synthase.

3.4.2. Comparison of aldosterone synthase mutant B2-D147E with wild type 11β-hydroxylase

As 11β-hydroxylation was increased, the effect of B2-D147E on the conversion of DOC to B with wild-type 11β-hydroxylase was compared. **Figure 3.4c.** shows that, as expected, the B:DOC ratio for the wild-type 11β-hydroxylase was high: 6.2 ± 0.4 compared to wild-type aldosterone synthase which was 0.5 ± 0.05 ($p<0.001$). The B: DOC ratio for B2-D147E was 3.0 ± 0.4 ($n=8$) suggesting that the 11β-hydroxylase function of the mutant is not as efficient as that of wild type 11β-hydroxylase.

3.4.3 Effect of B2-D147E on overall steroid production

The previous results show the effects of the mutants on the individual enzymatic functions of aldosterone synthase. The effects, in terms of overall steroid production, are shown in **figure 3.4d**. These were calculated as a percentage of total counts in each 1ml sample. Compared to wild-type aldosterone synthase, mutant B2-D147E increased B production 85%, 18-OH-B by 50% and aldosterone by 25%. The production of 18-OH-DOC, which can be synthesised from DOC by 11β-hydroxylase, was also measured. However, its production was not significantly affected by B2-D147E.

3.4.4. Conversion of 11-deoxycortisol to cortisol by aldosterone synthase mutant B2-D147E

As B2-D147E causes an increase in 11β-hydroxylation, its effect on the conversion of 11-deoxycortisol, the principal substrate of 11β-hydroxylase, to cortisol was also studied. **Figure 3.4e.** shows the effect of B2-D147E on the F:S ratio. The ratio of wild type 11β-hydroxylase was 9.6 ± 1.2 compared to 0.3 ± 0.02 for wild-type aldosterone synthase and 0.3 ± 0.03 for B2-D147E. ($n=6$; $P<0.001$).

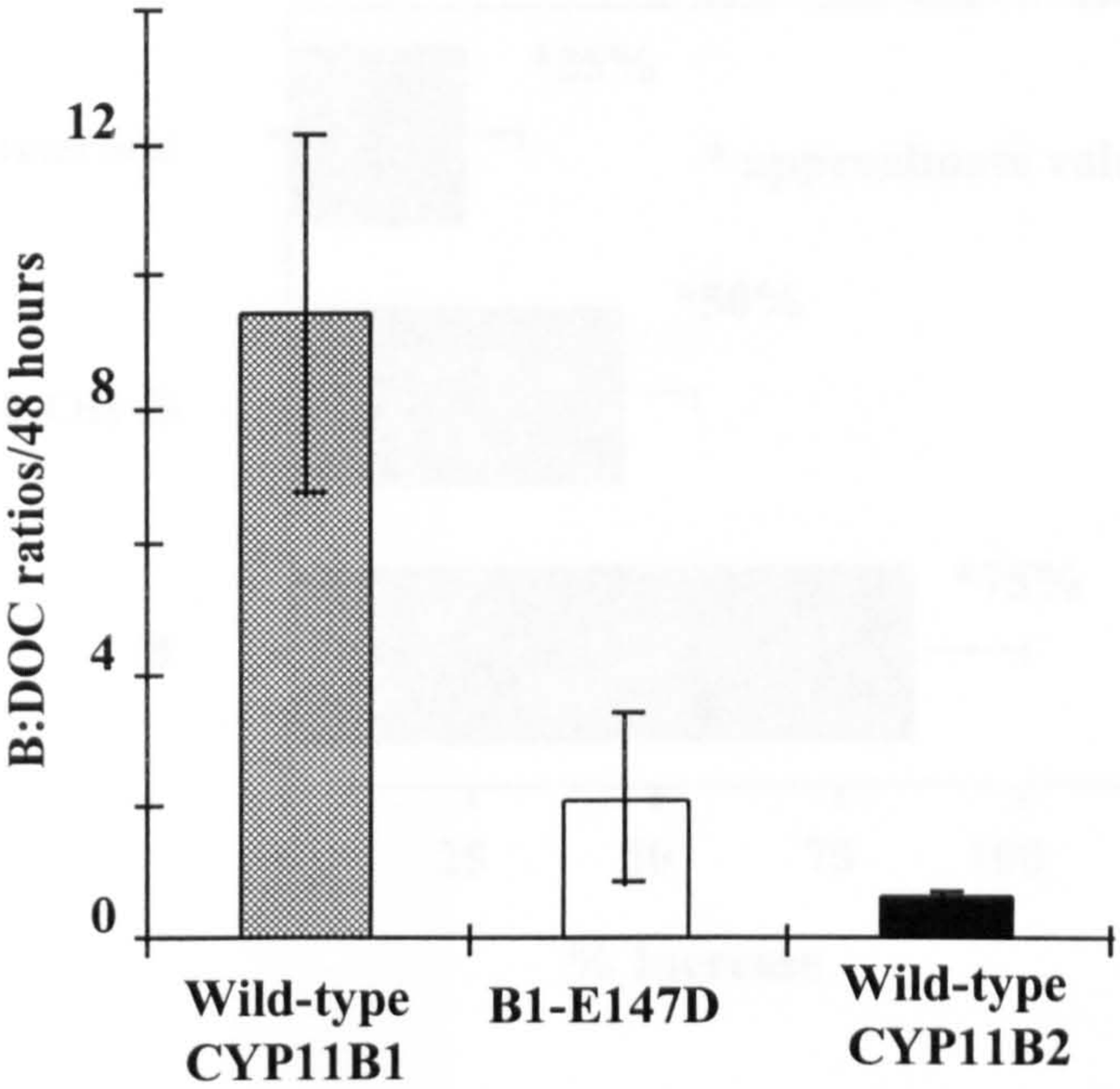


Figure 3.5a.

B:DOC ratio of 11β-hydroxylase mutant B1-E147D

Comparison of B:DOC ratio of 11β-hydroxylase mutant B1-E147D with wild-type 11β–hydroxylase (CYP11B1). COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with 5μM ^{3}H-DOC for 48 hours. ^{3}H-steroids from the medium were extracted and separated by TLC and analysed in duplicate by liquid scintillation counting. Results are expressed as ratio of product to substrate B:DOC, which is an index of 11β-hydroxylase activity. Results are mean ± SEM from four separate transfections, each done in duplicate. Statistical analysis was done by Mann-Whitney-U test.

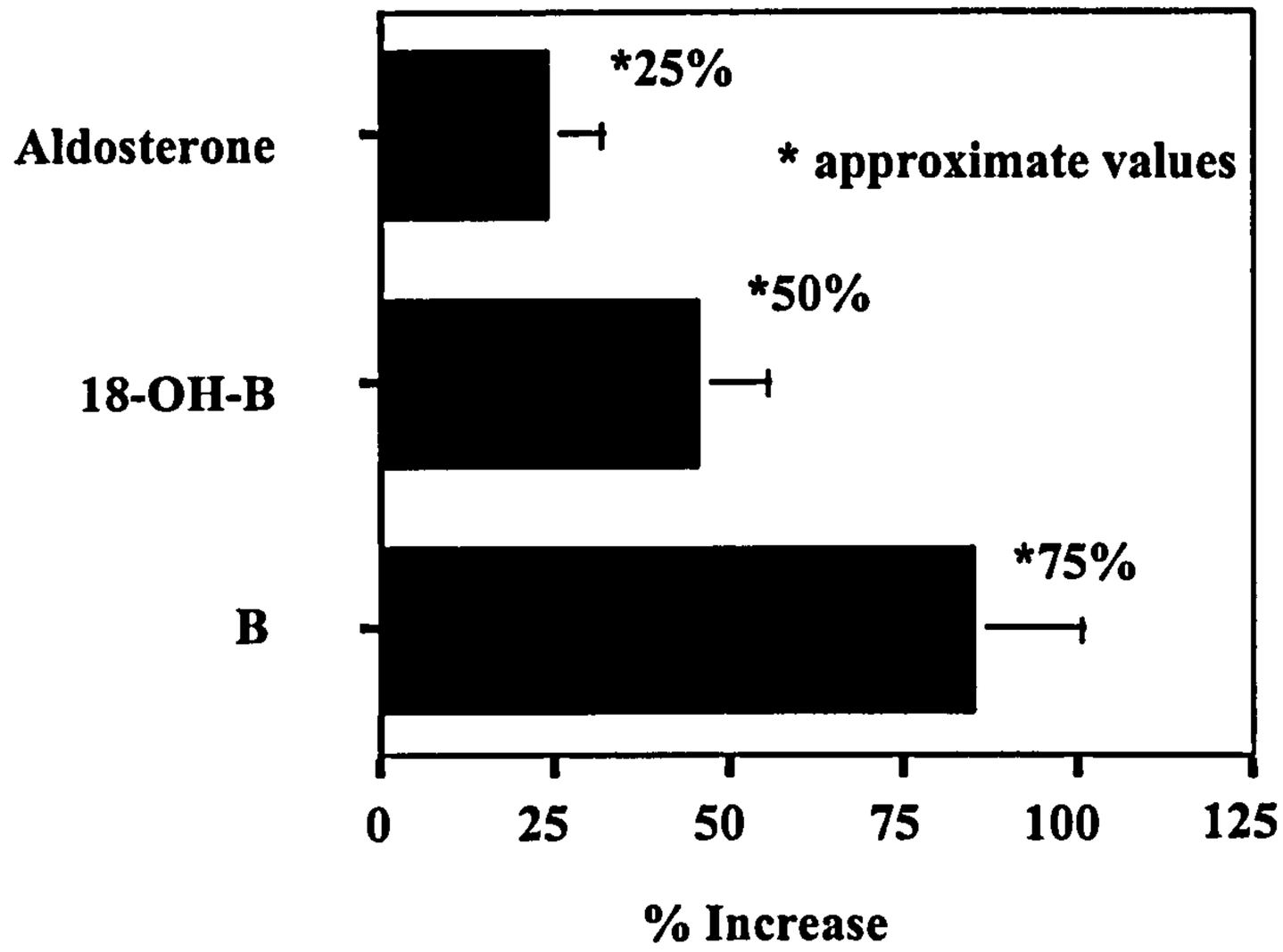


Figure 3.4d.
Effect of aldosterone synthase mutant B2-D147E on overall steroid production compared to wild-type aldosterone synthase (CYP11B2).
COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with 5μM ^{3}H DOCfor 48 hours. ^{3}H-steroids from the medium were extracted and separated by TLC and analysed in duplicate by liquid scintillation counting. Results are expressed a percentage of total counts in 1ml of steroid medim. Results are mean ± SEM from four separate transfections, each done in duplicate.

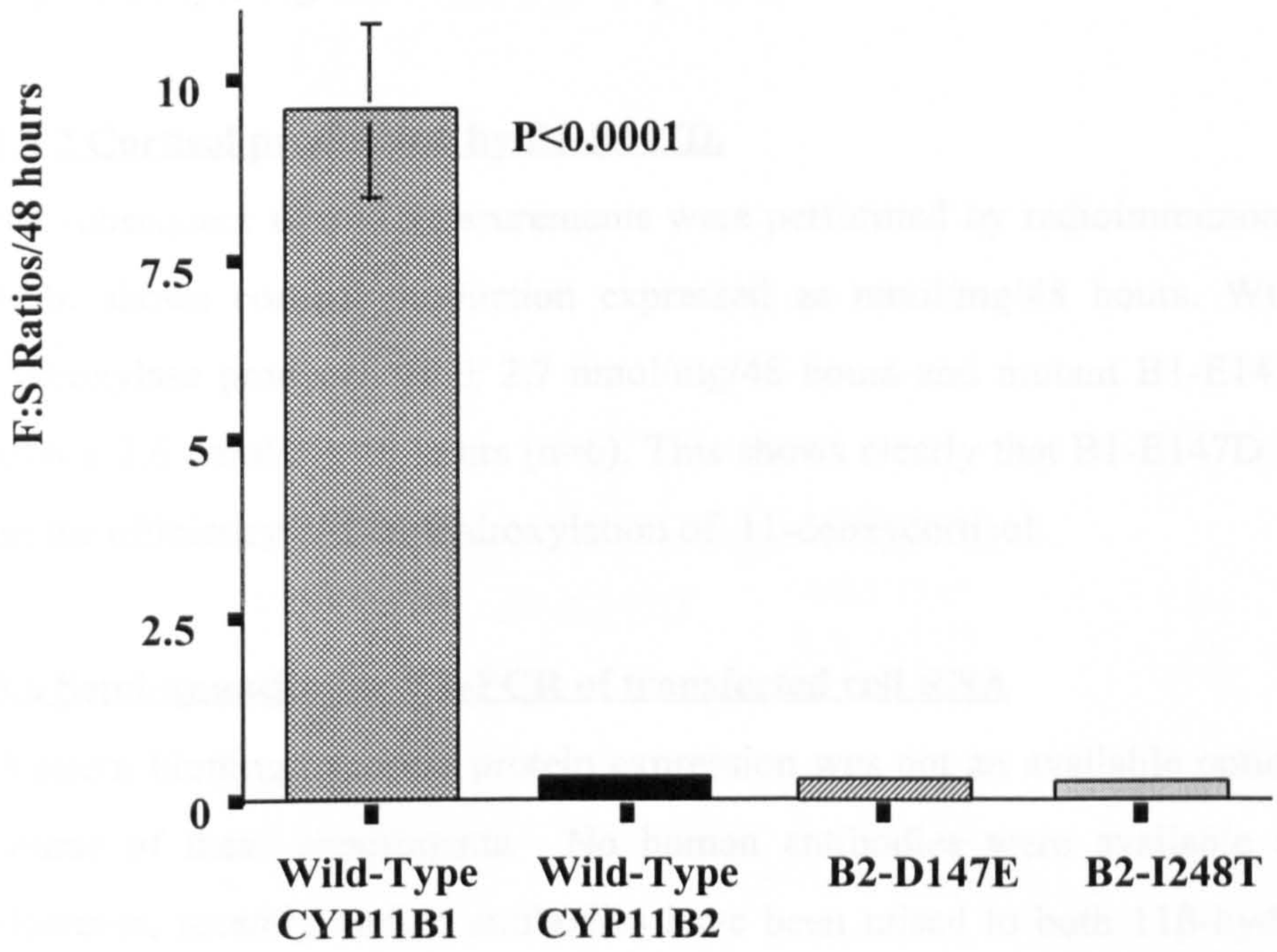


Figure 3.4e.

F:S ratios of aldosterone synthase mutants B2-D147E and B2-I248T.

The F:S ratios of B2-D147E and I248Tare shown compared to wild-type aldosterone synthase (CYP11B2) wild-type 11β -hydroxylase(CYP11B1). COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with 5μM ^{3}H S for 48 hours. ^{3}H-steroids from the medium were extracted and separated by TLC and analysed in duplicate by liquid scintillation counting. Results are expressed as ratio of product to substrate (cortisol) F:S which is an index of 11β-hydroxylase activity. Results are mean ± SEM from four separate transfections, each done in duplicate. Statistical analysis was done by the Mann-Whitney U test.

3.5.1 Effect of 11β-hydroxylase mutant B1-E147D on B:DOC steroid ratio

The 11β-hydroxylase mutant B1-E147D B:DOC ratio was significantly decreased compared to wild-type 11β-hydroxylase from 9.5 ± 2.7 to 2.1 ±1.3 (n=6) ($p<0.0001$). **Figure 3.5a.**. shows that, as expected, the B:DOC ratio for the wild-type 11β-hydroxylase was high (9.5 ± 2.7) compared to wild-type aldosterone synthase (0.6 ± 0.08; $p<0.001$). This suggests that the B1-E147D mutant decreased 11β-hydroxylase efficiency compared to wild type 11β-hydroxylase but that it is still more efficient at 11β-hydroxylating than aldosterone synthase.

3.5.2 Cortisol production by B1-E147D.

All subsequent steroid measurements were performed by radioimmunoassay. **Figure 3.5b.** shows cortisol production expressed as nmol/mg/48 hours. Wild-type 11β-hydroxylase produced 22 ± 2.7 nmol/mg/48 hours and mutant B1-E147D produced 20.6 ± 2.6 nmol/mg/48 hours (n=6). This shows clearly that B1-E147D has no effect on the efficiency of 11β-hydroxylation of 11-deoxycortisol.

3.6 Semi-quantitative RT-PCR of transfected cell RNA

Western blotting to assess protein expression was not an available option during the course of these experiments. No human antibodies were available at that time. However, recently human antibodies have been raised to both 11β-hydroxylase and aldosterone synthase which are currently being tested. As protein expression was not an option, semi-quantitative RT-PCR was used to measure the level of transcription.

For RT-PCR, the number of cycles used has to be within the linear part of the amplification curve. **Figure 3.6a** shows the RT.PCR amplification curve for GAPDH and CYP11B1/B2. In subsequent experiments , the optimised cycle number GAPDH and CYP11B1/B2, was 34 and 30 respectively.

Figure 3.6b shows representative RT-PCR amplification of GAPDH and CYP11B2 for wild-type aldosterone synthase and mutant B2-D147E. The levels of CYP11B2 expression (arbitrary units calculated by band intensity) were standardised by dividing by the levels of GAPDH expression, calculated in the same way.

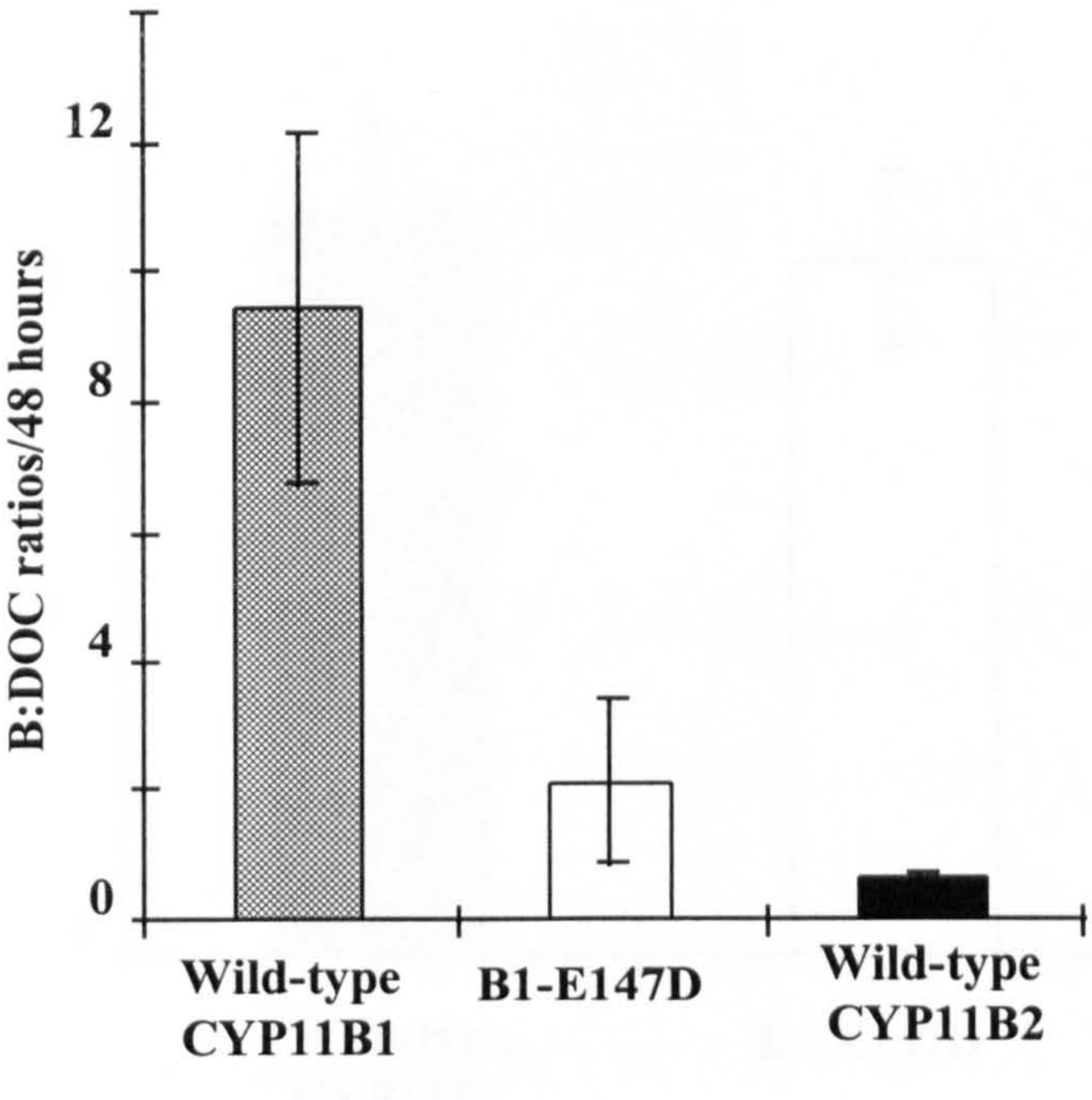


Figure 3.5a.

B:DOC ratio of 11β-hydroxylase mutant B1-E147D

Comparison of B:DOC ratio of 11β-hydroxylase mutant B1-E147D with wild-type 11β–hydroxylase (CYP11B1). COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with 5μM ^{3}H-DOC for 48 hours. ^{3}H-steroids from the medium were extracted and separated by TLC and analysed in duplicate by liquid scintillation counting. Results are expressed as ratio of product to substrate B:DOC, which is an index of 11β-hydroxylase activity. Results are mean ± SEM from four separate transfections, each done in duplicate. Statistical analysis was done by Mann-Whitney-U test.

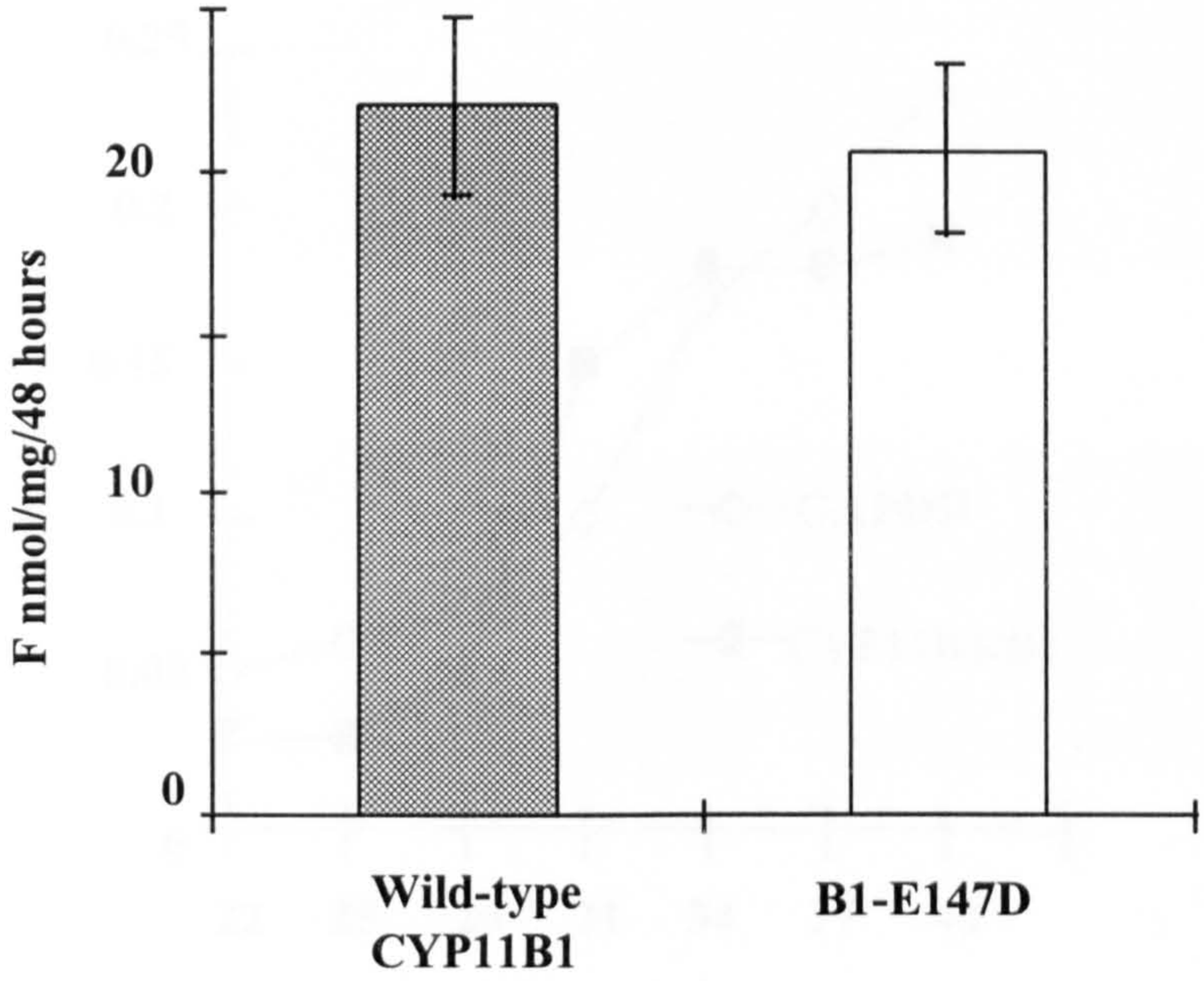


Figure 3.5b.
F production from 11β-hydroxylase mutant B1-E147D
Comparison of cortisol (F) production from 11β-hydroxylase mutant B1-E147D with wild-type 11β–hydroxylase. COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with 5μM 11-deoxycortisol (S) for 48 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are expressed as mean ± SEM from four separate transfections, each done in duplicate.

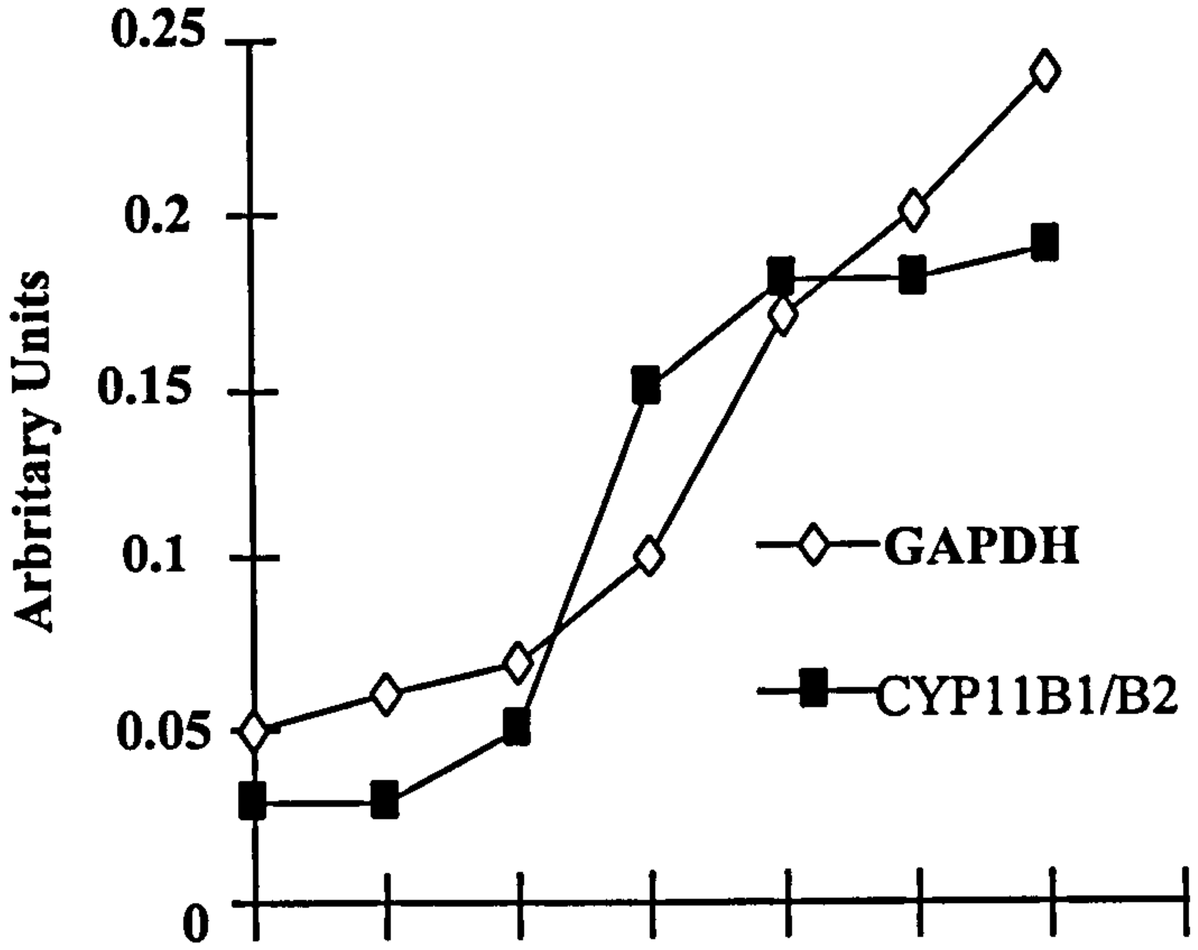


Figure 3.6a Optimisation of cycle number for RT-PCR
Phosphorimage analysis of CYP11B1/B2 and GAPDH PCR amplicon with varying cycle number. RT-PCR was carried out on RNA (1μg) for 22-40 cycles and the intensity of the band analysed. Subsequent RT-PCR was carried out using the number of cycles where the graph remained linear. For CYP11B1/B2 30 cycles were used and for GAPDH 34 cycles were used.

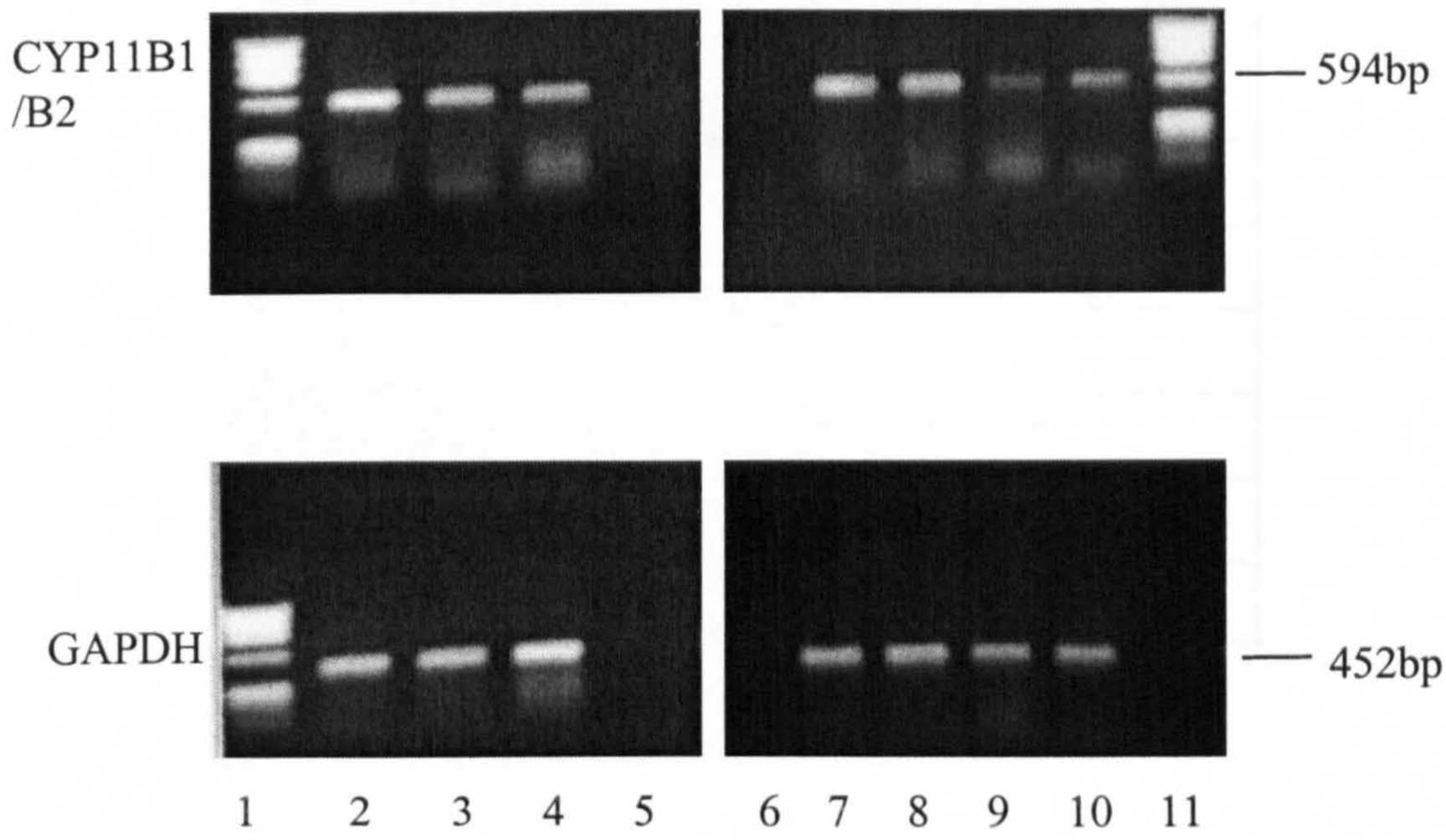


Figure 3.6b RT-PCR of RNA from cells transfected with wild-type CYP11B2 or aldosterone synthase mutant B2-D147E

Representative examples of semi-quantitative RT-PCR analysis of RNA (1μg) from COS-7 cells transfected with wild-type CYP11B2 or aldosterone synthase mutant B2-D147E. The top panel shows the CYP11B1/B2 amplicon (594bp) in 3 flasks transfected with B2-D147E (lanes 2-4) and 4 flasks transfected with wild-type CYP11B2 (lanes 7-10). The corresponding GAPDH amplicon (452bp) is shown in the bottom panel. Lanes 5 and 6 show water blanks. The intensity of the bands was analysed by phosphorimaging analysis.

Table 3.6 shows the range of CYP11B1/B2:GAPDH ratios for the wild-type constructs and the mutants tested. Mutant construct values are within the ranges of the wild-type constructs.

CONSTRUCT	CYP11B1/B2:GAPDH RATIOS
CYP11B1	0.88 to 1.82 (n=3)
CYP11B2	0.8 to 1.6 (n=4)
B2-D147E	0.7 to 1.2 (n=3)
B2-I248T	0.6 to 1.3 (n=3)
B2-K357N	0.6 to 0.7 (n=2)
B1-E147D	0.7 to 0.9 (n=2)

Table 3.6 CYP11B1/B2: GAPDH ratios for RT-PCR

The results clearly show that residue 147 of aldosterone synthase and 11β-hydroxylase are important for efficient 11β-hydroxylation of 11-deoxycorticosterone. Results from semi-quantitative RT-PCR show that the level of transcription was similar in transfected cells which suggests that the altered steroid production was due to an effect on enzyme activity and not to an increase in gene transcription. They did not, however, allow a decision on whether protein expression had been altered. If this was the case, all three activities of aldosterone synthase would be increased in similar proportions. However, only the 11β-hydroxylase function was affected. To assess whether these amino acid changes affect binding affinity for 11-deoxycorticosterone, kinetic analysis was performed.

3.7. Kinetic analysis of mutants B2-D147E and B1-E147D

Kinetic experiments were performed for mutants B2-D147E and B1-E147D using DOC as substrate at concentrations ranging from 0.01 to 10 and 15μM respectively. As before, the mutants and wild-type plasmids were transiently expressed in COS-7

cells with the necessary controls. Time course experiments were performed for B production, as measured by radioimmunoassay (2.8.3), to determine the end-point which was maximal but still in the linear range which follows first rate order kinetics. An end-point of 8 hours was chosen as determined by **figure 3.7a.**

3.7.1 Kinetic analysis of aldosterone synthase mutant B2-D147E compared to wild-type aldosterone synthase

Dose-response curves of steroid products: corticosterone (B), 18-hydroxycorticosterone (18-OHB) and aldosterone against 11-deoxycorticosterone (DOC) concentration for aldosterone synthase and the mutant B2-D147E are shown in **figures 3.7b, 3.7c and 3.7d.** B production by aldosterone synthase mutant B2-D147E was significantly increased at all concentrations of DOC compared to wild-type aldosterone synthase. Results were analysed by ANOVA followed by Student's t-test. Lineweaver-Burke analysis and derivation of Km was performed **(Figure 3.7e).** As Km is independent of enzyme concentration, it can be derived even though precise quantification of enzyme concentration was not possible. Vmax on the other hand largely depends on enzyme concentration. Therefore it is unacceptable to use this data set to obtain Vmax values. The Lineweaver-Burke plot for B analysis for wild-type aldosterone was $y = 0.0005x + 0.0001$ and for B2-D147E was $y = 0.00007x + 0.00005$. The data points show the mean ± SEM for four individual plates of cells. The values for Km for wild-type aldosterone synthase and B2-D147E, derived from these equations, were 5μmol/L and 1.4μmol/L respectively. These values differ significantly. Thus, mutant B2-D147E has a lower apparent Km for conversion of DOC to corticosterone than that of wild-type aldosterone synthase.

18-OHB production from B2-D147E was also increased compared to wild-type aldosterone synthase but to a lesser extent than B production. No attempt to derive Km values was made.

3.8. Kinetic analysis of 11β-hydroxylase mutant B1-E147D compared to wild-type 11β-hydroxylase

Dose-response curves of steroid products; B and 18-OHB against DOC concentration for 11β-hydroxylase and the mutant B1-E147D are shown in **figures 3.8a., and 3.8b.**

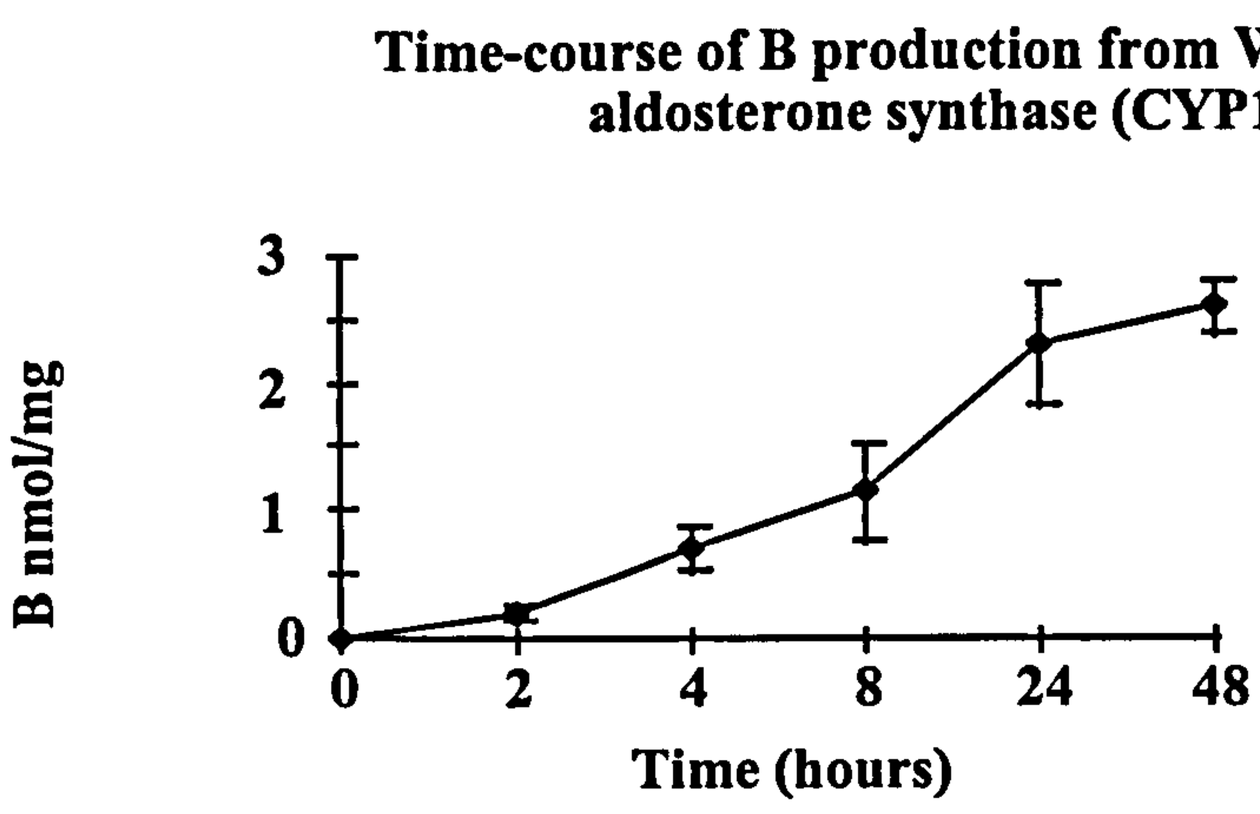


Figure 3.7a.

Time-course of B production from COS-7 cells transiently expressed with wild-type aldosterone synthase (CYP11B2) or 11β-hydroxylase (CYP11B1).

COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with 5μM DOC between 0 and 48 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from four separate transfections.

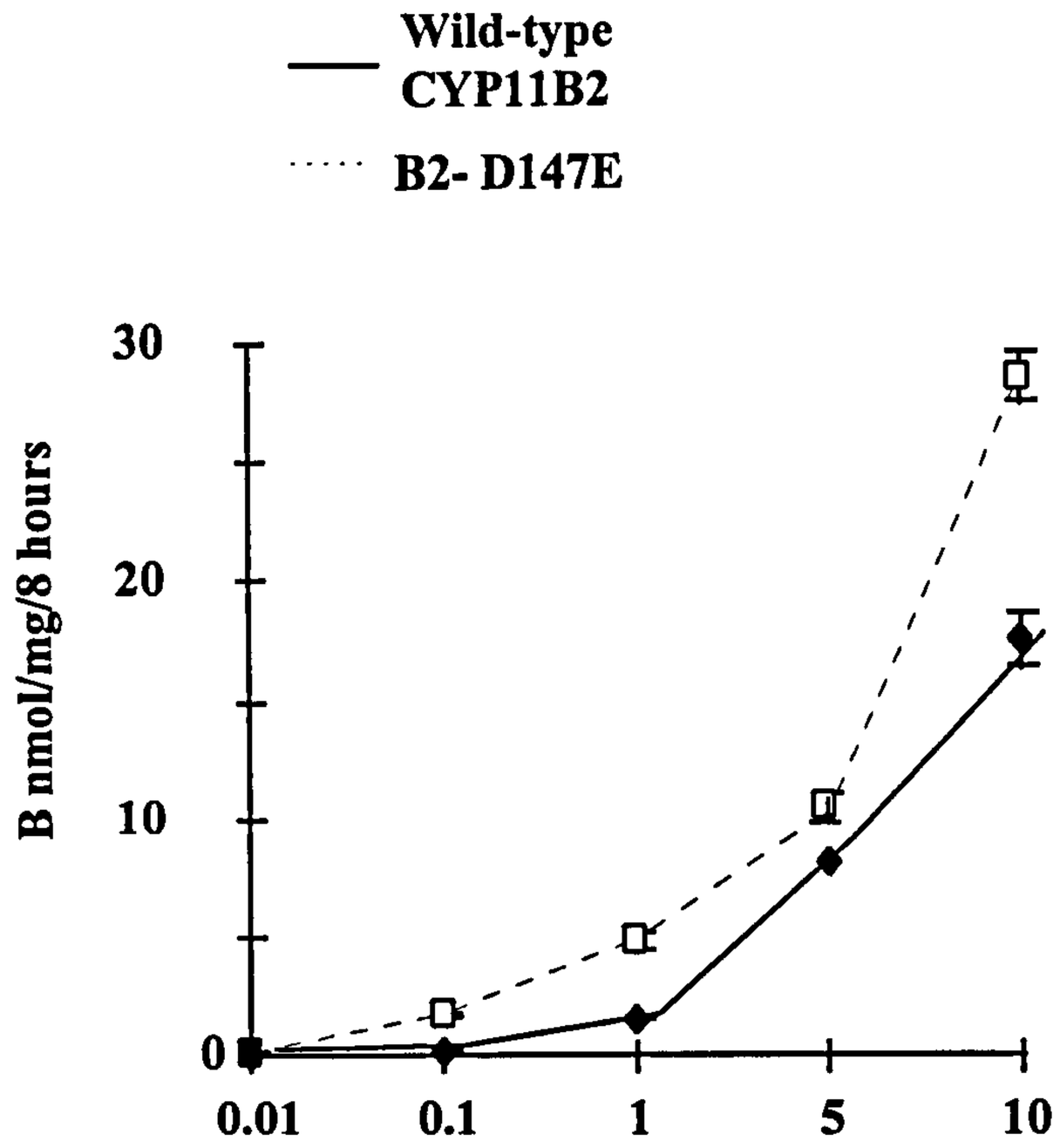


Figure 3.7b.
B production from various concentrations of DOC from aldosterone synthase mutant B2-D147E compared to wild-type aldosterone synthase (CYP11B2).
COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with varying concentrations DOC for 8 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from four separate transfections and were analysed by ANOVA. B production by B2-D147E was significantly higher that wild-type aldosterone synthase at all concentrations of substrate ($n=4$, $p<0.05$, Student's t-test)

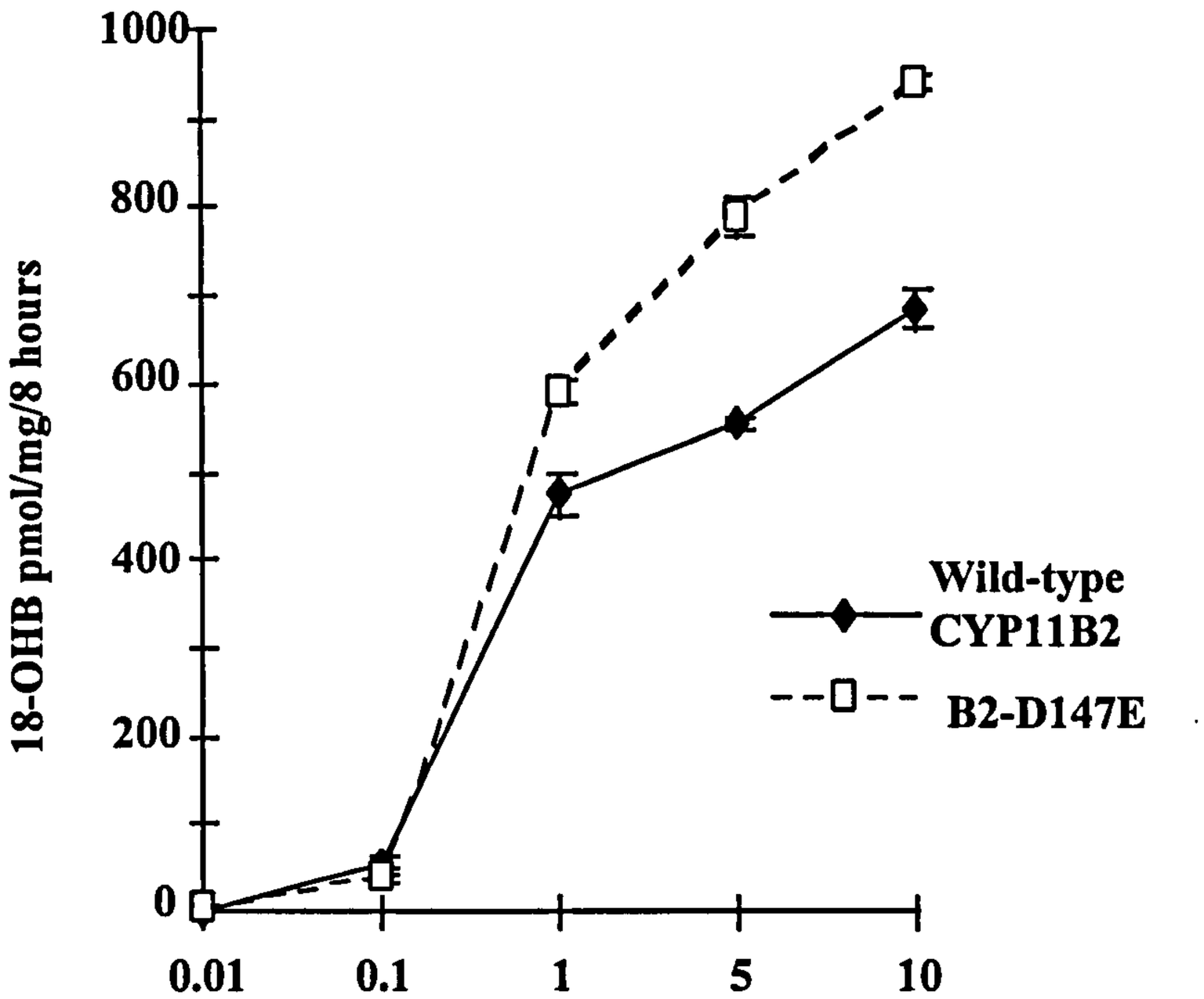


Figure 3.7c.
18-OHB production from various concentrations of DOC of aldosterone synthase mutant B2-D147E compared to wild-type aldosterone synthase (CYP11B2).
COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with varying concentrations DOC for 8 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from four separate transfections.

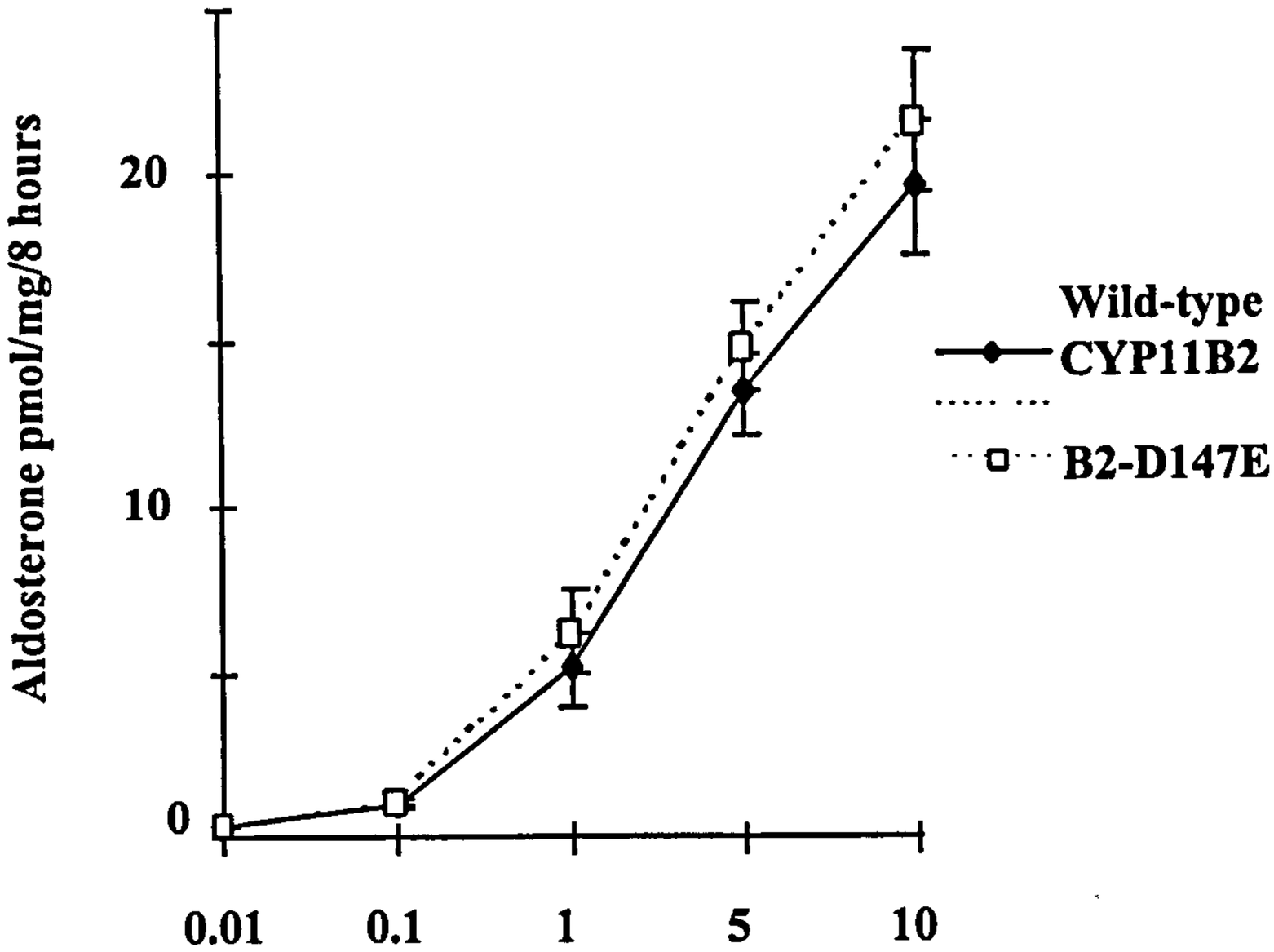


Figure 3.7d.
Aldosterone production from various concentrations of DOC of aldosterone synthase mutant B2-D147E compared to wild-type aldosterone synthase (CYP11B2).
COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with varying concentrations DOC for 8 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from four separate transfections.

Lineweaver-Burke plot of B production from B2-D147E

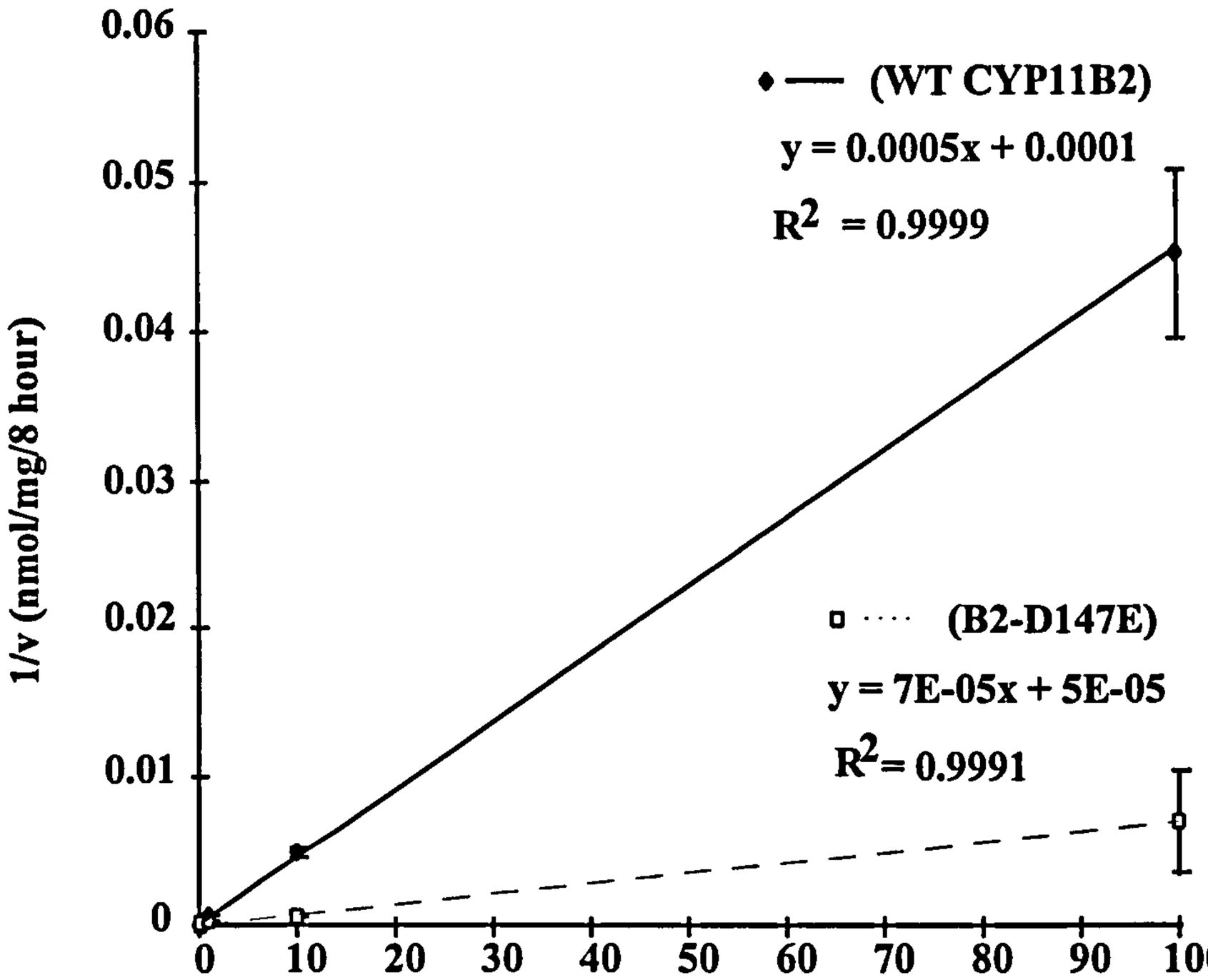


Figure 3.7e.
Lineweaver-Burke analysis and derivation of Km for aldosterone synthase mutant B2-D147E compared to wild-type aldosterone synthase (CYP11B2) for DOC to B conversion (data taken from figure 3.7b). The axes are the reciprocal of the substrate concentration in micromolar (1/S) and the reciprocal of the velocity of B production in nmol per mg/8 hours (1/V). COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with various concentrations of DOC for 8 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from four separate transfections.

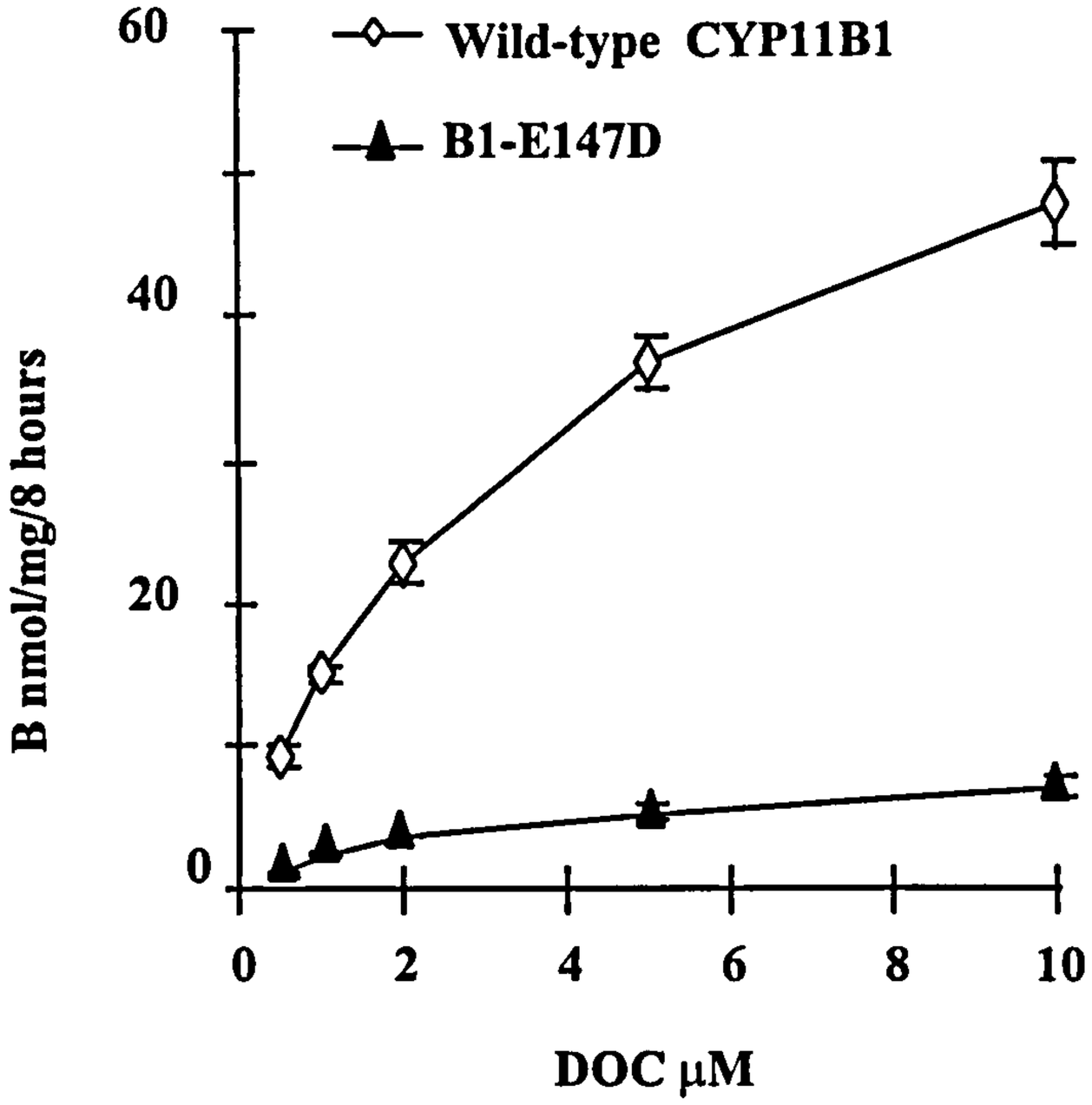


Figure 3.8a.
B production from various concentrations of DOC from 11β-hydroxylase mutant B1-E147D compared to wild-type 11β-hydroxylase (CYP11B1)
COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with varying concentrations DOC for 8 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from four separate transfections and were analysed by ANOVA followed by Student's t-test. B production by B1-E147D was significantly lower that wild-type aldosterone synthase at all concentrations of substrate ($n=4$, $p<0.0001$, Student's t-test).

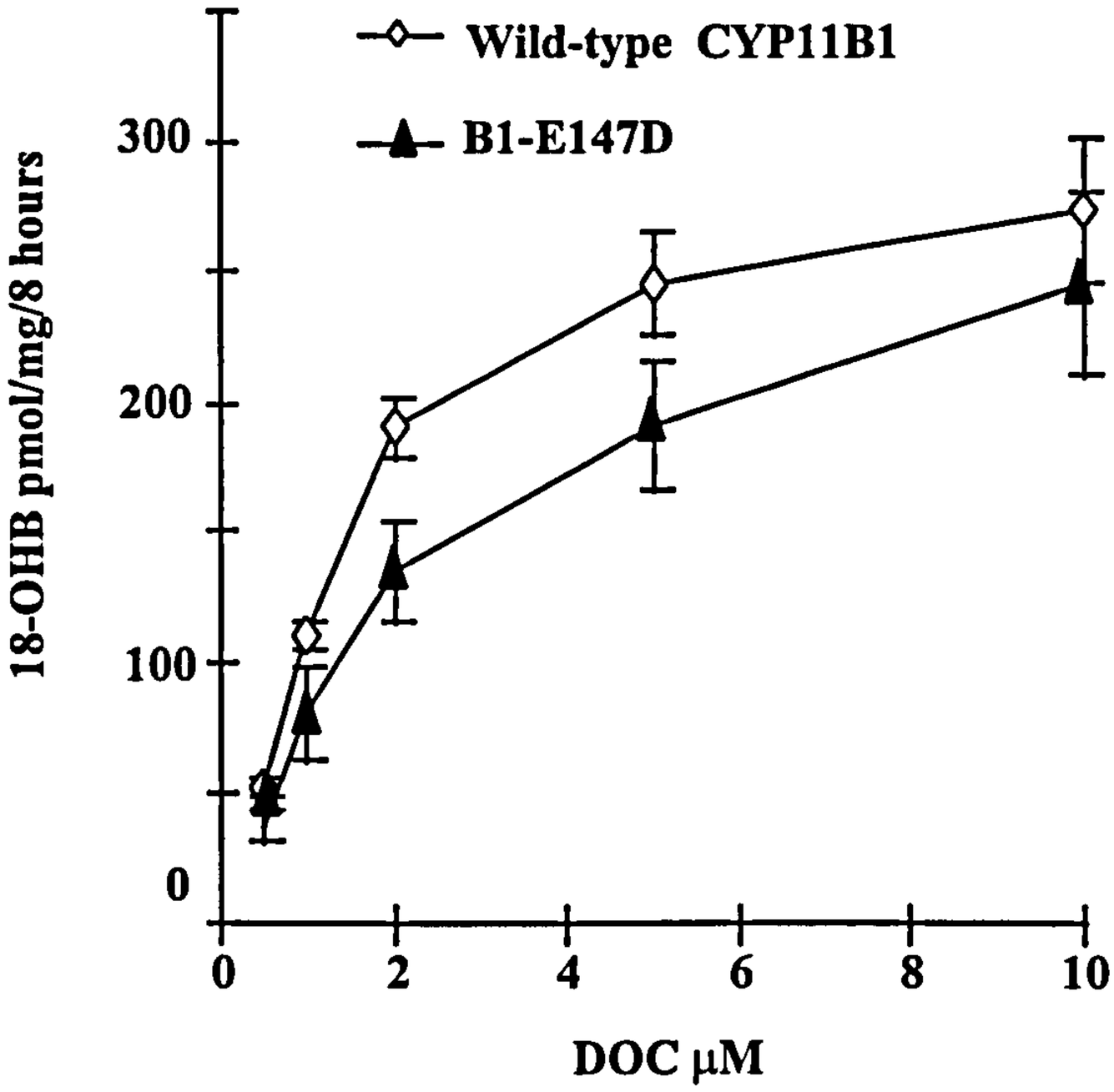


Figure 3.8b.
18-OHB production from various concentrations of DOC from 11β-hydroxylase mutant B1-E147D compared to wild-type 11β-hydroxylase (CYP11B1)
COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with varying concentrations DOC for 8 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from four separate transfections.

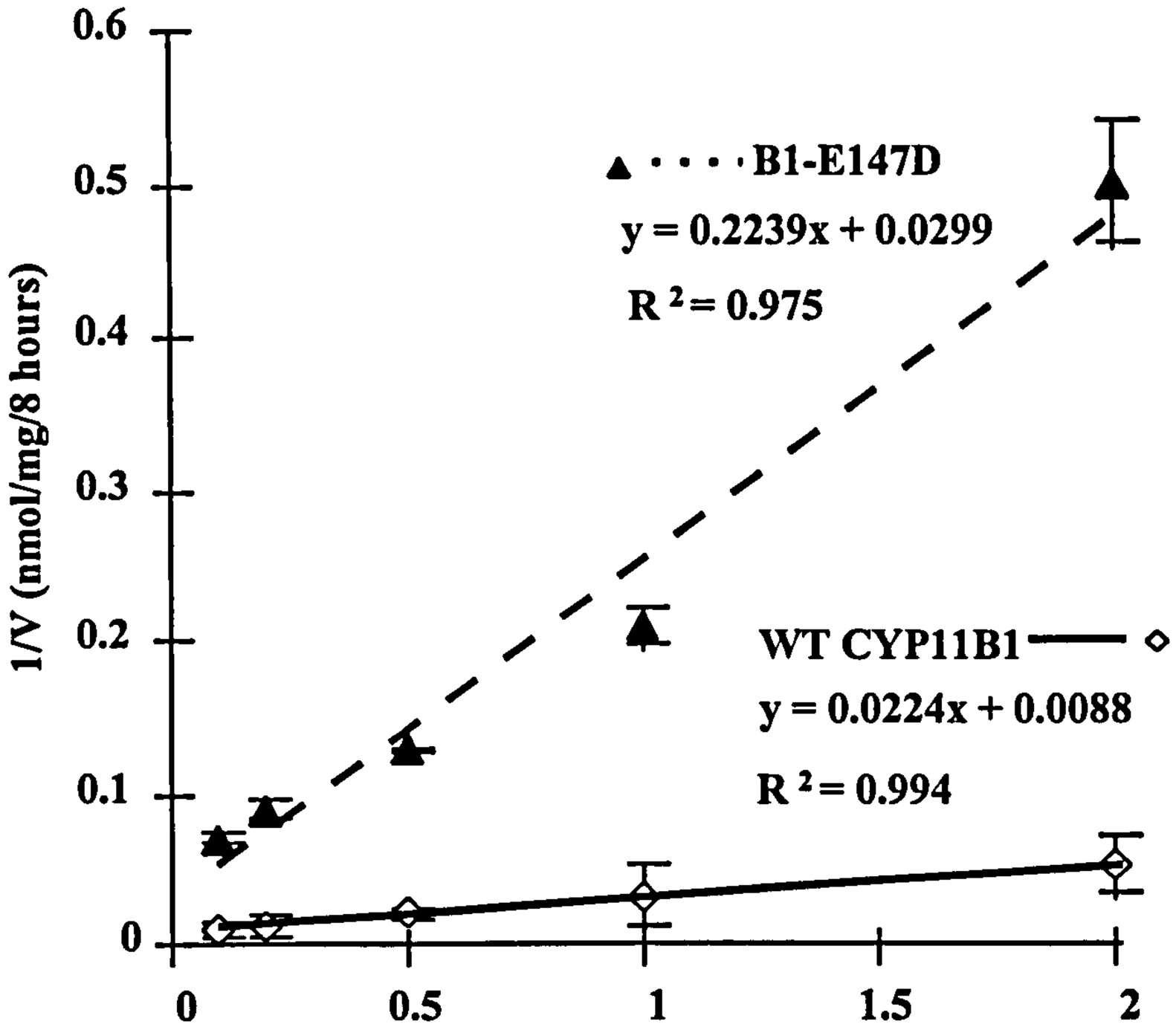


Figure 3.8c.
Lineweaver-Burke analysis and derivation of Km for 11β-hydroxylase mutant B1-E147D compared to wild-type 11β-hydroxylase (CYP11B1) for DOC to B conversion (data taken from figure 3.8a). The axes are the reciprocal of the substrate concentration in micromolar (1/S) and the reciprocal of the velocity of B production in nmoles per mg/8 hours (1/V). COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with various concentrations of DOC for 8 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from four separate transfections.

respectively. B production for 11β-hydroxylase mutant B1-E147D was significantly decreased at all concentrations of DOC compared to wild-type 11β-hydroxylase. Results were analysed by ANOVA followed by Student's t-test. Lineweaver-Burke analysis and derivation of the Km is shown in **figure 3.8c.** The Lineweaver-Burke regression analysis for wild-type 11β-hydroxylase was y= 0.0224x + 0.0088 and for B1-E147D was y=0.2239 + 0.0299. The values for Km for wild-type 11β-hydroxylase and the mutant B1-E147D, derived from these equations were 2.5 μmol/L and 7.5 μmol/L respectively. These values differ significantly. This shows that mutant B1-E147D has a higher apparent Km for conversion of DOC to corticosterone than that of wild-type 11β-hydroxylase which explains the decrease in corticosterone production. 18-OHB production from B1-E147D was decreased compared to wild-type 11β-hydroxylase but to a lesser extent than B production. No attempts to derive Km values was made.

3.5 Discussion

Aldosterone synthase and 11β-hydroxylase are key enzymes in the terminal stages of corticosteroid biosynthesis in the adrenal gland. Functionally these enzymes are quite different. 11β-Hydroxylase is expressed principally in the ZF and is regulated by ACTH (White et al. 1994). It catalyses the conversion of the mineralocorticoid, DOC to B, 18-OH-DOC or 19-OH-DOC. It also catalyses the conversion of 11-deoxycortisol (S) to cortisol (F). In contrast, aldosterone synthase is expressed solely in the ZG under the control of angiotensin II and potassium (White et al. 1994), and converts DOC to aldosterone via B and 18-OH-B. Despite these functional differences, the genes which encode these two enzymes are highly homologous. Only 7% of amino acid residues differ between these two enzymes and these account for the key differences in enzymatic activities and substrate specificity.

To investigate the molecular basis of these functional differences, specific amino acids of aldosterone synthase were mutated and the effects on steroid production studied. Molecular studies by other groups have already shown the absolute requirement of a glycine residue at position 288 and an alanine residue at position 320

for efficient 18-hydroxylation and subsequent 18-oxidation in aldosterone synthase (Curnow et al. 1997). Also 11β-hydroxylase specific residues 301, 302 and 320 have been shown to be important for 11β-hydroxylase activity (Bottner et al.1996). From the current study, it may be possible to gain further insight into the role played by other amino acid residues which differ between the two enzymes.

Four separate amino acid substitutions were examined, in each instance replacing an aldosterone synthase-specific residue with the 11β-hydroxylase equivalent and, in one case, replacing the 11β-hydroxylase-specific residue with the aldosterone synthase equivalent. The selection of which residues to study was guided, in part, by the example of the Dahl salt-resistant (R) and salt-sensitive (S) rat models which have been used to investigate the genetic component of salt-sensitive hypertension (see 1.8). In the S strain, there is increased 18-OH-DOC production which is thought to account for approximately 20% of the excess blood pressure in this model. Studies of the CYP11B1 gene in the R strain have revealed mutations which alter 11β-hydroxylase activity and decrease 18-OH-DOC production (Matsukawa et al. 1993). These mutations co-segregate with reduced adrenal capacity to synthesise 18-OH-DOC and resistance to the hypertensinogenic effects of salt (Cicila et al. 1993) . Studies of CYP11B2 gene have also identified 7 mutations in the Dahl R rat, 2 of which alter the predicted amino acid composition of the protein (Exon 3, Glu 136 to Asp and Exon 4, Gln 251 to Arg). These appear to encode an enzyme with a greater apparent Vmax and a lower apparent Km, resulting in an increased rate of conversion of DOC to aldosterone (Cover et al. 1995). Exact replication of these mutations in human aldosterone synthase, increased B and aldosterone production (Fardella et al. 1995). Further examination of exons 3 and 4 of the human genes showed 6 codons which differ between the CYP11B1 and B2 genes, 4 of which code for different amino acids. Two examples are codons 147 (exon 3) and 248 (exon 4), which code for the amino acids glutamate and isoleucine in aldosterone synthase and aspartate and threonine in 11β-hydroxylase. To determine the functional significance of these residues, which lie close to those identified in the Dahl R rat and have been shown to have major effects on enzyme activity, aldosterone synthase-specific residues were replaced with the 11β-hydroxylase equivalents. A converse mutant in 11β-

hydroxylase replacing was prepared where residue 147 was substituted with the aldosterone synthase equivalent. In addition, residues 43 and 493 which are found in exons 1 and 9 respectively, were mutated. These are distant from the putative active site of the enzyme and unlikely to exert a major effect on the enzyme activity of aldosterone synthase. A recent study, where chimeric proteins were constructed between 11β-hydroxylase and aldosterone synthase to investigate the importance of residues differing in their C-terminal regions, showed that residues 471,472,492, 493 and 494 were not important for steroid hydroxylation (Bottner et al. 1998). The different residues at this position in aldosterone synthase and 11β-hydroxylase do not therefore account for the differences in enzyme activity between these enzymes (Bottner et al. 1998). They provide a useful control for the two mutations in exons 3 and 4. The conversion of S to F was not assessed for these mutations and it is possible that they may affect cortisol production but this is unlikely. Whether analogous substitution of these residues in 11β-hydroxylase for the aldosterone synthase-specific residues affects enzyme activity of 11β-hydroxylase is not known.

The results showed clearly that alterations of residues 43, 248 and 493 of aldosterone synthase to the corresponding 11β-hydroxylase residues had no effect on steroid production. This strongly suggests that these amino acids do not confer functional specificity. Although they differ between the two enzymes, they may be naturally-occuring polymorphisms which have been conserved. It is relevant that, since this investigation was completed, there has been a further report suggesting that this is the case for residue 43 in aldosterone synthase (Curnow et al. 1997). Amino-acids 1-44, the mitochondrial anchor, have recently been shown to be necessary for insertion of mitochondrial cytochrome P450 enzymes into the membrane. Removal of these residues is not detrimental to enzyme function (Zvelebil et al. 1991). It is therefore to be expected that mutation of residue 43 does not alter enzymatic function. In addition, a recent study has identified a mutation in residue 173 of aldosterone synthase in a group of subjects with low renin essential hypertension. However, it had no demonstrable effect on the enzyme kinetics of aldosterone production in vitro (Fardella et al. 1996b). No attempt was made to assess whether it influenced B or 18-OHB production.

The major finding of the current study was that altering aspartate 147 residue (exon 3) of aldosterone synthase to the corresponding 11β-hydroxylase-specific glutamate residue caused a dramatic increase in B production and a smaller but significant increase in aldosterone production. Closer inspection of the individual enzymatic activities of aldosterone synthase revealed that there was a large increase in the ratio of B:DOC, a small but nonetheless significant decrease in the 18-OH-B:B ratio and no change in the aldosterone:18-OH-B or aldosterone:B ratios. This suggests that the increase in B and aldosterone production was due to an increase in the 11β-hydroxylase activity of aldosterone synthase and not increased 18-hydroxylase/oxidase activity. It seems likely that the small decrease in the 18-OH-B:B ratio was caused by increased availability of substrate (B) coming from the previous reaction, rather than an actual decrease in 18-hydroxylase efficiency. However, the use of B and 18-OH-B as substrates would provide a clearer picture of the effects of B2-D147E on 18-modifying functions of the enzyme. Unfortunately, studies by other groups have shown that these make poor substrates for aldosterone synthase (Vinson and Whitehouse 1970, Denner et al. 1995). To further emphasise the importance of this locus for 11β-hydroxylation, the converse mutation, where substitution of the 11β-hydroxylase-specific residue 147 was mutated to the aldosterone synthase equivalent, had the opposite effect on 11β-hydroxylase. Whereas B2-D147E increased 11β-hydroxylation of aldosterone synthase, B1-E147D dramatically reduced the 11β-hydroxylation efficiency of 11β-hydroxylase by reducing the ratio of B:DOC. It could be argued that the changes in enzyme function are due to altered levels of expression of the modified proteins. This seems extremely unlikely as only one function is majorly affected. If the effects were due to for example, an increase in expression of aldosterone synthase mutant B2-D147E, then a proportional increase in B, 18-OHB and aldosterone would be observed. That the 11β-hydroxylase mutant, B1-E147D, has the exact opposite effect and decreases 11β-hydroxylation is further evidence that the effects are not due to changes in protein expression. At the time of this study antibodies against human aldosterone synthase and 11β-hydroxylase were not readily available. These could have been used for Western blotting to demonstrate that protein expression was similar. Recently antibodies have become available which are currently being tested (Paul Stewart and Bill Rainey, personal communication). Semi-

quantitative RT-PCR was used to detect any obvious differences in transcriptional levels mutant cDNAs compared to wild-type cDNAs. No major differences were detected. Whilst it must be recognised that this technique does not indicate any changes at the expressional level, it does provide good evidence that transcription of the mutant and wild-type cDNAs are similar.

Having shown that B2-D147E increases the 11β-hydroxylase function of aldosterone synthase, this was compared with the ability of the wild-type 11β-hydroxylase to convert DOC to B. The B:DOC ratio of the wild-type 11β-hydroxylase is approximately 6, whereas for wild-type aldosterone synthase it is approximately 1. The B:DOC ratio for B2-D147E is between these two (approximately 3), suggesting that although residue 147 plays an important role in 11β-hydroxylation , there are other residues which contribute to the greater efficiency of 11β-hydroxylase activity in 11β-hydroxylase. Indeed, studies by other groups have shown that amino acid residues 301, 302 and 320 and 384 are also involved in 11β-hydroxylation (Curnow et al. 1993, Bottner et al. 1996). Similarly, the 11β-hydroxylase mutant B1-E147D was also compared to wild-type aldosterone synthase. The B:DOC ratio for wild-type aldosterone synthase was 0.88 ± 0.11 which was lower than that for the mutant B1-E147D. This suggests that, although mutation of residue 147 of 11β-hydroxylase decreases the B:DOC ratio, it remains higher than that of aldosterone synthase, again suggesting that other residues contribute to 11β-hydroxylase activity. Mutations of 11β-hydroxylase have been described in 11β-hydroxylase deficiency; these completely abolish enzyme activity (see section 1.8). This disorder results in severe hypertension due to the increased levels of DOC. It is possible that mutant B1-E147D, if found in vivo, may result in increased DOC levels.

As B2-D147E increases 11β-hydroxylation, its ability to convert 11-deoxycortisol, the principal substrate of 11β-hydroxylase, to cortisol was also studied. The F:S ratio for 11β-hydroxylase was approximately 10. In contrast, the F:S ratio with B2-D147E was not significantly different from that of wild-type aldosterone synthase, which does not convert S to F *in vivo*. This suggests that residue 147 plays a key role in the 11β-hydroxylation of DOC but not of S and, moreover, that some functions conferred by specific amino acids appear to be substrate-dependent. Similarly, mutant B1-E147D

produced levels of F comparable with those of wild-type 11β-hydroxylase, again showing that this residue exerts effects on 11β-hydroxylation which are substrate-specific for DOC. It is unlikely that 11β-hydroxylase mutant B1-E147D, if found *in vivo*, would cause cortisol deficiency which is a result of 11β-hydroxylase deficiency.

In the light of these findings, kinetic experiments which looked at 11β-hydroxylation of DOC revealed that mutation of residue 147 of aldosterone synthase reduced the Km value for DOC and mutation of residue 147 of 11β-hydroxylase increased the Km value for DOC compared to wild-type aldosterone synthase and 11β-hydroxylase respectively. Measurement of Km is independent of enzyme concentration and therefore any changes in Km reflect true changes in enzyme activity. Aldosterone synthase catalyses the conversion of DOC to aldosterone via three sequential steps. The efficiency of each individual step will therefore be directly affected by that of previous steps. To overcome this, the enzymes should be incubated with the intermediate precursors as substrates i.e corticosterone or 18-hydroxycorticosterone to determine Kms for the conversion of B to 18-OHB and 18-OHB to aldosterone respectively. However, as already stated both steroids are poor substrates. This may be why such kinetic experiments were not performed by Bottner et al. (1996) and Curnow et al. (1997). In this experiment, the efficiency of 11β-hydroxylation has been assessed; it was increased in mutant B2-D147E compared to wild-type aldosterone synthase (i.e lower Km for DOC). Increases in 18-OHB production were probably due to increased availability of B. This was particularly clear in the initial experiments where the ratio of 18-OHB:B was slightly decreased due to increased B production **(figure 3.4b)**. Aldosterone levels were not significantly increased for mutant B2-D147E compared to wild-type aldosterone synthase.

The reason for the effect of substituting residue 147 from CYP11B1 to CYP11B2 and vice versa on 11-hydroxylase activity is uncertain and surprising in that the amino acid change is conservative (acidic for acidic). In this regard, it is relevant that human aldosterone synthase and 11β-hydroxylase have not yet been crystallised and little is known about the structure of the protein. However, crystallised protein structures

have been determined for several bacterial P450 enzymes, for example P450cam, P450bm3 and P450terp, which show that they are composed of a number of helices (A to L) (see section 1.7). Although these enzymes share low homology with mammalian P450 enzymes, there are some highly conserved regions. One such region is the I helix, spanning amino acid residues 295-346 which runs through the core of the protein molecule and contains the haem and substrate binding domains. Putative models of the 3D-structure of aldosterone synthase and 11β-hydroxylase suggest that residue 147 may interact in some way with this active core, possibly to maintain the correct orientation/position of the substrate within the active site. That mutation of this residue only affects DOC conversion is unusual. DOC and S differ only by a 17α-hydroxy group in their steroid structure. It is possible that they have different contact points with the enzyme and that residue 147 affects contact with DOC and not S . From information obtained from protein modelling studies based on these known crystalline bacterial structures, it is possible to superimpose human aldosterone synthase and 11β-hydroxylase on them (see chapter 4). From this model, residue 147 is likely to be situated at the interface between the D- helix and a stretch of rope. It is possible that alteration of this residue, even conservatively, may alter the local environment such that the position of the helix with which it is closely associated may be slightly altered, possibly creating changes in the orientation of other nearby helices involved in particular substrate binding or recognition and, as a consequence, enhance or decrease the conversion of this substrate.

In conclusion, the data show clearly how a conservative change in amino acid composition can cause a profound change in enzymatic function and steroid production. It is likely that residues work synergistically to determine the activity or activities of a particular enzyme. Residue changes may have subtle or severe structural effects depending on their position and amino acid properties. This type of mutation plays a role in some forms of hypertension, where steroid ratios are altered. Mutant B2-D147E showed a small increase in aldosterone production. Although small and of borderline significance, small changes in aldosterone production may *in vivo* have a dramatic effect. For example at the peripheral level, tissues are extremely sensitive to small changes in aldosterone and it is possible that a mutation such as B2-D147E may

have a large effect on these tissues. A small increase in aldosterone in the brain, for example, may have dramatic effects on blood pressure (Gömez-Sanchez 1997). Mutant B1-E147D may, if found *in vivo*, cause hypertension due to the reduction in DOC to B conversion with resulting accumulation of DOC precursor. Mutations such as this may lead to a new form of 11β-hydroxylase deficiency where cortisol biosynthesis is unaffected. However, their contribution to essential hypertension remains to be determined.

CHAPTER 4

Chapter 4

4.1 Modelling of human aldosterone synthase and 11β-hydroxylase structures.

11β-Hydroxylase and aldosterone synthase are mitochondrial cytochrome P450 enzymes which insert molecular oxygen at positions 11 and 18 on the steroid molecule to form hydroxyl groups. Aldosterone synthase can also catalyse the formation of the C18 aldehyde group (see section 1.4). These enzymes belong to the cytochrome P450 superfamily, possessing characteristics attributable to the binding of haem to a highly conserved cysteine residue on the polypeptide which enables the iron-enzyme complex to absorb light at 450nm. It is obvious that the unique properties of these enzymes must depend on subtle differences in their three-dimensional structures. Several bacterial members of this family are soluble proteins (e.g. $P450_{cam}$, $P450_{BM3}$ and $P450_{terp}$). Their crystal structures have been determined (Poulos et al. 1987, Ravichandran et al. 1993, Hasemann et al. 1994). However, steroidogenic enzymes are associated with cell membranes. For example, side-chain cleavage enzyme,11β-hydroxylase and aldosterone synthase are mitochondrial and 21-hydroxylase and 17α-hydroxylase are microsomal. Membrane association has made crystallisation of these mammalian enzymes difficult and attempts to date have not been successful. However, since the chemical reactions are analogous, several functional domains have been conserved. It is possible therefore to model mammalian proteins on those crystal bacterial structures. This technique has proven to be a useful tool in determining regions of structural and functional importance.

Sequence alignment studies of the P450 superfamily proteins and modelling based on $P450_{cam}$ have revealed that all of these proteins have a similar folded structure (Nelson and Strobel 1989). They possess a common structural core on which the tertiary structures of all P450 proteins can be based. One such study modelled bovine P450scc, which is closely related to 11β-hydroxylase and aldosterone synthase, on the crystal structure of $P450_{cam}$. Sequence homology and physical properties were also considered. This showed that there were regions that were highly conserved such as the haem binding domain and regions involved in oxygen activation, and regions that were less well conserved such as the substrate binding regions. These latter regions will display greatest variabilty as they catalyse the conversion of substrates which

differ in size, shape, origin and chemistry. It is therefore to be expected that P450 enzymes which catalyse the same reactions and are from different species will display greatest homology. This being the case, it is possible to use the structure of a simple bacterial P450 to model the unknown structures of the more complex mammalian enzymes.

4.2 Methods

Protein sequences obtained from the Swissprot database. Accession numbers are listed below:

11β-hydroxylase	human p15538, sheep p51663, rat B1 p15393 & B3 p30100, mouse p15539, bovine p15150.
Aldosterone synthase	human p19099, rat p30099.
Side chain cleavage enzyme	human p05108, pig p10612, rat p14137, bovine p00189, oncmy Q07217.

The crystalline structures were obtained from pdb Brookhaven (Brookhaven National Laboratory Protein Databank) (Bernstein et al. 1977)

Accession numbers were:

$P450_{cam}$:	PDB:1CP4
$P450_{BM3}$:	PDB: 2HPD
$P450_{terp}$:	PDB: 1CPT
$P450_{NOR}$:	PDB: 1ROM
$P450_{ERY}$:	PDB: 10XA

Multiple Sequence Alignment

Sequence alignment was performed using the Multi-align Maxhom multiple sequence alignment. Human 11β-hydroxylase and aldosterone synthase amino acid sequences

were aligned using multi-align to the bacterial enzyme $P450_{cam}$, $P450_{BM3}$, $P450_{terp}$, $P450_{NOR}$ and $P450_{ERY}$ known crystal structures.

Human 11β-hydroxylase and aldosterone synthase each have 503 amino acids. The bacterial enzymes used in the alignment exercise have 60-100 fewer residues. Additional residues are distributed throughout the molecule but 30-50 residues at the start or N-terminus of 11β-hydroxylase and aldosterone synthase proteins constitute the mitochondrial sequence which inserts and anchors these enzymes into the inner mitochondrial membrane. As the crystallised bacterial structures are not membrane-bound, the alignment begins after this N-terminal sequence in human 11β-hydroxylase and aldosterone synthase. Gaps were introduced where regions lacked homology. The size and number of these gaps were varied by the program to optimise the positioning of gaps relative to highly conserved regions such as the oxygen binding site or the area surrounding the thiolate ligand. These gaps identify regions in human 11β-hydroxylase and aldosterone synthase where insertions may have occurred to accomodate the larger substrate. Human 11β-hydroxylase and aldosterone synthase and side chain cleavage enzymes can also be aligned to the same enzymes from other species. Again the introduction of gaps was necessary but to a lesser extent as these enzymes share greater homology.

Secondary structure prediction

Secondary structure prediction was performed by Procheck (Laskowski et al. 1993).

Modelling

The program Swiss-model was used to model the three-dimensional (3-D) structure of human 11β-hydroxylase and aldosterone synthase based on a consensus structure derived from the crystal structures of $P450_{cam}$, $P450_{BM3}$, $P450_{terp}$, $P450_{NOR}$ and $P450_{ERY}$ (Pietsch 1996). The predicted 3-D structure was superimposed on the crystalline structure of $P450_{cam}$. The model was refined manually to remove unrealistic features such as loops or wandering regions which, from their appearance, could contribute to or form a β-sheet or an α-helical structure.

4.3 Results

4.3.1 Sequence alignment

The results of sequence alignments are shown in **figures 4.3a and 4.3b.**

Sequence alignment of human 11β-hydroxylase and aldosterone synthase with the bacterial cytochrome P450 crystal structures **(figure 4.3a)** shows that there are 12 absolutely conserved residues between human 11β-hydroxylase and aldosterone synthase and all five crystal structures included in the alignment. The cysteine residue which forms the thiolate ligand is absolutely conserved in all cytochrome P450 enzymes and is marked with an asterisk. The threonine involved in oxygen activation is also indicated. Between human 11β-hydroxylase and aldosterone synthase and $P450_{cam}$,, there are 40 invariant residues. Absolutely conserved residues found in all the enzymes included in the alignment are in upper case in the consensus row and are shown in red.

Sequence alignment of human with other mammalian 11β-hydroxylase, aldosterone synthase and side chain cleavage enzymes **(figure 4.3b)** shows a greater number of absolutely conserved residues than that seen in **figure 4.3a**. There are 120 out of the 500 residues aligned within this group that are invariant. Finally between 11β-hydroxylase and aldosterone synthase enzymes from various species, 264 residues are invariant. The putative I-helix, steroid-binding domain and mitochondrial leader are indicated in **figure 4.3b.**

4.3.2 Secondary Structure prediction

The predicted secondary structure based on sequence and alignment alone is shown below:

Helix		**Sheet**		**Helix**
45-60	**A**	92-95		335-342 **K**
96-108	**B'**	160-162		375-380
122-130	**C**	262-271		402-410
135-155	**C/D**	352-360	β_3	420-440 **L**
165-175	**D**	363-372	β_4	
187-205		442-467	β_5	
215-220	**F**			
227-245	**G**			
267-317				
322-332	**I**			

Based on mutations and structure-function studies of these enzymes in the literature, residues 299-338 form part of the I-helix (Nonaka et al. 1989). Also mutation of residues 331, 337 and 427 disturb helices I, K and L respectively (Geley et al. 1996). Mutation of the leucine at position 464 in 11β-hydroxylase also disturbs the L-helix (Geley et al. 1996). Modelling, based on $P450_{cam,}$ of the closely related bovine side-chain cleavage enzyme (Vijaykamur and Salerno 1992) has identified particular helices (A, B', C, D, F, G, I and L) and β-sheet regions (β_3, β_4 and β_5). From these observations, it is possible to assign some of the helical regions and β-sheet regions from the secondary structure prediction of human aldosterone synthase and 11β-hydroxylase a specific locus nomination. These are indicated above.

4.3.3 Modelling based on known structures

A model three-dimensional structure of human aldosterone synthase and 11β-hydroxylase with the incorporated haem is shown in **figure 4.3c.** The model structure could be superimposed on that of P450cam indicating that in this model the positioning of the helices was conserved. The largest helix, the I-helix, runs through the centre of the molecule in close proximity to the haem. Residues of interest to this present study (chapters 3 and 5) are indicated on the structure.

From the model these residues can be assigned to particular regions of the molecule.

43	Rope/loop flanking helix A (exterior of the molecule)
107	Helix B' surrounding core
147	Flanking helix at interphase between rope/helix (between C and D).
186	At end of helix D.
248	Helix towards haem core (G-helix)
357	β-sheet exterior of molecule
493	β-sheet exterior of molecule

Using this method it is possible to assign residues to particular regions and hypothesise structural and functional roles.

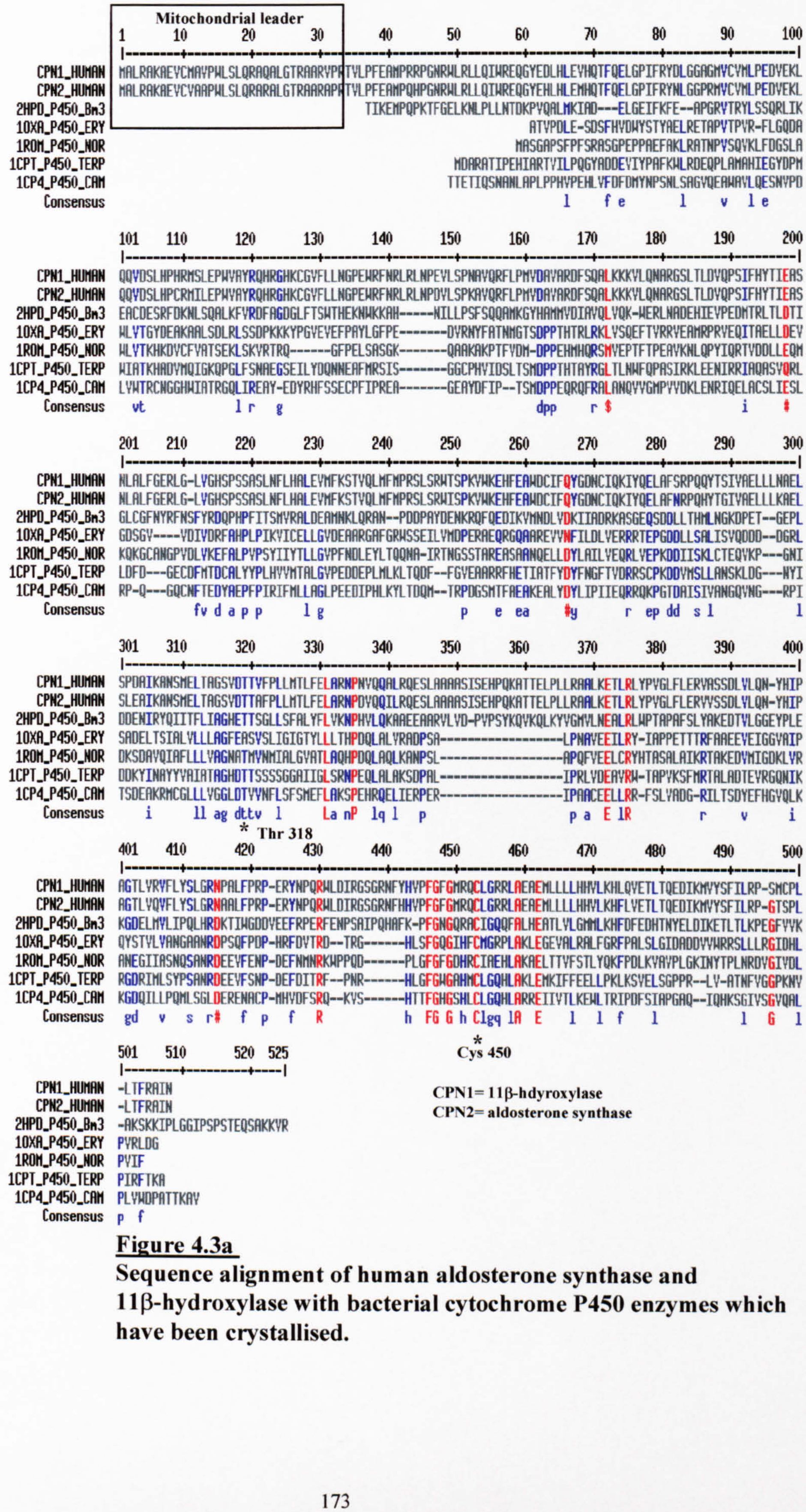


Figure 4.3a

Sequence alignment of human aldosterone synthase and 11β-hydroxylase with bacterial cytochrome P450 enzymes which have been crystallised.

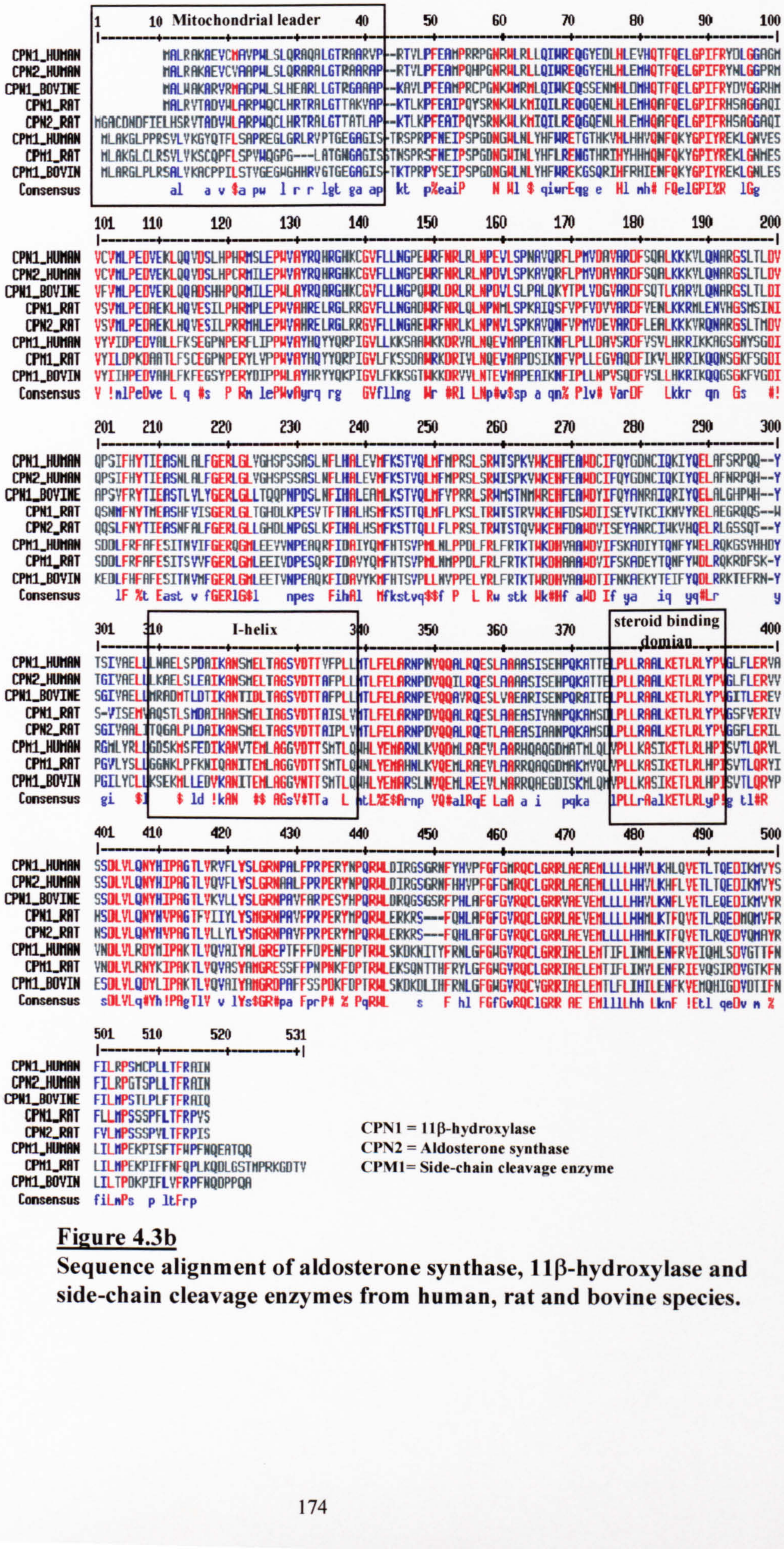


Figure 4.3b

Sequence alignment of aldosterone synthase, 11β-hydroxylase and side-chain cleavage enzymes from human, rat and bovine species.

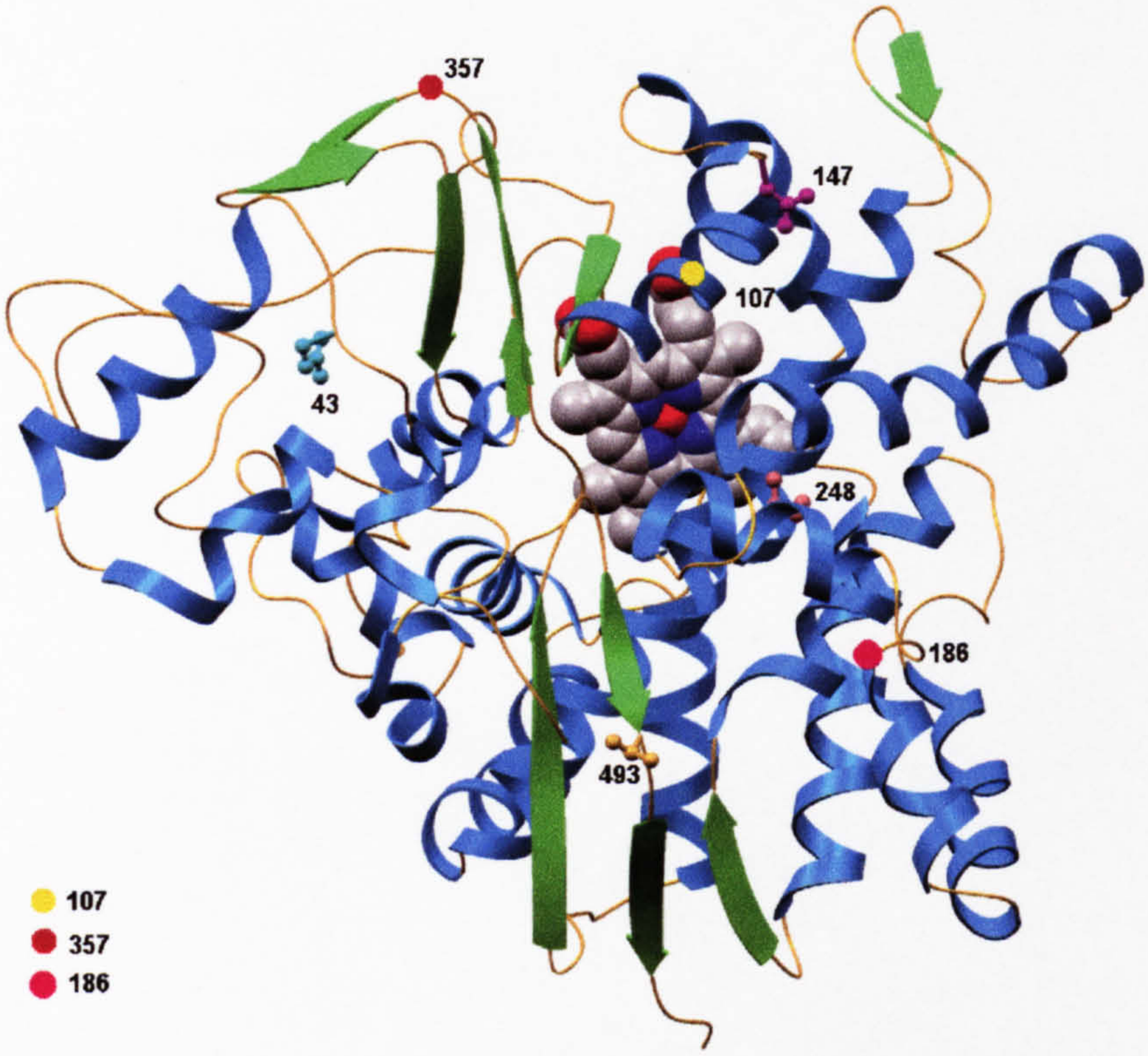


Figure 4.3c

3-Dimensional model of human aldosterone synthase and 11β-hydroxylase enzymes.

4.4 Discussion

It is clear that all P450's have a common folded structure despite having low sequence homology . From this present study, sequence alignment identified regions displaying a high degree of homology between bacterial protein sequences and those from mammals. Modelling based on known structures showed that the predicted three dimensional model of human 11β-hydroxylase and aldosterone synthase could be superimposed upon known structures such as P450cam. The overall sequence length is greater in mammalian enzymes than in the bacterial enzymes but these are distributed throughout the molecule as inserts in loops and are probably of little consequence.

The positioning of the helices remains the same in all P450's and is especially conserved surrounding the haem group. It is obvious that regions surrounding the haem and the I-helix, the key catalytic and functional centre, will display the greatest degree of structural similarity and that modelling should take this feature strongly into account to position the remaining helices.

Helices I and L form primary contact points with the haem and constitute the inner C-terminal domain (see section 1.7). The large I-helix is in the centre of the model running through the core in close proximity to the haem. The N-terminal domain is to the exterior of the molecule, controlling entry and binding of substrates, and is wrapped around the inner domain . These N-terminal regions are more difficult to model as they display the highest degree of variability between P450s. In this model of human aldosterone synthase and 11β-hydroxylase, the N-terminal regions required manual modification, indicating that there was significant structural differences due to the lack of homology between them and their bacterial relatives.

From secondary structure prediction and modelling studies, various residues of interest to this present study were assigned to a particular region. In chapter 3, several residues in aldosterone synthase were substituted for the 11β-hydroxylase equivalent at positions 43, 147, 248 and 493. Also, the analogous substitution was made in 11β-hydroxylase at position 147. These enzymes are highly homologous yet have strikingly different properties. Substitutions were performed to assess the functional

consequence of changes of particular residues. In chapter 5, screening of normotensive and hypertensive populations for mutations in the genes encoding aldosterone synthase (CYP11B2) and 11β-hydroxylase (CYP11B1) identified differences in the nucleotide sequence which result in amino acid changes at positions 107, 186 of 11β-hydroxylase and 357 of aldosterone synthase. Using the structural information obtained from modelling and results from structure-function studies, it was possible to suggest roles for these residues. Secondary structure prediction revealed whether they formed part of α-helices, β-sheets or of loops between them. The location of these structural segments is not exact; regions of helices, for example, may span shorter or longer amino acid distance than the secondary structure prediction suggests. Areas predicted as flanking helices, for example, may in fact form part of that helical structure itself. Progressing from secondary structure prediction to the three-dimensional model emphasises this.

Residue 43 clearly lies in a region of loop or rope. Structure-function studies revealed that substitution at this locus in aldosterone synthase to the 11β-hydroxylase residue (Q43R) had no effect on conversion of DOC (see chapter 3). This implies that a glutamate (Q) or arginine (R) at this locus are only accomodated in the structure and do not have a specific functional role. Residue 147 is positioned in the model at the immediate boundary of helix-D. Mutations at this locus may have functional implications. For example, mutations may alter the positioning of the adjacent helix and thus effect overall protein conformation which may have effects on enzyme activity. This residue of aldosterone synthase (D147) was replaced for the 11β-hydroxylase equivalent (E147) and was shown to increase 11β-hydroxylase activity which was specific for the substrate, DOC (chapter 3). The converse mutation decreased 11β-hydroxylase activity. It is therefore possible that 11β-hydroxylase-specific glutamic acid (E147) positions the helix in an orientation more favourable for the 11β-hydroxylation of the substrate DOC.

From these predictions residues 107, 186 and 248 lie within helical regions B', at the end of D and G respectively. Helix B' surrounds the active core and has been designated as SRS-1 in $P450_{cam}$ (Gotoh et al. 1992). This region and part of the F-G

regions are thought to form part of the substrate access channel. Residues within this region may form substrate-specific contact points. Residue 248 at the end of helix G, like residue 107, may play a role in substrate access and recognition. However, results from structure-function studies, where the aldosterone synthase specific residue (I248) was replaced by the 11β-hydroxylase equivalent (T248) (chapter 3), suggest that this is not the case and, like residue 43, 248 contributes to the framework of the molecule. It is possible that, in each enzyme, the isoleucine (I) and threonine (T) perform the same function. This is a relatively conservative amino acid change and perhaps alteration at this locus to a residue with properties distinct from those of isoleucine and threonine may exert demonstrable effects.

Residues 357 and 493 are within regions forming β-sheet which may form part of β_3, β_4 or β_5. Both residues are exposed at the surface of the molecule and so any functional roles they may have could involve interaction with adrenodoxin or with the substrate. β_3 is thought to be involved in adrenodoxin binding whereas β_4 is a substrate recognition sequence in $P450_{cam}$ (Gotoh et al. 1992). Mutation K357N did not alter substrate specificity or enzyme activity which suggests that this particular substitution, a lysine (K) for an asparagine (N), does not alter substrate interaction (chapter 5). Substitution of the aldosterone synthase-specific residue, T493 for the 11β-hydroxylase-specific residue M493, did not alter in vitro enzyme activity. That this conservative substitution does not alter activity does not preclude the idea that substitution at this locus with an amino acid with different properties may alter function.

Summary

Using modelling techniques, residues of interest pertaining to this present study have been assigned to particular regions of the enzyme molecule and their possible functional implications discussed. Although a valuable method, it is important to emphasise that the secondary structure and three-dimensional model are theoretical. To obtain an accurate picture of these enzymes it is necessary to obtain a high resolution x-ray structure .

CHAPTER 5

Chapter 5

5.1 Screening of coding regions of human CYP11B1 and CYP11B2 genes for mutations in normotensives and essential hypertensives.

Mutations of the CYP11B1 and CYP11B2 genes cause 11β-hydroxylase and aldosterone synthase deficiencies respectively (see 1.8.4 and 1.8.6). In 11β-hydroxylase deficiency, mutations completely abolish enzyme activity and are found in all exons but cluster in exons 6,7 and 8 (Curnow et al. 1993). Mutations consist of frameshift, insertions or nonsense mutations which ultimately result in a premature stop codon resulting in a truncated non-functional protein. Other mutations are missense which mutate critical functional residues and have deleterious effects on 11β-hydroxylase in this condition.

In aldosterone synthase deficiencies, CMO-I and II, mutations affect the 18-functions of aldosterone synthase causing accumulation of B or 18-OHB respectively. In comparison to 11β-hydroxylase deficiency, mutations causing aldosterone synthase deficiencies are less severe in their *in vivo* manifestations as there is not complete aberration of enzyme activity. When expressed *in vitro* however, CMO-I mutations inactivate the encoded enzyme whereas CMO-II mutations cause slight decreases in 11β-hydroxylation and loss of both 18-functions. In several instances multiple mutations have been identified in individual cases. The effects of these mutations individually and in combination have been assessed *in vitro*. It is clear that effects of mutations differ in their degree of severity. Milder mutations such as V386A may cause subtle changes in aldosterone production which are not obvious *in vivo*. It is possible that subtle steroid abnormalities may also exert detrimental effects, for example on blood pressure. Mutations which cause a mild increase in aldosterone production could be implicated in some forms of hypertension.

Essential hypertension is a multifactorial disease, the cause of which, unlike 11β-hydroxylase deficiency-induced hypertension, is unknown. Several studies have inferred that some essential hypertensives have differences in their urinary and plasma steroids compared to control subjects (De Simone et al.1985, Soro et al.1995) Whether these differences are due to mutations in the genes which produce these

steroids is not known. In this study, the CYP11B1 and CYP11B2 genes from hypertensive and normotensive control populations were screened exon by exon to look for mutations.

5.2 Methods

Genomic DNA was extracted from blood (2.3.2) and individual exons of the CYP11B1 and CYP11B2 genes amplified using PCR (2.3.3). Single stranded conformational polymorphism (SSCP) was used to detect sequence changes in exonic regions as determined by changes in electrophoretic mobility (2.3.6). As the sensitivity of SSCP is decreased greatly with PCR fragments greater than 300bp (Hayashi et al. 1991), digestions were performed to obtain fragments of suitable size (2.3.8). Where possible, primers were designed to specifically amplify CYP11B1 or B2. However, because of the high degree of homology, this was not always possible and some primer pairs amplified CYP11B1 and CYP11B2 within the same reaction. Therefore, to differentiate between the CYP11B1 and B2 amplified alleles, the PCR products were subcloned into T-vector which facilitated allele separation and sequencing (2.3.14). Where alteration in the nucleotides resulted in amino acid substitutions in the encoded protein, functional *in vitro* studies were performed using transient transfection in COS-7 cells (2.5). Mutations found in subjects were mimicked in pCMV4 B1 or B2 constructs by site-directed mutagenesis using primers containing the desired mutation (2.4).

5.3 Results

Screening of exonic regions of the CYP11B1 and CYP11B2 genes for mutations

5.3.1 PCR optimisation.

Prior to SSCP screening, PCR conditions were optimised for each pair of primers. The annealing temperatures were set 5-10°C below the melting temperature of primers and increased incrementally until PCR conditions were optimised. Typical examples for exons 5 and 6 of CYP11B2 are shown in **figure 5.3a.** PCR reactions were performed on 4 genomic DNA samples chosen at random where the primers used were T2141,T2138 and T2142, T2140 respectively. Amplification of exons 5 and 6 using these primers produced PCR amplicons of 337 and 224 bp respectively as determined

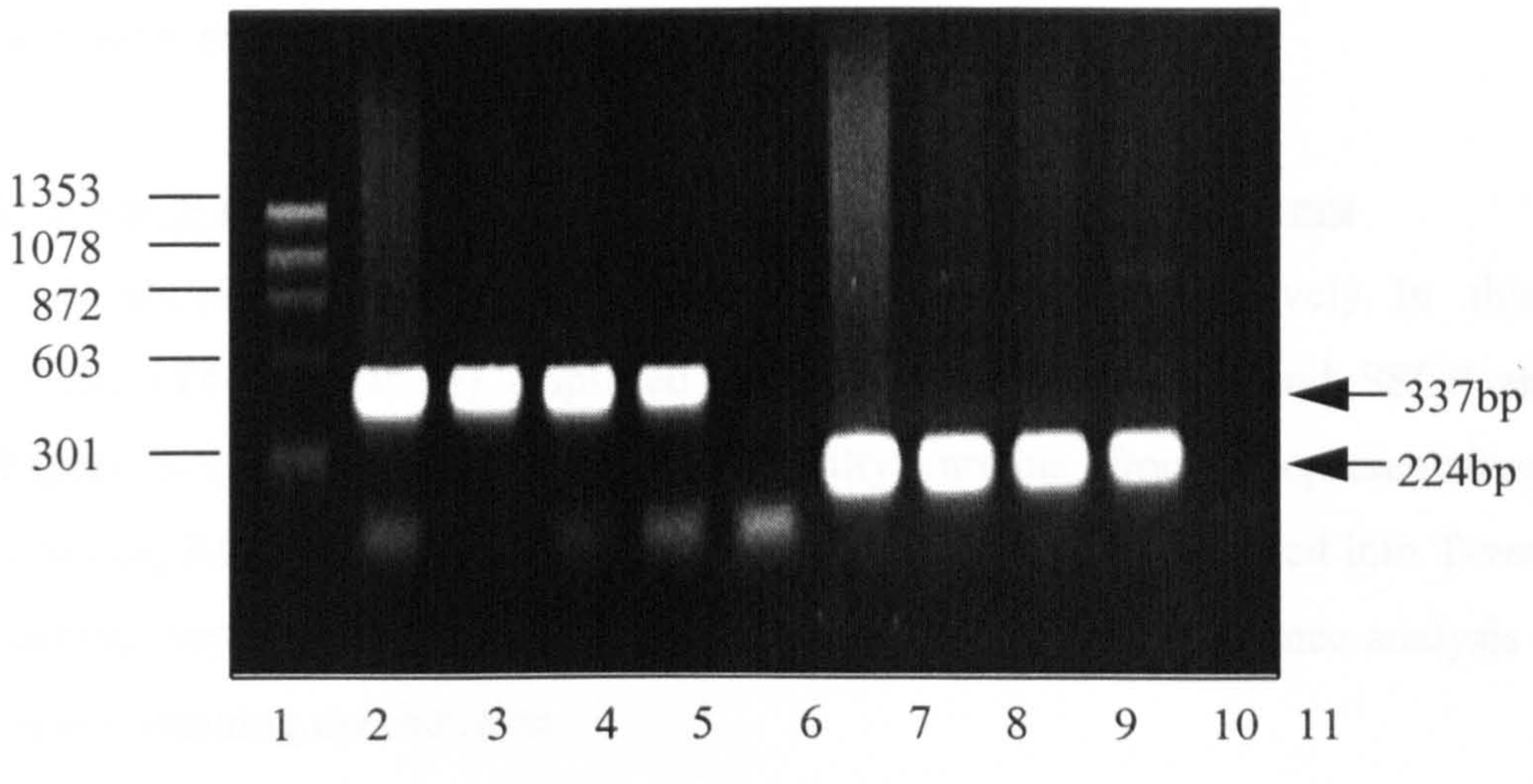


Figure 5.3a Optimised PCR reaction for exons 5 and 6 of CYP11B2

A 1% agarose gel showing optimised PCR of exons 5 and 6 of CYP11B2 using primers T2141, T2138 and T2142, T2140 respectively (see tables 3 and 4 appendix 2). PCR conditions for both were 94 for 1min, 60 for 1min, 72 for 1min for 30 cycles. Lane 1 shows λ DNA Hae III digest as a marker. Exon 5 amplicons were 337bp and are shown in lanes 2-5. Exon 6 amplicons were 224bp and are shown in lanes 7-10. Lanes 6 and 11 show water blank controls.

by agarose gel electrophoresis using λHae III digest as a marker. Primer sequences, specificity and annealing temperatures used are detailed in table 4 in appendix 3

5.3.2 SSCP analysis showing no electrophoretic mobility changes.

Figure 5.3b. Shows a representative area of SSCP analysis of PCR amplified exon 1 of the CYP11B2 gene. No electrophoretic mobility variants were detected in the sample number studied. This was also the case for exons 2, 3, 4, 5, 7 and 8 of CYP11B2 and also exons 4, 5, 7, 8 and 9 of CYP11B1. Exons 1 and 6 of CYP11B1 were not screened.

5.3.3 SSCP analysis of exon 2 of the CYP11B1 and CYP11B2 genes

It was not possible to amplify exon 2 of CYP11B1 or B2 selectively. In this case, primers (Y6265, Y6266) amplified this region of both genes and SSCP analysis **(Figure 5.3c.)** shows electrophoretic mobilty variants, from a representative area, following Rsa I digestion. Samples showing variants were subcloned into T-vector to facilitate separation of CYP11B1 and CYP11B2 alleles and sequence analysis of the allele containing the mutation.

5.3.4 SSCP analysis of exon 3 of the CYP11B1 and CYP11B2 genes

Again, due to the high degree of homology, exon 3 of CYP11B1 or B2 could not be selectively amplified. As described in 5.3.3 , both CYP11B1 and CYP11B2 exon 3 were amplified using primers Y6263, Y6264. SSCP analysis of a representative area **(Figure 5.3d.)** shows electrophoretic mobility variants following HhaI digestion. Those samples expressing differences in mobility, as indicated by the arrows, were sub-cloned into T-vector as described in 5.3.3.

5.3.5 SSCP analysis of exon 6 of the CYP11B2 gene

Exon 6 of the CYP11B2 gene was selectively amplified using primers T2142, T2140. As the amplicon size was less than 250bp no digestion was required. SSCP analysis is shown in **(figure 5.3e).** Electrophoretic mobility variants of exon 6 were detected in one single sample as shown by the arrows.

CYP11B2 exon 1 SSCP

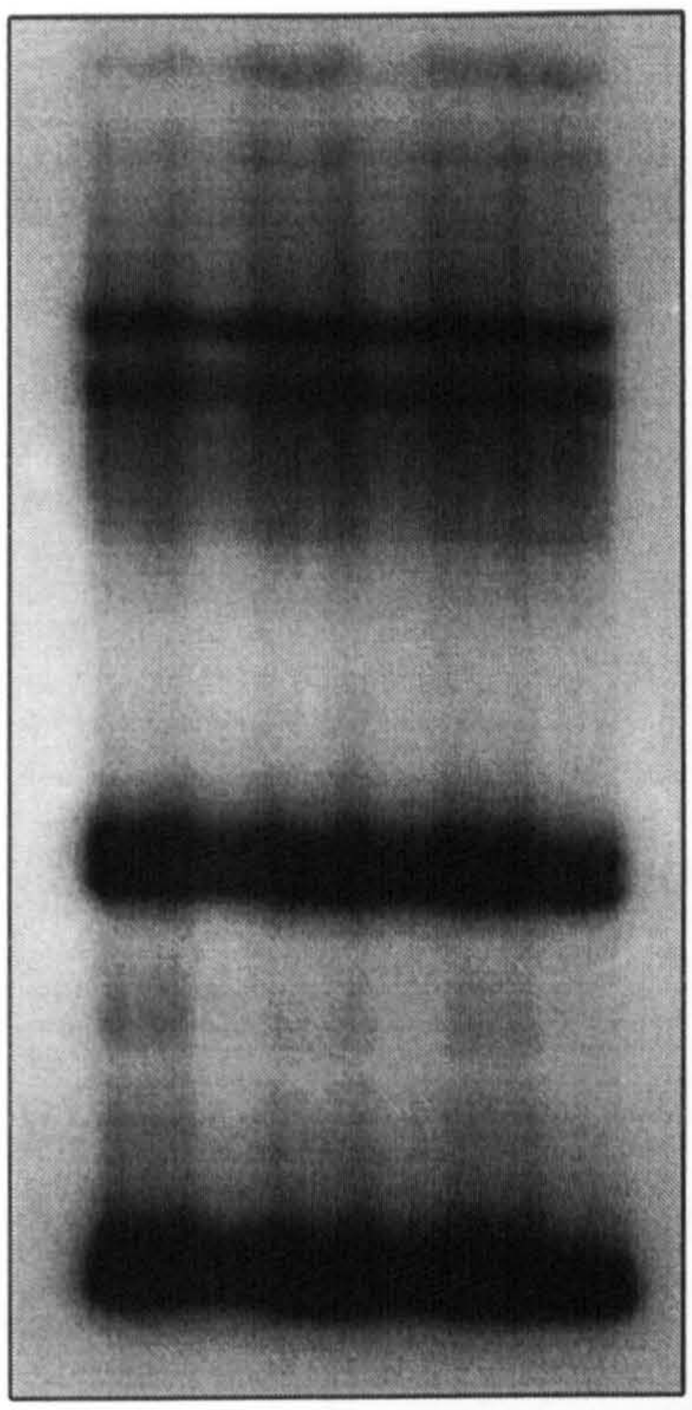


Figure 5.3b. CYP11B2 exon 1 SSCP

SSCP analysis of PCR amplified/ Hha I digested DNA fragments of exon 1 of the human CYP11B2 gene. PCR conditions and specific primers are shown in table 3 appendix 2. Non-denaturing acrylamide gel electrophoresis (6%) gel was carried out at room temperature (RT), 30W for 4/5 hours in the presence of 10% glycerol and 1X TBE. No electrophoretic variants were detected.

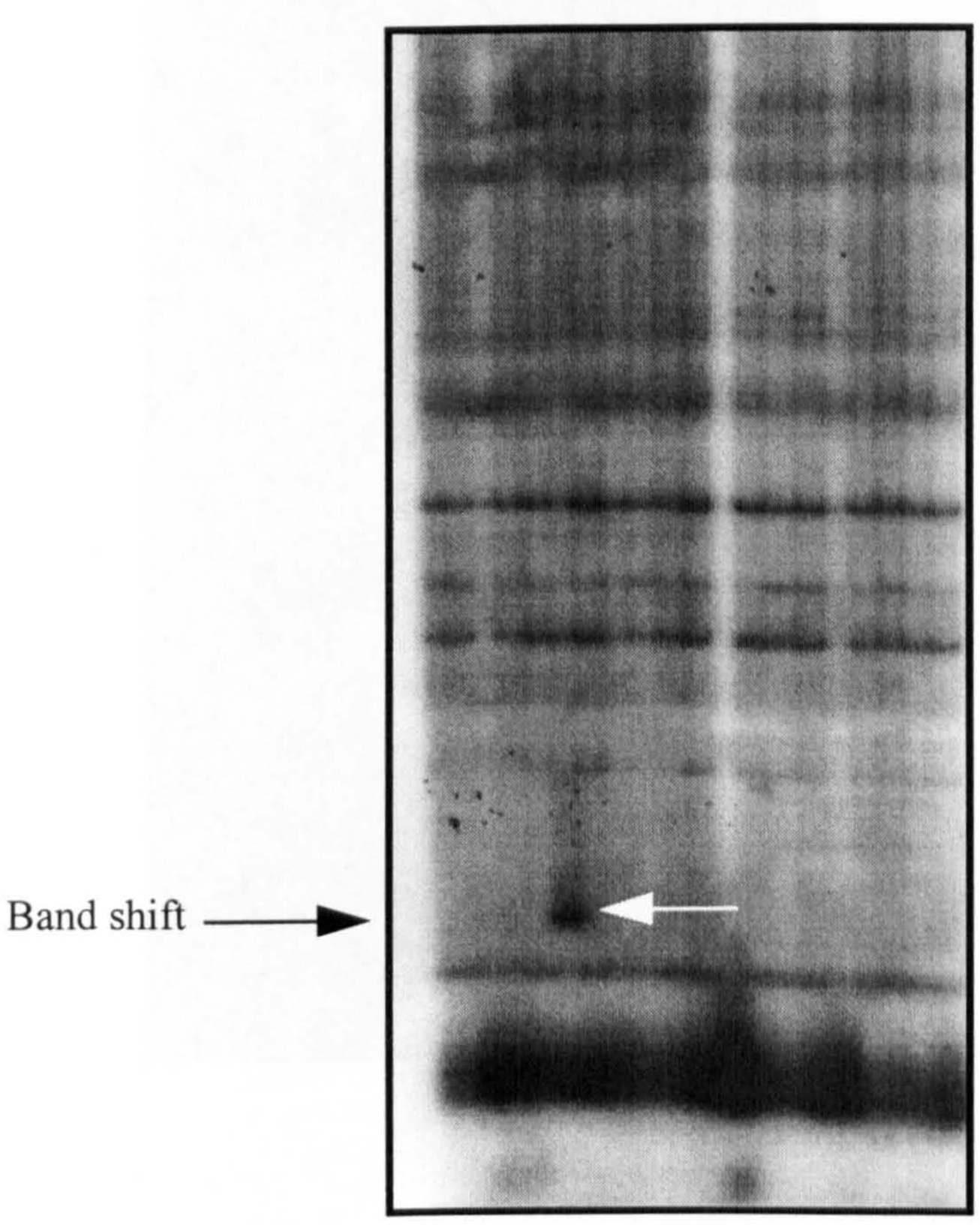


Figure 5.3c. CYP11B1 and CYP11B2 exon 2 SSCP

SSCP analysis of PCR amplified/ Rsa I digested DNA fragments of exon 2 of the human CYP11B1 and CYP11B2 genes. PCR conditions and specific primers are shown in table 3 appendix 2. Non-denaturing acrylamide gel electrophoresis (6%) gel was carried out at room temperature (RT), 30W for 4/5 hours in the presence of 10% glycerol and 1X TBE. Electrophoretic variants are shown by the arrows which were subsequently analysed.

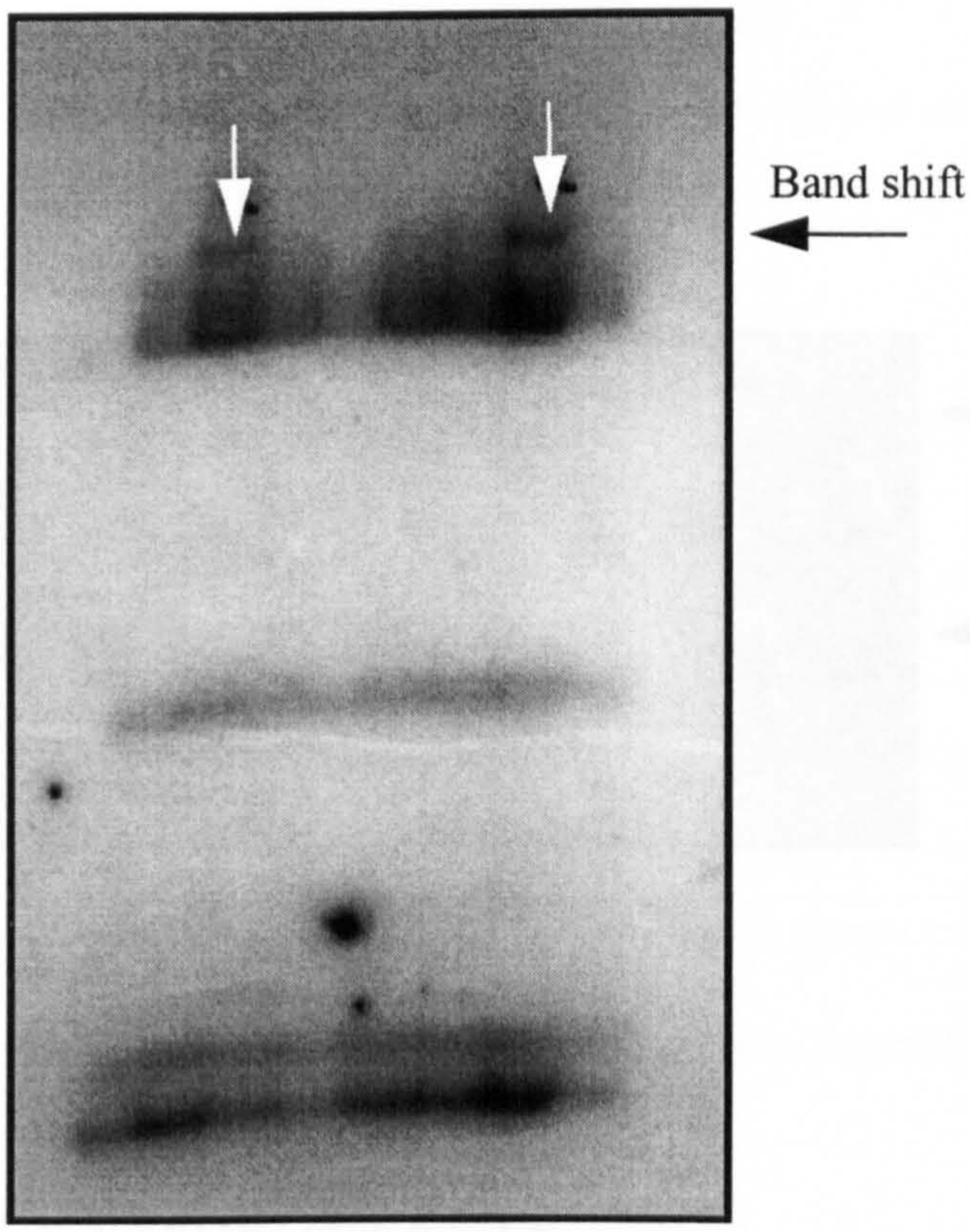


Figure 5.3d. CYP11B1 and CYP11B2 exon 3 SSCP
SSCP analysis of PCR amplified/ Hha Idigested DNA fragments of exon 3 of the human CYP11B1 and CYP11B2 genes. PCR conditions and specific primers are shown in table 3 appendix 2. Non-denaturing acrylamide gel electrophoresis (6%) gel was carried out at room temperature (RT), 30W for 4/5 hours in the presence of 10% glycerol and 1X TBE. Electrophoretic variants are shown by the arrows which were subsequently analysed.

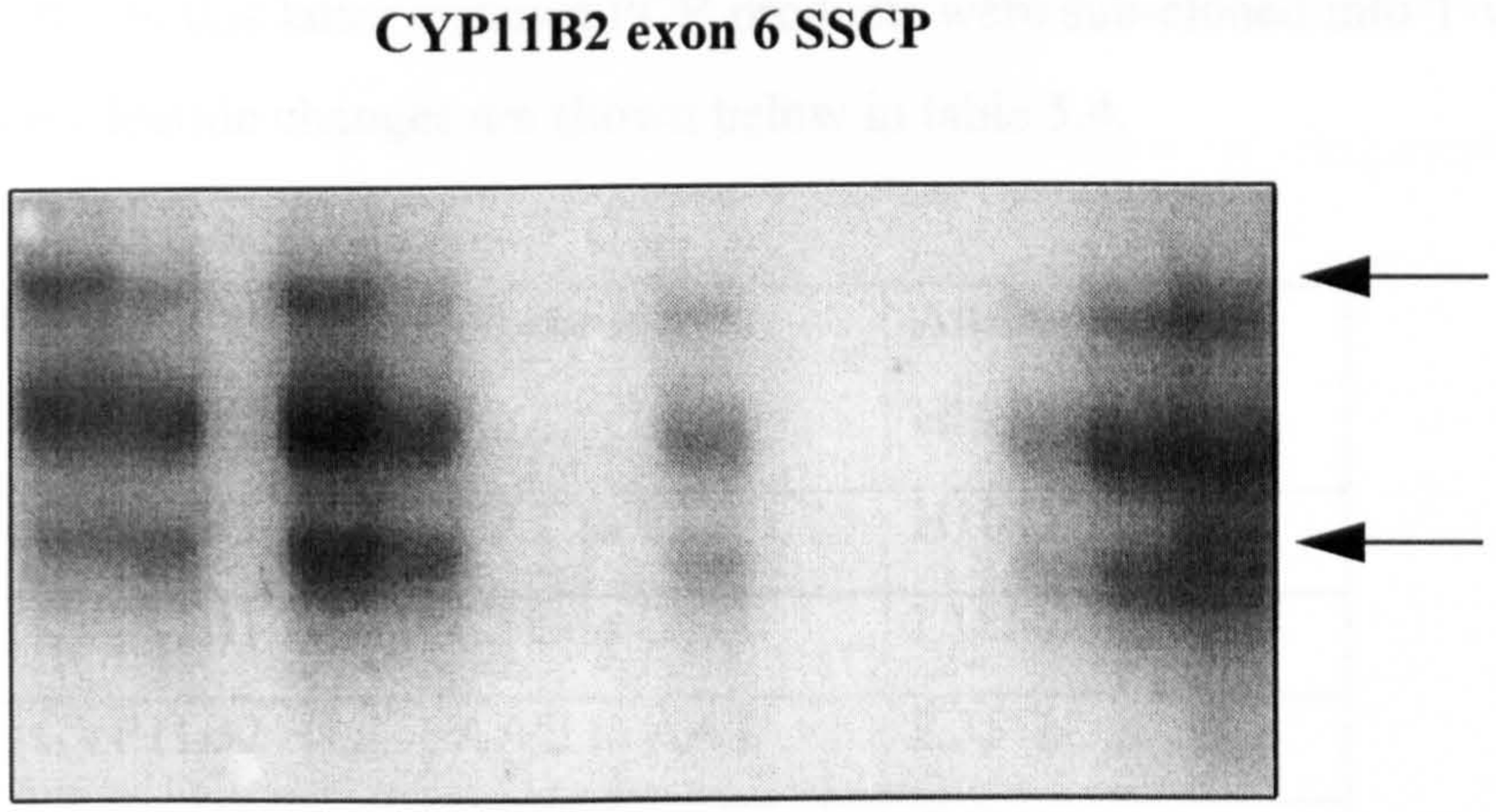


Figure 5.3e. CYP11B2 exon 6 SSCP

SSCP analysis of PCR amplified DNA of exon 6 of the human CYP11B2 gene. PCR conditions and specific primers are shown in table 3 appendix 2. Non-denaturing acrylamide gel electrophoresis (6%) gel was carried out at room temperature (RT), 30W for 4/5 hours in the presence of 10% glycerol and 1X TBE. Electrophoretic variants are shown by the arrows which were subsequently analysed.

5.3.6 SSCP analysis of exon 9 of the CYP11B2 gene

Exon 9 of CYP11B2 was selectively amplified using specific primers (S6719, M6641). The amplicon size was 403bp. SSCP analysis is shown in **figure 5.3f.** following Stu I digestion. A single variant is indicated with an arrow.

5.4 Analysis of electrophoretic mobility variants

Variants were detected in exons 6 an 9 of CYP11B2 and exons 2 and 3 of CYP11B1 or B2. In this latter instance PCR products were sub-cloned into T-vector. A summary of nucleotide changes are shown below in table 5.4.

EXON	Mutation	Amino acid change
2 CYP11B1	CAT to TAT	H107Y
3 CYP11B1	CTG to GTG	L186V
6 CYP11B2	AAG to AAT	K357N
9 CYP11B1	TGC to TTC	C494F

Table 5.4 Summary of nucleotide differences and resulting amino acid change

5.4.1 Sequence analysis of exon 6 of CYP11B2

Autoradiograph (**Figure 5.4a.**)shows the nucleotide sequence 5' to 3' of a section of exon 6 of the hypertensive sample compared to a normal CYP11B2. The sense primer T2142 used in the PCR amplification was used to sequence the region amplified. Using the antisense primer T2140 the region was also sequenced in the opposite direction (not shown) to ensure that the sequence change was incorporated on both strands. The sequence starts at the top with codon CAG (356) and ends with nucleotide A. The published sequence (see appendix 1) for this region is shown in the normal sequence CAG AAG GCA ACC A. The corresponding hypertensive sequence is CAG AAG/T GCA ACC A showing both AAT and AAG at codon 357. The presence of an aberrant T band indicates heterozygosity.

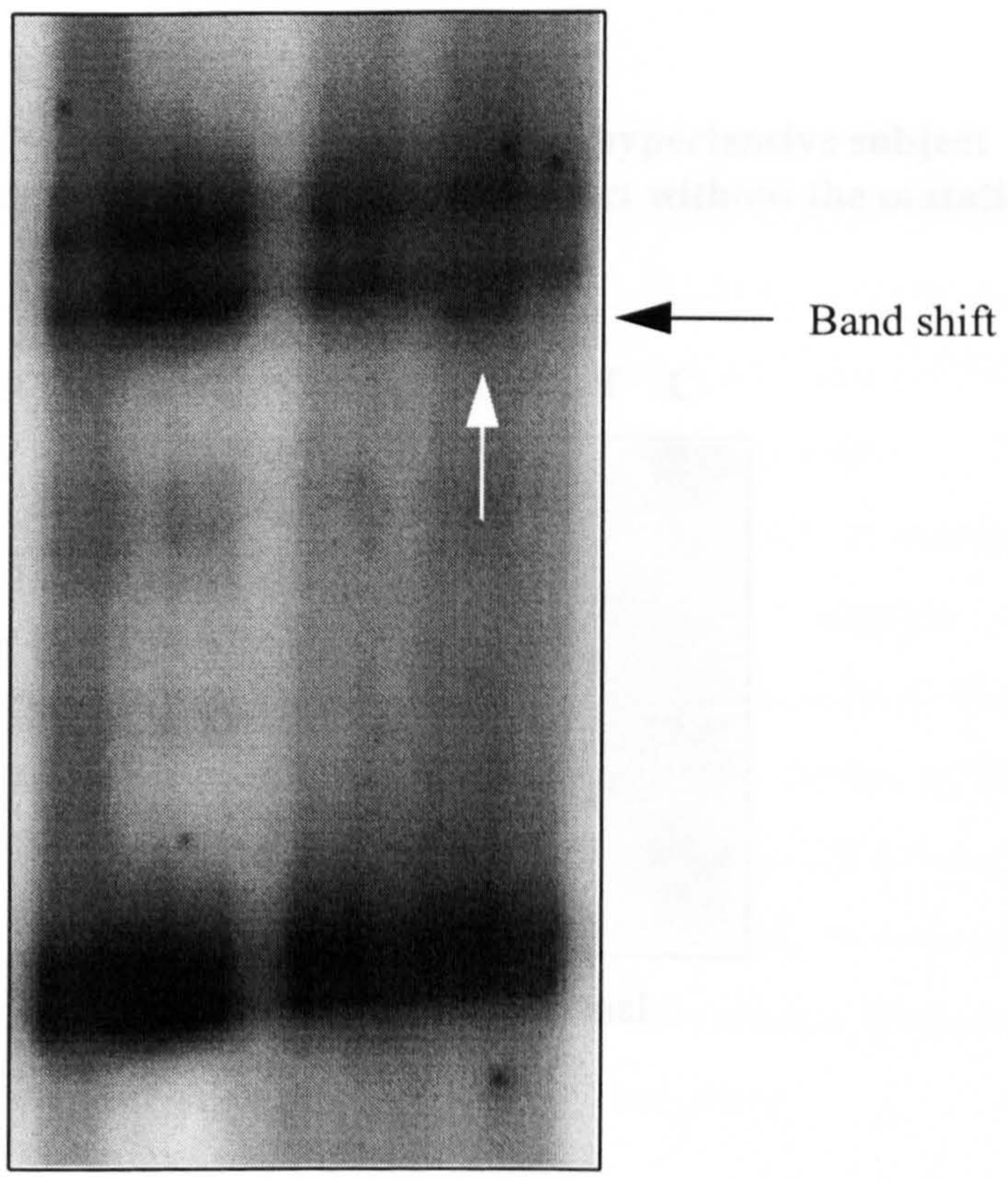


Figure 5.3f. CYP11B2 exon 9 SSCP

SSCP analysis of PCR amplified/ Stu I digested DNA fragments of exon 9 of the human CYP11B2 gene. PCR conditions and specific primers are shown in table 3 appendix 2. Non-denaturing acrylamide gel electrophoresis (6%) gel was carried out at room temperature (RT), 30W for 4/5 hours in the presence of 10% glycerol and 1X TBE. An electrophoretic variant is shown by the arrow which was subsequently analysed.

CYP11B2 exon 6- 357 sequence from hypertensive subject carrying the mutation and a normal subject without the mutation.

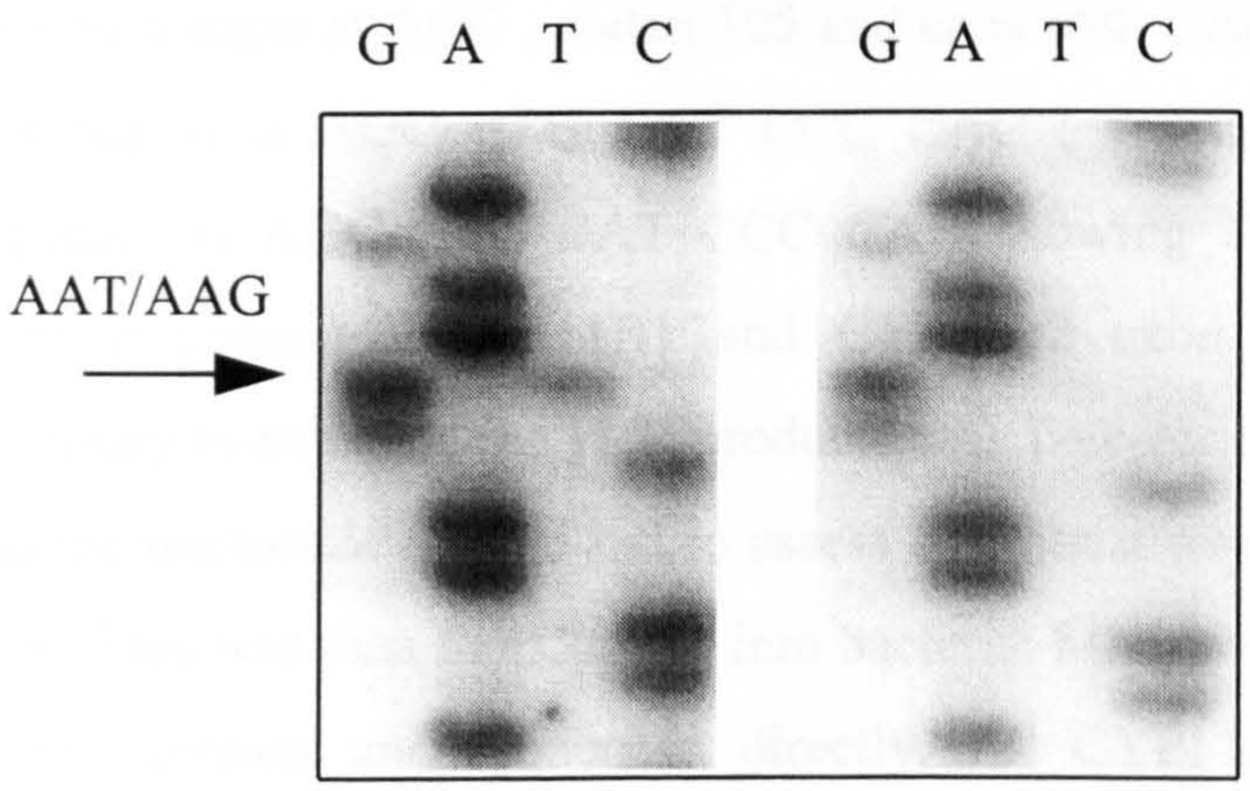


Figure 5.4a. CYP11B2 exon 6 DNA sequence

Nucleotide sequence analysis of a portion of exon 6 of CYP11B2. A portion of sequence ladders of exon 6 in CYP11B2 from a normal healthy individual (normal) and an essential hypertensive (hypertensive) patient are shown. The arrow denotes the point mutation at codon 357.

5.4.2 Sequence analysis of exon 9 of CYP11B2

Figure 5.4b. shows a region of exon 9 of CYP11B2 from a hypertensive patient. The sequence starts at CCC , codon 491 and ends with CTC. The published sequence for this region is CCC AGC ATG TGC CCC CTC CTC(see appendix 1). The hypertensive sequence is CCC AGC ATG TTC CCC CTC CTC showing a TTC at codon 494.

5.4.3 T-vector subcloning and subsequent sequence analysis of exon 2

Figure 5.4c. shows a region of exon 2 of CYP11B1 from a hypertensive patient. The sequence starts at AGC , codon 105 and ends with CAC. The published sequence for this region is AGC CTG CAT CCC CAC (see appendix 1). The hypertensive sequence is AGC CTG TAT CCC CAC showing TAT at codon 107. The PCR reaction contained CYP11B1 and CYP11B2 exon 2 regions. It was therefore necessary to subclone the PCR products into T-vector in order to identify which gene had the nucleotide change and to assess whether it was present on both alleles of the gene. This was then transformed into bacteria. Mini-prep DNA was prepared from 20 single colonies and sequenced directly. All CYP11B2 alleles had sequences as published. Only CYP11B1 alleles had the nucleotide change. Both normal and mutant CYP11B1 alleles were detected indicating heterozygosity.

5.4.4 T-vector subcloning and subsequent sequence analysis of exon 3

Figure 5.4d. shows a region of exon 3 of CYP11B1 from a hypertensive patient. The sequence starts at AGC , codon 183 and ends with TAG. The published sequence for this region is AGC CTG ACC CTG GAC GTC CAG (see appendix 1). The hypertensive sequence is AGC CTG ACC GTG GAC GTC TAG showing GTG at codon 186 followed by a premature stop codon. The PCR products were sub-cloned into T-vector as described in 5.4.3. All CYP11B2 alleles were normal and had sequences as published. Only CYP11B1 alleles had the nucleotide change. Both normal and mutant CYP11B1 alleles were detected indicating heterozygosity.

CYP11B1 exon 9- 494 sequence from hypertensive subject carrying the mutation.

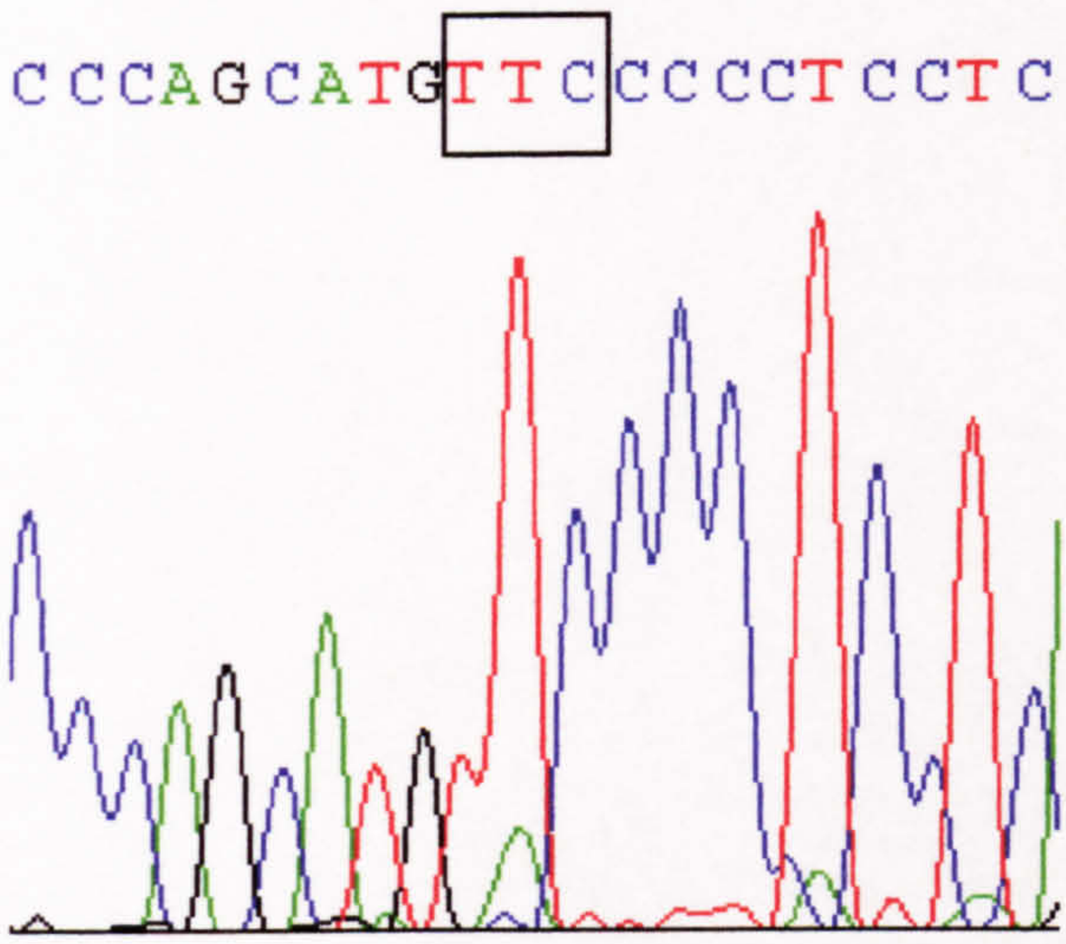


Figure 5.4b. CYP11B1 exon 9 DNA sequence

Nucleotide sequence analysis of a portion of exon 9 of CYP11B1. A portion of sequence of exon 9 in CYP11B1 from an essential hypertensive patient is shown. The box denotes the point mutation at codon 494.

CYP11B1 exon 2- 107 sequence from hypertensive subject carrying the mutation.

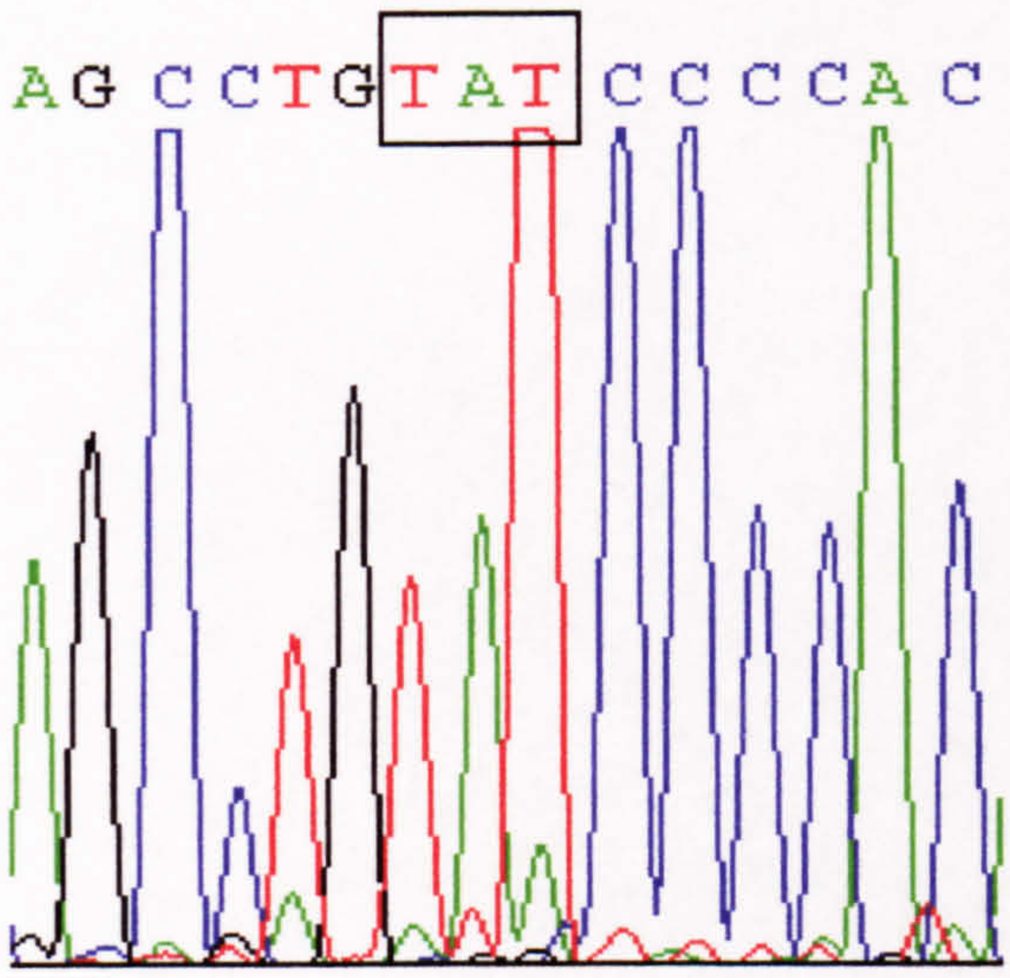


Figure 5.4c. CYP11B1 exon 2 DNA sequence

Nucleotide sequence analysis of a portion of exon 2 of CYP11B1. A portion of sequence of exon 2 in CYP11B1 from an essential hypertensive patient is shown. The box denotes the point mutation at codon 107.

CYP11B1 exon 3- 186 sequence from hypertensive subject carrying the mutation.

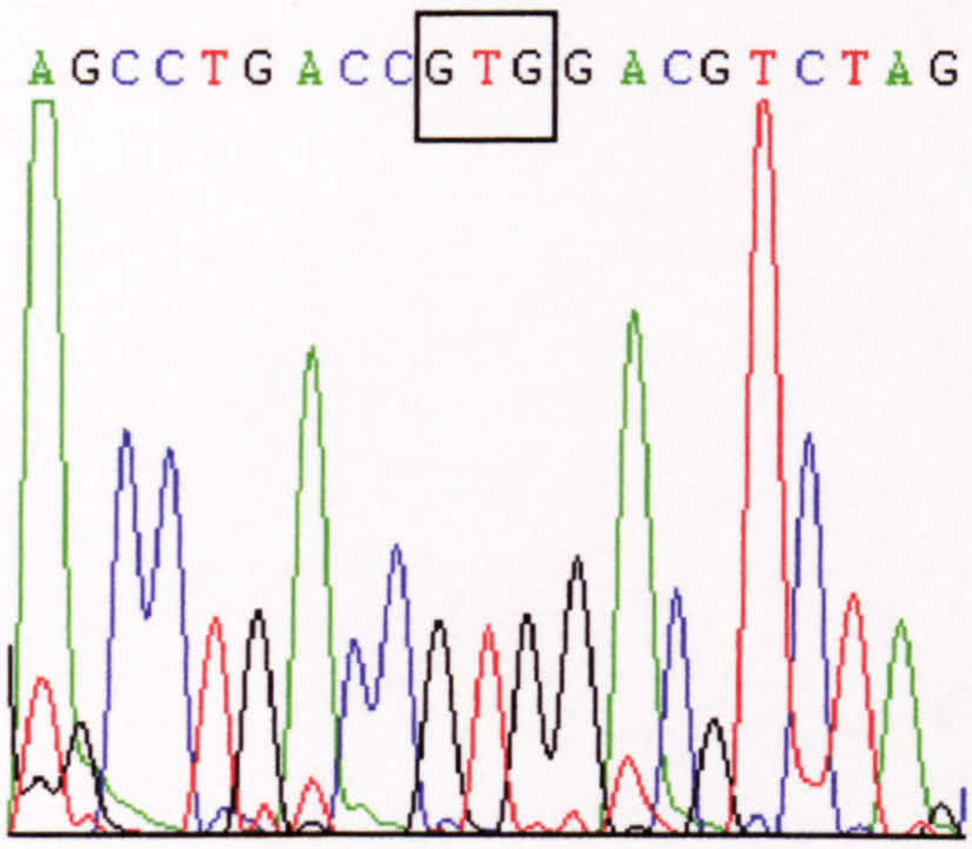


Figure 5.4d. CYP11B1 exon 3 DNA sequence

Nucleotide sequence analysis of a portion of exon 3 of CYP11B1. A portion of sequence of exon 3 in CYP11B1 from an essential hypertensive patient is shown. The box denotes the point mutation at codon 186.

5.4.5 Screening by restriction enzyme digestion

Mutations in exons 3 and 6 generate a restriction enzyme site. In future studies use of restriction digestion may facilitate screening of a large number of samples in an attempt to determine the frequency of these mutations.

EXON	Restriction enzyme	Recognition sequence	Supplier
CYP11B2 exon 6	Bsm I	5' GAATGCN^ 3' 3' CTTAC^GN 5'	Promega
CYP11B1 exon 3	Dsa I	5' C^CRYGG 3' 3' GGYRC^C 5'	Boehringer-Mannheim

Table 5.4.5 Screening by resrtiction enzymes

5.5 Functional Studies

5.5.1 Sequence analysis of wild-type plasmid $pCMV_4$ B2 and mutant construct B2-K357N

The incorporation of the mutations was verified by sequencing. Codon 357 in exon 6 was altered from AAG to AAT, thus changing a lysine (K) to an asparagine (N) residue (**figure 5.5a**). The sequencing primer used was Z0895. (see appendix 2 table 2 for sequences of primers).

5.5.2 Sequence analysis of mutant constructs B1-H107Y and B1-L186V

The incorporation of the mutations was verified by sequencing of exons 2 and 3 respectively using primers pCMV4 (s) and N4019. Codon 107 in exon 2 was altered from CAT to TAT, thus changing a histidine (H) to a tyrosine (Y) residue. Codon 186 in exon 3 (**figure 5.5b.**) was altered from CTG to GTG, thus changing a leucine (L) to a valine (V) residue. Wild-type plasmid $pCMV_4$ B1 sequence is shown in appendix 1.

5.5.3 B production from DOC by mutant B2-K357N

Corticosterone (B) production expressed as nmol/ mg/48 hours from 5μM DOC was measured by radioimmunoassay (**figure 5.5c**). Wild-type aldosterone synthase produced 62.3 ± 7.1 nmol/mg/48 hours and mutant B2-K357N produced 57.4 ± 5.6 nmol/ mg/48 hours (n=6). This shows that B2-K357N has no effect on 11β-

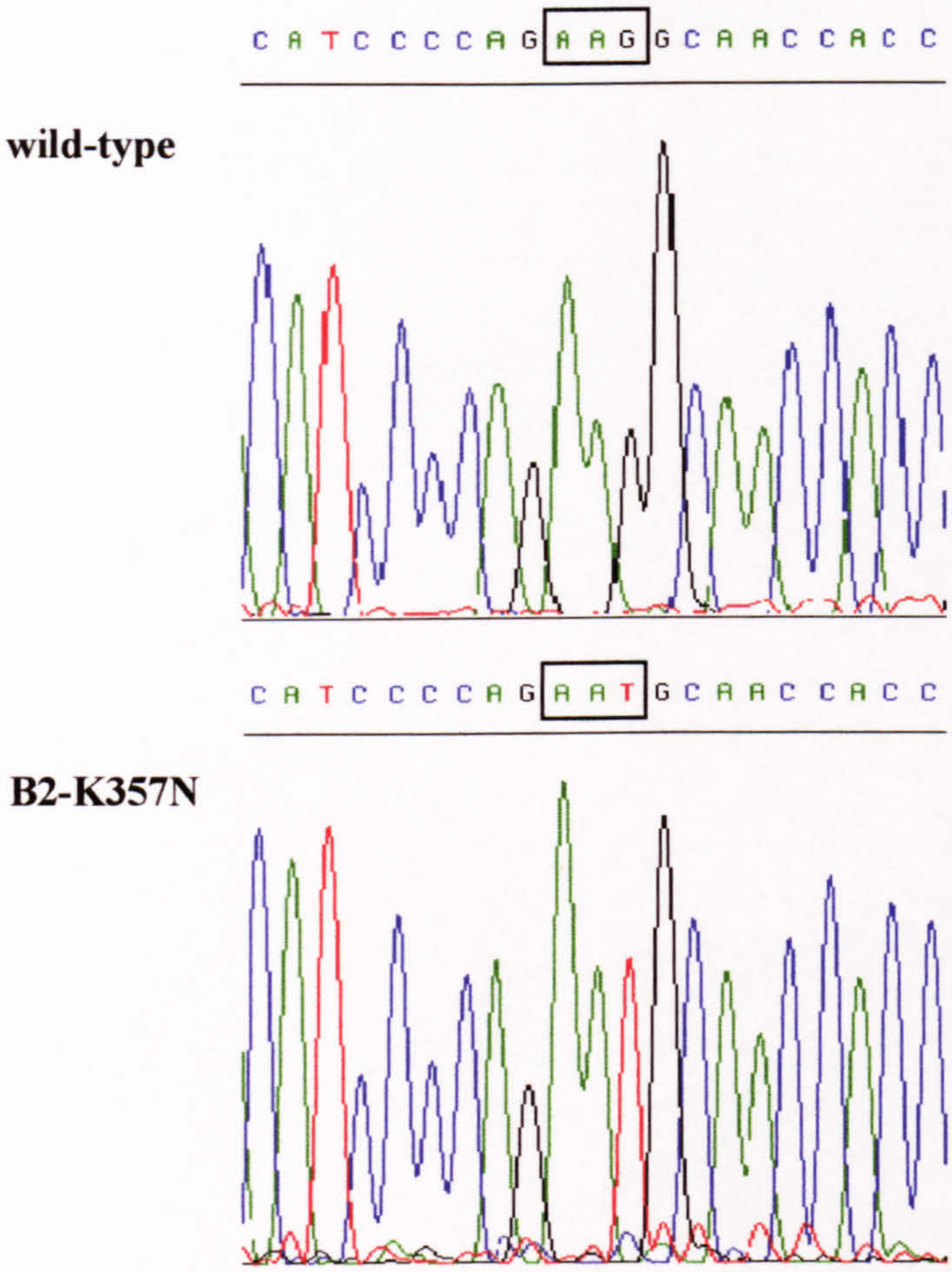


Figure 5.5a. Sequence of wild-type plasmid pCMV4B2 and mutant construct B2-K357N.

A portion of nucleotide sequence of exon 6 from wild-type pCMV4 B2 and from mutant construct B2-K357N are shown. The box indicates codon 357 where the mutation has been incorporated.

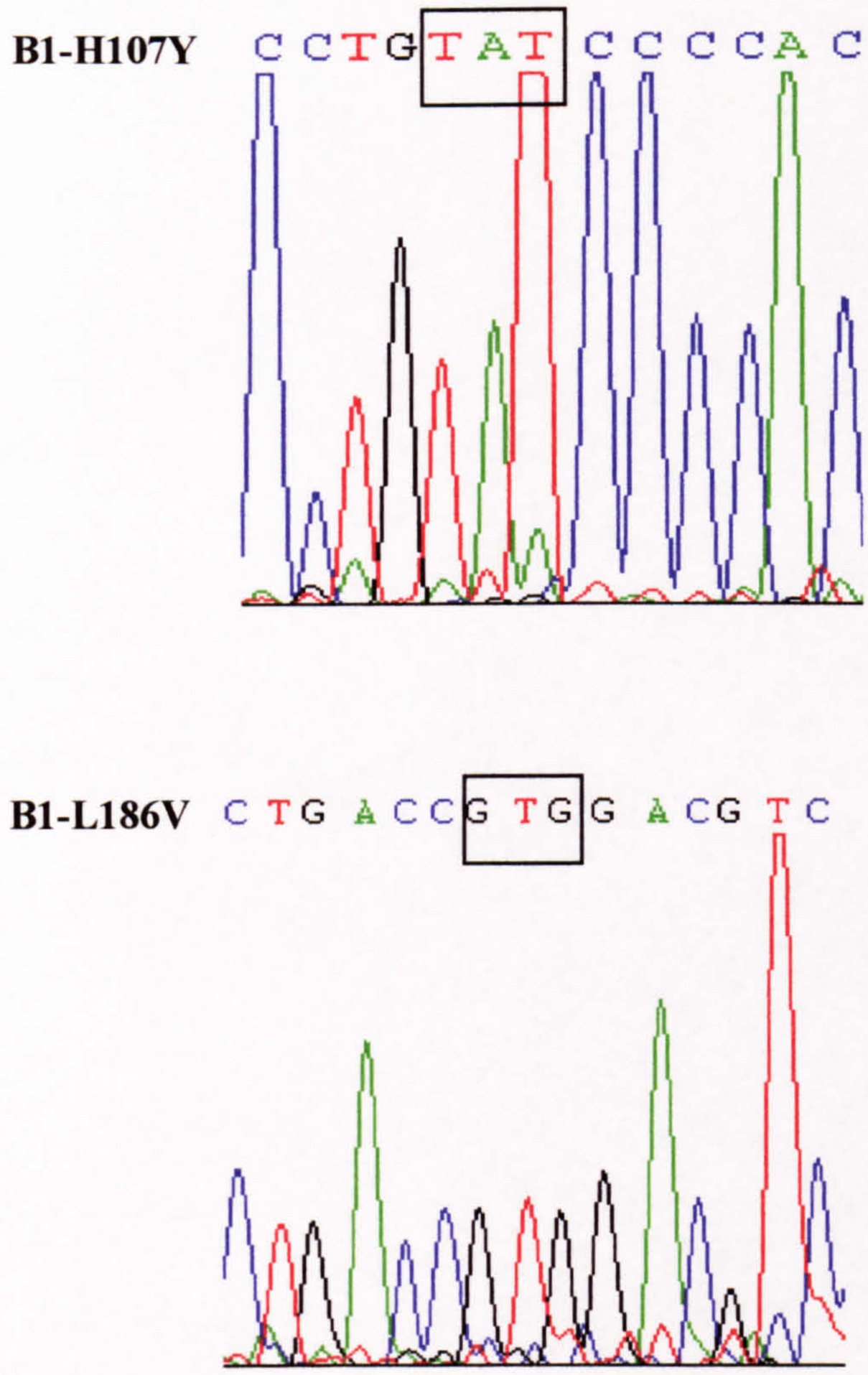


Figure 5.5b. Sequence of 11β-hydroxylase mutants B1-H107Y and B1-L186V.

A portion of nucleotide sequence of exon 2 and exon 3 from mutant constructs B1-H107Y and B1-L186V are shown. sequencing primers $pCMV_4$ (S) was used to sequence exon 2 and N4019 for exon 3. See table 2 of appendix 2 for details of primers. The boxes indicate codons 107 and 186 where the mutations have been incorporated.

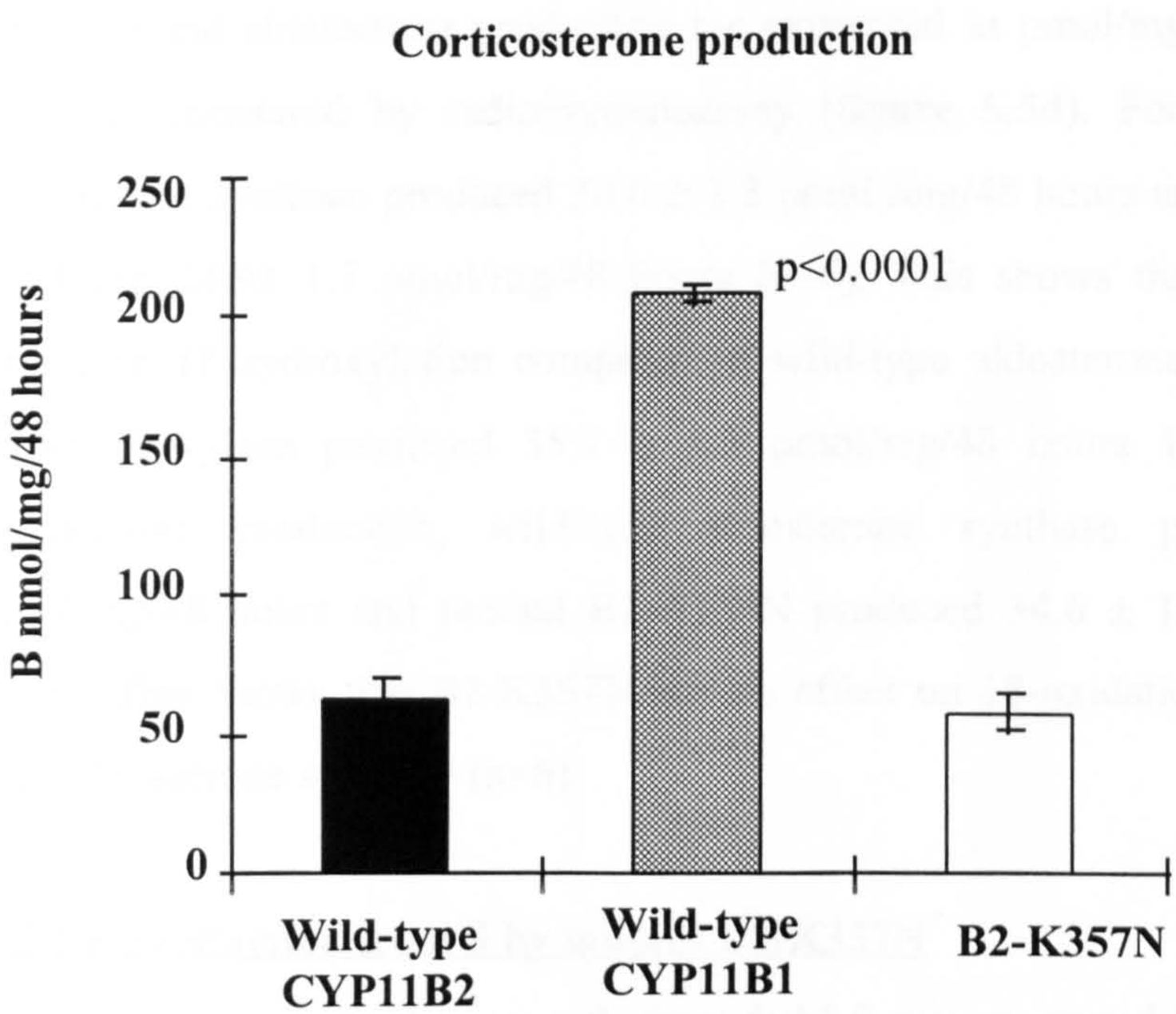


Figure 5.5c. B production from DOC from aldosterone synthase mutant B2-K357N.

Comparison of corticosterone (B) from aldosterone synthase mutant B2-357 with wild-type aldosterone synthase (CYP11B2) and 11β–hydroxylase (CYP11B1). COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with 5μM 11-deoxycorticosterone for 48 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are expressed as SI units. Results are mean ± SEM from three separate transfections, each done in duplicate. Statistical analysis was done by Student's t-test.

hydroxylation of the substrate DOC. Wild-type 11β-hydroxylase produced 208 ± 20.4 nmol/mg/48 hours which is significantly greater than wild-type aldosterone synthase or B2-K357N (n=6; $p<0.0001$, Student's t-test).

5.5.4 18-OHB and aldosterone production from DOC by mutant B2-K357N

18-OHB and aldosterone production are expressed as pmol/mg/48 hours from 5μM DOC as measured by radioimmunoassay (**figure 5.5d**). For 18-OHB, wild-type aldosterone synthase produced 30.6 ± 1.3 pmol /mg/48 hours and mutant B2-K357N produced 24.9± 1.7 pmol/mg/48 hours (n=6). This shows that B2-K357N has no effect on 18-hydroxylation compared to wild-type aldosterone synthase. Wild-type 11β-hydroxylase produced 35.7 ± 4.9 pmol/mg/48 hours 18-OHB (n=6). For aldosterone production, wild-type aldosterone synthase produced 40 ± 2.9 pmol/mg/48 hours and mutant B2-K357N produced 34.6 ± 1.6 pmol/mg/48 hours (n=6). This shows that B2-K357N has no effect on 18-oxidation compared to wild-type aldosterone synthase (n=6).

5.5.5 F production from S by mutant B2-K357N

F production expressed as nmol/L from 5μM S was measured by radioimmunoassay (**figure 5.5e**) Wild-type aldosterone synthase produced 69.7 ± 6 nmol/mg/48 hours and mutant B2-K357N produced 80 ± 9.2 nmol/mg/48 hours (n=6). This shows that B2-K357N has no effect on 11β-hydroxylation of the substrate S compared to wild-type aldosterone synthase. Wild-type 11β-hydroxylase produced 246 ± 41 nmol/mg/48 hours which is significantly greater than wild-type aldosterone synthase or B2-K357N (n=6; $p<0.0001$, Student's t-test).

5.6 F production from 11β-hydroxylase mutant B1-H107Y

F production expressed as nmol/mg/48 hours from 5μM S was measured by radioimmunoassay (**figure 5.6**) Wild-type 11β-hydroxylase produced 10.1 ± 1.7 nmol/mg/48 hours whereas mutant B1-H107Y produced 69.4 ± 7.1 nmol/mg/48 hours which is significantly greater than wild-type 11β-hydroxylase

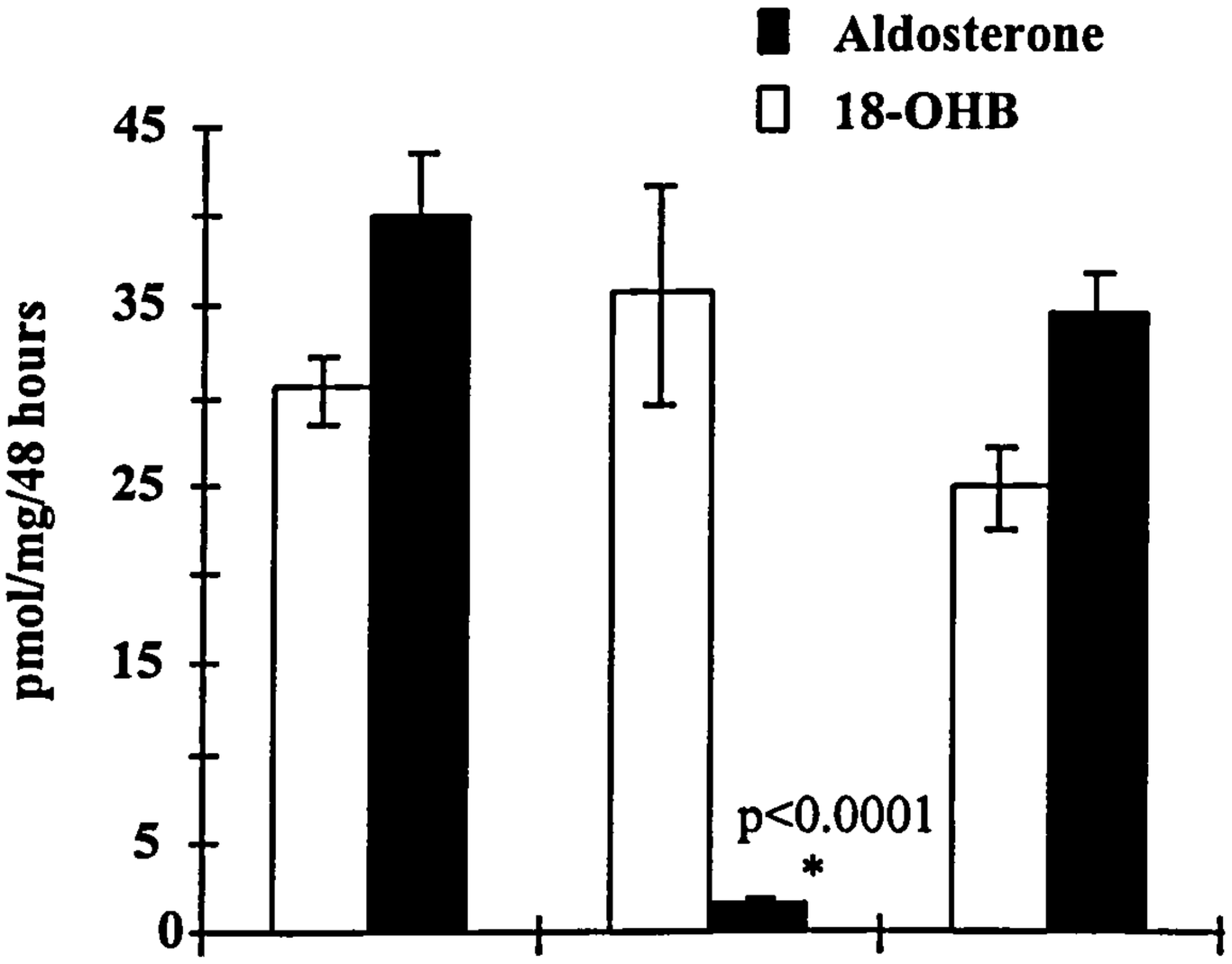


Figure 5.5d. 18-OHB and aldosterone production from DOC from aldosterone synthase mutant B2-K357N.

Comparison of 18-OHB and aldosterone production from aldosterone synthase mutant B2-K357N with wild-type aldosterone synthase (CYP11B2) and 11β–hydroxylase (CYP11B1). COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with 5μM 11-deoxycorticosterone (DOC) for 48 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are expressed as SI units. Results are mean ± SEM from three separate transfections, each done in duplicate. Statistical analysis was done by Student's t-test.

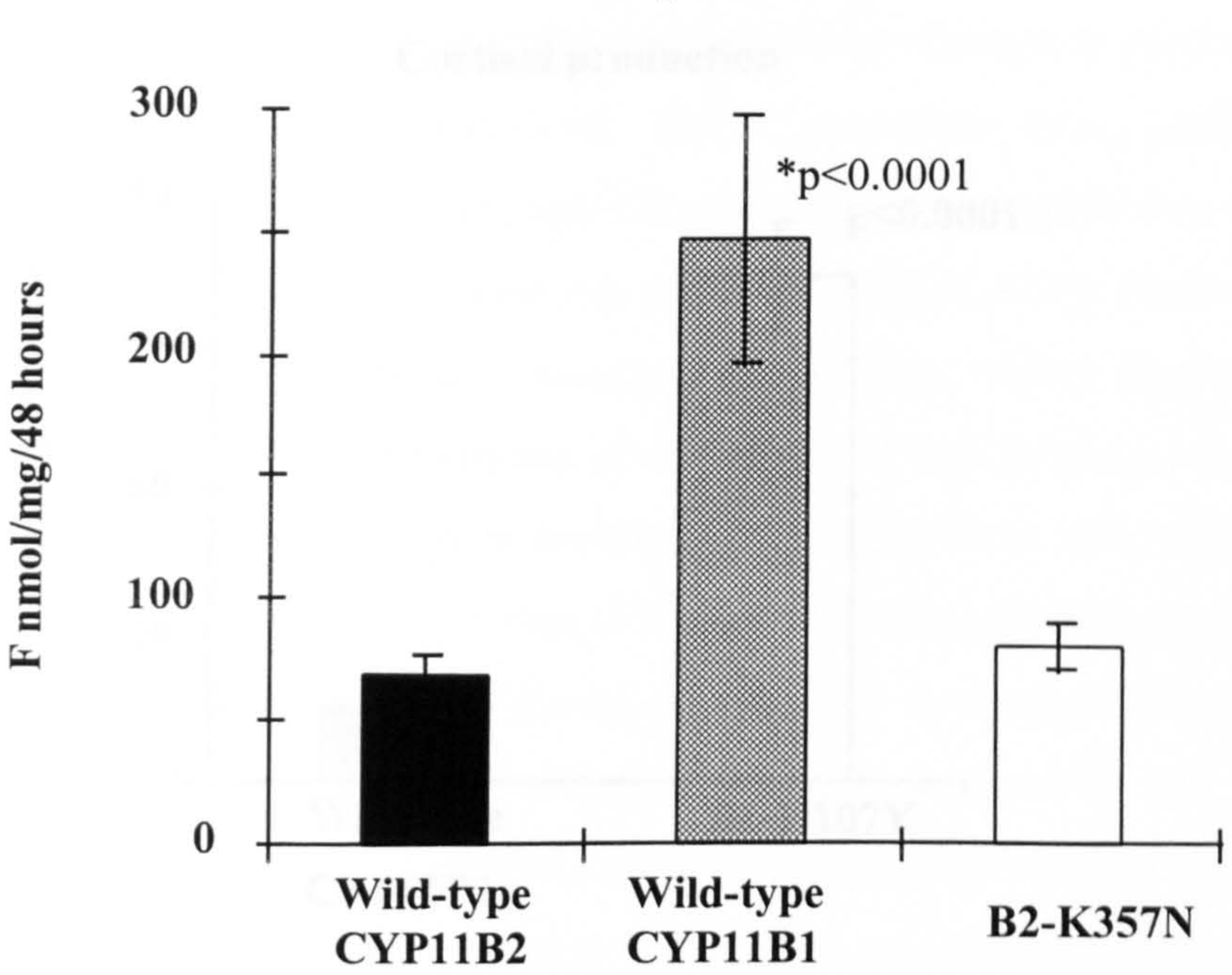


Figure 5.5e. F production from aldosterone synthase mutant B2-K357N.

Comparison of cortisol (F) from aldosterone synthase mutant B2-K357N with wild-type aldosterone synthase (CYP11B2) and 11β–hydroxylase (CYP11B1). COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with 5μM 11-deoxycortisol (S) for 48 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are expressed as SI units. Results are mean ± SEM from three separate transfections, each done in duplicate. Statistical analysis was done by Student's t-test.

Figure 5.6. F production from 11β-hydroxylase mutant B1-H107Y.

Comparison of cortisol (F) production from 11-deoxycortisol (S) from 11β-hydroxylase mutant B1-H107Y with wild-type 11β–hydroxylase (CYP11B1). COS-7 cells transfected with 10μg of pCMV4 expression vector, 5μg of pCD-Adx and 10μg of pSV-β-gal were incubated with 5μM 11-deoxycortisol (S) for 48 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are expressed as SI units. Results are mean ± SEM from three separate transfections, each done in duplicate. Statistical analysis was done by Student's t-test.

(n=6; p<0.0001, Student's t-test).

5.7 Discussion

In this study, single stranded conformational polymorphism (SSCP) was used to detect mutations in the CYP11B1 and CYP11B2 genes in normotensive and hypertensive subjects. Whilst this is a convenient, PCR-based technique which enables rapid detection of nucleotide variants by changes in electrophoretic mobility, it does have some limitations. SSCP can show false positives. Firstly, Taq polymerase may misincorporate nucleotides during amplification. Secondly, DNA strands having identical sequences can have different stable conformations which can present as electrophoretic variants. In addition, false negatives which are a consequence of the limitations of sensitivity of this technique can occur. SSCP is approximately 97% sensitive for sequences ranging from 100-300bp in its detection of nucleotide differences, leaving 3% of nucleotides which could potentially avoid detection. The gel used in this study is by far the most informative but varying the gel composition (i.e glycerol concentration and acrylamide) and temperature may occassionally identify mutations which have previously gone undetected. One added problem in this study was the high degree of homology of the CYP11B1 and CYP11B2 genes which made it difficult to design primers which would selectively amplify the desired region of either gene, a problem which necessitated sub-cloning of amplicons into T-vector.

Using the SSCP conditions described, several differences in coding regions of CYP11B1 and CYP11B2 were detected which alter amino acids in the encoded proteins which may have functional implications. Out of 40 hypertensive patients and 10 normal patients, an AAG to an AAT was identified in a single case at codon 357 of exon 6 in CYP11B2. This alters a lysine (K) to an asparagine (N) in the protein. In order to rule out the possibility of false negatives, several other hypertensives and normals, chosen at random, were also sequenced but were not different from the published sequence (see appendix 1). Manual sequencing analysis of the subject with

the exon 6 variant demonstrated that the patient was heterozygous as shown by co-existance of wild-type and mutant alleles.

Residue 357 in aldosterone synthase is located in a region which, from secondary structure prediction and 3-D modelling, forms β-sheet (see chapter 4). In the closely related bovine side shain cleavage enzyme, amino acid regions 353-359 and 363- 370 (bovine numbering) form sheets denoted β-3 and β-4 as predicted from modelling based on P450cam (Vijayakamur & Salerno 1992). β-4 is a substrate recognition sequence in P450cam (Gotoh et al. 1992). It is obvious from the model of human aldosterone synthase and 11β-hydroxylase based on known crystal structures and the bovine side chain cleavage enzyme model (see chapter 4 figure 4.3c), that this residue is exposed to the exterior of the molecule. This suggests that any role it may have may be involved with interaction with substrates or electron donors. Indeed, in bovine side chain cleavage enzyme, the closeby K-helix is known to be involved in adrenodoxin interaction where mutation of residues K377 and K381 dramatically reduce binding of adrenodoxin (Wada & Waterman 1992).

To assess the functional significance of this residue change (K357N), the mutation was incorporated in CYP11B2 cDNA in plasmid $pCMV_4$-B2 by site-directed mutatgenesis. When expressed in COS-7 cells, the mutant cDNA had no effect on the conversion of DOC to B, 18-OHB and aldosterone compared to wild-type aldosterone synthase nor on S to F conversion. DOC is the preferred substrate for aldosterone synthase. Incubations with S were performed to assess whether the mutation caused a switch in substrate specifity to improve or decrease S to F conversion. That this substitution does not have any detrimental effects on enzyme conversion suggests that it does not appear to have a role in substrate interaction. Indeed the amino acid substitution is lysine (K) to asparagine (N) which changes a basic to an uncharged residue. This may not be sufficient to alter the chemical properties at the surface of the molecule to alter substrate recognition or interaction with other cofactors.

Although there was no effect of mutant 357 *in vitro, in vivo,* it is possible that the B2 357 mutation may be found in in combination with other as yet unidentified mutations

which may also change amino acid residues. These mutations may interact to effect protein conformation and subsequent enzyme function. Several mutations identified in these genes have been shown *in vitro* to exert very little, if any effect, on enzyme activity. However, in combination with another mutation known to reduce activity, they exacerbate the detrimental effect (Pascoe et al. 1992, Portrat-Doyen et al. 1998). Indeed, all mutations/polymorphisms of the CYP11B1 and CYP11B2 genes described in this chapter have been confined to 5 patients, suggesting that single subjects may have several mutations/ polymorphisms on the same allele. Sequencing of the complete coding region of both genes is necessary to establish this followed by functional studies using constructs containing all mutations.

In addition, a number of other mutations were identified. In the CYP11B1 gene, three missense mutations resulted in amino acid differences in the encoded protein. These included nucleotide changes in CYP11B1 which resulted in amino acid differences; L83S*, H107Y and H125R*(exon 2), L186V (exon 3) and C494F (exon 9). * Those indicated were identified by Christine Holloway (Blood Pressure Unit). . To determine the effects of these mutations on enzyme activity, *in vitro* functional studies are required. Preliminary *in vitro* functional analysis was performed for mutant B1-H107Y.

11β-Hydroxylase mutant B1-H107Y increases conversion of 11-deoxycortisol to cortisol compared to wild-type 11β-hydroxylase *in vitro.* From these studies it is not possible to state whether this is purely a functional effect or may be due to an increase in protein expression. *In vivo,* an increase in cortisol production may affect overall adrenal secretion rate to switch off ACTH drive. Indeed, higher secretion rates of DOC and S have been reported in cases of essential hypertension where 11β-hydroxylation is abnormal, indicating that this may be a key locus (deSimone et al. 1985). The effect on DOC conversion has yet to be assesssed.

Nonsense mutations were also identified in the CYP11B1 gene at codon 189. A nucleotide deletion was identified in one subject and a stop codon identified in another. Both would be expected to result in premature termination of the coding

regions and a truncated non-functional product. The stop 189 was found in a subject also carrying L186V. If this subject were to be homozygous then the stop would determine phenotype and not the L186V mutation. However, this would most definitely result in a severely altered steroidogenic profile which would have been detected in the clinic. It is also possible that subjects may have the L186V polymorphism/mutation on its own. Effects of this on enzyme activity have yet to be assessed.

In CYP11B2 exon 8, a missing codon (464) was detected in a single patient. It is interesting that in another study, a 3bp insertion (homozygous) was detected at this codon in a patient with 11β-hydroxylase deficiency (Geley et al. 1996), suggesting that this codon or those in the immediate vicinity are important for enzyme function.

As yet it is unclear whether the mutations found in this study are unique to the patients studied or whether they occur frequently. Large population studies are necessary to determine the frequency of a mutation. Mutations whose frequency are greater than 2% are generally termed polymorphic. The polymorphism may or may not change an amino acid and/or the function of the enzyme. Although not carried out in this study, readily available and simple techniques such as restriction enzyme digestion and allele specific oligonucleotide should enable rapid and high throughput screening. Indeed restriction enzymes have been identified for some of mutations found in this study.

Other polymorphisms have been identified in the CYP11B2 gene, for example Q43R, R173K, B296K, V386A (Mornet et al. 1989, Kawamoto et al. 1990, Fardella et al. 1996b, Portrat -Doyan et al. 1998) and also C494F which, from our findings and comparison with the published sequence can encode a phenylalanine or a cysteine at this codon in CYP11B1. The functional significance of this substitution has not been established. V386A, although characterised as a polymorphism, has been reported by some groups to cause a small decrease in enzyme activity *in vitro* (Pascoe et al. 1992).

Although some mutations may cause only mild effects on enzyme activity and steroid production and may not lead to significant alterations in circulating corticosteroid concentrations, they may still have important implications *in vivo*. Indeed, there is now increasing evidence to suggest that the CYP11B1 and CYP11B2 genes are also expressed in other tissues such as the CNS, heart and vascular system where they are thought to be involved in cardiovascular/blood pressure homeostasis. In the CNS, intracerebroventricular (icv) administration of aldosterone at doses too low to act systemically, cause dramatic increases in blood pressure in rats (Gomez-Sanchez 1997). Therefore, it is possible that subtle changes in aldosterone production caused by mutations in the CYP11B2 gene may result in higher local concentrations in tissues such as the brain and cause significant changes in phenotype.

Polymorphisms may be markers of certain forms of hypertension. In a recent study, codon 173 of CYP11B2 was shown to be polymorphic in a Chilean population encoding an arginine(R) or a lysine(K) (Fardella et al. 1996b). The R173 variant of CYP11B2 had a higher frequency in patients with low-renin hypertension (Fardella et al. 1996b) which is characterised by suppressed renin activity which may result from elevated aldosterone secretion. It is also possible that polymorphisms may be in linkage disequilibrium with other genotypic variants. To assess this proposal, a larger number of subjects would have to be screened to determine whether polymorphisms occur only in hypertensives and thus predict succeptability to hypertension. Screening the CYP11B1 and B2 genes in a well-characterised group of hypertensive subjects for example, low-renin hypertensives, may lead to the identification of mutations with greater functional significance.

Whilst this study has concentrated on the exonic-coding regions of these genes, other studies have shown an intronic conversion and a SF-1 polymorphism in the non-coding and 5' untranslated regions of the CYP11B2 gene (see 1.6) (Brand et al. 1998 Davies et al. 1999, White et al. 1991). There was a significant association of the -344T allele of the SF-1 site with essential hypertension (Brand et al. 1998, Davies et al. 1999). Screening of the promoter and intronic regions of both genes may also be informative.

In summary, a number of mutations have been identified in this study. *In vitro* functional implications for some of them have been assessed. Whether these *in vitro* observations are applicable to the *in vivo* situation is not known. No attempt has been made to compare genotype with phenotype, as all of the hypertensive patients studied were anonymous. However, future studies should examine how these mutations affect circulating concentrations of components of the renin-angiotensin-aldosterone system and blood pressure response to therapy.

CHAPTER 6

Chapter. 6.

6.1 Effects of 18-OXOF, 18-OHF and 18-OHDOC on 11β-hydroxylation of 11-deoxycortisol and 11-deoxycorticotserone by human aldosterone synthase and 11β-hydroxylase in vitro in stably transfected V79 cells.

In the rare autosomal dominant disorder, glucocorticoid-suppressible hyperaldosteronism (GSH) aldosterone synthase activity is ectopically expressed in the adrenal zona fasciculata and is subject to ACTH control (see section 1.8). In addition to aldosterone, the chimeric enzyme also catalyses the synthesis of 18-hydroxycortisol (18-OHF) and 18-oxocortisol (18-OXOF) from cortisol (F). These are secreted in large quantities. It has been suggested that the impaired 11β-hydroxylation observed in this condition (Jamieson et al. 1996) is due either to competitive or to non-competitive inhibition by these metabolites. A number of other 18-hydroxy compounds are made in the normal adrenal cortex which may also exert endogenous inhibitory control. In this study, the effect of 18-OXOF, 18-OHF and 18-OH-DOC on the conversion of 11-deoxycorticosterone (DOC) to corticosterone (B) and 11-deoxycortisol (S) to cortisol (F) was investigated using cell lines stably transfected with CYP11B1or CYP11B2.

6.2 Methods.

Stably transfected cell lines were a kind gift from Prof Rita Bernhardt and were handled as described in section (2.9.1). CYP11B1 and CYP11B2 cells stably express human 11β-hydroxylase and aldosterone synthase respectively. Steroid incubations (24hours) were performed as described in section (2.9.4). 18-OXOF and 18-OHF were synthesised and subsequently purified by CE Gomez-Sanchez. They were then re-purified by paper chromatography. Steroid products were measured by radioimmunoassay (section 2.8). Cell lysates were prepared (section 2.6.1) and assayed for protein (section 2.6.3) All steroid measurements were corrected and are expressed as nmol/pmol per mg of protein per 24 hours.

6.3 Results

Effects of 18-OHF and 18-OXOF on B and F production from DOC and S in CYP11B1 and CYP11B2 stably transfected cells.

Time course experiments were performed to determine the point of maximum steroid conversion. An end-point of 24 hours was chosen **(figure 6.3a)** for both B and F production from DOC and S respectively in both cell lines.

Stably transfected CYP11B1 cells were incubated with (1μM) DOC in the presence and absence of 10 and 20μM 18-OXOF or 18-OHF. *In vivo* 18-OHF urinary levels in patients with GSH are approximately 10 times the upper limit of the normal range. The results are shown in **figure 6.3b**. Neither 18-OXOF nor 18-OHF had any effect on B production from cells stably transfected with CYP11B1 at these concentrations.

Figure 6.3c shows the results of incubating cells stably transfected with CYP11B1 with (1μM) S in the presence and absence of 10 and 20μM 18-OXOF or 18-OHF. These steroids had no effect on F production.

Stably transfected CYP11B2 cells expressing human aldosterone synthase were treated as decribed in **figures 6.3b and 6.3c**. Similar results were obtained **(figures 6.3d and 6.3e).** In stably transfected CYP11B2 cells incubated with DOC, 18-hydroxycorticosterone (18-OHB) and aldosterone were also measured. 18-OXOF or 18-OHF had no effect on 18-OHB or aldosterone production from DOC in these cells **(figure 6.3f and 6.3g.)**

Summary

From these results, 18-OXOF and 18-OHF at concentrations of 10 and 20μM do not affect B or F production from DOC and S respectively by human aldosterone synthase or 11β-hydroxylase, nor do they affect 18-OHB or aldosterone production from DOC by human aldosterone synthase.

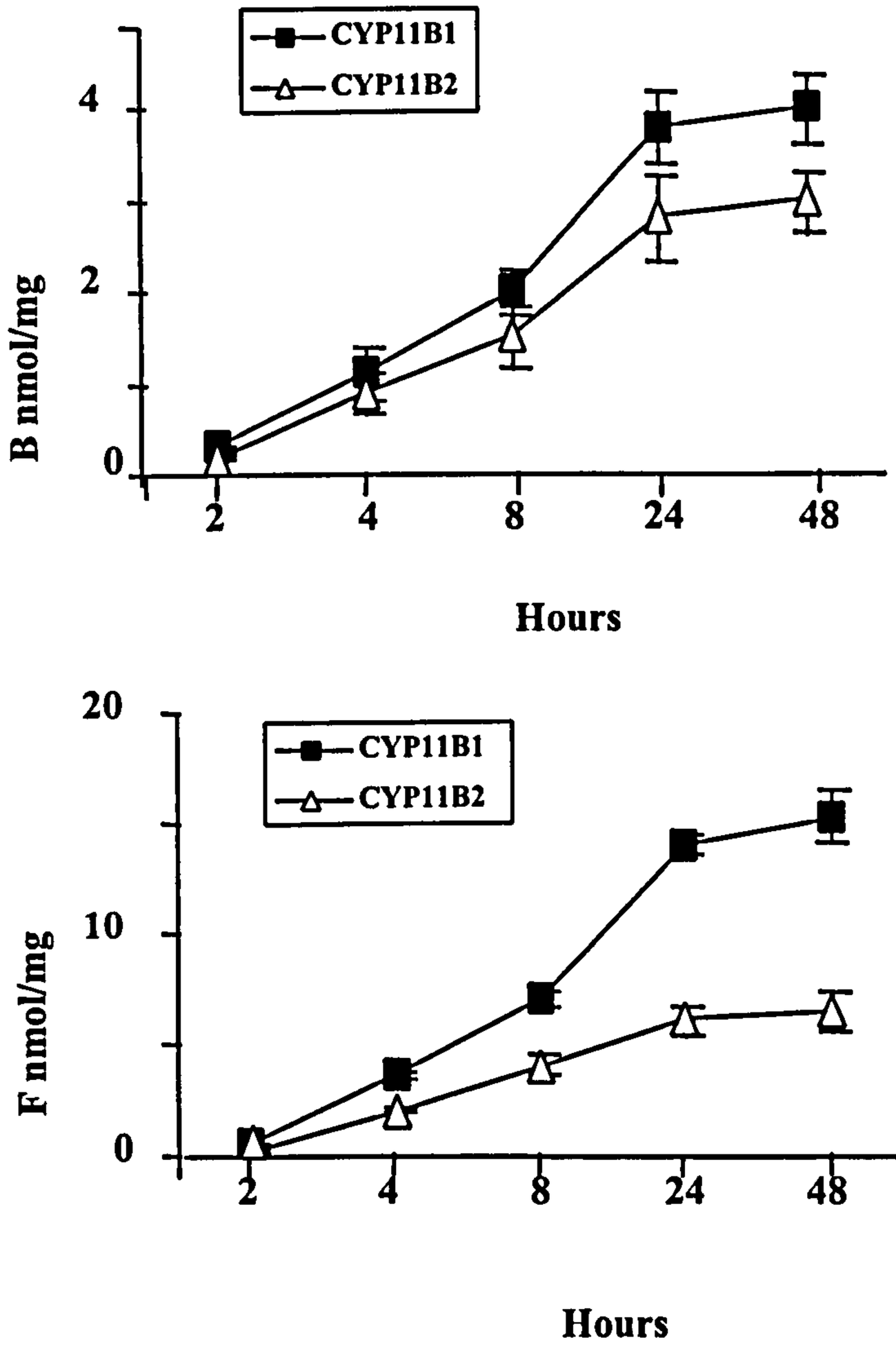
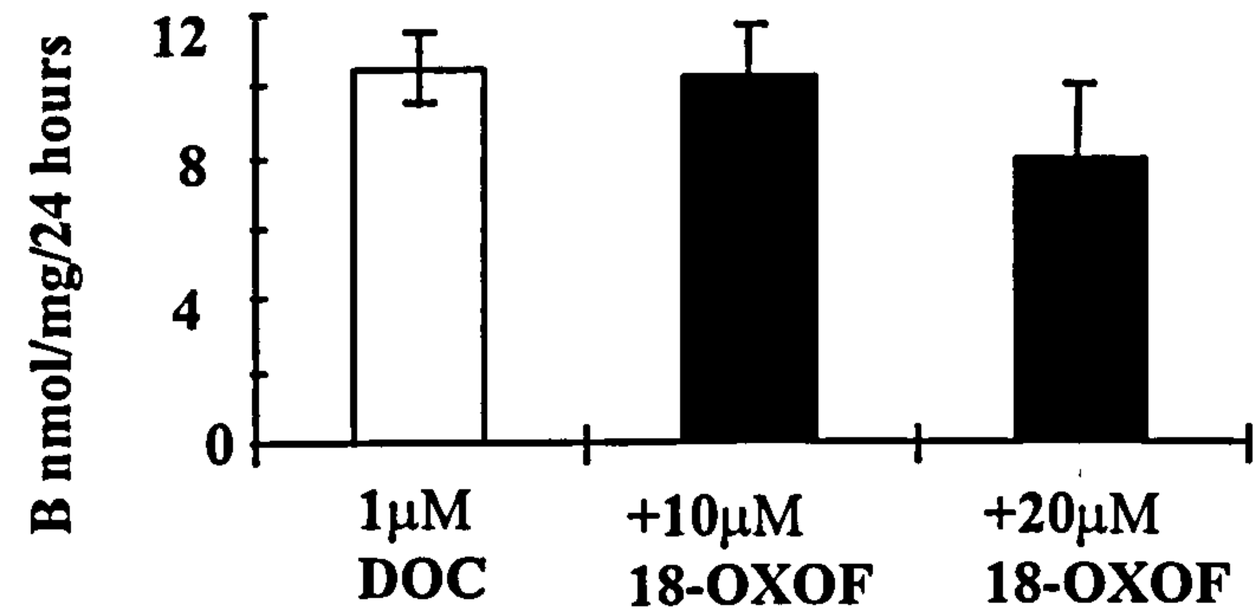


Figure 6.3a

Time course of corticosterone (B) and cortisol (F) production from CYP11B1 and CYP11B2 stably transfected V79 cells.

V79 cells (c10^6) were incubated with 1μM of 11-deoxycorticosterone (DOC) or 11-deoxycortisol (S) for 0 to 48 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.



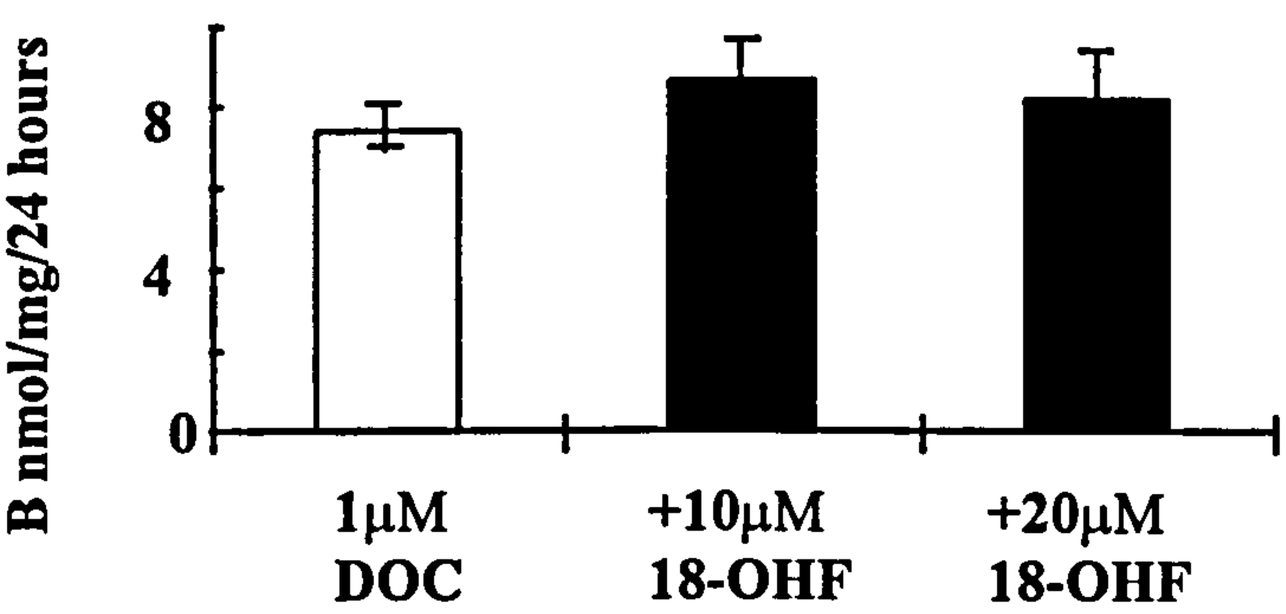


Figure 6.3b

Corticosterone (B) production from 1μM 11–deoxycorticosterone (DOC) in the presence and absence of 10 and 20μM of 18-oxocortisol (18-OXOF) and 18-hydroxycortisol (18-OHF) in V79 cells stably transfected with CYP11B1.

V79 cells (c10^6) were incubated for 24 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.

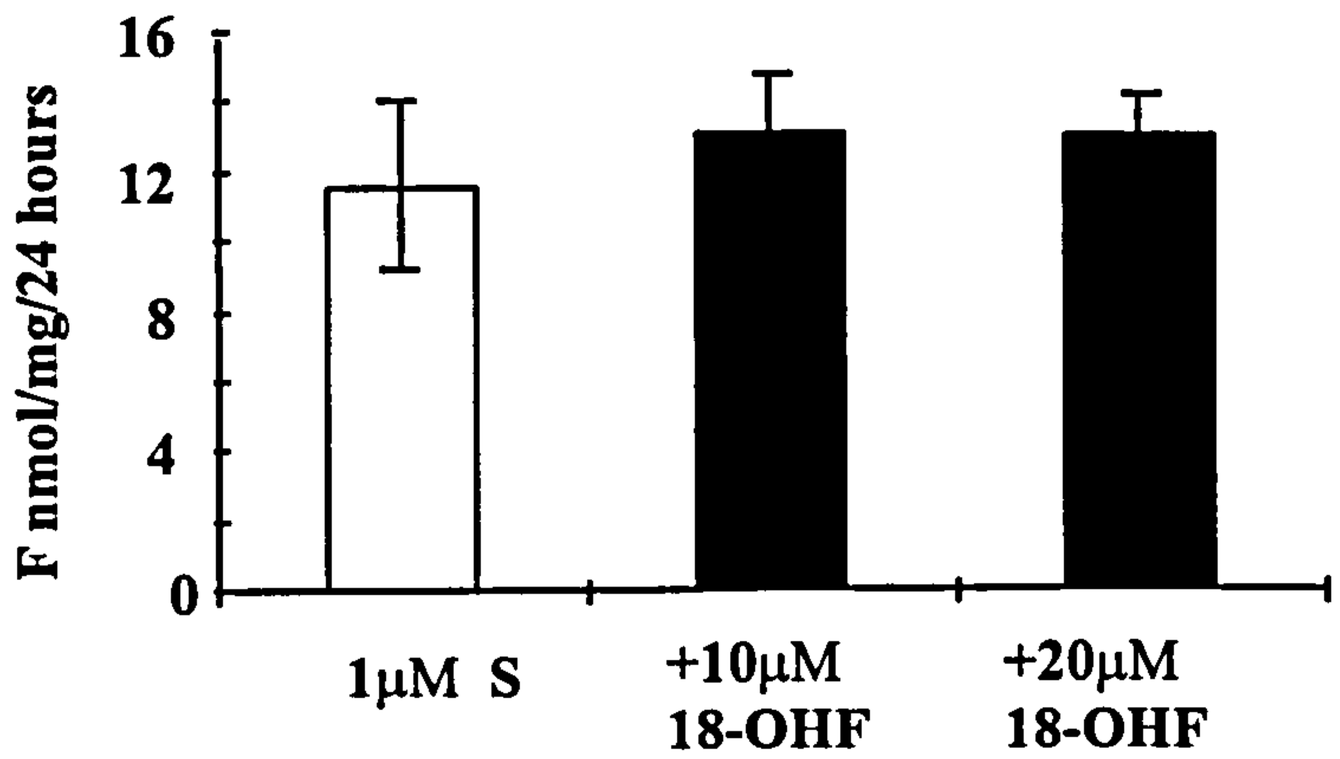
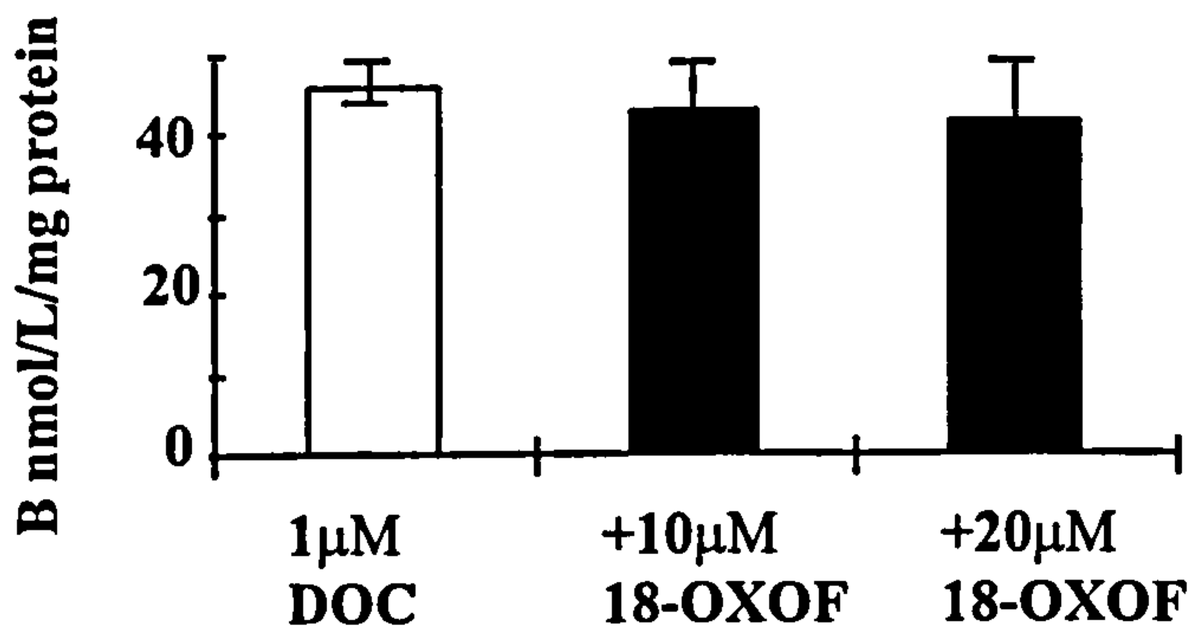


Figure 6.3c

Cortisol (F) production from1μM 11-deoxycortisol (S) in the presence and absence of 10 and 20μM of 18-oxocortisol (18-OXOF) and 18-hydroxycortisol (18-OHF) in V79 cells stably transfected with CYP11B1. V79 cells (c10^6) were incubated for 24 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.



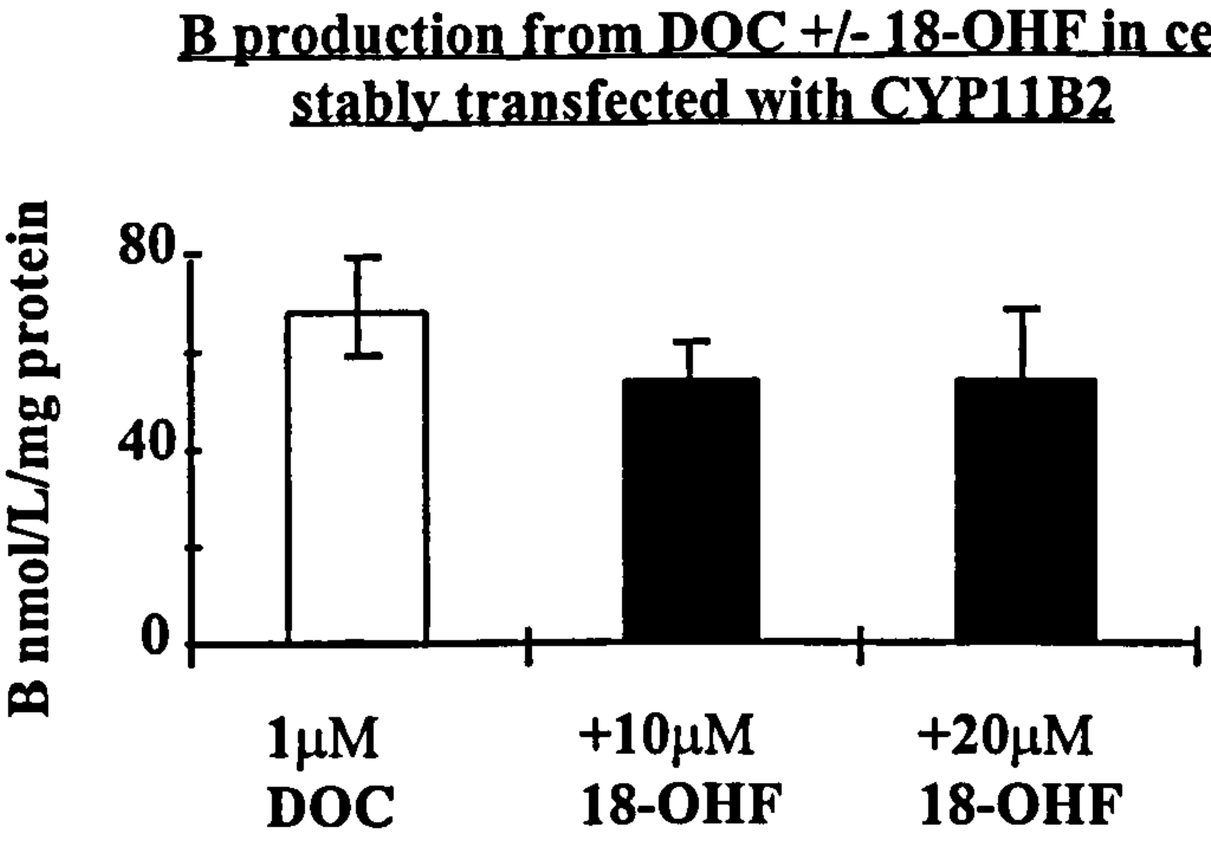
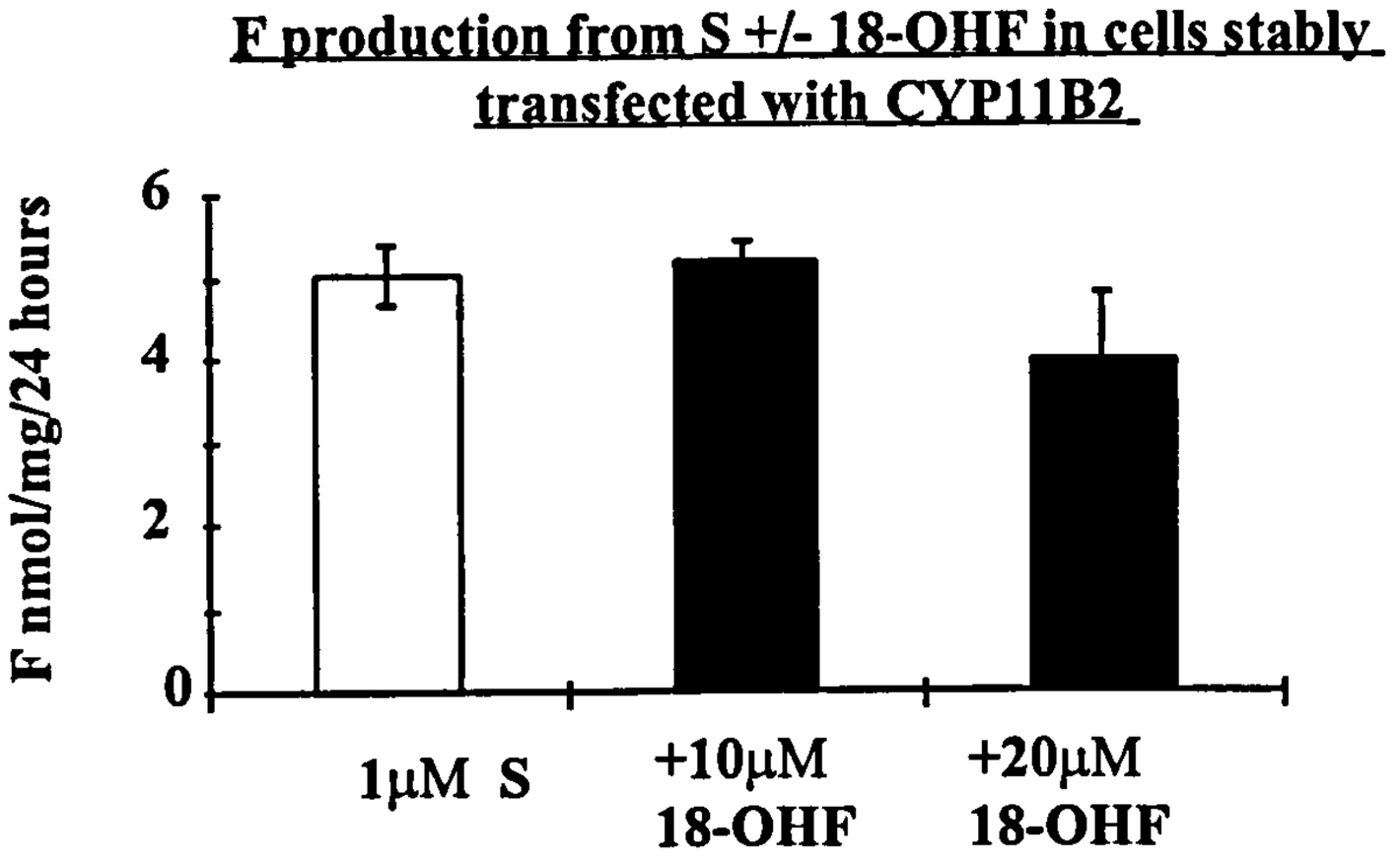


Figure 6.3d

Corticosterone (B) production from 1μM 11-deoxycorticosterone (DOC) in the presence and absence of 10 and 20μM of 18-oxocortisol (18-OXOF) and 18-hydroxycortisol (18-OHF) in V79 cells stably transfected with CYP11B2. V79 cells (c10^6) were incubated for 24 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.



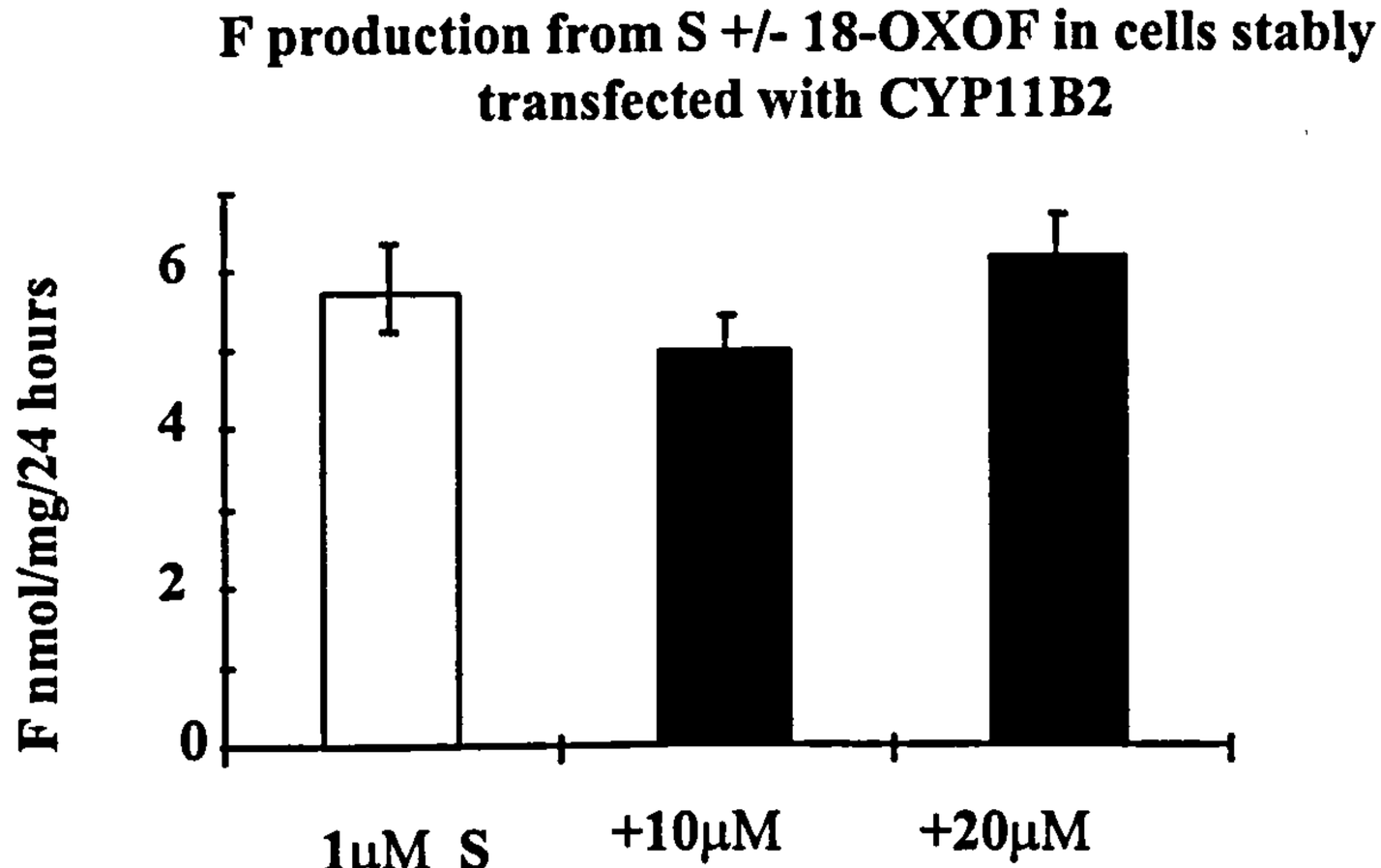
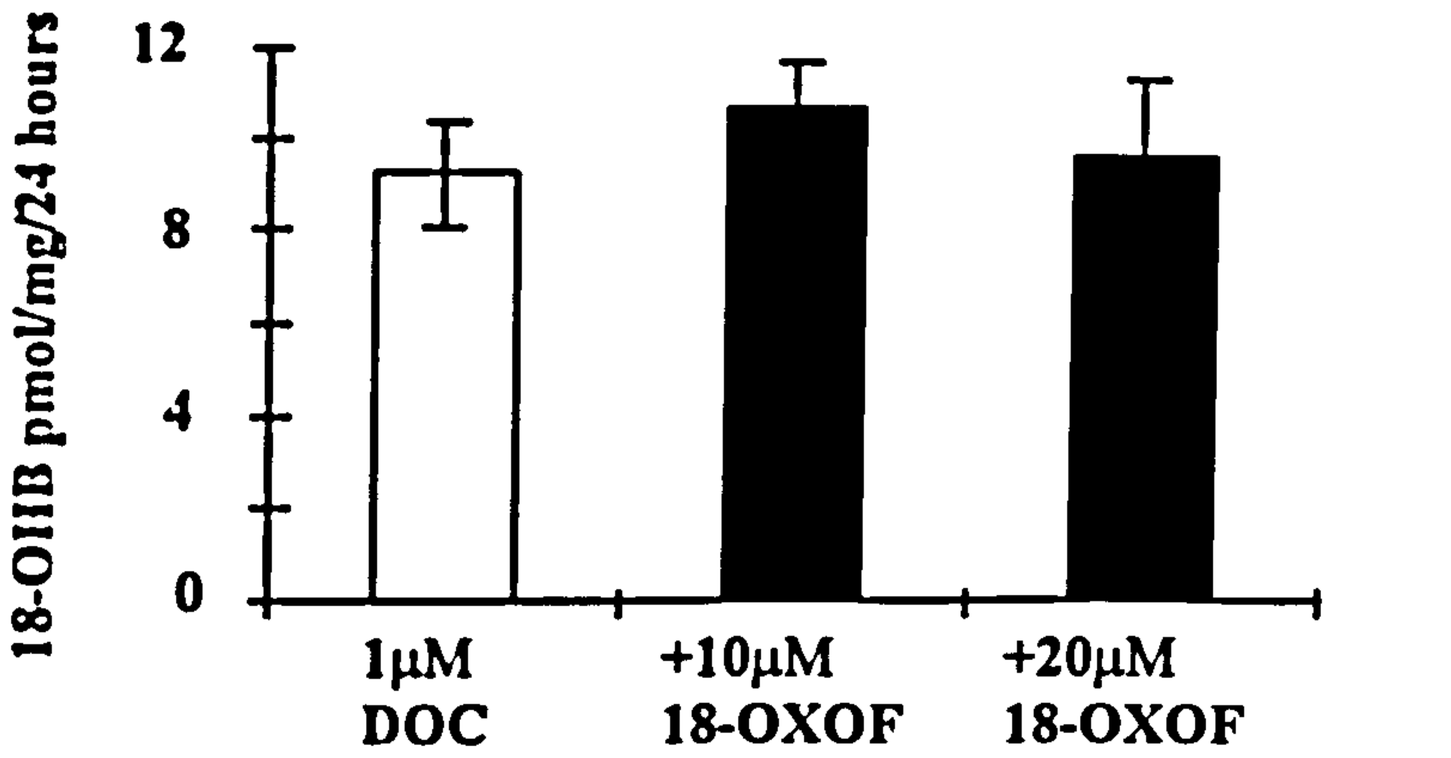


Figure 6.3e
Cortisol (F) production from1μM 11-deoxycortisol (S) in the presence and absence of 10 and 20μM of 18-oxocortisol (18-OXOF) and 18-hydroxycortisol (18-OHF) in V79 cells stably transfected with CYP11B2. V79 cells (c10^6) were incubated for 24 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.



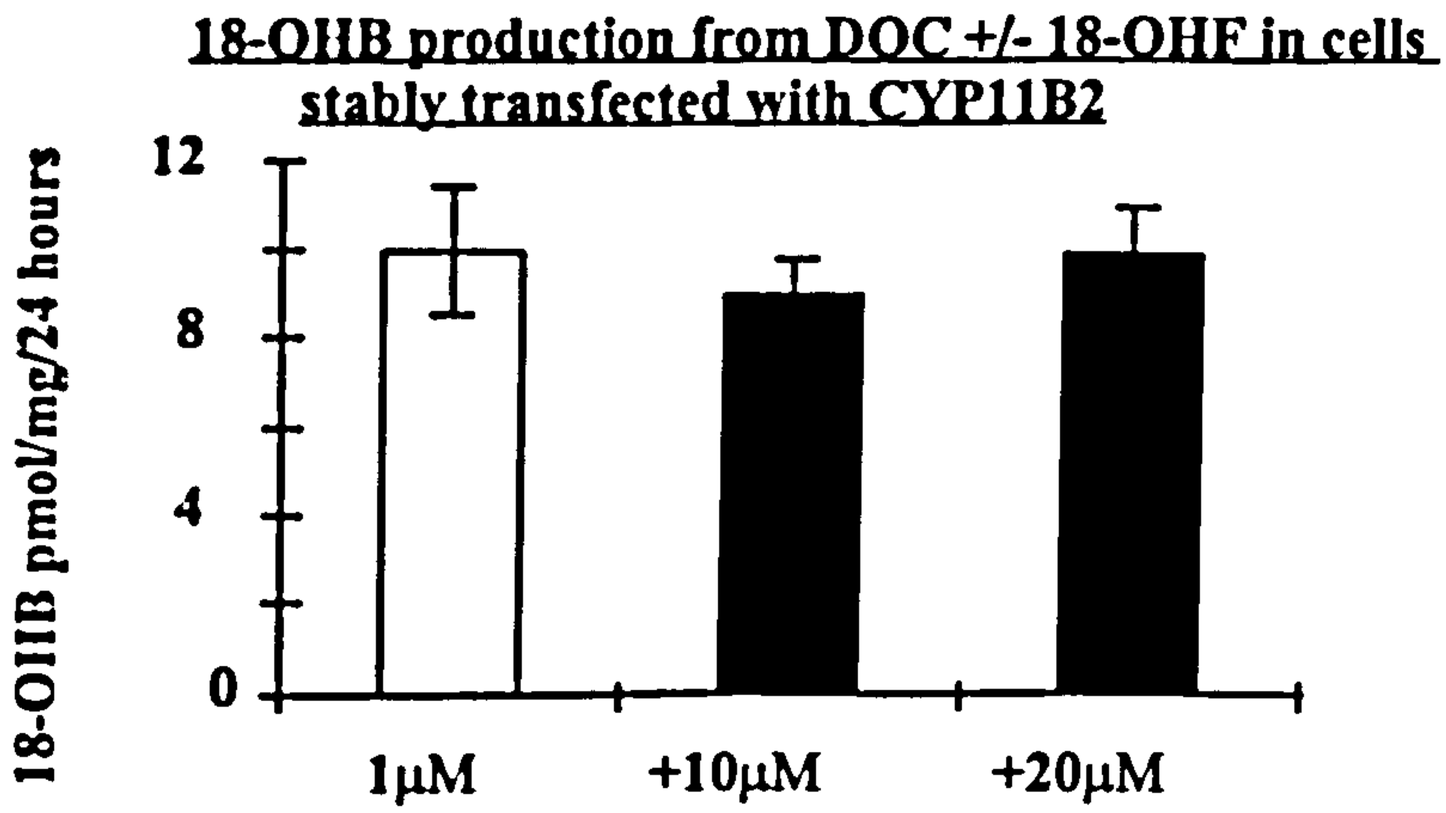
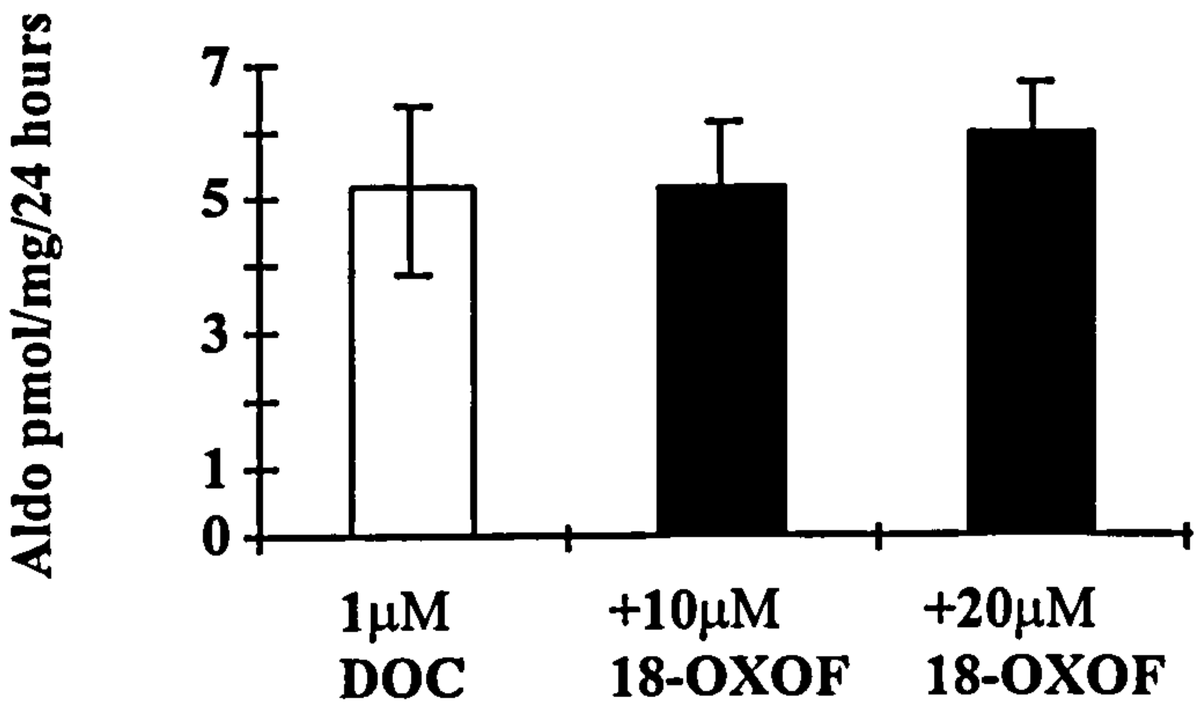


Figure 6.3 f

18-hydroxycorticosterone (18-OHB) production from 1μM 11-deoxycorticosterone (DOC) in the presence and absence of 10 and 20μM of 18-oxocortisol (18-OXOF) and 18-hydroxycortisol (18-OHF) in V79 cells stably transfected with CYP11B2. V79 cells (c10^6) were incubated for 24 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.



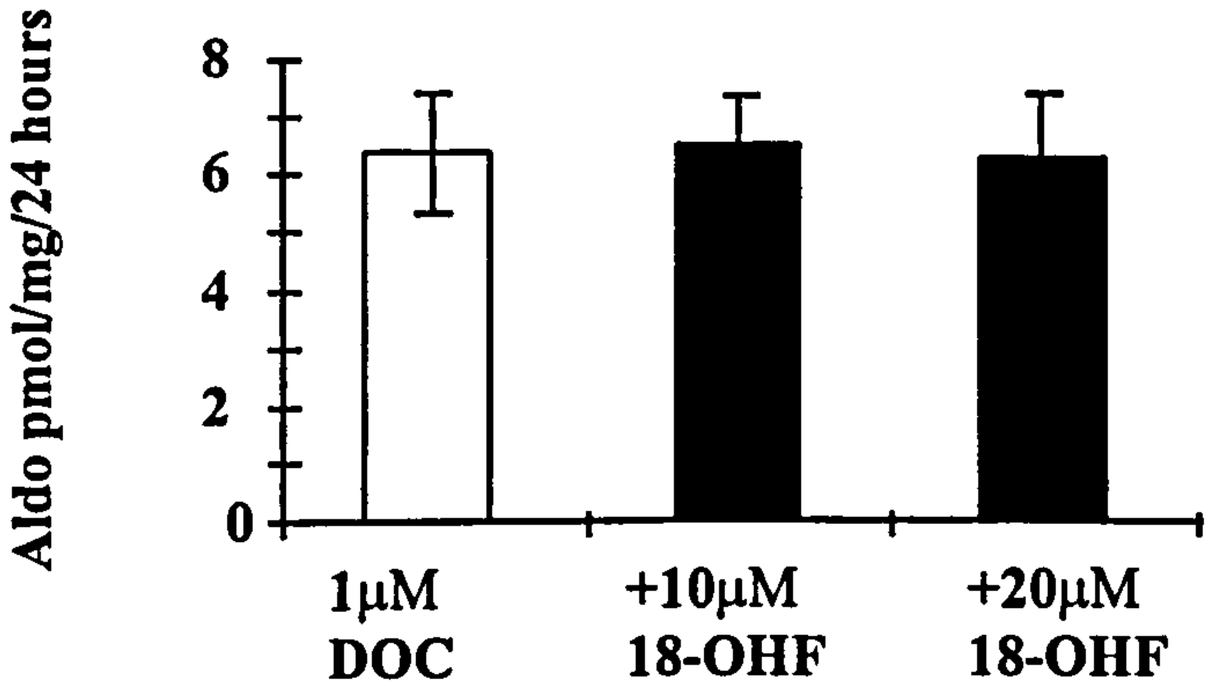


Figure 6.3 g

Aldosterone (Aldo) production from 1μM 11-deoxycorticosterone (DOC) in the presence and absence of 10 and 20μM of 18-oxocortisol (18-OXOF) and 18-hydroxycortisol (18-OHF) in V79 cells stably transfected with CYP11B2. V79 cells ($c10^6$) were incubated for 24 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.

6.4 Effects of 18-hydroxydeoxycorticosterone (18-OHDOC) on B and F production from DOC and S in CYP11B1 stably transfected cells.

18-OHDOC was also investigated as a potential inhibitor of B and F production. Initial studies using (1μM) DOC or (1μM) S with 10μM 18-OHDOC showed a reduction of B and F production by both cell types in the presence of 18-OHDOC. To characterise this inhibition further, a range of substrate concentrations between 0.001 and 1μM were used in the presence and absence of 10μM 18-OHDOC.

Figure 6.4a shows B production from DOC in the presence and absence of 10μM 18-OHDOC from cells stably transfected with CYP11B1. At concentrations less than 0.01μM steroid concentrations were at the limits of detection of the radioimmunoassay. At all other concentrations of substrate, there was a significant reduction in B production of about 40-50% in the presence of 18-OHDOC ($n=4; p<0.05$; unpaired Student's t-test). At 0.01, 0.1 and 1μM substrate concentrations, B production was reduced from 0.08 ± 0.11 to 0.04 ± 0.03, 1 ± 0.1 to 0.4 ± 0.05 and 4.8 ± 0.7 to 2.2 ± 0.2 nmol/mg/24 hours respectively.

Figure 6.4b shows F production from S in the presence and absence of 10μM 18-OHDOC in cells stably transfected with CYP11B1. There was a significant reduction of about 40-50% in F production in the presence of 18-OHDOC ($n=4; p<0.05$). At 0.01, 0.1 and 1μM substrate concentrations, F production was reduced from 0.7 ± 0.09 to 0.1 ± 0.02, 2.4 ± 0.2 to 0.7 ± 0.1 and 14.0 ± 1.9 to 7.3 ± 0.9 nmol/mg/24 hours respectively.

6.5 Effects of 18-OHDOC on B and F production from DOC and S in CYP11B2 stably transfected cells.

Cells stably transfected with CYP11B2 were treated as described in **figures 6.4a and 6.4b.** Similarly, B and F production from DOC and S respectively, were significantly reduced in the presence of 18-OHDOC **(figures 6.5a and 6.5b).**

B production from DOC +/- 10μM 18-OHDOC from stably transfected CYP11B1 cells

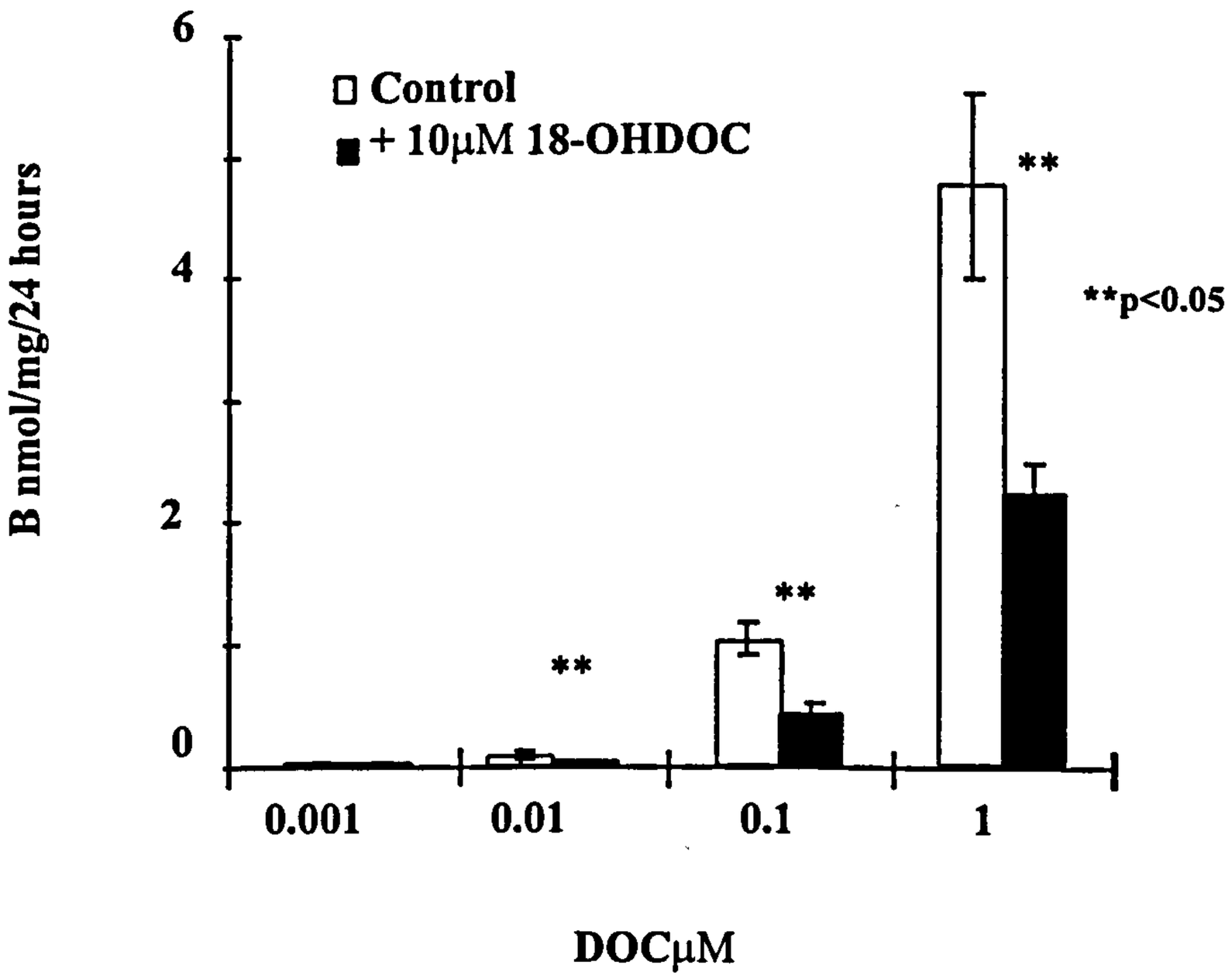


Figure 6.4 a

Corticosterone (B) production from 0.001 to 1μM 11-deoxycorticosterone (DOC) in the presence and absence of 10 μM of 18-hydroxydeoxycorticosterone (18-OHDOC) in V79 cells stably transfected with CYP11B1. V79 cells ($c10^6$) were incubated for 24 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.

F production from S +/- 10 μM 18-OHDOC in cells stably transfected with CYP11B1

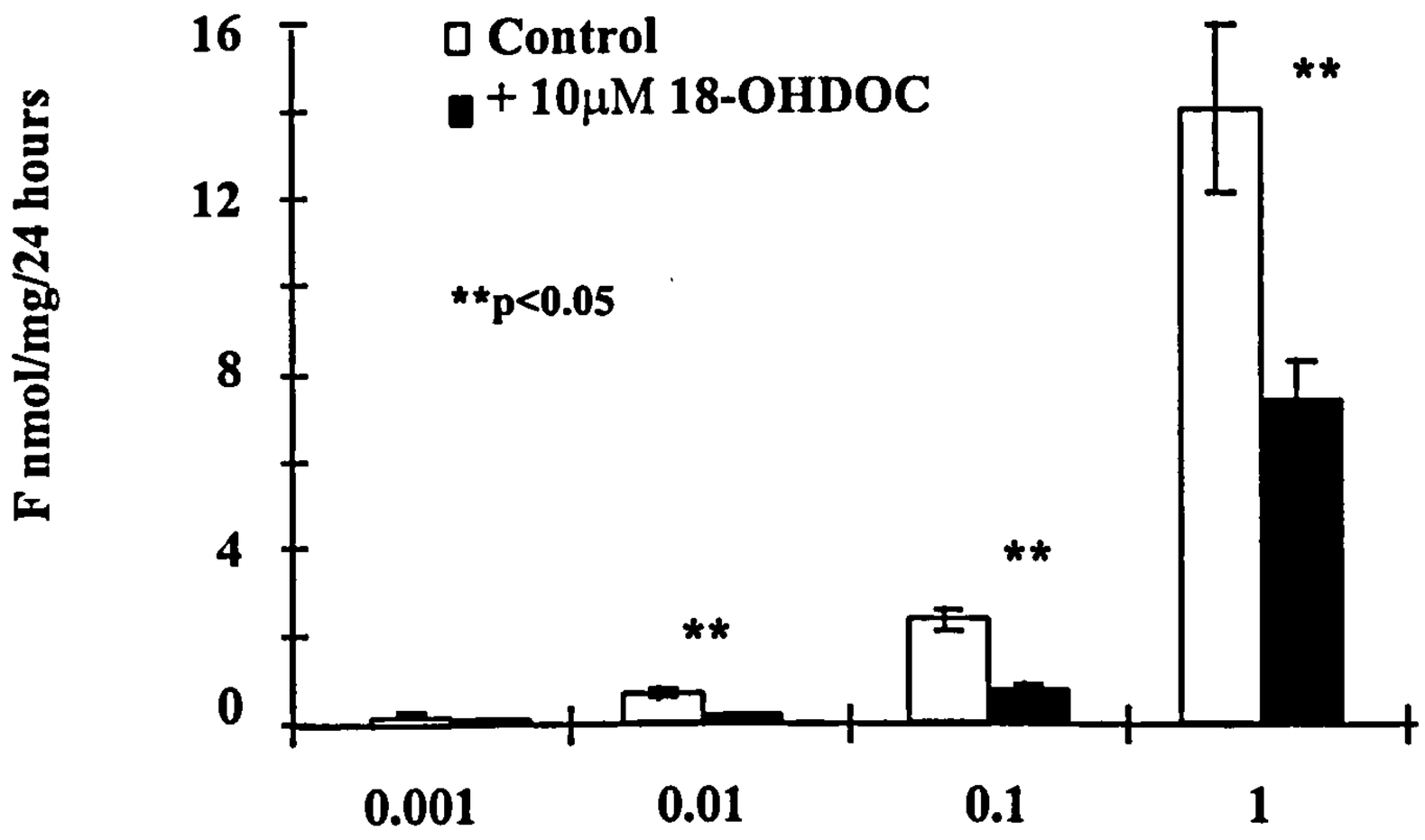


Figure 6.4 b

Cortisol (F) production from 0.001 to 1μM 11-deoxycortisol (S) in the presence and absence of 10 μM of 18-hydroxydeoxycorticosterone (18-OHDOC) in V79 cells stably transfected with CYP11B1. V79 cells ($c10^6$) were incubated for 24 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.

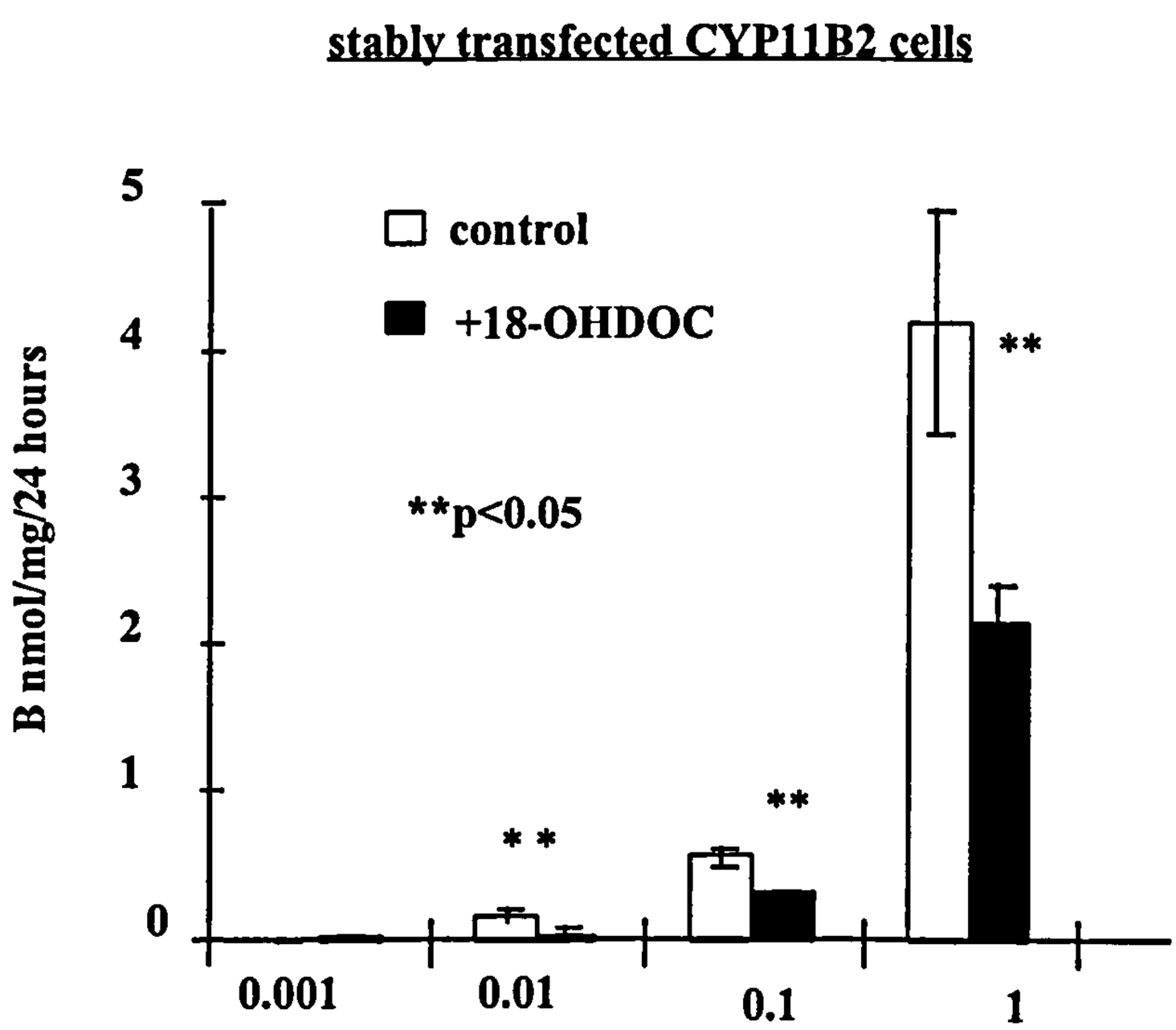


Figure 6.5 a

Corticosterone (B) production from 0.001 to 1μM 11-deoxycorticosterone (DOC) in the presence and absence of 10 μM of 18-hydroxydeoxycorticosterone (18-OHDOC) in V79 cells stably transfected with CYP11B2. V79 cells ($c10^6$) were incubated for 24 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.

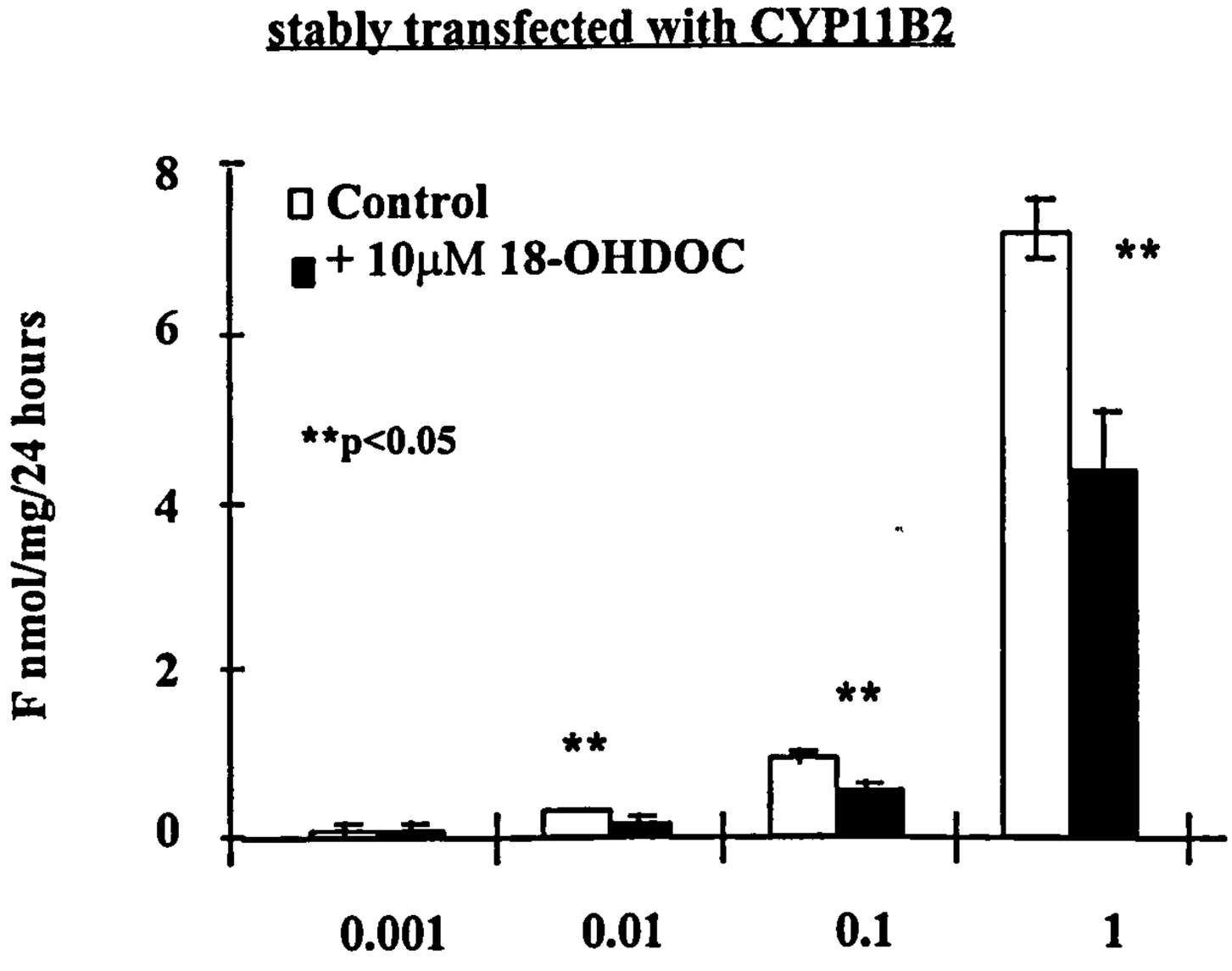


Figure 6.5 b

Cortisol (F) production from 0.001 to 1μM 11-deoxycortisol (S) in the presence and absence of 10 μM of 18-hydroxydeoxycorticosterone (18-OHDOC) in V79 cells stably transfected with CYP11B2. V79 cells ($c10^6$) were incubated for 24 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.

At 0.01, 0.1 and 1μM substrate concentrations B was reduced from 0.2 ± 0.01 to 0.05 ± 0.02, 0.5 ± 0.06 to 0.3 ± 0.02 and 4.2 ± 0.7 to 2.1 ± 0.2 nmol/mg/24 hours respectively. Similarly, F production was reduced from 0.3 ± 0.02 to 0.18 ± 0.06, 1 ± 0.16 to 0.5 ± 0.06 and 7.2 ± 0.4 to 4.4 ± 0.7 nmol/mg/24 hours respectively.

In CYP11B2 cells, 18-OHB and aldosterone production from DOC in the presence and absence of 10μM 18-OHDOC were measured. As shown in **figure 6.5c. and 6.5d,** 18-OHDOC caused a significant increase in 18-OHB and aldosterone production at all concentrations. At 0.001, 0.01, 0.1 and 1μM substrate concentrations, 18-OHB production was increased from 0.4 ± 0.08 to 5.2 ± 1.2, 1.3 ± 0.1 to 6.1 ± 0.6, 4.0 ± 0.3 to 9.3 ± 0.7 and 11.9 ± 2.1 to 21.6 ± 2.1 pmol/mg/24 hours respectively. Similarly, aldosterone production was increased from 0.8 ± 0.6 to 6.6 ± 0.7, 2.9 ± 0.5 to 7.8 ± 0.7, 4.2 ± 0.9 to 6.7 ± 0.5 and 5.3 ± 0.7 to 11.3 ± 3.2 pmol/mg/24 hours respectively.

6.6 18-OHDOC as a substrate for aldosterone biosynthesis in CYP11B2 stably transfected cells.

Figure 6.6a shows 18-OHB and aldosterone production from concentrations of 18-OHDOC ranging from 0.5 to 10μM in CYP11B2 cells expressing human aldosterone synthase.

With increasing concentrations of 18-OHDOC, there was a dose-dependent increase in 18-OHB and aldosterone production. This shows that 18-OHDOC can act as a substrate for human aldosterone synthase. Aldosterone production from DOC and 18-OHDOC was compared and is shown in **figure 6.6b.** There was a dose dependent increase in aldosterone production with increasing concentrations of substrate. Results were analysed by ANOVA followed by Student's t-test. Aldosterone production from DOC was significantly higher than aldosterone production from 18-OHDOC at all substrate concentrations ($n=4$; $p<0.05$; Students' t-test).

18-OHB production from DOC +/- 10 μM 18-OHDOC in cells stably transfected with CYP11B2

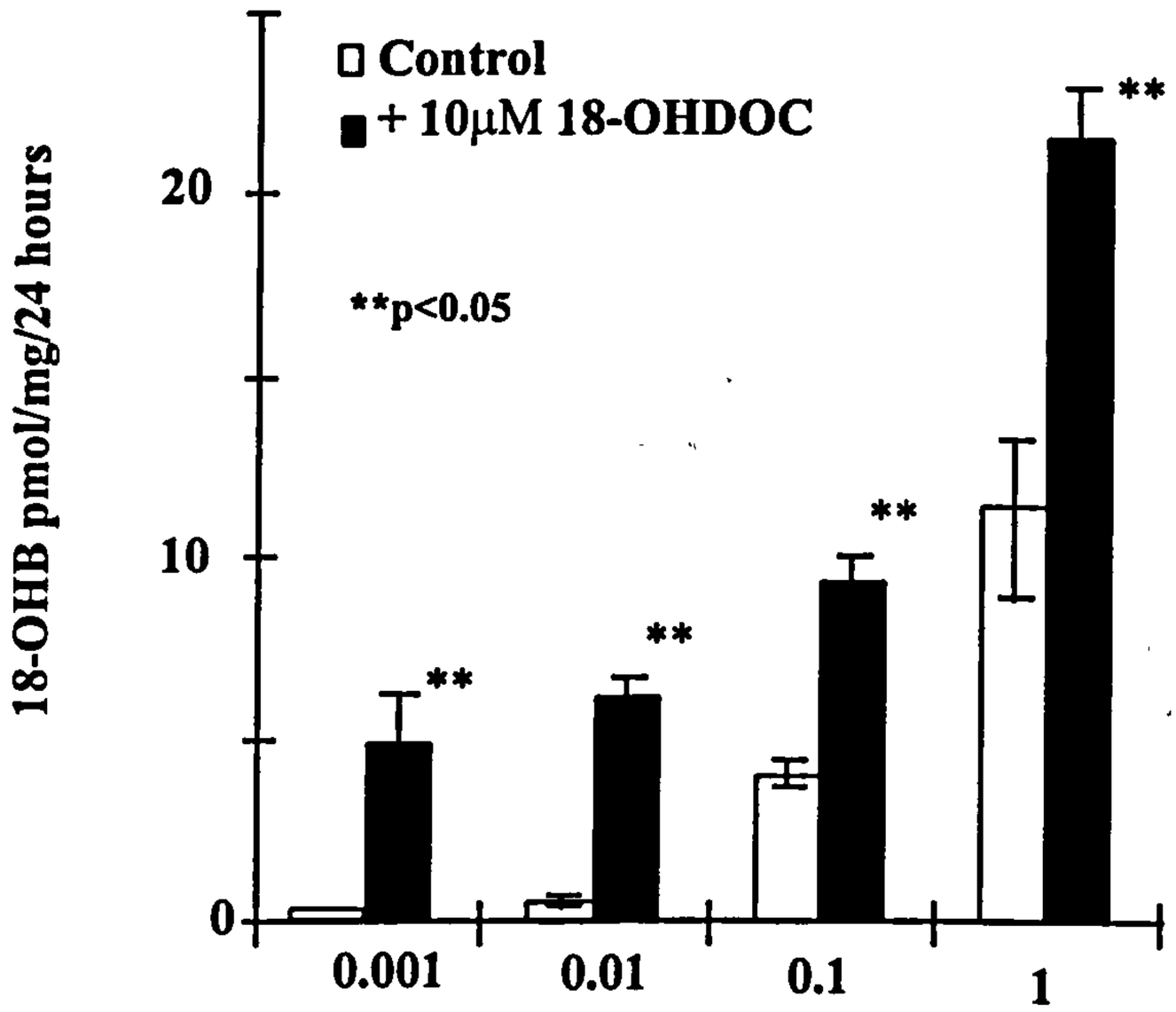


Figure 6.5 c

18-hydroxycorticosterone (18-OHB) production from 0.001 to 1μM 11-deoxycorticosterone in the presence and absence of 10 μM of 18-hydroxydeoxycorticosterone (18-OHDOC) in V79 cells stably transfected with CYP11B2. V79 cells ($c10^6$) were incubated for 24 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.

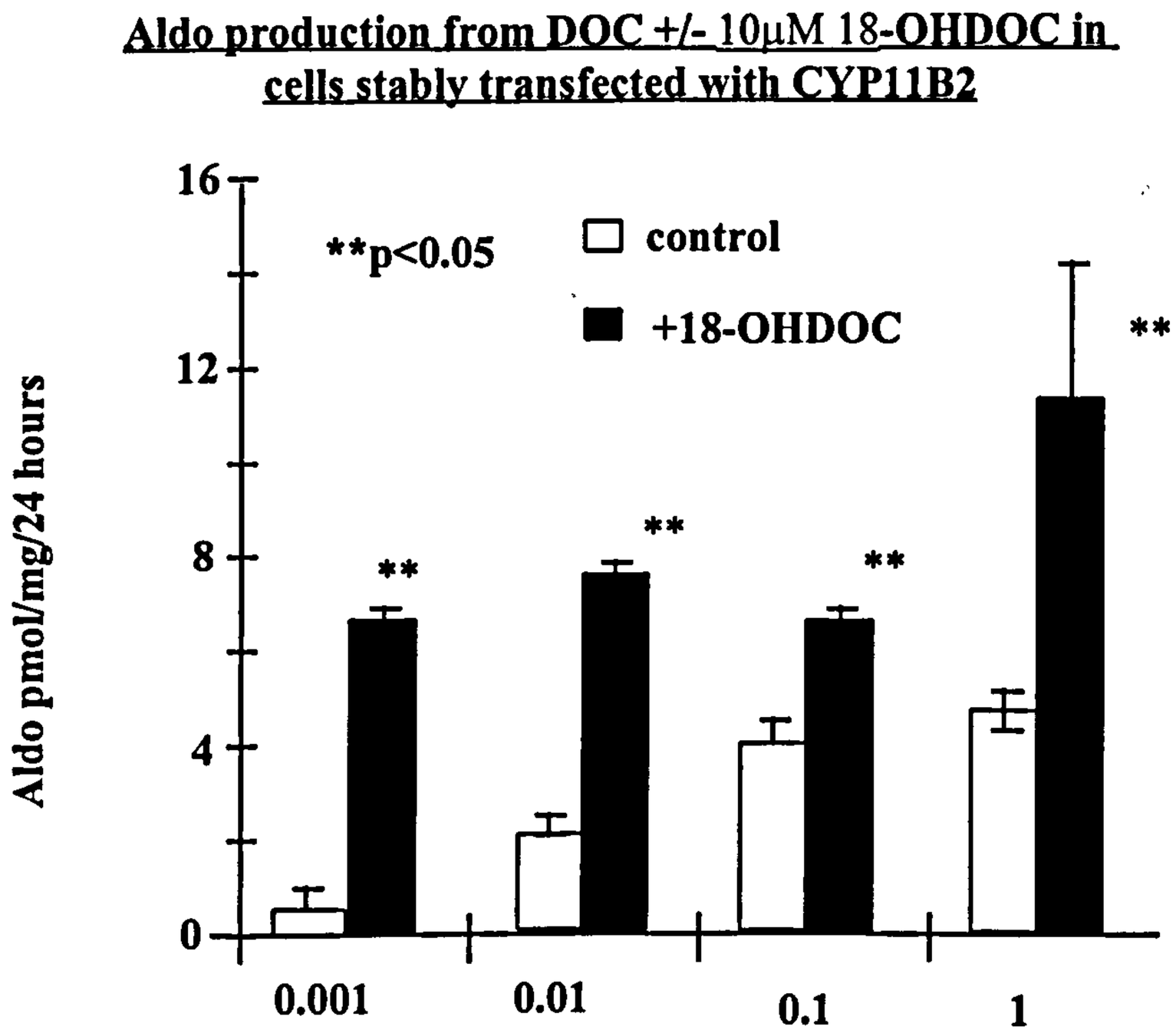


Figure 6.5 d

Aldosterone (Aldo) production from 0.001 to 1μM 11-deoxycorticosterone (DOC) in the presence and absence of 10 μM of 18-hydroxydeoxycorticosterone (18-OHDOC) in V79 cells stably transfected with CYP11B2. V79 cells ($c10^6$) were incubated for 24 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.

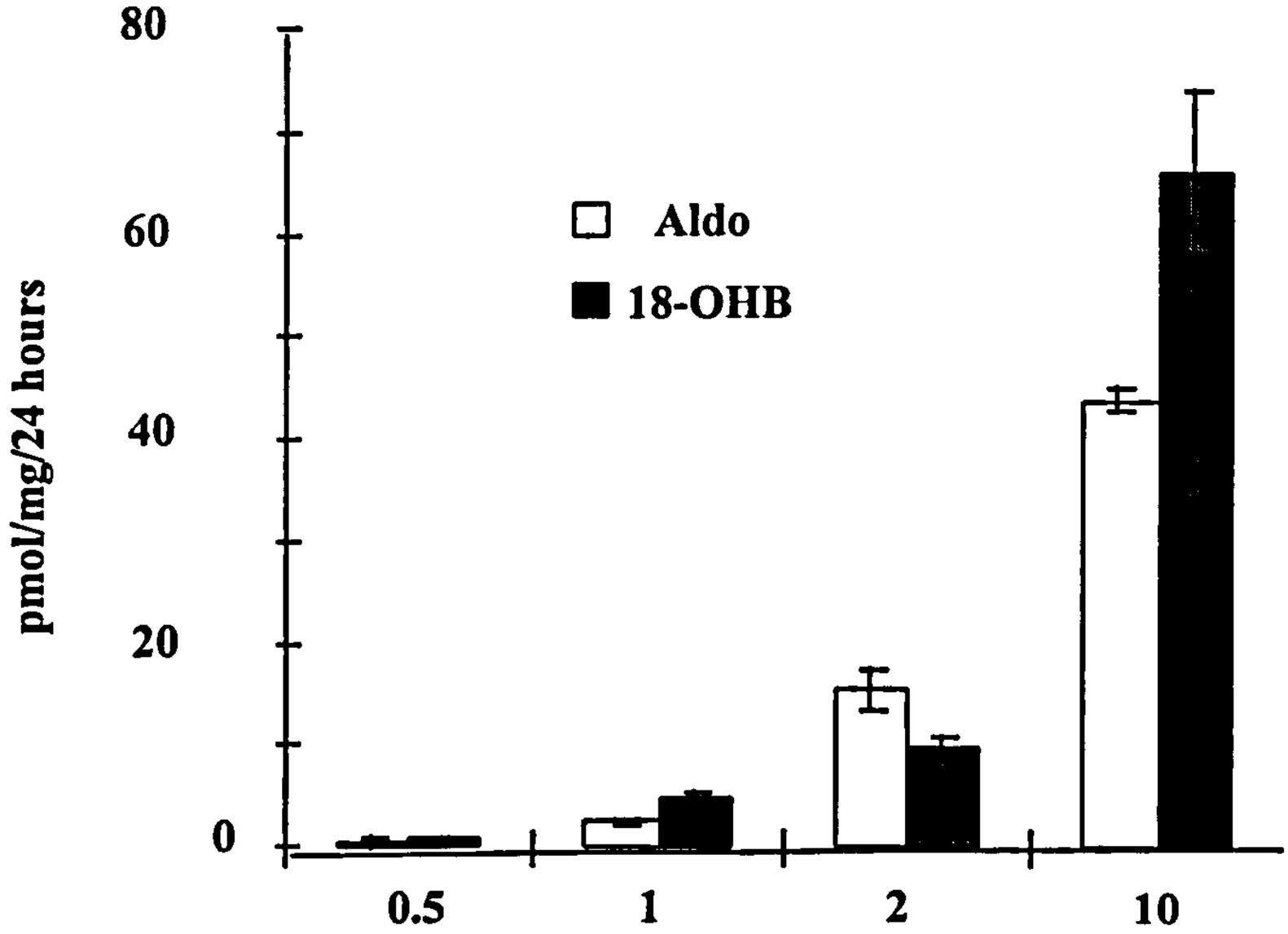


Figure 6.6 a
Aldosterone (Aldo) and 18-hydroxycorticosterone (18-OHB) production from 0.5 to 10μM 18-hydroxydeoxycorticosterone (18-OHDOC) in V79 cells stably transfected with CYP11B2. V79 cells ($c10^6$) were incubated for 24 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.

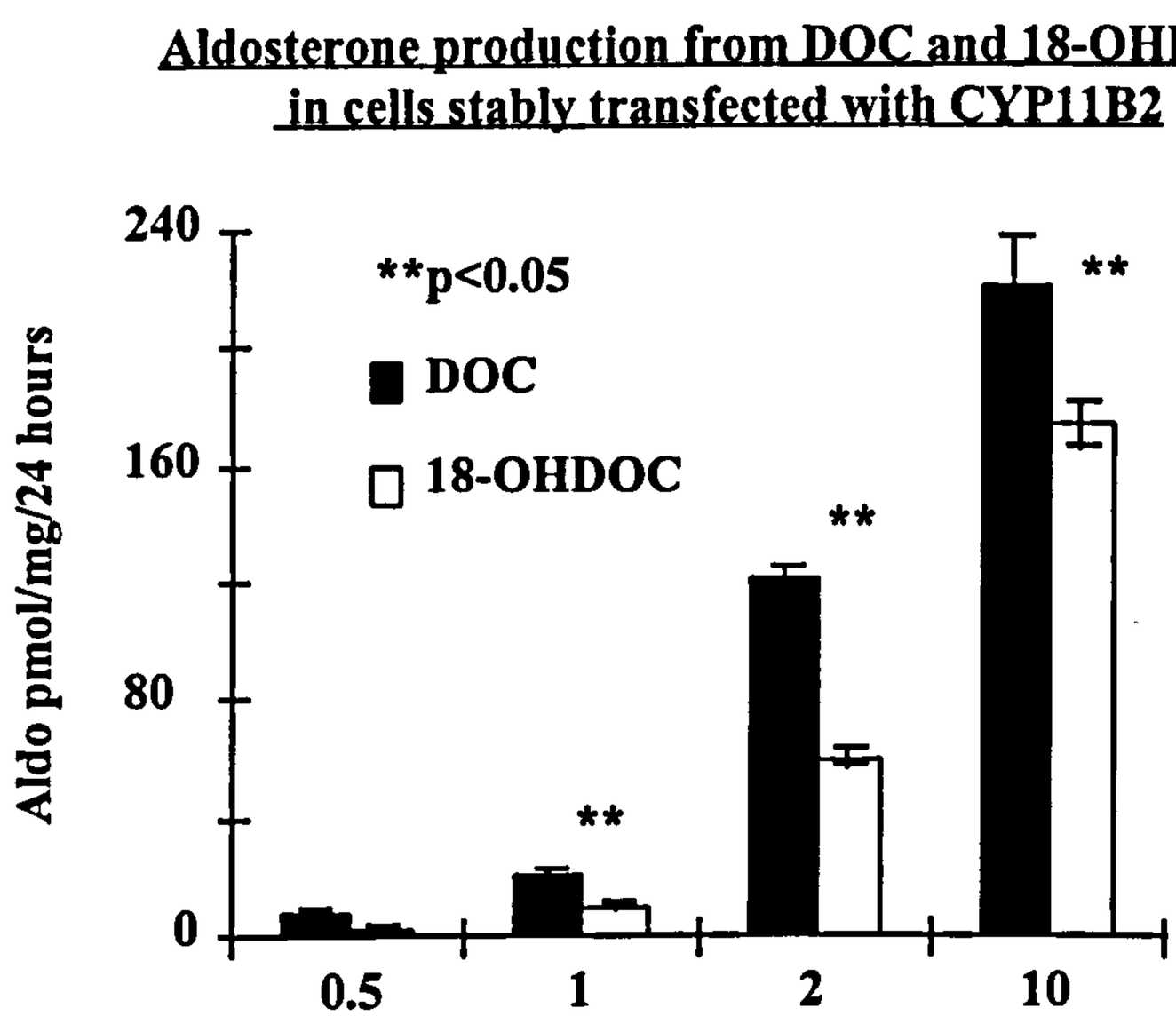


Figure 6.6 b

Aldosterone (Aldo) production from 0.5 to 10μM 18-hydroxydeoxycorticosterone (18-OHDOC) and 11deoxycorticosterone (DOC) in V79 cells stably transfected with CYP11B2. V79 cells ($c10^6$) were incubated for 24 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.

The approximate Km values for aldosterone production from DOC and 18-OHDOC were determined. Lineweaver-Burke regression analysis and equations are shown in **figure 6.6c**. The Km for 18-OHDOC was 9.09 μmol/L. The Km value for DOC was 1.73 μmol/L. These Km values are significantly different suggesting that human aldosterone synthase uses DOC more effectively than 18-OHDOC.

6.7 18-OHDOC as a substrate for 18-OHB production in CYP11B1 stably transfected cells.

Figure 6.7 shows 18-OHB production from concentrations of 18-OHDOC and DOC ranging from 0.5 to 10μM in CYP11B1 cells expressing human 11β-hydroxylase. 18-OHB production from 18-OHDOC was very poor indicating that 18-OHDOC is not a good substrate for human 11β-hydroxylase *in vitro*. Results were analysed using ANOVA followed by Student's t-test. There was a dose dependent increase in 18-OHB production with increasing concentrations of DOC which was significantly higher, at all concentrations, than 18-OHB production from 18-OHDOC (n=4; $p<0.0001$; Student's t-test).

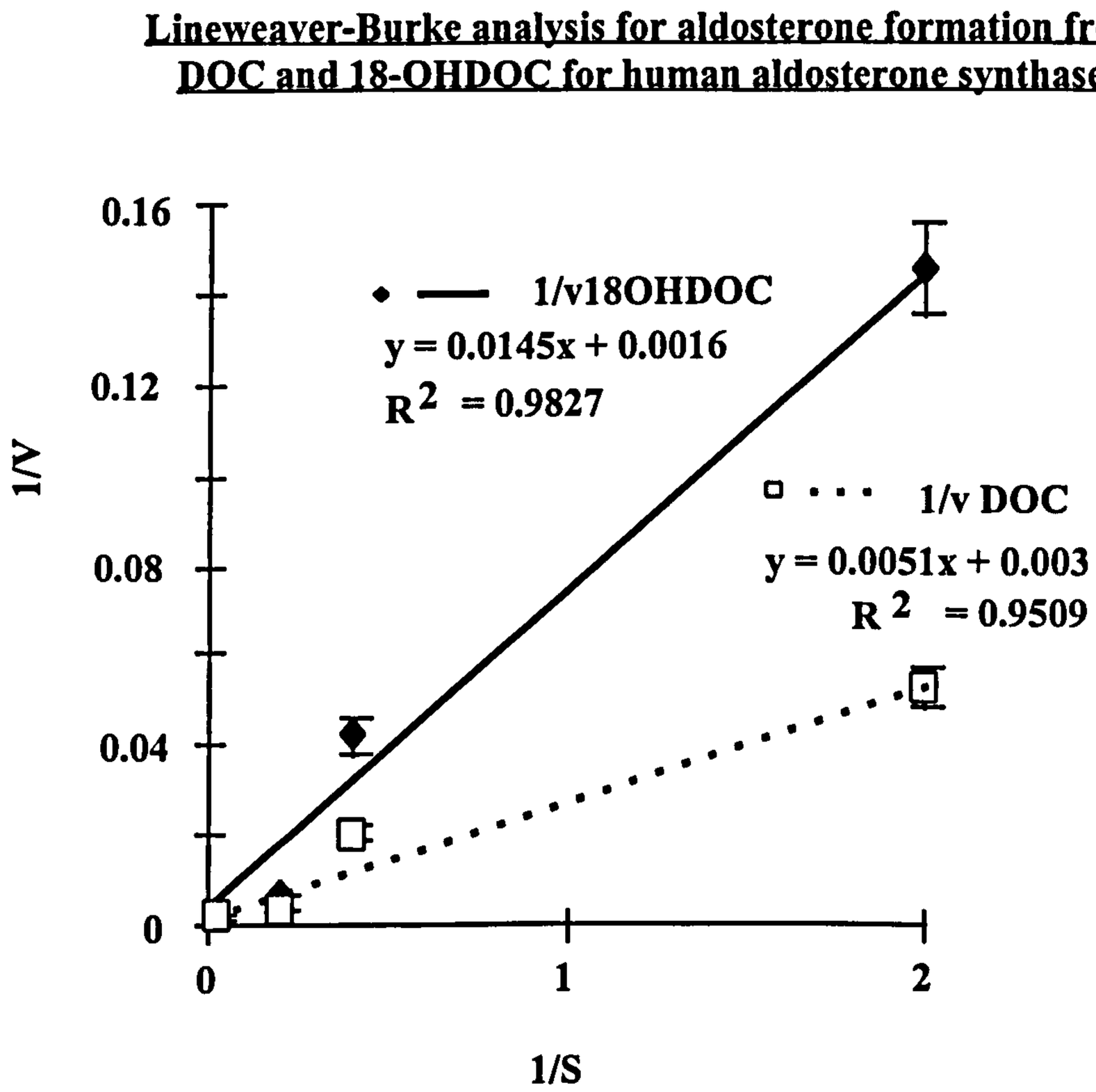


Figure 6.6 c

Lineweaver-Burke analysis and derivation of the Km for human aldosterone synthase for 11-deoxycorticosterone (DOC) and 18-hydroxydeoxycorticosterone (18-OHDOC) conversion to Aldosterone (Aldo). The axes are the reciprocal of the substrate concentration in micromolar (1/S) and the reciprocal of the velocity of aldosterone production in pmol per mg per 24 hours (1/V).
Stably transfected V79 CYP11B2. cells (c10^6) were incubated for 8 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.

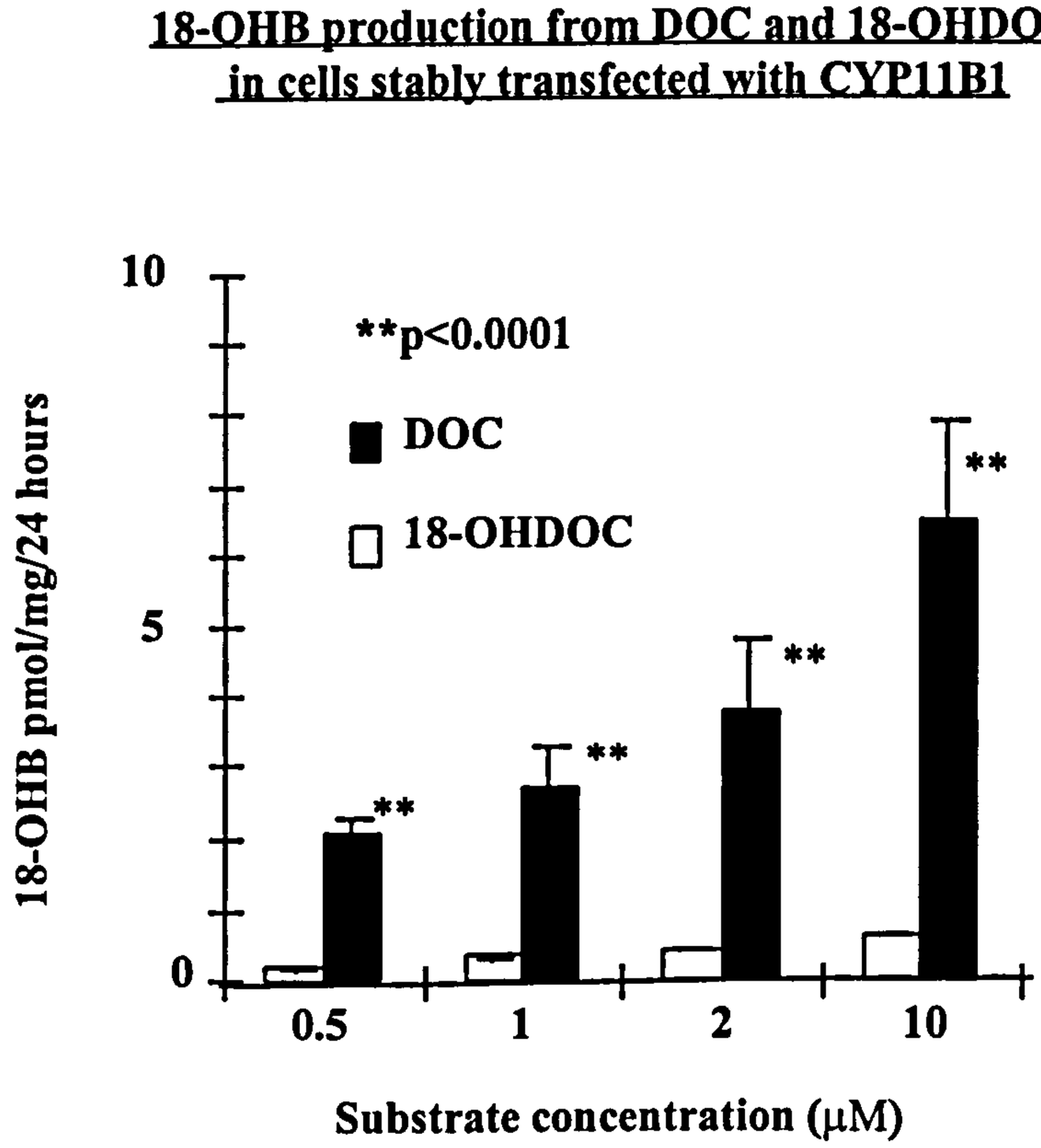


Figure 6.7

18-OHB production from 0.5 to 10μM 18-hydroxydeoxycorticosterone (18-OHDOC) and 11deoxycorticosterone (DOC) in V79 cells stably transfected with CYP11B1. V79 cells ($c10^6$) were incubated for 24 hours. Steroids from the medium were extracted and separated by paper chromatography and analysed in duplicate by radioimmunoassay. Results are mean ± SEM from quadruplicate incubations.

6.8 Discussion

In GSH, aldosterone biosynthesis is dysregulated. In all kindreds studied, all affected individuals have inherited a chimeric gene consisting of the 5' regulatory regions of CYP11B1 fused to the 3' coding regions of CYP11B2 (see section 1.8). High levels of 18-OHF and 18-OXOF are produced in GSH due to further metabolism of cortisol via the 18-hydroxylase and 18-oxidase activities of the hybrid enzyme. 18-OHF is produced in normal subjects through metabolism of cortisol by 11β-hydroxylase which also has 18-hydroxylase activity. However, urinary levels in GSH patients are some 10-fold greater than those observed in normal subjects (Ulick et al. 1982, Gomez -Sanchez et al. 1984)

In a recent study, the indices of 11β-hydroxylation, S:F and DOC:B ratios, were measured in patients with GSH after ACTH-stimulation and under resting conditions (Jamieson et al. 1996). Compared to control subjects, both resting and ACTH-stimulated, both indices of 11β-hydroxylation were altered in patients, suggesting that there was inhibition of DOC to B and S to F conversion. The explanation for this is not known but one suggestion was that 18-OXOF and 18-OHF may inhibit 11β-hydroxylation. In this study, 18-OXOF, 18-OHF and also 18-OHDOC, another 18-hydroxy derivative, were assessed *in vitro* for effects on 11β-hydroxylation by human aldosterone synthase and 11β-hydroxylase. As stated above, urinary levels of 18-OHF are some 10-fold greater than normal levels in GSH patients. Comparable concentrations of 18-OXOF and 18-OHF did not affect 11β-hydroxylation of S or DOC by either 11β-hydroxylase or aldosterone synthase *in vitro.* In addition, neither steroid had any effect on DOC conversion to 18-hydroxycorticosterone or aldosterone by aldosterone synthase.

It is possible that higher concentrations are necessary to exert inhibitory action but higher concentrations may exceed the pathophysiological range. However, the question arises as to whether urinary excretion rates reflect levels in the blood or more importantly local tissue levels within the adrenal cortex. It is probable that adrenal levels of 18-OHF and 18-OXOF are higher than those observed in urine as,

upon leaving their adrenal site of synthesis, they become diluted. That these steroids exert a synergistic inhibitory effect is unlikely. Therefore it remains possible that local concentrations *in vivo*, in close proximity to 11β-hydroxylase and aldosterone synthase, may indeed alter S:F and DOC:B ratios by inhibiting 11β-hydroxylation.

Alternatively, these *in vitro* observations may reflect the *in vivo* situation. Since 18-OXOF and 18-OHF themselves do not directly inhibit 11β-hydroxylation, what other explanations might account for the defective 11β-hydroxylation observed in patients with GSH? There are several possibilities. One is the consequence of the high rate of expression of the chimeric gene itself in the ZF and also in the ZG (Pascoe et al. 1995). CYP11B2 expression is down-regulated due to suppression of the renin-angiotensin system and therefore DOC to B and S to F conversion must be due to the sum of the activities of the chimeric enzyme and 11β-hydroxylase. Levels of chimeric mRNA in the ZF are greater than levels of CYP11B1 mRNA (Pascoe et al. 1995). The chimera responds more sensitively to ACTH and is more easily switched off by dexamethasone administration. This may explain why, although CYP11B1 and the chimera have the same 5' regulatory regions, the chimera is expressed at higher levels. As stated previously, levels of aldosterone, 18-OXOF and 18-OHF are abnormally high in GSH. It is possible that high aldosterone concentrations *in vivo* cause product inhibition of the further conversion of DOC by the chimeric aldosterone synthase. It then also follows that 18-OHF may similarly inhibit 11β-hydroxylase or the chimera to prevent further conversion of S to F and 18-OHF. This mechanism has been described; exogenously administered aldosterone has been shown to inhibit formation of corticosterone in cultured adrenocortical cells (Vinson and Whitehouse. 1979). It is possible that the defective DOC to B and S to F conversion observed in GSH is totally attributable to the chimera steroid products and that 11β-hydroxylase is converting DOC to B and S to F normally. However, it is not possible to distinguish the contributions of the chimera and 11β-hydroxylase *in vivo* as both are responsive to ACTH. The situation *in vivo* is far more complex and it is probable that other factors may contribute to the lower apparent 11β-hydroxylase activity.

The unequal crossover event between CYP11B1 and CYP11B2 has occurred at meiosis. The alternative chimera which in theory could occur which has the 5' regions of CYP11B2 fused to the 5' regions of CYP11B1 to date, has not been found. That it would have any major effect on steroidoigenesis is unlikely.

A closely related 18-hydroxysteroid was also used in this study. 18-OHDOC decreased production of B and F by inhibiting 11β-hydroxylation of S and DOC by aldosterone synthase and 11β-hydroxylase. The mechanism is probably competitive inhibition. 18-OHDOC can act as a substrate for aldosterone synthase where 11β-hydroxylation converts it to 18-OHB and subsequent 18-oxidase activity produces aldosterone. 11β-Hydroxylase can convert 18-OHDOC to 18-OHB but produces only small quantities. Therefore, 18-OHDOC may reduce the conversion of S to F and DOC to B by competing as a substrate for 11β-hydroxylation with both DOC and S. However, this is unlikely as compared to DOC, 18-OHDOC is a poor substrate of 11β-hydroxylase and not likely to compete with DOC or S for the active site. It was not possible to perform Lineweaver-Burke regression analysis on the data as too few concentration points were used. Studies of adrenal steroids displaying inhibitory effects have been reported (Matkovic et al. 1995). In mitochondrial fractions from rat adrenals, cortisol completely inhibited the conversion of B, 18-OHB or 18-OHDOC to aldosterone but had no effect on conversion of DOC to 18-OHDOC or B (Matkovic et al. 1995). This may have been due to F competing for 18-hydroxylase and 18-oxidase activities with B, 18-OHB and 18-OHDOC which were used as substrates in this study. DOC to B conversion is performed by an 11β-hydroxylation. Cortisol already has an 11β-hydroxyl group. This may explain why it did not inhibit DOC to B conversion.

18-OHDOC was shown unequivocally in these experiments to be a substrate for human aldosterone synthase and, to a much lesser extent, 11β-hydroxylase *in vitro*, producing 18-OHB and aldosterone and small quantities of 18-OHB respectively. Whether it is an important substrate in vivo remains to be established. In the rat, 18-OHDOC is said to be produced by 11β-hydroxylase, sequestered within the adrenal cortex and available as an alternative substrate for aldosterone biosynthesis (see

below).

18-OHDOC was first isolated and identified in 1961 (Birmingham and Ward 1961, Peron 1961). As early as 1979, 18-OHDOC was thought to contribute to some forms of hypertension by acting as a mineralocorticoid (Nicholls et al. 1979). In the rat, high rates of secretion are associated with increases in blood pressure. Thus, in the DahI R rat, mutations in CYP11B1 result in an enzyme which produces 2-fold less 18-OHDOC than the DahI S rat *in vitro* (Matsukawa et al. 1993) and this is now an accepted explanation of the difference in sensitivity of blood pressure to salt (see 1.8.10). In man, 18-OHDOC levels are slightly greater than those of aldosterone (Melby et al. 1972, Messeril et al. 1976, Ulick et al. 1976) but its mineralocorticoid potency is much lower. There have been occasional reports of mildly raised levels of 18-OHDOC in some forms of hypertension. However, that the associated mineralocorticoid effects observed in these patients with higher 18-OHDOC levels may be attributable to consequent small increases in aldosterone production through 18-OHDOC conversion by aldosterone synthase, while possible, is unlikely.

The question arises as to whether 18-OHDOC is an important substrate *in vivo* for aldosterone synthase or 11β-hydroxylase in human subjects. There are several factors which suggest that it is not. The local tissue concentrations of adrenal steroids are not known. For the sake of argument, one may assume that the relative concentrations in plasma and urine excretion rates reflect their relative local concentrations. Normal plasma/ urine ranges for DOC, B and 18-OHDOC are 80 to 500 pM, 2.3 to 23 nM and 0.6 to 4.6 nM respectively. Taking these as indices of their relative local adrenal concentrations, it then follows that 18-OHDOC concentration may be some 10-fold higher than that of DOC. This would suggest that 18-OHDOC would be a readily accessible substrate for aldosterone synthase. This study however, shows that DOC has a much lower Km for aldosterone synthase than does 18-OHDOC. Thus, despite somewhat higher concentrations, that 18-OHDOC can compete effectively with DOC for the aldosterone synthase active site seems unlikely. The Km of 11β-hydroxylase for 18-OHDOC was not assessed as conversion to 18-OHB from 18-OHDOC was so poor. 18-OHDOC was converted to 18-OHB but the yield of 18-

OHB was much lower than from DOC. This suggests that 18-OHDOC is probably not an important substrate for 11β-hydroxylase *in vivo*. The efficiency of 18-OHDOC conversion to aldosterone by cloned rat aldosterone synthase *in vitro* has been shown to be poor (Zhou et al. 1995). Also, bovine 11β-hydroxylase catalyses aldosterone synthesis and 18-OHDOC has recently been shown not to be a preferred substrate (Imai et al. 1998).

Another important uncertainty is whether 18-OHDOC is sequestered by protein binding in man as it is in the rat (Vinson et al. 1992) and also whether this pool of 18-OHDOC is readily available to the enzymes. In the rat, 18-OHDOC can only be released from ZF-sequestered stores *in vitro* by trypsinisation of cultured adrenal sections or by prior sodium depletion (Vinson et al. 1995). The mechanism by which ZF-produced 18-OHDOC finds its way to the ZG is not known but may involve a carrier and active transport. Whether in human subjects, ZF-produced 18-OHDOC has access to the ZG is not known. That 18-OHDOC is made by human aldosterone synthase *in situ* in the ZG in realistically useful quantities is also unlikely. In the rat, aldosterone synthase produces only very low levels of 18-OHDOC (Okamato & Nonaka 1992). Moreover, to use sequestered 18-OHDOC, aldosterone synthase, which has a relatively low affinity for this steroid, must compete with the putative binding protein which, from *in vitro* evidence at least, binds it strongly. Therefore *in vivo*, 18-OHDOC is probably not an important substrate for aldosterone biosynthesis in man. Recent studies have claimed that 18-OHDOC may be utilised in aldosterone synthase deficiency (Portrat-Doyan et al. 1998). This too is unlikely as conversion of 18-OHDOC to aldosterone requires 11β-hydroxylation and 18-oxidation. This latter function requires aldosterone synthase. In summary, it is clear from the present *in vitro* study that 18-OHDOC inhibits DOC to B and S to F conversion and also that it can be converted to aldosterone and 18-OHB by human aldosterone synthase and 11β-hydroxylase respectively. However, the affinity of 18-OHDOC for these enzymes is much lower than that of DOC, indicating that it is not a preferred substrate.

CHAPTER 7

7. General discussion

Enzyme function, like that of any catalyst, is structurally dependent. Substances such as substrates and cofactors are brought into close proximity with the enzyme active site and manipulated into the precise orientations appropriate for reaction and transformation. In the globulin protein enzyme molecule, this complex but precise topography is derived from the folding of a linear polypeptide comprising α-helices and β-sheets with linking loops. Clearly, the effect of sequence differences - i.e. mutations or polymorphisms will depend on the extent to which they may change conformation. This is relevant to the studies of 11β-hydroxylase as aldosterone synthase described in this thesis in two ways.

1) As emphasised frequently in previous sections, aldosterone synthase and 11β-hydroxylase have extensive structural homology but functions which, while overlapping, are different. While both catalyse 11β- and 18-hydroxylation, only aldosterone synthase is capable of inducing the second hydroxylation at C18 necessary for aldosterone biosynthesis. Moreover, studies in this thesis have shown that substrate specificities are quantitatively different (see chapter 3, 5 and 6). This difference in potential lies mainly in the haem and substrate binding regions encoded by exons 6, 7 and 8. Evidence in this thesis corroborates and extends that of previous structure-function studies. This information was obtained by site-directed mutagenesis of specific codons of CYP11B1 and B2 and by expressing the altered genes *in vitro*. Careful evaluation in this study of the effects of mutations on the kinetics of each component reaction using each of the physiological substrates was accomplished using specific steroid analysis. Less predictable, but of great potential interest, was the finding that a relatively conservative mutation at some distance from domains apparently directly involved in catalysis (eg. the I helix, K-helix and β-sheets 3 and 4, see section 1.7, 1.8 and 1.9) had significant quantitative and qualitative effects on the reactions. In order to establish the reasons for such effects, it is necessary to know their relative positions. Mutation of residues which appear to be in significant regions may have functional implications. In chapter 4, an attempt has been made to study this using modelling techniques based on simpler bacterial

cytochrome P450 enzymes. Residues at positions 43, 107, 147, 186, 248, 357 and 493 have been highlighted in this study. The regions to which they have been assigned by modelling techniques shows: residue 43 is within the membrane anchor, residues 107,147 and 186 and 248 lie within or flank helices surrounding the haem while residues 357 and 493 may form part of β-sheets 3 or 4. Substitutions K357N and T493M which lie within β-sheets 3 and 4, regions involved in substrate/adrenodoxin interaction, did not alter function. However, it is probable that the chemical properties of an amino acid are important to the function. While these substitutions did not alter function, it remains possible that different amino acids at the same locus might do so. It is also possible that the modelling is imprecise and that residues are wrongly positioned resulting in a false expectation of the effects of a mutation. The shortcomings of this approach are obvious and have been emphasised. In order to obtain reliable information, it will be necessary to obtain the crystal structure of these enzymes. Milligram quantities are necessary for protein crystallography techniques. This will require large-scale protein expression using for example, a Baculovirus system, and subsequent isolation and purification.

2) The practical implications of these findings, the second way in which the structure-function studies are important, relate to their possible clinical significance. As described in sections 1.6 and 1.8 and chapters 3 and 5, the aetiology of essential hypertension is unknown but there is strong evidence of an inherited component in the blood pressure rise and this may, at least in part, be explained by changes in corticosteroid synthesis and catabolism. Moreover, recent evidence obtained from twins (Inglis et al. 1998) suggests that secretion of many corticosteroids is strongly heritable. Screening DNA from patients with essential hypertension in our department has already revealed a number of polymorphisms in CYP11B1 and CYP11B2 (chapter 5). Their clinical significance is not established. Using the techniques established in this study to prepare these altered genes and carry out kinetic analysis it will be possible to assess their potential influence on endogenous hormone levels. Used in conjunction with the results of crystallographic analysis, it may in the future be possible to predict their effects and perhaps also to develop pharmaceutical agents to control gene function.

The results outlined and discussed in previous chapters could be improved and extended in several ways. For example, in chapter 3, since the principal aim was to investigate the specific basis of aldosterone synthesis, only those residues potentially important to this activity were studied in detail. Residue 147 was found to be important in the 11β-hydroxylation component of aldosterone synthesis; it was also important to the activity of 11β-hydroxylase. However, although manipulation of residues 43, 248, 357 and 493 was without effect on aldosterone synthase activity, it cannot be assumed that they are similarly inconsequential to 11β-hydroxylase. This must be tested experimentally. The choice of residues to mutate may have been affected had the computer modelling been investigated before the experimental work began. This may have identified more key residues involved in specific enzyme activities.

As explained in chapter 1, 11β-hydroxylase also catalyses 18- and 19-hydroxylation. Studies in the DahI rat strongly indicate that it is the balance of these activities that determines physiological and pathophysiological outcome. Moreover, it is possible that the timecourses of these various transformations are different. Future studies should therefore follow the effect of induced or naturally-occurring mutations of 11β-hydroxylase on the ratio of 11β: 18: 19-hydroxy products, not at a single time point (48h) but as it changes with time.

The enzymes studied interact with their respective substrates with characteristic affinities (chapter 3). It is important to emphasise that they also interact with co-factors, notably adrenodoxin. In the studies described herein, cells were cotransfected with bovine adrenodoxin whereas the human protein may have been more appropriate. While there are reports that such species-compatible constructs are more efficient, a thorough study of species and dose-response relationships would be valuable.

No evidence that either 18-oxocortisol or 18-hydroxycortisol inhibit aldosterone synthase or 11β-hydroxylase was obtained (chapter 6). This conclusion was based on the use of a single concentration (10μM), a choice in turn based on the levels of

18-hydroxycortisol in GSH patents compared to control subjects. Again, it must be emphasised that the local in vivo tissue concentrations of these compounds in GSH or in normal subjects are unknown, nor is it known whether the fact that they are intracellular in vivo and extracellular in vitro may alter their action. Certainly, the low conversion rates of substrate steroids in this in vitro system suggest that it may not have been optimal. Further studies of tissue concentrations would be helpful.

Finally, consideration of the crystallographic structure of human aldosterone synthase and 11β-hydroxylase were necessarily speculative although it produced some interesting ideas. More detailed analysis is required and hence determination of the crystal structure.

Like all good research endeavours, this work commenced with a small series of related questions. Some have been answered; some have not. Importantly, the research has raised a larger series of important questions to be addressed in the future.

APPENDICES

APPENDIX 1

Nucleotide Sequences of CYP11B1 and CYP11B2

Listed below are the nucleotide sequences for CYP11B1 and CYP11B2 obtained from the Human Genome databank.Accession numbers for the sequences are as follows:

	CYP11B1	CYP11B2
5' flanking region and exon 1	D10169, D90428, X55765	D10170, D90429
Exon 1 and 2	M32863, J01540	M32864, J05140
Exon 3 8	M32878, J01540	M32880, J05140
Exon 9	M32879 J01540	M32881 J05140

Exons are indicated by bold type. The position of oligonucleotides listed in appendix III are indicated by bold letters above the relevant sequence and underlined. Sense and antisense oligonucleotide are indicated by > and < respectively.

CYP11B1

```
TTTTCTAGTTCTTTTAATTGTGATGTTAGGGTGTCAGTTTTGGATCTTTCCTGCTTTCTC 60

TTGTGGGCATTTAGTGCTATAAATTTCCCTCTACACACTGCTTTGAATGTGTTCCAGAGA 120

TTCTGGTATGCTGTGTCTTTGTTCTCGTTGGTTTCAAGAACATCTTTATTTCTGCCTTCA 180

TTTTGTTACGTACCCAGTAGTCATTCAGGAGCAGGTTGCTCAGTTTCCATGTAATTGAGC 240

GGTTTTGAGTGAGTTTCTTAATCCTGAGTTCTAGTTTGATTGCACTAAAATTTTTAAAAA 300

GTAAAAAAAATACATGTGGTTTAATACAATTCATGCCAACTCATTCCCTCGTTTTTTGCT 360

ATAAACCTTGCAAGGAGATGAATAATCCAAGGCTCTTGGATAAGATAAGGGCCCCATCCA 420

TCTTGCTCCTCTCAGCCCTTGGAGGAGGAGGGAGAGTCCTTTTCCCCTGTCTACGCTCAT 480

GCACCCCCAATGAGTCCCTGCCTCCAGCCCTGACCTCTGCCCTCGGTCTCTCAGGCAGAT 540

CCAGGGCCAGTTCTCCCATGACGTGATCCCTCTCGAAGGCAAGGCACCAGGCAAGATAAA 600

AGGATTGCAGCTGAACAGGGTGGAGGGAGCATTGGAATGGCACTCAGGGCAAAGGCAGAG 660 ex 1

GTGTGCATGGCAGTGCCCTGGCTGTCCCTGCAAAGGGCACAGGCACTGGGCACGAGAGCC 720

GCCCGGGTCCCCAGGACAGTGCTGCCCTTTGAAGCCATGCCCCGGCGTCCAGGCAACAGG 780

TGGCTGAGGCTGCTGCAGATCTGGAGGGAGCAGGGTTATGAGGACCTGCACCTGGAAGTA 840
```

CACCAGACCTTCCAGGAACTGGGGCCCATTTTCAGGTAAAGCCCTCCCTGGCCCTCGCTG 900

GAACACCCAGTGCCCTGCCCTTGCTGCCCAGGACCCTGCCGGGCACTCAGCACTGCCATT 960

CCCAGCAGGTCCCGGCACTCTGCATCCTTTGGAAGAGGGAAGATCGAGCACGTGCTGTCT 1020

GTGCGCTGCAGGGCAGGGCATGTGCAGAGCAAATGGGAGCTCGGCTGCAGAGAGGGCAGG 1080

ACTCAGAGGCACTGAAGTTAAGAGGTTCCGGGCAGTCAGCAAGAGGGCGTTTAGCTGTGA 1140

Y6265>

AGCCGCTAATCCAGGAGAGGGGAGGGTGGACAGGAGACACTTTGGATTGGGACTGCAGGG 1200

TGGGGCCAGCAGGGACTAGACCCCGTCCAGCAGGGCCTCCTGCTTGGCCCCACAGGTACG 1260 ex 2

ATTTGGGAGGAGCAGGCATGGTGTGTGTGATGCTGCCGGAGGACGTGGAGAAGCTGCAAC 1320

C3130> C3131<

AGGTGGACAGCCTGCATCCCCACAGGATGAGCCTGGAGCCCTGGGTGGCCTACAGACAAC 1380

C0222>

ATCGTGGGCACAAATGTGGCGTGTTCTTGCTGTAAGCGGCGAGCTGAGAGCTGGGAGCAG 1440

C0223<

GGTGGGCAGCCTGGGTGTAGGGGGGAGGCGAGAGAGGCAGGAAAAGCTTGACAACAGGGG 1500

TCAGTTCCTTTCTTGCAGAAAATCCCTCCCCCCTACTACAGGGAGGGCCCGCATGGGTGA 1560

GGTGGTGCCAGACTTGGGGCGCCAGGTCCCGGGAATGACCTCAGTTACCCTGTCAGCACC 1620

TGTGGGCAGAAGCTACCATCTCATCCCTGCTTAGACCTGAGTGGCCTTTGTCCAGCACCT 1680

GGAGGCCGTCTGAGAAAAGGCTGCAGCTCGAACACAAACAGGCAGCTTCTACCAGGGCCC 1740

Y6263

CCAGTCAGCTCCCTGCAGGCCGATTCCCCTTGGGACAAGGAGGATGGGATACGGGTCAGG 1800

GCCTGTGTTTTGCTGGGGCGGCCTCACAAGCTCTGCCCTGGCCTCTGTAGGAATGGGCCT 1860 ex 3

C8489< Y6130> Y6131<

GAATGGCGCTTCAACCGATTGCGGCTGAATCCAGAAGTGCTGTCGCCCAACGCTGTGCAG 1920

AGGTTCCTCCCGATGGTGGATGCAGTGGCCAGGGACTTCTCCCAGGCCCTGAAGAAGAAG 1980

GTGCTGCAGAACGCCCGGGGGGAGCCTGACCCTGGACGTCCAGCCCAGCATCTTCCACTAC 2040

Y6264<

ACCATAGAAGGTGTGGGCCACATGGGTTGATCCAGCCTCAGAGACCCTGGAGTGGCCAGG 2100

GACGGGGATGGGGGACTGAAGGGAGTGTGGGGAGGCAGCCAGGAGGCCCGGTTCCCTTGT 2160

GCTCAGCAGTGCATCCTCCCCGCAGCCAGCAACTTGGCTCTTTTTGGAGAGCGGCTGGGC 2220 ex 4

S6718>

CTGGTTGGCCACAGCCCCAGTTCTGCCAGCCTGAACTTCCTCCATGCCCTGGAGGTCATG 2280

TTCAAATCCACCGTCCAGCTCATGTTCATGCCCAGGAGCCTGTCTCGCTGGACCAGCCCC 2340

AAGGTGTGGAAGGAGCACTTTGAGGCCTGGGACTGCATCTTCCAGTACGGTGAGGCCAGG 2400

B4251<

GACCCGGGCAGTGCTATGGGGAAGGACACCATCGGGCCCCCAATTTCTCCCTCTCCACCA 2460

CCCAGTGGGGAATGGAGGCCACAGGGAGGGGTCGGGGATTCCTCACCGTCCTGCCAGGGA 2520

GATTGGTGTGAGGCTGGGGCTGGGCTGGGCTGATCCGGAGAATTTGGGATGAGAGCAGGG 2580

M6642>
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AGTCTGAGGGCTGCCTCCCGCTCCCCGGATAGGCGACAACTGTATCCAGAAAATCTATCA 2700 ex 5

GGAACTGGCCTTCAGCCGCCCTCAACAGTACACCAGCATCGTGGCGGAGCTCCTGTTGAA 2760

TGCGGAACTGTCGCCAGATGCCATCAAGGCCAACTCTATGGAACTCACTGCAGGGAGCGT 2820

GGACACGGTCAGGCCGGCAACCAGCCCCACCCAGAGAGGGTGATGCCAAGCCTGCCTCCC 2880

M6643<
AGGCACTGCCTGCCAATGTCACACGGCGCCCACGTGTCCCATGCCCAGGCTATGGGCCCC 2940

ACATTTCTTACTTGGGATTGTGATGTGATAAACACGTTTGCAGGTTGCCATGGTTGGAAT 3000

GGGGGGTTCCTTTCCTTCTGTGGAGGACTCAGGGAAACGGGGTTTGGATGGGCATTAGGA 3060

TTTGAAGTCTTGGGCTCTGTCGTGCTCAGGGTATGCATGTCTGCACCCCTCACAGGGAGG 3120

TTGTCCTGGGAGGGGTGTCCCGGGGGCTGAGTCCTCCTGTGCAAGGTCTGACCCTGCAGC 3180

TGTGTCTCCTGCAGACGGTGTTTCCCTTGCTGATGACGCTCTTTGAGCTGGCTCGGAACC 3240 ex 6

CCAACGTGCAGCAGGCCCTGCGCCAGGAGAGCCTGGCCGCCGCAGCCAGCATCAGTGAAC 3300

ATCCCCAGAAGGCAACCACCGAGCTGCCCTTGCTGCGTGCGGCCCTCAAGGAGACCTTGC 3360

T2140<
GGTGGGTGCTGGCTGAGGCCTCCCTGTGGCCCTGGCCCTGCTGGAGAGTCAGCCCCCACT 3420

GGGTGGTTGCAGACAGAATCTGGGCTATAAACACCTACCCAGCAGCCATCCTGACTGCTC 3480

TCTCGCGTCAAGGACAGGGAGCTCTTCTTCCTCTGGAATCCCTCTTCAACGCCCTGGGGA 3540

TTAACGTGGGGGCATGTCCTTCTGCGCTCGGGGCTGCTTAAGTTAGGGGAGGTTTGGCCG 3600

GGCTCAGCAGGTGCAAGGAAGCACTTCCTACACCTGGGCTTCCCATGGATCTGGGACCTC 3660

TGCGGGGTCTTCGGTAGGAAGGGTGCAGAGAGCACAGGAACCCCATCCCAGCTGAGACCC 3720

Y6259>
TTTCTATGGATGCCCCCACCTCCAGGCTCTACCCTGTGGGTCTGTTTCTGGAGCGAGTGG 3780 ex 7

CGAGCTCAGACTTGGTGCTTCAGAACTACCACATCCCAGCTGGGGTGAGTGAGCCCCACA 3840

Y6261>
CCCTCGAGCTGAGAACCTCCCTCCCCAGTCATTCCCTGATCCCCGCTCTGCTCCGTCCGC 3900

AGACATTGGTGCGCGTGTTCCTCTACTCTCTGGGTCGCAACCCCGCCTTGTTCCCGAGGC 3960 ex 8

CTGAGCGCTATAACCCCCAGCGCTGGCTAGACATCAGGGGCTCCGGCAGGAACTTCTACC 4020

ACGTGCCCTTTGGCTTTGGCATGCGCCAGTGCCTTGGGCGGCGCCTGGCAGAGGCAGAGA 4080

TGCTGCTGCTGCTGCACCATGTGAGCAGGCCCGGGGAATTCTGGGCCTGGGCTGTAAGGT 4140

M6635>
GGGGCTGGTCAGGAATGAAACAGGTTGGAGGCCAGGCTGCTGTTCCCCCTTCAGCATAAT 4200

CTCTGCAACTTTGAGGGTCTGAGAAGGCTGCACCACGTCGATGGGCTGCGGACCAAGCCA 4260

GATGGAAACCCGGCTTCTGTCCTAGGTGCTGAAACACCTCCAGGTGGAGACACTAACCCA 4320 ex 9

AGAGGACATAAAGATGGTCTACAGCTTCATATTGAGGCCCAGCATGTGCCCCCTCCTCAC 4380

M6637<

CTTCAGAGCCATCAACTAATCACGTCTCTGCACCCAGGGTCCCAGCCTGGCACCAGCCTC 4440

CCTTTCTGCCTGACCCCAGGCCACCCCTCTTCTCTCCCACATGCACAGCTTCCTGAGTCA 4500

CCCCTCTGTCTAACCAGCCCCAGCACAAATGGAACTCCCGAGGGCCTCTAGGACCAGGGT 4560

TTGCCAGGCTAAGCAGCAATGCCAGGGCACAGCTGGGGAAGATCTTGCTGACCTTGTCCC 4620

CAGCCCCACCTGGCCCTTTCTCCAGCAAGCACTGTCCTCTGGCAGTTTGCCCCCATCCCT 4680

CCCAGTGCTGGCTCCAGGCTCCTCGTGTGGCCATGCAAGGGTGCTGTGGTTTTGTCCCTT 4740

GCCTTCCTGCCTCTAGTCTCACATGTCCCTGTTCCTCTTCCCCTGCCAGGGCCCCTGCGC 4800

AGACTGTCAGAGTCATTAAGCGGGATCCCAGCATCTAGAGTCCAGTCAAGTTCCCTCCTG 4860

CAGCCTGCCCCCTAGGCAGCTCGAGCATGCCCTGAGCTCTCTGAAAGTTGTCGCCCTGGA 4920

ATAGGGTCCTGCAGGGTAGAATAAAAAGGCCCCTGTGGTCACTTGTCCTGACATCCCCAT 4980

TTTCAAGTGATACAACTGAGTCTCGAGGGACGTGTGTTCCCCAGCTGATCGTGTCAGCCT 5040

CATGCCCCTGGCCTCATCTTTCATGGACCAGGCCTTGTTCCAGGAGTGGGCGTTGGGTCC 5100

TCTGCTTCCTGTGCTGTCCCCTGGGGAAGGTCCCAAGGATGCTGTCAGGAGATGGAAGAG 5160

TCATGTGGGGTGGGAACCTGGGGTGTGGTTCCAGAAATGTTTTTGGCAACAGGAGAGACA 5220

GGATTGGGCCAACAAGGACTCAGACGAGTTTTATTGACTATTCTCTGACA 5270

CYP11B2

TTTGTC-TACATGTGTTCAAAACCCACAGCATGTTGACCACCAGGAGGAGACCCCATGTGACTCCAG 60

-6 1+

GGCCCCTGGTTGATAACAACGTATCGAGATTCCTCACATGGAACCAGTGCGCTTCTGTGG 120

TGGAGGGTGTACCTGTGTCAGGGCAGGGGGTACGTGGACATTTTCTGCAGTTTTTGATCA 180

ATTTTGCAATGAACTAATCTCTGGTATAAAAATAAAGTCTATTAAAAGAATCCAAGGGGC 240

CCTCTCATCTCACGATAAGATAAAGTCCCCATCCATTTTGCTCCTCTCAGCCCTGGAGAA 300

AGGAGAGGCCAGGTCCCACCACCTTCCACCAGCATGGACCCCCAGTCCAGACCCCACGCC 360

TTTTCTCAGCATCCTCAGACCAGCAGGACTTGCAGCAATGGGGAATTAGGCACCAGACTT 420

T2137>

CTCCTTCATCTACCTTTGGCTGGGGGCCTCCAGCCTTGACCTTCGCTCTGAGAGTCTCAG 480

GCAGGTCCAGAGCCAGTTCTCCCATGACGTGATATGTTTCCAGAGCAGGTTCCTGGGTGA 540

GATAAAAGGATTTGGGCTGAACAGGGTGGAGGGAGCATTGGA**ATGGCACTCAGGGCAAAG** 600 **ex 1**

GCAGAGGTGTGCGTGGCAGCGCCCTGGCTGTGCCTGCAAAGGGCACGGGCACTGGGCACT 660
V5311> V5312<
AGAGCCGCTCGGGCCCCTAGGACGGTGCTGCCGTTTGAAGCCATGCCCCAGCATCCAGGC 720

AACAGGTGGCTGAGGCTGCTGCAGATGTGGAGGGAGCAGGGTTATGAGCACCTGCACCTG 780

GAGATGCACCAGACCTTCCAGGAGCTGGGGCCCATTTTCAGGTAAAGCCCTCCCTGGCCC 840
T2139<
TCGCTGGGAACACCCAGATCCCTCCCCCTGCTGCCCAGGACACTGCCAGGCACTCAGCAC 900

TGCCATTCCCAGCAGGTCCCGGCACTCTGCATCCTTTGGAGGATGGGGAAGGAGTGCAGC 960

ACATGCTGGTCTGTGGTGCTGCCAGGGCAGGGGATAGTGCAGAGAAAACCCCAGCTCACT 1020

GCAGAGAGGGCAGGACTCAGAAGCACTAAAGTTGAAAGGTTCCAGGGAGCCAGCAGGAGG 1080

GCTTTAGCTGTGAAGCCGCTAATCCAGGAGCAGGGAGGGTGGACAGGAGACACTTTGGAT 1140

TGGGACTGCAGGGTGGGGCCACGAGGGACATGACCCCGTCCAGCAGGGCCTCCTGCTTGG 1200

CCCCACAGGTACAACTTGGGAGGACCACGCATGGTGTGTGTGATGCTGCCGGAGGATGTG 1260
ex 2

GAGAAGCTGCAACAGGTGGACAGCCTGCATCCCTGCAGGATGATCCTGGAGCCCTGGGTG 1320
N4019>
GCCATCAGACAACATCGTGGGCACAAATGTGGCGTGTTCTTGTTGTAAGCGGCGAGTTGG 1380
Y6266<
GAGCTGAGAGCTGGGAGCAGGGTGGGCAGCCTGGGTGTAGGGGGGAGGCGAGAGAGGTAG 1440

GACCCAAAAGCACATCTGCCCTGGGCCCCTGTGGTGGGCAGTGAGGGTGAGCACCCGGCC 1500

CAGAGGACGGCCATCCTGTGGGGTCGCGTCTGCACTGTGGGTTGGGGAAGCAGGGCGGTG 1560

GTGGAGAAATGGGCAGGGGCACCTCTGCAGAGAAGACGCAGAGCAATGAGCCCTTCTGTG 1620

TAGTGAGAACCCGCTCTGCACCAACCTCGGCGGCTGCTTTCTCTTGCGGTCTGGGGACTC 1680

TCCTTCCCATAGGTCAGAAAACTGAGGCCCTGAGAAGGGGACTTCCACTGGCCCAGGTCA 1740

CAGGCTGAGTACTGAGCCTGGTGTTCGCCGGGGCCACAGCCTCCCTCAGGGCGCTCAGGG 1800

TCCCTGCAGAACAGGGGTCACCTCCTTTCTTGGAGAAAAGCCCTACCCTGTTACTACAGG 1860

GAGGGCCTGCATGGGTGAGGTGGTGCCAGACTTGGGTCGCCAGGTCCCAGGAATGACCTC 1920

AGTTACCCTGTCAGCACCTGTGGGCAGAAGCTACAGTCTCATCCCTGCTTAGACCTGAGC 1980

GGCCTTTGCCCAGCACCTGGAGGTCGCTCTGAGAAAAGGTCTGCAGCTCGAACACAAACA 2040
Y6263>
GGCAGCTTCTACCAGGGCCCCAGTCACTCCTGCAGGCCGATTCCCCTTGGGTACAAGGAG 2100

GATGGGATACGGGGTCAGGGCCTGTGTCTTGCTGGGGCGGCCTCACAAGCTCTGCCCTGG 2160
T3900> T3901<
CCTCTGTAGGAATGGGCCTGAATGGCGCTTCAACCGATTGCGGCTGAACCCAGATGTGCT 2220
ex 3

GTCGCCCAAGGCCGTGCAGAGGTTCCTCCCGATGGTGGATGCAGTGGCCAGGGACTTTTC 2280

CCAGGCCCTGAGGAAGAAGGTGCTGCAGAACGCCCGGGGGAGCCTGACCCTGGACGTCCA 2340

GCCCAGCATCTTCCACTACACCATAGAAGGTGTGGGCCATGCGGGAAGGTCCAGCCCCAG 2400
Y6264<
AGACCCTGGAGTGGCCAGGGATGGGGATGGAGGACTGAAGGGAGTGTGGGGAGGCAGCCA 2460
B4250>
GGAGGTCCGGGGCTGCCTTGTGCTCAGCAGTGCATCCTCCCCGCAGCCAGCAACTTAGCT 2520
ex 4
S6718>
CTTTTTGGAGAGCGGCTGGGCCTGGTTGGCCACAGCCCCAGTTCTGCCAGCCTGAACTTC 2580

CTCCATGCCCTGGAGGTCATGTTCAAATCCACCGTCCAGCTCATGTTCATGCCCAGGAGC 2640
T3898> T3899<
CTGTCTCGCTGGATCAGCCCCAAGGTGTGGAAGGAGCACTTTGAGGCCTGGGACTGCATC 2700

TTCCAGTACGGTGAGGCCAGGGACCCGGGCAGTGCTATGGGGAAGGGACACCATGGGGGC 2760
B4251<
CCAATTTCTCCCTCTCCACCACCCAGTGGGGAATGGAGGCCACAGGGAGGGGTCGGGGAT 2820

TCCTCACCTTCCTGCCGGGGAGATTGGTGCGAGGCTGGGGCTGGGCTGGGCTGATCCGGA 2880
T2138>
GAATTTGGGATGAGAGCAGGGAGATTTGGGTGTCGGGGCAGTCTCGGCAGGAGGAGGACA 2940

CTGAAGGATGCTTCCCAGCACCAAGATCTAGGGCTGTCCCCTGCTCCCTGTACAGGTGAC 3000
ex 5
Z0895>
AACTGTATCCAGAAAATCTACCAGGAACTGGCCTTCAACCGCCCTCAACACTACACAGGC 3060

ATCGTGGCAGAGCTCCTGTTGAAGGCGGAACTGTCACTAGAAGCCATCAAGGCCAACTCT 3120

ATGGAACTCACTGCAGGGAGCGTGGACACGGTCAGGCCAGCAACCAGCCCCACCCAGAGA 3180
T2141<
GGGTGATGCCAAGCCCTGCCTCCCAGCACTGCCTGCCAATGCCACACGGCACCCACGTTC 3240

CCCATCCCCAGGCTACAGGCCCCACATTTCTGTTGCCCTCAGCCTTCCCCCTCCTTTGTT 3300

AAGGGATGAGATTTGCAGGGGAGGGGAAATGTGAGCTCCCCCTCACATGAGACTGAGTTT 3360

GCAGTTACCTGTGTGGGGATCCATGCTCCAGGCTGGAAGAAAGTTGGATGAGGCCCTGGA 3420

CACACAGCAGCTCTGTCCCCACTGGAAAGCTCTGGGTGTACAAGGAGAAGGAGGGTTGAG 3480

AGGCAGCTGGAGGACTCCACTGGGCACCCTTCCCAGTGTGCCCGGTCACCTTGGGCCAGA 3540

AATGTACATGCATGGGAGGGCAGGGTTGTGGGGAAGGCAGCAGCACGGGCTCCAGCCAGT 3600

GCAGAGGGGCCTGTGGGTGCACAGTGGGGAGAACTCAATGGAAGCAGAGGGAGCTGGGGC 3660

TCCAGAACTCCCAGGATGATGCTGAGGTCTGGCCCCCTTTTCTAAGGTGGCTGTGAGAAC 3720

CCGCCTGAAGAGGCTGCAGGGGACCTGGGCCTTGGTGGAGATGGGGGTCAGCTTTGCGTG 3780

AAGAAGTCAGGGAATCTGGCCCAAGTGGTCATCAAGGTTTCAGATCCGGCGTCCCAGGGC 3840

TCTGTCGTGCTCAGGGCATGGATGTCTCCACCCCTCAGAGGGAGGTTGTCCTGGCTGGGG 3900
T2142>

TGTCCCGGGGGCTGAGTCCTCCTGTGCAAGGTCAGACCCTGCAGACATGGCTTCTGTAGA 3960
ex 6

CAGCGTTTCCGTTGCTGATGACGCTCTTTGAGCTGGCTCGGAACCCCGACGTGCAGCAGA 4020
Y6132>

TCCTGCGCAAGGAGAGCCTGGCCGCCGCAGCCAGCATCAGTGAACATCCCCAGAAGGCAA 4080
Y6133<
CCACCGAGCTGCCCTTGCTGCGGGCGGCCCTCAAGGAGACCTTGAGGTGGGTGCTGGATG 4140

AGGCCTCCCTGTGGCCCTGGCCCCCTGCTGGAGAGCAGCCCCCACTGGGTGGTGGCAGAC 4200

AGAATCTGGGGCTGATAAACAGCGTCACCCAGCAGCCCATTCCCCTGCACCTGCTCTTCC 4260

TCCCCCTCAAGGTCTGGGAGCTCTTCTTCCTCTGAATCCCTCTTCAACACCCTGGGGATT 4320

AACGTGGGGCATGTCCTTCTGCGCTTGGGGCTTCTCAAGTTAGGGGAGGTTTGGCTGGGC 4380

TCAGCAGGTGCAAGGAAGCACTTCGTCACGACCTGGGCTTCCCATGGGCCAGGGAGCTGT 4440

GCGGGGTCTTCGGTAGGAAGGGTGCAGAGAGCACAGGGAGCCCCATCCAGCTGAGGACCC 4500
Y6259>
TTTCTGTGGATGCCCCCACCTCCAGGCTCTACCCTGTGGGTCTGTTTTTGGAGCGAGTGG 4560
ex 7

TGAGCTCAGACTTGGTGCTTCAGAACTACCACATCCCAGCTGGGGTGAGTGAGCCCCCAC 4620
Y6261> Y6260<
ACCCCTCGAGCTGAGAACCTCCCTCCCCAGTCATTCCCTGATCCCTGCTCTGCACCGTCC 4680

GCAGACATTGGTACAGGTTTTCCTCTACTCGCTGGGTCGCAATGCCGCCTTGTTCCCGAG 4740
ex 8

GCCTGAGCGGTATAATCCCCAGCGCTGGCTAGACATCAGGGGCTCCGGCAGGAACTTGCA 4800

CCACGTGCCCTTTGGCTTTGGCATGCGCCAGTGCCTCGGGCGGCGCCTGGCAGAGGCAGA 4860
Y6262<
GATGCTGCTGCTGCTGCACCACGTAAGCAGGCCTGGGCCCCTTCAGCATAATTGTTGCAC 4920
S6719>
CTGGGACGATGGGAGGAAGCTGCCCCAGGTCCATGGGCTACTGACCAGCGCTGATGGAAA 4980

CCCAGCCTCTGTCCTAGGTGCTGAAGCGCTTCCTGGTGGAGACACTAACTCAAGAGGACA 5040
ex 9
V5315 V5316
TAAAGATGGTCTACAGCTTCATATTGAGGCCTGGCACGTCCCCCCTCCTCACTTTCAGAG 5100

CGATTAACTAGTCTTGCATCTGCACCCAGGGTCCCAGCCTGGCCACCAGCTTCCCTCTGC 5160
M6641<
CTGACCCCAGGCCACCTGTCTTCTCTCCCACGTGCACAGCTTCCTGAGTCACCCCTCTGT 5220

CCAGCCAGCTCCTGCACAAATGGAACTCCCCAGGGCCTCCAGGACTGGGGCTTGCCAGGC 5280

TTGTCAAATAGCAAGGCCAGCGCACAGCTGGAGCGATCTTGCTGCAGGCCTGCCTTGTCC 5340

CCAGCCCCACCTGGCCCCTTCTCCAGCAAGCAGTGCCCTCTGGACACTTGACTCTACTCC 6000

TCCCAGCGCTGGCTCCAGGCTCCTCATGAGGCCATGCAAGGGTGCTGTGATTTTGTCCCZ 6060

DTTGCCTTCCTGCZZZZCTAGTCTCACATGTCCCTGTCCCTCTCGCCCTGGCCAGGGCCT 6120

CTGTGCAGACAGTGTCAGAGTCATTAAGCGGGATCCCAGCATCTCAGAGTCCAGTCAAGT 6180

TCCCTCCTGCAGCCTGACCCCAGGCAGCTCGAGCATGCCCTGAGCTCTCTGAAAGTTGTC 6240

ACCCAGAAATACGATCCTGCAGGGTAGACTAAAAAGGCCCCTGTGGTCACTTATACTGAC 6300

ACATTTTAAGTGATACAACTGAGTCTCGAGGGGCGTGTGTTCCCCAGCTGATCATGTCAG 6360

CCTCATGCCCCAGGCCTCGTCTTTCATGGACCAGGTCTTGTTCAAGCAGCGAGTGTTGGG 6420

TCCTCTGCTTCCTGAGCTGTCCCCTGGAAAAGGTCCCGAGGATGCTGTCAGGAGATGGAA 6480

GAGTCATGTGGGGTGGGAACCTGGGGTGTGGTTCCAGAAATGTTTTTGGCAACAGGAGAG 6540

ACAGGATTGGGCCAACAAGGACTCAGATGAGTTTATTGACTCATTCCTCTGGAAGATACG 6600

CAGC

A	Ala	Alanine
B	Asx	Asparagine or Aspartic acid
C	Cys	Cysteine
D	Asp	Asparticacid
E	Glu	Glutamicacid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
Z	Glx	Glutamine or Glutamic acid

Amino acid names, abbreviations and symbols.

TTT Phe	TCT Ser	TAT Tyr	TGT Cys
TTC Phe	TCC Ser	TAC Tyr	TGC Cys
TTA Leu	TCA Ser	TAA Stop	TGA Stop
TTG Leu	TCG Ser	TAG Stop	TGG Trp
CTT Leu	CCT Pro	CAT His	CGT Arg
TCT Leu	CCC Pro	CAC His	CGC Arg
CTA Leu	CCA Pro	CAA Gln	CGA Arg
CTG Leu	CCG Pro	CAG Gln	CGG Arg
ATT Ile	ACT Thr	AAT Asn	AGT Ser
ATC Ile	ACC Thr	AAC Asn	AGC Ser
ATA Ile	ACA Thr	AAA Lys	AGA Arg
ATG Met	ACG Thr	AAG Lys	AGG Arg
GTT Val	GCT Ala	GAT Asp	GGT Gly
GTC Val	GCC Ala	GAC Asp	GGC Gly
GTA Val	GCA Ala	GAA Glu	GGA Gly
GTG Val	GCG Ala	GAG Glu	GGG Gly

Amino acid codons

Appendix 2
Primers

The melting temperature of primers was calculated by:

Tm °C = 2(A +T) + 4(G +C)

The annealing temperature was 5°C below the melting temperature.

CYP11B2 MUTATIONS			
Exon	**Amino acid change**	**SENSE**	**ANTISENSE**
1	B2-Q43R	5'GAAGCCATGCCCCGGCATC CAGGCAAC 3' **V5311**	5'GTTGCCTGGATGCCGGGGCA TGGCTTC3' **V5312**
3	B2-D147E	5'GCGGCTGAACCCAGAAGT GCTGTCGCC3' **T3900**	5'GGCGACAGCACTTCTGGGTT CAGCCGC3' **T3901**
4	B2-I248T	5'CTGTCTCGCTGGACCAGCC CCAAGGTG3' **T3898**	5'CACCTTGGGGCTGGTCCAGC GAGACAG3' **T3899**
6	B2-K357N	5'GTGAACATCCCCAGAATGC AACCACCGAGC3' **Y6132**	5'GCTCGGTGGTTGCATTCTGG GGATGTTCAC3' **Y6133**
9	B2-T493M	5'GAGGCCTGGCATGTCCCCC CTCCTCAC3' **V5315**	5'GTGAGGAGGGGGGACATGCC AGGCCTC3' **V5316**
		CYP11B1 MUTATIONS	
Exon	**Amino acid change**	**SENSE**	**ANTISENSE**
2	B1-H107Y	5'GGTGGACAGCCTGTATCCC CACAGGATG3' **C3130**	5'CATCCTGTGGGGATACAGGC TGTCCACC3' **C3131**
2	B1-L186V	5'GGGAGCCTGACCGTGGAC GTCCAGC3' **C0222**	5'GCTGGACGTCCACGGTCAGG CTCCC3' **C0223**
3	B1-E147D	5'GCGGCTGAATCCAGATGTG CTGTCGCC3' **Y6130**	5'GGCGACAGCACATCTGGATT CAGCCGC3' **Y6131**

Table 1 Primers for site-directed mutagenesis

SEQUENCING PRIMERS		
EXON	**Name**	**PRIMER**
*$pCMV_4$ (S)	**V6776**	5'TAGTGAACCGTCAGAATTG3'
*$pCMV_4$ (AS)	**V6777**	5'TAGAGGACACTAGTCAGAC3'
2 (S)	**C8489**	5'CAATCGGTTGAAGCGCCATTC3'
2 (S)	**N4019**	5'AGACAACATCGTGGGCACAAATG3'
4 (S)	**S6718**	5'AGTTCTGCCAGCCTGAACTTC3'
5 (S)	**Z0895**	5'TACACAGGCATCGTGGCAGAG3'

* primers flanking either side of the $pCMV_4$ cloning site

Table 2 Sequencing Primers

EXON	Primer Pairs (S) (AS)		Size	Digestion	Fragments
CYP11B12	Y6265	Y6266	260bp	Rsa I	77, 183bp
3	Y6264	Y6263	349bp	Hha I	115, 234bp
4	B4250	B4251	306bp	Stu I	210, 96bp
5	M6642	M6643	312bp	Rsa I	123, 189bp
7	Y6260	Y6259	147bp		
8	Y6261	Y6262	262bp	Hae III	119, 143bp
9	M6635	M6637	224bp		
CYP11B21	T2137	T2139	487bp	HhaI	199, 288bp
2	Y6265	Y6266	260bp	Rsa I	77, 183bp
3	Y6264	Y6263	349bp	Hha I	116, 233bp
4	B4250	B4251	306bp	Stu I	210, 96bp
5	T2138	T2141	337bp	Rsa I	148, 189bp
6	T2142	T2140	224bp		
7	Y6260	Y6259	147bp		
8	Y6261	Y6262	262bp	Hae III	119, 143bp
9	S6719	M6641	403bp	Stu I	78, 100, 210, 15bp

Table 3 Primer pairs for SSCP, amplicon sizes and digests

SSCP PRIMER	Sequence
T2137	5' TCCTTCATCTACCTTTGGCTG3'
T2139	5'GAATGGCAGTGCTGAGTGCC3'
Y6265	5'TTTGGATTGGGACTGCAGGG3'
Y6266	5'CCCACCCTGCTCCCAGCTCT3'
Y6264	5'TGGCCACTCCAGGGTCTCTG3'
Y6263	5'CTGCAGGCCGATTCCCCTTG3'
B4250	5'CCTTGTGCTCAGCAGTGCAT3'
B4251	5'GTGGTGGAGAGGGAGAAATT3'
M6642	5'AGGAGGACACTGAAGGATGTT3'
M6643	5'GACACGTGGGCGCCGTGTGAC3'
T2141	5'GAACGTGGGTGCCGTGTGGC3'
T2138	5'ATTTGGGTGTCGGGGCAGTCT3'
T2140	5'AGGGCCACAGGGAGGCCTCA3'
T2142	5'GACCCTGCAGACATGGCTTC3'
Y6260	5'AATGACTGGGGAGGGAGGTT3'
Y6259	5'TGGATGCCCCCACCTCCAGG3'
Y6262	5'ACATGGTGCAGCAGCAGCAGC3'
Y6261	5'CCCTCGAGCTGAGAACCTCC3'
M6635	5'CTGTTCCCCCTTCAGCATAAT3'
M6637	5'GAGACGTGATTAGTTGATGGC3'
S6719	5'TACTGACCAGCGCTGATGGAAAC3'
M6641	5'CTGTGCACGTGGGAGAGAAGA3'

Table 4 SSCP primer sequence

	SEQUENCE	
GAPDH (S)	5'ACCACAGTCCATGCCATCAC3'	P5817
GAPDH (AS)	5'TCCACCACCCTGTTGCTGTA3'	P5818
CYP11B1/B2 (S)	5'AGACAACATCGTGGGCACAAATG3'	N4019 Ex 2
CYP11B1/B2 (AS)	5'GCTCCCTGCAGTGAGTTCCAT3'	C065 Ex 6

Table 5 Primers for RT-PCR

Appendix 3
Reagents

Unless otherwise stated all standard chemicals and reagents were purchased form Sigma (Sigma-Aldrich Co Ltd).

Bacterial media and plates

LB medium	10g bacto-tryptone , 5g bacto-yeast extract, 10g NaCl to a final volume of 1 litre with deionised water, adjust to pH 7.0 with 5N NaOH. Autoclave (20 minutes at 15lb/sq.in. on liquid cycle)
SOC medium	20g bacto-tryptone , 5g bacto-yeast extract, 0.5g NaCl to a volume of 950 ml of deionised water. Dissolve and add 10ml of 250mM KCl, adjust to pH 7.0 with 5N NaOH and adjust volume to 1 litre with deionised water. Autoclave as above. Before use add 5ml of 2M $MgCl_2$ and 20 ml of a filter-sterilised 1M glucose solution.
LB agar	10g NaCl, 10g Tryptone, 5g yeast extract, 20g of agar to a final volume of 1litre with deionised water, adjust to pH 7.0 with 5N NaOH
L-amp plates	1 liter of LB agar, add 20mg of filter-sterilized ampicillin (Sigma)
Miller transformation solution	LB broth containing: 10% (w/v) PEG 4000 5% (v/v) DMSO 50mM $MgSO_4$, pH 6.5. Chung et al. 1989.

Plasmid preparation alkaline lysis

Modified version of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981)

Solutions I	50mM glucose, 25mM Tris-Cl (pH 8.0), 10mM EDTA (pH 8.0)
Solution II	0.2N NaOH, 1% SDS freshly prepared
Solution III	60ml of 5M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml of deioinised water. Resulting solution is 3M with respect to potassium
Lysozyme	10mg/ml lysozyme, 10mM Tris pH 8.0
Ethidium bromide EtBr(Sigma)	10mg/ml preapred in sterile water

Buffers

TE buffer	10mM Tris-HCl, 1mM EDTA, pH 7.5.
TAE buffer	40mM Tris-acetate, 1mM EDTA pH 8.0

TBE Buffer 45mM Tris-phosphate, 1mM EDTA pH8.0

Loading dye/agarose gel blue 1.5g Ficoll, 25mg Bromophenol Blue, 25mg Xylene Cyanol, room tempertaure

Genomic DNA extraction (Sambrook et al. 1989)

Cell lysis mix 10% sucrose, 10mM Tris pH 7.5, 5mM $MgCl_2$, 1% Triton® X-100 (Sigma).

Nucleic lysis mix 10mM Tris pH 8.2, 0.4M NaCl, 2mM EDTA.

Proteinase K (Sigma) 10mg/ml Proteinase K, 1% SDS, 2mM EDTA

PCR

Taq DNA polymerase (Promega)

Storage buffer: 50mM Tris-HCl (pH 8.0),100mM NaCl, 1mM DTT, 50% Glycerol, 1% Triton® X-100.

PCR 10X reaction buffer w/o $MgCl_2$:(Promega)

$MgCl_2$ (Promega) 25mM

dNTPs (Promega) 10mM each

SSCP

Ammonium persulphate (APS) (Sigma) 25%w/v in sterile water

Tetramethylethylenediamine (TEMED) (Sigma)

30% Acrylamide/Bis Solution 37.5:1 (Biorad)

[α-^{32}P] dCTP 10mCi/ml (Amersham)

SSCP Stop Blue 9.5mls formamide, 0.2mls EDTA (0.5M pH8.0), 5mg Bromophenol Blue, 5g Xylene cyanol

Site directed mutagenesis

Quick change site-directed mutagnesis kit (Stratagene)

Pfu DNA polymerase (2.5U/µl) (Stratagene)

10X reaction buffer (Stratagene)

Dpn I restriction enzyme (10 U/µl) (Stratagene)

Reagents not supplied in kit:

Primers see table 1 appendix 2

NZY+ broth (Sigma)	10g of NZ amine (casein hydrolysate), 5g of yeast extract, 5g of NaCl, 12.5ml of 1M $MgCl_2$, 12.5ml of 1M Mg SO_4, 10ml of 2M filter sterilised glucose solution per litre.
LB-amp met agar plates	1 liter of LB agar, add 20mg of filter-sterilized ampicillin (Sigma), 80mg of filter-sterilized methicillin. (Sigma)

Transfections
DOTAP (Boerhinger-Mannheim)

HEPES (Sigma)

PBS 1X buffer (Mg^{2+} and Ca^{2+} free) (Sigma)

DMEM with 4500mg glucose/L, L-glutamine, pyroxidine , HCl and $NaHCO_3$ (Sigma)

Fetal bovine serum (FCS) Heat inactivated, cell culture tested and sterile filtered (Sigma)

Antibiotic-Antimycotic 10 000 units/ml penicillin G sodium, 10 000 µg/ml streptomycin sulphate and 25 µg/ml amphotericin B as Fungizone ® (Gibco BRL)

Trypsin-EDTA solution (1X) (Sigma)

Steroids
Working solutions prepared in 100% ethanol (Sigma)

Paper Chromatography
Whatman2 paper.
Solvent system 1 was used for separation of B and solvent system 2 was used for separation of F, 18-OHB or aldosterone.

Solvent system 1:	Water	300ml
	Methanol	700ml
	Petrolium Ether (80-100°C)	500ml
	Toluene	500ml

Solvent system 2:	Hexane	200ml
	Toluene	1800ml
	Methanol	1000ml
	Water	500ml
	Triethylamine	3.5ml

Thin layer Chromatography

Whatman Silica glass-backed fluorescent plates

Solvent system: Methylene Chloride: Methanol:water
Ratio 300:20:1

Ecoscint A Scintillation solution (National diagnostics, Atlanta Georgia 30336)

β-gal assay (Promega)
Reporter lysis buffer (Promega)

2X assay buffer	200mM sodium phosphate buffer, pH 7.3, 2mM $MgCl_2$, 100mM β-mercaptoethanol, 1.33mg/ml ONPG
β-Galactosidase	(1U/μl) (Promega)

1M Sodium carbonate

Biorad Protein assay kit I
Biorad protein assay dye reagent concentrate

Bovine serum albumin (BSA).1.42mg/ml

Based on method of Bradford (Bradford 1976)

RNA and RT-PCR
Primers see table 4 appendix 2

RNA isolation RNAzol B (Biogenesis)

Gene Amp RNA PCR Core Kit (Perkin Elmer)

Reagents supplied in kit:

Reverse trancriptase MuLV (50U/μl)
RNase Inhibitor (20U/μl)
10X PCR buffer
dNTPs (10mM) each
Taq DNA polymerase (5U/μl)
$MgCl_2$ (25mM)
Oligo $d(T)_{16}$ primer (50μM)

DEPC(Sigma) Diethyl pyrocarbonate 10μg/100ml sterile water

Markers:
1Kb, 100bp ladder (Promega)

λHind III, λHae III (New England Biolabs)

DNA sequencing
Urea (Sigma)

40% Acrylamide/Bis Solution 19:1 (Biorad)

TEMED and APS as for SSCP.

[^{35}S]dATPαS 10mCi/ml (Amersham)

DNA Sequencing Kit (Sequenase Version 2.0)

Storage Buffer	13U/μl in 20mM KPO_4, pH7.4, 1mM DTT, 0.1mM EDTA, 50% glycerol.
Enzyme dilution buffer	10mM Tris-HCl, pH7.5, 5mM DTT, 0.5mg/ml BSA.
Sequenase buffer (5X)	200mM Tris-HCl, pH 7.5, 100mM $MgCl_2$, 250mM NaCl.
Labelling mix (5X)	7.5μM dGTP, 7.5μM dCTP, 7.5μM dTTP
Termination mixes (5X)	
ddG	80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 40μM ddGTP, 50mM NaCl.
ddA	80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 40μM ddATP, 50mM NaCl.
ddT	80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 40μM ddTTP, 50mM NaCl.
ddC	80μM dGTP, 80μM dATP, 80μM dCTP, 80μM dTTP, 40μM ddCTP, 50mM NaCl.
Stop Solution	95% formamide, 20mM EDTA, 0.05% Bromophenol blue 0.05% Xylene Cyanol FF.

Autonmated Cycle Sequencing
ABI Prism BigDye Terminator Cycle Sequencing Reaction Kit (PE Applied Biosystems, Foster City, CA, USA)

Ready Reaction Mix
Amplitaq® DNA Polymerase, FS
R*Tth* pyrophosphatase
Fluorescently labelled 2',3'-dideoxynucleotides

2'-deoxynucleotides
$MgCl_2$
Buffer- Tris-HCl pH 9.0

ddNTPs	Excitation emission λ_{max} (nm)
ddGTP- Dichloro R110	532
ddATP- Dichloro [R6G]	560
ddTTP- Dichloro TAMRA	594
ddCTP- Dichloro ROX	620

Gel 5% polyacrylamide (Long Ranger Gel Solution, FMC, Rockland, Maine, USA)

Apppendix 4
Enzymes

BamHI (Promega) 10U/µl Recognition site G^GATCC
CCTAG^G

Storage buffer
10mM Tris-HCl, 300mM KCl, 0.1mM EDTA, 1mM DTT, 0,5mg/ml BSA, 50% glycerol, pH 7.4.

10X Reaction buffer E
6mM Tris-HCl, 6mM $MgCl_2$, 100mM NaCl, 1mM DTT, pH 7.5.

SpeI (Boehringer- Mannheim) 10 U/µl Recognition site A^CTAGT
TGATC^A

Storage buffer
20mM Tris-HCl, 100mM NaCl, 0.1mM EDTA, 10mM 2-mercaptoethanol, 0.2% (v/v) Triton® X-100, 50% (v/v) glycerol, pH 8.0.

10X Reaction buffer
50mM Tris-HCl, 100mM NaCl, 10mM $MgCl_2$, 1mM DTT, pH 7.5.

Bgl II (Promega) 10 U/µl Recognition site A^GATCT
TCTAG^A

Storage buffer
10mM Tris-HCl pH 7.3, 300mM NaCl, 0.1mM EDTA, 1mM DTT, 0.5mg/ml BSA, 50% (v/v) glycerol.

10X Reaction Buffer D
6mM Tris-HCl, 150mM NaCl, 6mM $MgCl_2$, 1mM DTT, pH 7.9.

EcoR I (Promega) 8-12U/µl Recognition site G^AATTC
CTTAA^G

Storage buffer
10mM Tris-HCl pH 7.4, 400mM NaCl, 0.1mM EDTA, 1mM DTT, 0.5mg/ml BSA, 50% (v/v) glycerol.

10X Reaction Buffer H
90mM Tris-HCl, 50mM NaCl, 10mM $MgCl_2$, pH 7.5.

Hae III (Promega) 10 U/µl Recognition site GG^CC
CC^GG

Storage buffer
10mM Tris-HCl pH 7.4, 300mM NaCl, 0.1mM EDTA, 1mM DTT, 0.5mg/ml BSA, 50% (v/v) glycerol.

10X Reaction Buffer C
10mM Tris-HCl, 50mM NaCl, 10mM $MgCl_2$, 1mM DTT, pH 7.9.

Hha I (Promega) 10 U/µl Recognition site GCG^C
C^GCG

Storage buffer
10mM Tris-HCl pH 7.4, 50mM NaCl, 0.1mM EDTA, 1mM DTT, 0.5mg/ml BSA, 50% (v/v) glycerol.

10X Reaction Buffer C
10mM Tris-HCl, 50mM NaCl, 10mM $MgCl_2$, 1mM DTT, pH 7.9.

Rsa I (Promega) 10 U/µl Recognition site GT^AC
CA^TG

Storage buffer
10mM Tris-HCl pH 7.4, 300mM NaCl, 0.1mM EDTA, 1mM DTT, 0.5mg/ml BSA, 50% (v/v) glycerol.

10X Reaction Buffer C
10mM Tris-HCl, 50mM NaCl, 10mM $MgCl_2$, 1mM DTT, pH 7.9.

Stu I (Promega) 10 U/µl Recognition site AGG^CCT
TCC^GGA

Storage buffer
10mM Tris-HCl pH 7.4, 50mM NaCl, 0.1mM EDTA, 1mM DTT, 0.5mg/ml BSA, 50% (v/v) glycerol.

10X Reaction Buffer B
6mM Tris-HCl, 50mM NaCl, 6mM $MgCl_2$, 1mM DTT, pH 7.5.

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