# CLINICAL, BIOCHEMICAL AND MOLECULAR GENETIC FEATURES OF GLUCOCORTICOID-SUPPRESSIBLE HYPERALDOSTERONISM

by

ANDREW JAMIESON MB ChB (Hons.), MRCP (UK)



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Department of Medicine and Therapeutics
UNIVERSITY OF GLASGOW
Western Infirmary, Glasgow

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

## Abstract

Human hypertension is a multifactorial disorder affecting up to 20% of the population of developed countries. The hypertensive phenotype varies between individual s and both environmental and genetic factors may be important in determining an individuals blood pressure. Although the majority of cases of hypertension are labelled as primary (or essential) implying that the aetiology of the hypertension is unknown, a genetically inherited form of hypertension, glucocorticoid-suppressible hyperaldosteronism (GSH), has been characterised and studied in detail in this thesis.

GSH is an inherited form of hypertension characterised by elevated plasma aldosterone concentrations with suppression of plasma renin activity, hypertension, hypokalaemia, and an autosomal dominant mode of inheritance. A chimeric 11ß-hydroxylase/aldosterone synthase gene has been described in patients with GSH. This gene, presumably a result of unequal crossing-over at meiosis, has ACTH-inducible aldosterone synthase activity demonstrable *in vivo* which explains the observed clinical and biochemical phenotype of GSH.

A technique for detection of individuals with GSH was devised based on the detection of the chimeric gene by probing of a Southern blot of BamHI digested genomic DNA. Normal individuals possess two hybridising species, a 8.4 kb band (11ß-hydroxylase, CYP11B1) and a 4.4 kb band (aldosterone synthase, CYP11B2) when probed with a $^{32}$P-labelled CYP11B1 exon 2-5 probe . Affected individuals possess the two normal bands and a third 6.3 kb band corresponding to the chimeric gene. This method was used to screen five kindreds affected by GSH and to detect 19 affected individuals.

Treatment of affected individuals with the oral glucocorticoid dexamethasone 0.5 mg q.d.s. resulted in a fall in blood pressure and plasma aldosterone concentrations, and a normalisation of plasma renin concentration and statistically significant rise in plasma potassium concentration. Further evaluation of kindreds with GSH revealed a wide variation in the clinical phenotype of GSH, particularly in terms of blood pressure.

A novel observation on the role of parental origin of the disease gene was made. Patients inheriting the chimeric gene from their mother had higher blood pressures and higher plasma aldosterone concentrations than those inheriting the condition from their father.

The exact nucleotide sequence of the chimeric genes in the 5 kindreds was determined by the dideoxy- chain termination method. Three kindreds possessed chimeric genes indistinguishable by sequencing, whilst the other two kindreds had unique crossovers. All crossover events occurred within the exon 3 - intron 4 region of the chimeric genes, in keeping with previously published observations. The structure of the chimeric gene, i.e. the position of the crossover site, has no effect on the clinical phenotype.

In addition to determining the site of the crossover region in the kindreds, a diallelic polymorphism in the promoter region of the aldosterone synthase gene was identified. The C/T single nucleotide polymorphism lies within the Steroidogencic Factor-1 binding region of the promoter at position -340. One allele of this polymorphism was shown to be in linkage disequilibrium with GSH suggesting a degree of allelic bias in the origin of the chimeric genes causing GSH.

Individuals with GSH show impaired basal and ACTH-stimulated 11ß-hydroxylation ability compared with normal controls. Basal molar plasma ratios of DOC:B and S:F are raised in patients with GSH, but ACTH-stimulation results in differential effects on the 17-hydroxy- and 17-deoxy- pathways. In the 17-hydroxy- pathway (cortisol synthesis) the impaired 11ß-hydroxylation is exacerbated by ACTH, whereas in the 17-deoxy- pathway (aldosterone synthesis) ACTH stimulation acts to rectify the abnormality. Basal molar plasma B:ALDO ratio (an index of aldosterone synthase activity) was lower in patients with GSH than controls. ACTH stimulation resulted in a rise in B:ALDO ratio in patients with GSH and a fall in normal controls. The reasons for the observed differences are unclear but may arise as a result of pseudosubstrate inhibition of 11ß-hydroxylation by compounds such as 18-hydroxycortisol, or as a result of altered gene expression in the adrenal cortex in GSH.

Finally, a study of the effect of lifelong hyperaldosteronism on echocardiographically determined left ventricular mass index was performed. The study revealed that GSH did not predispose to premature or excessive left ventricular hypertrophy despite evidence that aldosterone can produce myocardial fibrosis. However, a significant positive correlation between left ventricular mass and plasma aldosterone concentration in patients with GSH was noted.

The study of GSH provides a framework for assessing the possible factors involved in the development of human essential hypertension. The observations contained in this thesis shed light on how a single gene disorder may cause hypertension and how other factors such as intrauterine influences may modulate the genetic

component of hypertension. Further studies of GSH may reveal the mechanisms underlying altered corticosteroidogenesis in essential hypertension.

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## Abbreviations

Abbreviations used are as recommended in the Biochemical Journal Instructions to Authors, 1989, with the following additions:

| | |
|---|---|
| Ang I | angiotensin I |
| Ang II | angiotensin II |
| ACE | angiotensin-I converting enzyme |
| ACTH | adrenocorticotrophin |
| ALDO | aldosterone |
| cAMP | adenosine 3', 5'-cyclic monophosphate |
| ASO | allele-specific oligonucleotide |
| B | corticosterone |
| CYP11B1 | gene encoding cytochrome $P450_{11\beta}$ |
| CYP11B2 | gene encoding cytochrome $P450_{aldo}$ |
| $CytP450_{aldo}$ | cytochrome P450 aldosterone synthase |
| $CytP450_{11\beta}$ | cytochrome P450 11ß-hydroxylase |
| DNA | deoxyribonucleic acid |
| DTT | dithiothreitol |
| EDTA | ethylene diamine tetracetate |
| F | cortisol |
| GSH | glucocorticoid-suppressible hyperaldosteronism |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| RFLP | restriction fragment length polymorphism |
| S | 11-deoxycortisol |
| SDS | sodium dodecyl sulphate |
| SF-1 | Steroidogenic factor-1 |
| SSC | sodium chloride/sodium citrate |
| SSCP | single-strand conformational polymorphism |
| TE | TRIS-HCl/EDTA buffer |
| TEMED | N,N,N,N'-tetramethylethylenediamine |

## Steroid Nomenclature

| | |
|---|---|
| Aldosterone | 4-pregnen-11ß,21-diol-18al-3,20-dione |
| Androstenedione | 4-androsten-3,17-dione |
| Cholesterol | 5-cholesten-3ß-ol |
| Corticosterone | 4-pregnen-11ß,21-diol-3,20-dione |
| Cortisol | 4-pregnen-11ß,17α,21-triol-3,20-dione |
| Cortisone | 4-pregnen-17α,21-diol-3,11,20-trione |
| Dehydroepiandrosterone | 5-androsten-3ß-ol-17-one |
| 11-Deoxycorticosterone | 4-pregnen-21-ol-3,20-dione |
| 11-Deoxycortisol | 4-pregnen-17α,21-diol-3,20-dione |
| Dexamethasone | 1,4-pregnadien-9fluoro-16α-methyl-11ß,17α,21-triol-3,20-dione |
| 18-Hydroxydeoxycorticosterone | 4-pregnen-18,21-diol-3,20-dione |
| 18-Hydroxycorticosterone | 4-pregnen-11ß,18,21-triol-3,20-dione |
| 17α-hydroxypregenolone | 5-pregnen-3ß,17α-diol-20-one |
| 17α-hydroxyprogesterone | 4-pregnen-17α-ol-3,20-dione |
| Pregnenolone | 5-pregnen-3ß-ol-20-one |
| Progesterone | 4-pregnen-3,20-dione |

## Publications

The work contained in this thesis has been published in peer reviewed journals as follows:

**Jamieson A**, Connell JMC, Fraser R. Glucocorticoid-suppressible hyperaldosteronism: from confusion to conclusion? **Journal of Molecular Endocrinology** 1993; 10(1): 3-5.

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**Jamieson A**. Dissecting hypertension: The role of the 'New Genetics'. **Journal of the Royal College of Physicians** 1994; 28(6):512-519.

**Jamieson A**, Slutsker L, Inglis GC, Campbell M, Fraser R, White PC, Connell JMC. Clinical, biochemical and genetic features of five extended kindred's with glucocorticoid-suppressible hyperaldosteronism. **Endocrine Research** 1995; (In press).

Connell JMC, Inglis GC, Fraser R, **Jamieson A**. Dexamethasone-suppressible hyperaldosteronism: clinical, biochemical and genetic studies. **Journal of Human Hypertension** 1995; (In press)

**Jamieson A**, Murdoch DL, Kennedy JA, Connell JMC. Left ventricular mass in glucocorticoid-suppressible hyperaldosteronism. **Journal of Hypertension** 1995; (In press).

**Jamieson A**, Slutsker L, Inglis GC, Campbell M, Fraser R, White PC, Connell JMC. Glucocorticoid-suppressible hyperaldosteronism: Effects of crossover site and parental origin of chimaeric gene on phenotypic expression. **Clinical Science** 1995; (In press).

# Chapter 1

## Introduction

### 1.1 Blood Pressure: Overview

It is over 300 years since William Harvey first correctly described the circulation of blood (Harvey, 1949) and some 250 years since the Reverend Stephen Hales made the first quantitative measure of blood pressure by inserting a brass tube in to the crural artery of a horse and connecting this to a vertical glass tube to observe that the value was 8 feet 3 inches (Hales, 1733). Important as the observation of Hales was, it was clearly not a practical method for application to the study of the blood pressure in man and it was another 172 years before Korotkoff reported a reliable, simple and reproducible method of measuring systolic and diastolic blood pressure in man using an inflatable cuff to compress an upper limb artery and listening to the sounds created by the arterial flow on release of the compression (Korotkoff, 1905).

Ninety years on from Korotkoff's initial observations, his is still the principal method of measuring blood pressure in man. However, almost every other aspect of our understanding of the circulation and its pathology has advanced by a considerable degree. Advances, initially in the clinical description of the effects of untreated high blood pressure, then in its physiology and biochemistry and most recently in the molecular biology and genetics of the cardiovascular system have led to a revolution in our understanding of high blood pressure. Observations in large populations of the effects of high blood pressure have identified hypertension as a major health problem in the developed world and have highlighted the importance of treating high blood pressure to minimise its consequences.

### 1.2 The Importance of Human Hypertension

In the majority of developed countries the leading cause of death is cardiovascular disease (Whelton, 1984). In addition, cardiovascular disease is a principal cause of major morbidity, loss of income and social disruption (Stamler et al., 1986). There are many "risk factors" for cardiovascular disease, i.e. factors affecting an individual which can influence his or her chance of developing cardiovascular disease, one of the most important of which is blood pressure. Blood pressure is of particular importance because it is one risk factor which can be modified with relative ease by the use of medication with consequent benefits for the individual (Stamler et al, 1986).

### 1.2.1 Prevalence of Hypertension

There has always been controversy surrounding the definition of hypertension, particularly in regard to the distribution of blood pressure in the population. Platt argued that hypertension was an entity distinct from normotension whilst Pickering argued that blood pressure was a continuous spectrum and hypertension was a quantitative deviation from the norm (Pickering, 1961, Platt, 1959). Time has borne out Pickering's arguments. Therefore, when examining the blood pressure spectrum it has been difficult to decide where hypertension begins.

Any distinction between hypertension and normotension is therefore arbitrary and one disadvantage of labelling the remainder of the population as normotensive is that the normotensive group are assumed to be at no risk from cardiovascular disease which is not true since the risk of blood pressure-related cardiovascular disease increases throughout the range of normal blood pressure (Working Group on Primary Prevention, 1994). In the United States the prevalence of hypertension, as defined by the Fifth Report of the Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure, has been reported by the Third Health and Nutritional Examination Survey of the National Centre for Health Statistics (Joint National Committee, 1993) (Table 1.2 a). This group defined an individual as hypertensive if his or her systolic blood pressure is >= 140 mmHg and diastolic blood pressure is >= 90 mmHg, the readings being the mean of three readings taken on a single visit. Using the cut-off points in this study the prevalence of hypertension rises dramatically from 4% at 18-29 years to 65% at >80 years. These blood pressure measurements could represent a considerable overestimate of the prevalence of hypertension because they are taken on a single visit, but it has been shown that even a single measurement of blood pressure has prognostic significance (Stamler et al, 1986).

Table 1.2 b lists the prevalence of hypertension in the United States when a more conservative definition of hypertension (systolic blood pressure >=160 mmHg, or diastolic blood pressure >=95 mmHg) similar to that used in the United Kingdom (Gordon, 1964). The estimates of overall prevalence from this survey are less than the corresponding age-specific estimates in Table 1.2 a, but in both cases there was a clear association between age and prevalence of hypertension. Of particular interest in this regard is the change in nature of the hypertension in the different age groups. Young hypertensives tend to have raised diastolic blood pressures whilst elderly hypertensives have raised systolic blood pressures with little or no elevation in diastolic pressures.

Indeed, by the end of the seventh decade, isolated systolic hypertension accounts for more than 60% and 70% of the hypertension seen in men and women, respectively (Whelton et al., 1994).

| Age in Years | Percentage Hypertensive |
|---|---|
| | |
| 18-29 | 4 |
| 30-39 | 11 |
| 40-49 | 21 |
| 50-59 | 44 |
| 60-60 | 54 |
| 70-79 | 64 |
| >80 | 65 |

**Table 1.2 a Prevalence of hypertension [a] by age in the general population of the United States, 1988-91. Adapted from** (Joint National Committee, 1993)

| Age (years) | Male | Female |
|---|---|---|
| | | |
| 18-24 | 1.6 | 1.2 |
| 25-34 | 4.8 | 3.1 |
| 35-44 | 13.4 | 8.3 |
| 45-54 | 19.0 | 17.9 |
| 55-64 | 23.4 | 30.4 |
| 65-74 | 30.3 | 49.8 |
| 75-79 | 41.7 | 46.0 |

**Table 1.2 b Prevalence (%) of adult male and female residents of the United States with definite hypertension [b] as classified before the wide spread use of antihypertensive medications. Adapted from** (Gordon, 1964)

### 1.2.2 Incidence of Hypertension

There are relatively few studies which provide information on the incidence of hypertension in the general population. However, these studies do suggest a consistent tendency for incidence to rise with age (Andre et al., 1982, Buck and Donner, 1987, Dannenberg et al., 1988, Dunn et al., 1970, Garrison et al., 1987, Paffenberger et al., 1983). Typically, the annual incidence of hypertension has been less than 1-2% in the second and third decades rising to 4-8% in the sixth and seventh decades (Andre et al, 1982, Buck and Donner, 1987, Dannenberg et al, 1988, Garrison et al, 1987, Paffenberger et al, 1983). This incidence is affected by other factors such as body weight, gender, alcohol intake and physical activity

[a] Average of three blood pressure measurements, at a single visit, >=140 mmHg systolic, or 90 mmHg diastolic, or current treatment with antihypertensive drugs.
[b] Systolic BP >= 160 mmHg or diastolic >=95 mmHg

and accruing evidence suggests that weight reduction, increased physical activity and reduction of alcohol intake can reduce the incidence of hypertension (Dannenberg et al., 1987, Working Group on Primary Prevention, 1994).

### 1.2.3 Morbidity and Mortality from Hypertension

As stated previously, hypertension is a major cause of morbidity and mortality in the developed countries of the world (Whelton, 1984). Blood pressure has been identified as the major reversible risk factor for cerebrovascular accidents (CVA) and as an important modifiable risk factor in the genesis of myocardial infarction (Kannel and Stokes, 1985). In addition, elevated blood pressure leads to a variety of so-called "end-organ" effects, i.e. the presence of hypertension has deleterious effects on a number of important tissue in the body exposed to the elevated pressure. Organs susceptible to the effects of hypertension include the heart, kidney, retinal vessels, the cerebral circulation and the systemic arterial circulation in general. Table 1.2 c lists the potential effects of hypertension on various organs. The effect of prolonged elevation of blood pressure on the primary incidence of stroke and has been estimated from the pooled data of 9 prospective observational studies (MacMahon, 1994). These studies have shown that the association between blood pressure and stroke is relatively constant throughout the full range of blood pressures, i.e. the risk of stroke at a given level of blood pressure rises in proportion to the level of blood pressure studied. In addition, these studies suggested that there was no lower limit of blood pressure associated with a reduced risk of stroke and that even in individuals with blood pressure levels regarded as normotensive there was a direct relationship between blood pressure level and stroke risk (MacMahon, 1994). If an individual has a 5mm Hg lower diastolic blood pressure accompanied by a 9mm Hg lower systolic pressure the risk of stroke is 34% lower whereas a 10mm Hg lower diastolic pressure accompanied by an 18-19mm Hg lower systolic pressure confers a 56% reduction in the risk of stroke (Table 1.2 d).

| Organ | Effect |
|---|---|
| **Brain** | Microaneurysm formation in arterioles resulting in small areas of cerebral infarction, major areas of cerebral haemorrhage leading to disability and death, hypertensive encephalopathy |
| **Heart** | Left ventricular hypertrophy, ventricular dysrhythmmias, heart failure |
| **Kidney** | sclerosis of arterioles with no clinical sequelae, progressive renal impairment, acute renal failure in malignant phase hypertension |
| **Retinal Vessels** | Minor thickening of arterioles with narrowing of vessel lumen, leakage of plasma and ischaemia of retina, vascular occlusion (arterial and venous) with loss of sight |
| **Arterial Circulation** | Generalised arteriosclerosis, thickening of arterial media, dissection of aorta. |

**Table 1.2 c Effects of hypertension on end-organ tissues in man.**

A similar analysis of the effect of blood pressure on the incidence of coronary heart disease (CHD) revealed a trend in the incidence of CHD events and blood pressure level similar to that observed for stroke disease, although the slope of the association was less steep and there was the same trend in risk of CHD events throughout the normotensive range of blood pressure.

| Difference in | | Difference in risk of | |
|---|---|---|---|
| SBP | DBP | Stroke | CHD |
| | | | |
| 9 | 5 | 34% | 21% |
| 14 | 7.5 | 46% | 29% |
| 19 | 10 | 56% | 37% |

**Table 1.2 d Estimates of percentage differences in stroke and coronary event risk associated with prolonged differences in systolic blood pressure (SBP) and diastolic blood pressure (DBP) adjusted for age, smoking and blood cholesterol. Adapted from (MacMahon, 1994).**

The analysis of CHD events showed that if an individual has a 5mm Hg lower diastolic blood pressure accompanied by a 9mm Hg lower systolic pressure, the CHD event risk is 21% lower whereas a 10mm Hg lower diastolic pressure

accompanied by an 18-19mm Hg lower systolic pressure confers a 37% reduction in the risk of CHD (Table 1.2 d).

The effect of prolonged hypertension on the incidence of heart failure is also considerable. A blood pressure in the hypertensive range (systolic BP >= 160, diastolic BP >= 95 mmHg) is associated with a six-fold increase in risk of heart failure compared to normotensive subjects (Stokes et al., 1989). Similarly, an effect of blood pressure on the incidence of peripheral vascular disease (incidence in hypertensive group twice that in normotensive group) (Hughson et al., 1978) and renal impairment have been detected (Lindeman et al., 1984). These risks can perhaps be better appreciated in terms of the number of deaths due to stroke and CHD that could be avoided by a 9/5 mm Hg reduction in population (Table 1.2 e) (Wagner et al., 1991, World Health Organisation, 1991, World Health Organisation, 1992).Any means of producing a significant reduction in the prevalence of hypertension and/or lowering the blood pressure of the general population should lead to a significant reduction in the incidence of stroke and coronary disease.

| | | Annual mortality | | Deaths avoided by 9/5 mm Hg reduction in population BP* | |
|---|---|---|---|---|---|
| **Country** | | **Stroke** | **CHD** | **Stroke** | **CHD** |
| | | | | | |
| UK | | 76308 | 169481 | 26000 | 36000 |
| USA | | 149972 | 509412 | 51000 | 107000 |

**Table 1.2 e Annual mortality from stroke and coronary disease, and number of deaths likely to be avoided by a reduction in population blood pressure of 9/5 mm Hg (MacMahon, 1994). *Based on the risk reduction stated in Table 1.2 d. Adapted from (MacMahon, 1994)**

### 1.2.4 Effect of Drug Treatment on Hypertension

There have been many trials of drug therapy in hypertension, initially in the treatment of so-called malignant phase hypertension, i.e. hypertension associated with end-organ damage, rapid deterioration in renal function and a high mortality (2 year survival 8%), and subsequently severe hypertension, i.e. a systolic blood pressure >- 180 mmHg and a diastolic blood pressure >=105 mmHg, associated with lesser degrees of end-organ damage and lesser mortality (Harrington et al., 1959, Hodge et al., 1961, Sokolow and Perloff, 1960). These trials showed that drug therapy reduces the risk of death, stroke and to a lesser extent, coronary

disease. The effect of drug treatment on the reduction of stroke and CHD event risk in individuals with less severe forms of hypertension has been less encouraging. Drug treatment of mild-moderate high blood pressure by a variety of treatments, principally diuretics and ß-blocker drugs, has been assessed by a number of trials. A meta-analysis of these trials has shown that lowering of diastolic blood pressure by an average of 6 mm Hg leads to a reduction in stroke incidence (fatal and non-fatal) of 38% and a reduction in CHD event incidence of 16% (Collins and Peto, 1994, Collins et al., 1990).

From the above data it can be appreciated that the burden of disease attributable to blood pressure is considerable and the reduction of this burden by drug treatment is limited. An understanding of the cause of hypertension may lead to a more rational framework for the treatment and prevention of hypertension and its consequences.

## 1.3 Causes and Classification of Hypertension

Hypertension is clearly a common and important problem. However, the underlying cause in the majority of cases of hypertension is unknown, so-called 'essential' or 'primary' hypertension. This group constitutes the vast majority of all cases of hypertension and accounts for between 95-99% of all cases of hypertension. The remaining cases are classified as 'secondary' hypertension, i.e. hypertension is the consequence of some biochemical or mechanical pathology which is potentially reversible and the hypertension curable.

### 1.3.1 Secondary Hypertension

Secondary causes of hypertension are relatively uncommon and published series have reported a prevalence of 1.1-11.0% (Berglund et al., 1976, Gifford, 1969, Lewin et al., 1985, Sinclair et al., 1987). The wide variation in prevalence takes into account the different populations investigated (Table 1.3 a).

Screening those patients referred to a specialist clinic with refractory hypertension is likely to yield a greater number of secondary cases than screening the general population just as screening an entire population will involve screening many normotensive as well as hypertensive individuals and thus lessen the diagnostic yield of the procedure.

| | (Gifford, 1969) | (Berglund et al, 1976) | (Lewin et al, 1985) | (Sinclair et al, 1987) |
|---|---|---|---|---|
| | | | | |
| **Patients** | 4939 | 689 | 5485 | 3783 |
| **Source** | **Referred** | **Population Screen** | **Population Screen** | **Referred** |
| | | | | |
| **Secondary HT** | 11 | 5.8 | 1.1 | 7.9 |
| | | | | |
| **Renal** | 54 | 3.6 | 1.0 | 5.6 |
| **Renovascular** | 4.5 | 0.6 | 0.1 | 0.7 |
| **Aldosteronism** | 0.5 | 0.1 | 0.1 | 0.3 |
| **Pill** | - | - | 0.1 | 1.0 |

**Table 1.3 a Prevalence (percentage) of secondary hypertension in four published series. HT - hypertension, Aldosteronism - Primary Hyperaldosteronism, Pill - Oral Contraceptive Pill.**

Similarly, the form of the screening test will affect the estimation of the prevalence of a particular cause in the screened population, e.g. intravenous urography will fail to detect very few cases of renal artery stenosis but will over-estimate the

incidence in a clinic population and underestimate the incidence in the general population (Sinclair et al, 1987). However, most estimates agree that secondary hypertension is uncommon although the causes are many. The majority of causes of secondary hypertension are due to hormone excess, renal disease or to some vascular disease such as renal artery stenosis or coarctation of the aorta (Table 1.3 b).

Many of these conditions illustrate the effects of the extremes of physiology with respect to the physiological system involved. For example, in cases of phaeochromocytoma excessive catecholamine secretion from the adrenal gland results in a constellation of signs and symptoms which are an exaggeration of the physiological effects of catecholamines *in vivo* (Hall and Ball, 1992).

Given the potential for these gross abnormalities of the mechanisms controlling blood pressure to give rise to hypertension, it is conceivable that less severe alterations in the physiology of those systems which regulate blood pressure might be responsible for causing the more common form of essential hypertension.

| Organ | Example |
|---|---|
| **Kidney** | Autosomal Dominant Polycystic Disease<br>Renal Parenchymal Disease,<br>e.g. chronic glomerulonephritis, chronic interstitial nephritis, reflux nephropathy, analgesic nephropathy, amyloidosis |
| **Arteries** | Renal Artery Stenosis - either uni- or bilateral, e.g. atheromatous or secondary to fibromuscular dysplasia |
| **Adrenal** | Medulla - Phaeochromocytoma<br>Cortex - Cortisol overproduction<br>i.e. ACTH producing pituitary tumour, ectopic ACTH production, cortisol-secreting adrenal tumour<br>Aldosterone overproduction<br>i.e. Adrenal adenoma (Conn's Syndrome), idiopathic hyperaldosteronism, glucocorticoid suppressible hyperaldosteronism<br>Inborn errors of metabolism, i.e. 11ß-hydroxylase deficiency, 17α-hydroxylase deficiency, apparent mineralocorticoid excess |
| **Drugs** | Oral contraceptive pill,<br>glucocorticoids, e.g. prednisolone, dexamethasone,<br>mineralocorticoids, e.g. 9α-fludrocortisone,<br>cyclosporine, non-steroidal anti-inflammatory drugs, erythropoietin, Liquorice and carbenoxolone |
| **Others** | Acromegaly, Hyperthyroidism, Acute intermittent porphyria, Raised intracranial pressure<br>Rare tumours - renin, angiotensin, endothelin and medullolipin-secreting tumours have hypertension as a feature of the syndrome |

**Table 1.3b Secondary causes of hypertension**

### 1.3.2 Essential Hypertension

Essential hypertension (Primary hypertension) is the name given to the most common form of hypertension. This accounts for >95% of all cases of hypertension and excludes all those forms where a remediable cause can be found (Table 1.3 b). Thus, diagnosis is a negative process. In an attempt to provide a positive basis for diagnosis the physiology of virtually every human biological system described has been studied in hypertensive patients. Many have been found to be deranged when compared with normal subjects. Indeed, many types of secondary hypertension represent extremes of the physiological norm and similar but milder abnormalities can be demonstrated in patients with essential hypertension, e.g. steroid 11ß-hydroxylase deficiency in children with hypertension and impaired 11ß-hydroxylase activity in patients with essential hypertension. A complex interaction of environment, in particular dietary electrolyte intake, genetic background and lifestyle appear to determine the lifetime risk of hypertension.

Below is a brief account of some of the factors thought to be important in the pathogenesis of hypertension. Evidence of genetic influences is summarised in 1.4.

### 1.3.3 Overview of essential hypertension

Essential hypertension is a multifactorial disorder thought to be caused by the interaction of an individual's genetically determined risk of developing hypertension with his or hers environment, including diet, intrauterine environment and other modifiable factors (Swales, 1994).

Environmental influences have been assessed in detail in hypertensive populations and factors such as high dietary sodium intake, obesity, excessive alcohol intake, lack of exercise and psychosocial stress have all been implicated as potentially reversible risk factors in the development and maintenance of hypertension (Swales, 1994). These may account for some of the population-dependant variations in blood pressure observed. For example, the high sodium intake of Japanese populations results in a high proportion of hypertensive subjects whereas in the USA where salt intake is lower, other factors such as obesity may play a greater role in the development of hypertension (Swales, 1994). These factors together with certain genetic factors (See 1.4) may be considered as primary determinants, i.e. they are directly responsible for the elevation of blood pressure, in contradistinction to secondary phenomena. Secondary phenomena are those abnormalities of physiology, biochemistry and molecular biology found in

hypertensive but not in normotensive people which may be a consequence the hypertensive state and which may amplify the blood pressure response, e.g. vascular hypertrophy.

An example of a consequence of hypertension is left ventricular hypertrophy, whilst other examples of secondary phenomena include enhanced activity of the sympathetic nervous system, and increased lipid membrane viscosity. In addition, chronic elevation of arterial pressure also leads to hypertrophy of arterial resistance vessels in hypertension (Folkow, 1978, Folkow, 1982).

It is not easy in some cases to distinguish primary and secondary roles and some systems may contribute in both capacities. For example, a case has been made for vascular hypertrophy as the primary cause of hypertension (Folkow, 1978, Folkow, 1982).There is strong evidence of both primary and secondary effects on blood pressure by the adrenal gland. A number of studies (See 1.3.4-5, 1.4) have hinted at the possibility of primary genetic lesions predisposing to the development of essential hypertension but observed changes of regulation of adrenal steroid secretion may reflect secondary adaptation.

### 1.3.4 Adrenal corticosteroids in essential hypertension

As long ago as 1956, functional abnormalities of the adrenal cortex were suggested as a cause of essential hypertension (Genest et al., 1956). A number of abnormalities of urinary excretion rate, measured secretion rate, plasma levels and clearance of several adrenal steroids were noted in hypertensive patients (Brown et al., 1972a, Nowaczynski et al., 1971, Nowaczynski et al., 1975). However, no single defect in steroid biosynthesis has so far been detected in patients with essential hypertension to account for the development of essential hypertension. The possibility of other unconventional steroids (e.g. 16ß-hydroxydehydroepiandrosterone, 18-hydroxyDOC, 18-hydroxycorticosterone, 19-nor-aldosterone) having an important role in the causation of essential hypertension, particularly in the 'low-renin' forms of essential hypertension, has been suggested but has not yet been substantiated (Bennett et al., 1975, Tan and Mulrow, 1979).

Two studies have indicated that impaired activity of the enzyme steroid 11ß-hydroxylase (see 1.5.2) may be an important feature of patients with essential hypertension. In the first study of 10 patients with hypertension classified on the basis of their plasma renin as having either low- or normal-renin hypertension (Honda et al., 1977). Patients were given intravenous ACTH and the plasma

concentrations of a variety of steroid hormones measured. Patients with essential hypertension had higher post-ACTH plasma aldosterone, DOC and 11-deoxycortisol (S) concentrations, and lower plasma corticosterone (B) concentrations than a normotensive control group (Table 1.3 c). Calculation of molar plasma ratios of DOC:B and S:F serve a an index of adrenal cortex 11ß-hydroxylation ability. High ratios imply impaired conversion of DOC and S to B or F respectively. In patients with essential hypertension, the DOC:B ratio following ACTH was higher than in normotensive controls.

Secondly, a study of 15 patients with essential hypertension and 15 normotensive controls demonstrated significant differences in basal and ACTH-stimulated plasma steroid concentrations between the two groups (De Simone et al., 1985). Hypertensive subjects had significantly higher basal plasma DOC and S concentrations than normotensive controls. In addition, basal DOC:B and S:F ratios were also higher in the hypertensive group and ACTH-stimulation resulted in higher DOC:B and S:F ratios in hypertensive than normotensive patients (Table 1.3 c).

In both of these studies, no abnormality of aldosterone synthesis was detected suggesting the defect lay within the zona fasciculata region of the adrenal cortex (See 1.5).

| | DOC:B | | | | S:F | | | |
|---|---|---|---|---|---|---|---|---|
| | Basal | Basal | Post-ACTH | Post-ACTH | Basal | Basal | Post-ACTH | Post-ACTH |
| | NT | HT | NT | HT | NT | HT | NT | HT |
| Honda | 0.018 | 0.015 | 0.02 | 0.06‡ | N/A | N/A | N/A | N/A |
| De Simone | 0.013 | 0.04† | 13.1* | 37.4*† | 0.003 | 0.011† | 3.4* | 10.2*† |

† $p<0.001$, ‡ $p<0.05$
*ACTH stimulation areas, N/A - not available

**Table 1.3 c Impaired 11ß-hydroxylation ability in essential hypertension**

Both studies provide evidence of alteration in adrenal cortex 11ß-hydroxylase activity in essential hypertension. The exact nature of the abnormality is not clear and previous authors have suggested that these differences may arise from unknown, genetically-determined partial deficiency of steroid-11ß-hydroxylase (De Simone et al, 1985, Honda et al, 1977). A syndrome of complete steroid 11ß-hydroxylase deficiency has been described, one feature of which is hypertension (See 1.8.2), but heterozygotes for mutations giving rise to clinical 11ß-hydroxylase

deficiency are not hypertensive nor do they have abnormalities of their basal steroid profiles similar to those in patients with essential hypertension (Pang et al., 1980). Therefore, in patients with essential hypertension the genetic mutation is unlikely to be similar to that giving rise to clinical 11ß-hydroxylase deficiency. Abnormal regulation of the expression of 11ß-hydroxylase in the adrenal cortex is one possibility (See chapter 5). Evidence that this apparent deficiency may not merely be a mild classical form has been reported (R Fraser, Personal communication). In a large population of hypertensive subjects, 11ß-hydroxylase activity was impaired in the 17α-hydroxy pathway (cortisol synthesis) but not in the 17-deoxy pathway (corticosterone synthesis).

A further abnormality relating to corticosteroids in hypertensive patients has been the demonstration of possible alterations in the activity of the enzyme 11ß-hydroxysteroid dehydrogenase (11ß-OHSD, see 1.5.6) in hypertensive patients. Studies in hypertensive patients have shown that there is reduced cortisol clearance and increased vascular sensitivity to cortisol compared with normal subjects (Walker et al., 1991a, Walker et al., 1991b) suggesting that 11ßOHSD activity is reduced and that cortisol is able to exert mineralocorticoid effects in target tissues (1.5.6). More recently clear evidence of impaired 11ß-OHSD activity in hypertensive patients has been demonstrated by examining their urinary steroid excretion patterns (Soro et al., 1995). Patients with essential hypertension have higher ratios of 11ß-hydroxy metabolites to 11-dehydro metabolites of cortisol (tetrahydro- + allotetrahydrocortisol / tetrahydrocortisone), consistent with impairment of 11ßOHSD activity, but also higher ratios of 5α- to 5ß- reduced metabolites (allotetrahydrocortisol/tetrahydrocortisol), consistent with impairment of 5ß-reductase activity, than normotensive subjects. This may point to a possible common link between 11ßOHSD and the enzyme 5ß-reductase *in vivo*. The rare syndrome of apparent mineralocorticoid excess (SAME) is due to the congenital absence of 11ßOHSD function. The urinary steroid abnormalities in essential hypertension are similar to, but much less profound than those found in SAME but do not exclude a role for a partial deficiency of 11ßOHSD in essential hypertension.

### 1.3.5 Abnormalities of aldosterone secretion in essential hypertension

The aldosterone secretory and pressor response to AngII infusion are abnormal in patients with essential hypertension (Chinn and Düsterdieck, 1972, Kaplan and Silah, 1964, Kisch et al., 1976). Angiotensin II is a key regulator of aldosterone secretion in man (Fraser et al., 1979), and has an important pressor effect.

Patients with essential hypertension have enhanced pressor and aldosterone secretory responses to intravenous AngII infusion (Kisch et al, 1976, Oelkers et al., 1974), both when assessed as the dose necessary to raise systolic blood pressure by 20mmHg or as the absolute rise in blood pressure in response to a fixed amount of infused AngII (Chinn and Düsterdieck, 1972, Zoccali et al., 1983).

Both the pressor response and aldosterone-secretory response to Ang II are sodium dependent, i.e. manipulation of body sodium content can alter the relationship. Sodium restriction results in both increased basal plasma aldosterone concentrations and enhanced AngII-induced aldosterone secretion in both normal and hypertensive subjects (Hollenberg et al., 1974, Kisch et al, 1976, Oelkers et al, 1974, Zoccali et al, 1983); hypertensive subjects have a somewhat greater response to sodium depletion than normotensive subjects but this response and the response to Ang II may be inconsistent (Zoccali et al, 1983). Those patients with a below average rise in plasma aldosterone in response to sodium restriction have also been shown to have a reduced aldosterone secretory response to AngII infusion when salt-deplete and have been termed 'non-modulators' (Williams et al., 1982).

Hypertensive patients can be defined as modulators or non-modulators on the basis of their change in plasma aldosterone concentration in response to sodium restriction and change in p-amminohippurate clearance following AngII infusion in the salt-loaded state (Williams et al., 1992). Using this classification gives a bimodal distribution in the hypertensive population of modulators and non-modulators. Non-modulators tend to be older than modulators and there is evidence to suggest that non-modulation has a genetic basis. In one study of patients with a positive family history (FH+) of hypertension, 81% of FH+ hypertensive patients were non-modulators (Lifton et al., 1989). In another study of hypertensive sibling-pairs, non-modulation was shown to aggregate within families and was independent of sodium intake (Dluhy et al., 1988).

Therefore, there are clearly abnormalities in the control of aldosterone secretion in essential hypertension which may have a heritable component although the physiological differences giving rise to these abnormalities is unclear.

## 1.4 Genetic Factors in Human Hypertension

Since the discussions of Platt and Pickering in the 1950's and 60's, there has been a general awareness of the role of inheritance in hypertension (Pickering, 1961, Platt, 1959). Most clinicians will observe aggregation of hypertension within a family, but this does not prove or disprove a genetic role in its causation as environmental influences may readily explain such observations. In order to study the genetic contribution to hypertension, one starting point is to find evidence of familial aggregation of blood pressure. If such aggregation occurs, it must be determined if there is a genetic component, a shared environment or both. If there is a genetic component present, does it arise from a minor effect of many contributory genes or are there a few genes with a major effect? Subsequently, the effect of environmental influences on a major gene and blood pressure should be determined and individuals within a family with differing genotypes studied to identify the specific environmental influences altering the blood pressure response.

### 1.4.1 Blood pressure phenotype

A positive family history is commonly used as a measure of familial aggregation of blood pressure and as a surrogate measure of undefined risk in family members. Significant associations between a family history of hypertension and the development of hypertension in a family member have been demonstrated (Hunt and Williams, 1994). The strength of the association varies depending on the definition of 'family history' and the risk attributable to a positive family history of hypertension can be detected after 13 years of follow-up (Hunt and Williams, 1994). Table 1.4 a outlines the effect of sex and number of affected family members on an individuals relative risk of developing hypertension. Similar findings have been reported in children with a positive family history of hypertension. Young adults with a systolic blood pressure over the age- and gender-specific 90th percentile had a greater prevalence of positive family histories of hypertension and more ischaemic heart disease and strokes (Hunt and Williams, 1994). Taken together, the majority of evidence suggests that a positive family history of hypertension, properly defined, is a significant indicator of hypertension risk in an individual. Even in two families where the cause of hypertension is very different, for example adult polycystic kidney disease and glucocorticoid-suppressible hyperaldosteronism, this will hold true despite the genetic basis being very different.

| | | Age | | | | |
|---|---|---|---|---|---|---|
| Defn of FH | %with +FH | 20-39 | 40-49 | 50-59 | 60-69 | ≥ 70 |
| Males | | | | | | |
| 1+Affected | 53 | 2.5 | 1.8 | 1.7 | 1.2 | 0.9 |
| 1 + age<55 | 32 | 2.8 | 2.1 | 1.8 | 1.1 | 0.8 |
| 2+Affected | 24 | 3.8 | 2.4 | 2.3 | 1.2 | 0.6 |
| 2 + age<55 | 11 | 4.1 | 2.5 | 2.4 | 1.0 | 0.8 |
| FHS ≥ 1.0 | 14 | 3.9 | 2.5 | 2.9 | 1.4 | 1.2 |
| FHS ≥ 2.0 | 7 | 4.0 | 3.2 | 3.3 | 1.4 | 2.8 |
| | | | | | | |
| Females | | | | | | |
| 1+Affected | | 2.8 | 2.0 | 1.5 | 1.2 | 1.0 |
| 1 + age<55 | | 3.2 | 2.3 | 1.5 | 1.0 | 0.8 |
| 2+Affected | | 3.8 | 2.4 | 2.3 | 1.2 | 0.6 |
| 2 + age<55 | | 5.0 | 3.5 | 1.5 | 0.7 | 0.8 |
| FHS ≥ 1.0 | | 4.4 | 3.3 | 2.0 | 1.0 | 1.2 |
| FHS ≥ 2.0 | | 4.4 | 3.9 | 2.0 | 1.0 | 1.3 |

Defn of FH - definition of family history of hypertension, % with FH - percentage of patients with a positive family history. FHS - family history score as defined by FHS= (observed number of hypertensives - expected number)/√expected, with a 0.5 correction in the numerator

**Table 1.4 a Relative risks of hypertension for different definitions of a positive family history of hypertension for males and females based upon 13 years of follow-up in 15200 families. Adapted from (Hunt and Williams, 1994)**

| Location | Systolic Blood Pressure | Diastolic Blood Pressure |
|---|---|---|
| Michigan, USA | 0.17 | 0.12 |
| Georgia, USA | 0.20 | 0.17 |
| Framingham, USA | 0.18 | 0.17 |
| Utah, USA | 0.16 | 0.21 |
| United Kingdom | 0.33 | 0.20 |
| Turkmenistan | 0.27 | 0.11 |
| Norway | 0.22 | 0.23 |
| Brazil | 0.27 | 0.32 |
| Tokelau | 0.17 | 0.13 |

**Table 1.4 b Sib-sib blood pressure correlations. Adapted from (Hunt and Williams, 1994)**

| SBP | | | DBP | | |
|---|---|---|---|---|---|
| | | | | | |
| $r_{(mz)}$ | $r_{(dz)}$ | $h^2$ | $r_{(mz)}$ | $r_{(dz)}$ | $h^2$ |
| | | | | | |
| 0.61 | 0.29 | 0.63 | 0.61 | 0.32 | 0.66 |

r - correlation between twins, mz - monozygotic, dz - dizygotic, $h^2$ - heritability

**Table 1.4 c Intraclass blood pressure correlations and heritability estimates. Adapted from (Hunt and Williams, 1994).**

Many large population studies have shown familial correlations of blood pressure (Table 1.4 b). These correlations are generally in the 0.1-0.3 range and are broadly similar amongst the various sib-sib pairings. Sib-sib correlations are in turn generally slightly higher than parent-offspring correlations (Hunt and Williams, 1994). There is also data which suggest that mother-offspring correlations are stronger than those of father-offspring and that this maternal effect is present from the first year of life (Levine et al., 1982). Estimates of polygenic heritability can be obtained from familial correlations These estimates cluster around 20% for family studies (Hunt and Williams, 1994), but are much higher in twin studies at around 60%(Table 1.4 c). In particular, monozygous twins show a higher correlation in blood pressure and blood pressure heritability than dizygous twins and both were higher than siblings (Hunt and Williams, 1994). The effect of a common environment in these groups has also been examined and has revealed that monozygous twins have a much more similar environment than dizygous twins or common sibs but correction for this still leaves the twin groups with a much higher blood pressure correlation suggesting a definite component derived from inheritance (Hunt and Williams, 1994).

Adoption studies have shown that in families with both adoptive and natural children, the correlation in blood pressure between parent and natural child was greater than that between parent and adoptive child and the correlation between natural sibs was greater than that between adoptive and natural sibs (Hunt and Williams, 1994).

The factors determining the inherited tendency to hypertension may be determined either directly from the genes inherited from the patients parents. However, the possibility that adverse conditions during intrauterine life may programme subsequent adult blood pressure has been raised. The potential role of *in utero* influences has been reviewed recently (Law and Barker, 1994) and references therein.

A number of studies have demonstrated an association between low fetal growth rate and increased death rate from coronary heart disease and stroke. Similarly, blood pressure shows an inverse relationship with birthweight across the range of blood pressures in both sexes (Law and Barker, 1994).

A number of studies have demonstrated 'tracking' of blood pressure from early childhood, suggesting that hypertension is programmed into an individual from an early age. In addition, it has been noted that those children born thin and undernourished have higher blood pressure at age 4 years than those with normal

body proportions, as have those adults who had large placentas at delivery (a sign of adaptation to fetal undernutrition) (Law et al., 1991), and the association is found in children who are 'light for dates' rather than premature. Finally, studies of the growth of children from birth have shown that it is the degree of undernutrition at birth rather than the accelerated growth (catch-up growth) after birth which determines subsequent blood pressure suggesting that the individuals blood pressure is programmed *in utero* (Law et al, 1991, Lever and Harrap, 1992).

Such programming of blood pressure has been demonstrated in protein deprived pregnant rats whose offspring had elevated systolic blood pressures compared with those receiving a normal protein diet (Law and Barker, 1994), and in the offspring of rats receiving glucocorticoids during pregnancy (Benedicktsson et al., 1993). Human evidence is less prevalent but the study of the Dutch Hunger Winter showed that the women whose mothers were malnourished during pregnancy themselves had babies with retarded intra-uterine growth (Law and Barker, 1994).

The difference in blood pressure in people of low and high birthweight is small in childhood but increases progressively with age (Law and Barker, 1994). One interpretation is that the process started *in utero* is amplified throughout life, perhaps through effects on blood vessel structure and function (Folkow, 1978, Folkow, 1982). It may be the case therefore, that alterations in fetal arterial structure secondary to the effects of undernutrition may lead to changes in arterial compliance which in turn lead to changes in vessel structure which in turn alter arterial compliance. Thus, a vicious circle is set up to maintain and amplify the process started *in utero*.

Thus, it appears from studies of heritability that hypertension has an important genetic component in the region of 20-30%. In order to relate this variance to the setting of human physiology, a number of the factors regulating blood pressure in man have been examined to determine whether genetic variation occurs in these systems and if they influence blood pressure.

### 1.4.2 Renin-angiotensin-aldosterone system

The renin-angiotensin system is central to the control of blood pressure in normal man (Figure 1.4 a). Variations in the plasma concentrations of certain components of the system have been detected in normal populations and a genetic basis for these variations has been postulated. Differences have also been detected in hypertensive patients and in animal models of hypertension . The role of the renin-

angiotensin system in the control of aldosterone secretion in man is reviewed in detail in 1.5.5.

The renin-angiotensin system is a good place to start looking for genetic variation resulting in hypertension because it contains many components central to the control of blood pressure in normal individuals and the actions of certain components, e.g. AngII, have been implicated in the mechanisms which sustain hypertension after the initial stimulus. The idea of a candidate gene, i.e. a gene for a protein or enzyme directly involved in a central physiological process, has been most readily studied in the renin-angiotensin system.

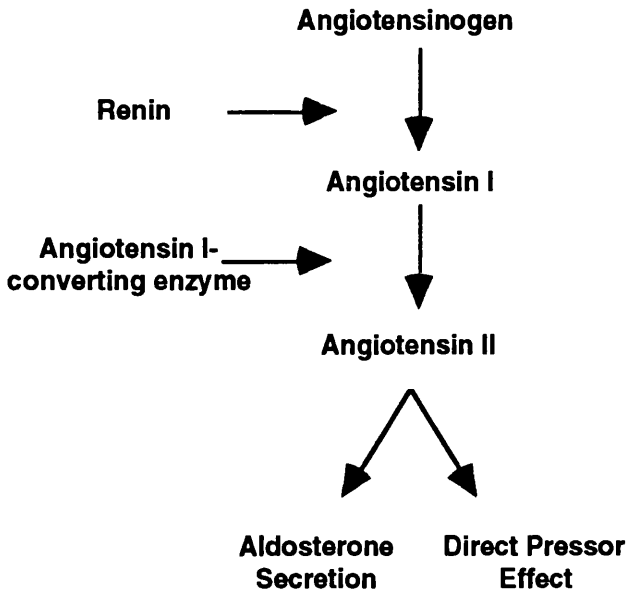



**Figure 1.4 a Renin-angiotensin system**

The renin gene has been cloned in man and mapped to chromosome 1. A number of studies have shown no evidence of linkage of the renin locus to human hypertension . This is not the case in rat hypertension where polymorphisms in the renin gene have been shown to co-segregate with the hypertensive phenotype (Hunt and Williams, 1994).

The gene for angiotensinogen (AGT) has been cloned in man, mapped to chromosome 1q42-43, and a number of polymorphic regions of this gene detected. Polymorphisms of this gene are associated with differences in plasma

angiotensinogen levels and hypertension (Jeunemaitre et al., 1992b). Sib-pair analysis has shown that the certain alleles of the gene predispose to hypertension, particularly more severe hypertension (defined as those cases requiring ≥2 antihypertensive drugs to control blood pressure), in males. No linkage was found in female subjects, perhaps due to the effects of oestrogen on plasma angiotensinogen levels. One marker polymorphism, M235T in exon 2 of the AGT locus has been associated with hypertension in both males and females in one study performed in Utah and France, where the frequency of M235T was 0.51 in severe hypertensives and 0.36 in controls (Jeunemaitre et al, 1992b), but not in a population studied in the United Kingdom (Caulfield et al., 1994). However, a second polymorphism, a GT-repeat in the 3' untranslated region of the gene, has been found to be in linkage with hypertension in both males and females in the British study (Caulfield et al, 1994).

A similar association between the GT-repeat and pre-eclampsia has been demonstrated in Scottish and Icelandic women (Angrimmson et al., 1993), and the M235T variant and pre-eclampsia in a study from Utah (Ward et al., 1993).

The gene for angiotensin-I converting enzyme (ACE) has been cloned, mapped to chromosome 17 and a polymorphism in intron 16 of the gene characterised (Hubert et al., 1991). This polymorphism consists of the presence or absence of an *Alu* repeat which can be detected by PCR amplification of intron 16 (insertion (I)/deletion (D) polymorphism) . The I/D polymorphism accounts for approximately 50% of the variance in plasma ACE levels in normal populations, with DD homozygotes having the highest plasma ACE levels, II homozygotes the lowest and heterozygotes intermediate levels (Rigat et al., 1990). However, a number of studies have shown that in man there is no association between the ACE I/D polymorphism and hypertension (Harrap et al., 1993, Jeunemaitre et al., 1992a, Schmidt et al., 1993), whilst only one suggest a possible role in the development of hypertension (Zee et al., 1992). There is however, one study that suggests that the ACE I/D polymorphism may be important in determining whether left ventricular hypertrophy develops in hypertensive subjects (Schunkert et al., 1994).

However, there is evidence that the ACE I/D polymorphism may be an important independent risk factor for the development of myocardial infarction, particularly in the absence of conventional risk factors (Cambien et al., 1992).

### 1.4.3 Glucocorticoid receptor polymorphism

Steroid hormones are important regulators of blood pressure in man. One major class of steroid hormones is the glucocorticoids, excess of which can give rise to hypertension. The principal human glucocorticoid is cortisol which, like all other steroid hormones, binds to a nuclear receptor to produce its effect. Cortisol binds to the glucocorticoid receptor (GR)*in vivo* and initiates a series of intracellular processes one of which is to alter vascular tone and ultimately raise blood pressure (Walker et al, 1991b) and 1.5.6. The gene for the GR has been cloned and mapped to chromosome 5 and a diallelic BclI RFLP in the region of the gene described (Murray et al., 1987). This polymorphism has been associated with a hypertensive tendency and abnormalities of steroid synthesis in the 'Ladywell Study' population (Watt et al., 1992). In this study the offspring of hypertensive and normotensive parents were studied and analysed by the '4-corners' method. The offspring were sub-divided into four groups on the basis of their parents blood pressure (high or low) and the offspring's blood pressure (high-normal or low-normal) thus deriving the four corners of the study group (Figure 1.4 b). When the two extremes of phenotype, the high-high and low-low groups, were compared it was found that there was an excess of homozygotes for the large allele of the RFLP in the high-high group compared with the low-low group (27% vs. 9%, $p<0.01$). Offspring of hypertensive parents with high normal blood pressures also had significantly higher plasma levels of cortisol, 18-hydroxycorticosterone and angiotensinogen compared with the low normal offspring.

Physiological studies have shown a trend towards increased affinity of dexamethasone for GR, reduced GR number, *in vitro*, and higher plasma cortisol concentrations in normal subjects homozygous for the large fragment of the BclI RFLP genotype (Panarelli and Fraser, 1994). It is not clear, however, if this is a direct effect of the polymorphism or is of relevance to the development of hypertension in man.

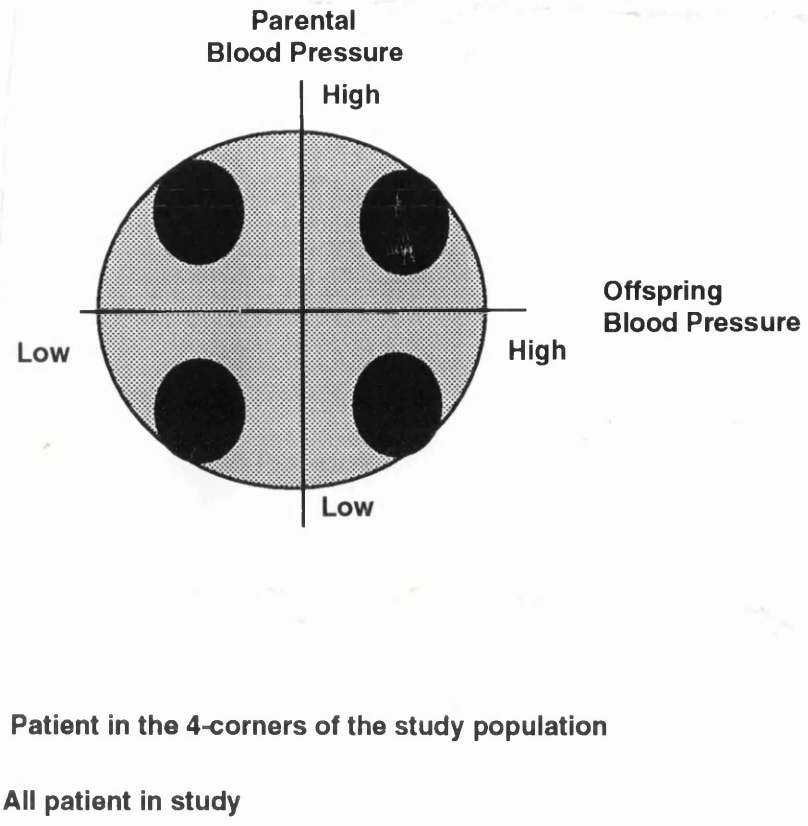



**Figure 1.4 b Four corners approach to analysis of inheritance of hypertension**

Thus it would appear that the GR locus or a locus nearby may have exert an effect on blood pressure in man but further studies are required.

### 1.4.4 Sodium-lithium countertransport

In man the presence of an erythrocyte sodium transporter which promotes intracellular to extracellular transport of sodium ions has been described. This pump also transports lithium ions and a measure of the degree of sodium-lithium countertransport (SLC) has been studied and found to be raised in patients with essential hypertension when compared with normotensive controls (Canessa et al., 1980).

Studies designed to test for the presence of a genetic effect on SLC have been reviewed in detail (Hunt and Williams, 1994). These studies demonstrate high parent-offspring (0.52) and sib-sib correlations (0.39) in SLC and twin studies estimate the polygenic heritability to be between 60-98%. Major gene segregation for SLC has also been demonstrated (Table 1.4 d)

| Study | Gene Frequency | Normal Mean | High Mean | $h^2$ |
|---|---|---|---|---|
| 1 | 0.45 | 0.31 | 0.48 | 0.45 |
| 2 | 0.28 | 0.29 | 0.45 | 0.17 |
| 3 | 0.26 | 0.29 | 0.60 | 0.42 |
| 4 | 0.23 | 0.26 | 0.53 | 0.70 |

$h^2$ - heritability

**Table 1.4 d Comparison of genetic model estimates of Na-Li countertransport (adapted from (Hunt and Williams, 1994)**

All four studies found evidence for a recessive major gene for high SLC levels along with a significant polygenic effect. However, the confounding effects of gender and plasma triglyceride levels on SLC results in a significant blunting of the bimodal distribution of SLC in the population. Further analysis of the pedigrees have shown that a subset of the all the kindreds studied show a high degree of segregation of SLC and hypertension and can reject a non-transmissible cause of the bimodality in SLC levels.

As yet no genetic marker for the SLC locus has been found and the *in vivo* function of the SLC mechanism is unknown. However, one study in sib-pairs has rejected the possibility that the SLC mechanism is in fact the Sodium-Hydrogen pump (gene on chromosome 1) with a LOD score of -5.91 for linkage between SLC and the Sodium-Hydrogen pump.

### 1.4.5 Kallikrein

Kallikrein is an enzyme which converts inactive kininogens to vasodilator kinins. The kallikrein-kinin system tends to counteract the renin-angiotensin system as ACE inactivates kinins as well as producing AngII. Therefore a defect in the kallikrein system leading to low production of active kinins may lead to the development of hypertension (Figure 1.4 c). The data relating to the genetics of kallikrein in human hypertension has been reviewed in detail (Hunt and Williams, 1994) and references therein. Urinary kallikrein levels are low in patients with essential hypertension and there is a strong familial aggregation in adults and children. Intrafamily correlations of urinary kallikrein excretion were high: adult siblings - 0.34, youth siblings - 0.55, and parent-offspring correlations for adults and youths were 0.43 and 0.50 respectively. Genetic modelling of the available data has shown that a recessive major gene effect with additional polygenic influences is the best fit. The major gene explained 51% of the variance in urinary kallikrein excretion with polygenes explaining another 27%.

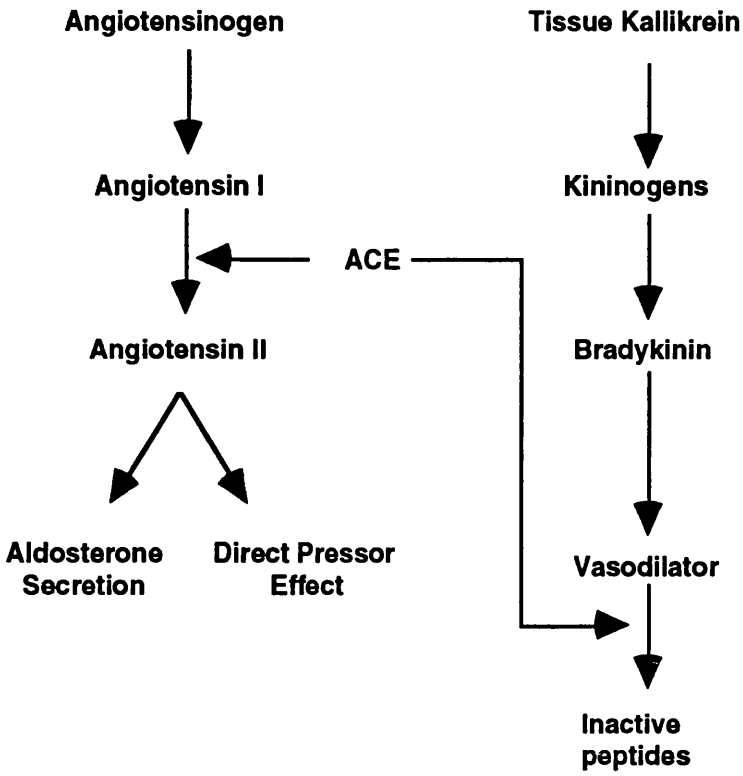



**Figure 1.4 c Kallikrein-Kinin and Renin-Angiotensin systems**

A study of monozygotic twins revealed that the strongest environmental determinant of urinary kallikrein excretion was urinary potassium excretion. Incorporation of the effects of environmental influences into the previous model allowed the model to be refined and clearly defined the effect of major genes and environment on urinary kallikrein excretion (Table 1.4 e).

A significant gene-environment interaction was found for the underlying variables represented by urinary kallikrein and potassium levels. Urinary potassium excretion reflects dietary potassium intake in the steady state whilst urinary kallikrein excretion is thought to reflect local renal kallikrein levels. The interaction suggested that subjects homozygous for the low kallikrein gene had low levels of renal kallikrein that are not modified by changes in potassium intake. Subjects homozygous for the high kallikrein gene may be protected from the development of hypertension by maintaining high kallikrein levels as a result of being more responsive to changing potassium levels or other alterations that might lower kallikrein levels. However heterozygotes, who account for 50% of the population studied, showed a very significant positive relationship between urinary kallikrein

and potassium. A decrease in urinary potassium in this population of 1.3 standard deviations results in a fall in urinary kallikrein levels to that seen in the low homozygote group. Similarly a rise in dietary potassium leading to a rise in urinary potassium excretion of 0.8 standard deviations would be expected to raise kallikrein levels to those of the high homozygote group. This finding would seem to fit well with the epidemiological evidence that dietary potassium supplementation may reduce blood pressure.

| | | Urinary Potassium | |
|---|---|---|---|
| **Source** | **Low** | **Average** | **High** |
| **Major Gene** | 47 | 38 | 62 |
| **Polygenes** | 0 | 0 | 0 |
| **Recent Environment** | 12 | 14 | 9 |
| **Individual Variation** | 30 | 35 | 21 |

**Table 1.4 e Sources of variation in urinary kallikrein levels by urinary potassium level (%). Adapted from** (Hunt and Williams, 1994)

The human kallikrein gene has been cloned but as yet no studies in man have been published showing co-segregation or linkage of this locus to hypertension.

### 1.4.6 Abnormalities of steroid metabolism

Adrenal steroids can cause hypertension when present in excess and there are a number of well defined inherited abnormalities of steroid metabolism which have hypertension as a feature of the clinical syndrome (1.8.2-4, 1.9). Three of these disorders, steroid 11ß-hydroxylase deficiency, steroid 17α-hydroxylase deficiency and the syndrome of apparent mineralocorticoid excess have an autosomal recessive mode of inheritance and are rare causes of hypertension. Glucocorticoid-suppressible hyperaldosteronism is inherited as an autosomal dominant trait and although thought to be rare, its incidence may be greater than first thought (1.9). In addition, there have been reports of aldosterone-secreting adrenal adenomata occurring within families although the genetic basis for this is unknown (Stowasser et al., 1992).

Impairment of 11ß-hydroxylase activity in patients with essential hypertension has been noted previously (De Simone et al, 1985, Honda et al, 1977) and the steroid synthetic defect in these patients may be genetically determined in that polymorphisms have been described within the coding region of the gene for 11ß-hydroxylase (Lifton et al., 1992). The enhanced pressor and aldosterone secretory response to AngII infusion in hypertensive patients may also have a genetic basis

because polymorphisms have been detected in the regulatory regions of the gene for aldosterone synthase which could affect the aldosterone secretory response to Ang II (Chapter 5), (Lifton et al, 1992).

In summary, there are clearly many ways in which hereditary influences may determine blood pressure. Three candidate genes appear to be linked to the hypertensive phenotype, angiotensinogen, sodium-lithium countertransport and urinary kallikrein excretion. Other loci, in particular the glucocorticoid receptor polymorphism and polymorphisms in the adrenal steroidogenic enzyme genes, may be of importance and further study will elucidate this.

The likelihood that a few genes exerting a small, additive effect on blood pressure in an individual would appear therefore to be the case in human hypertension, although the effects may vary on the combination of genes an individual has, the environmental influences encountered and other less well defined influences such as the *in utero* environment.

## 1.5 The Adrenal Gland

The first anatomical description of the adrenal gland was made in 1563 by Eustachius (Eustachius, 1774). The first differentiation of the adrenal gland into cortex and medulla was made by Huchke (Schoenwetter, 1968) and in the 19th century Arnold first described the concentric zones we now use to describe the adrenal cortex (Arnold, 1866). Ideas about the function of the adrenal cortex lagged behind the anatomical description of the gland. In 1716 the Académie des Sciences de Bordeaux offered a prize for the answer to the question, "What is the purpose of the suprarenal glands?" However the answers were so unsatisfactory that the prize was not awarded and the judge forced to say "Perhaps some day chance will reveal what all of this work was unable to do" (Gaunt, 1975). The first evidence of a physiological role for the adrenal glands came from the clinical observations of Thomas Addison in 1849 (Addison, 1849). It was six years later that Addison published his authoritative account of the effects of adrenal disease in man (Addison, 1855). Experimental confirmation of the clinical findings came the following year from Brown-Séquard who performed adrenalectomies on several species of animal and noted the detrimental effect (Brown-Séquard, 1856). It was not until 1930, however, that the efficacy of adrenal cortex extracts in maintaining the health and growth of adrenalectomised cats was noted (Swingle and Pfiffner, 1931) and these extracts were then used to successfully treat patients with Addison's disease (Rowntree et al., 1930).

### 1.5.1 Anatomy of the Adrenal Gland

Each adrenal gland consists of two functionally distinct endocrine glands within a single fibrous capsule. The adrenal gland is situated on the superior pole of the kidney and weighs about 4 grams in healthy adults. The outer capsule consists of loose fibrous tissue and surrounds the outer cortex of the adrenal. The cortex accounts for 90% of the weight of the adult adrenal and is derived from the mesenchymal cells adjacent to the urogenital ridge. The inner adrenal medulla is derived from cells of the neural crest which migrate into the adrenal during the third month of fetal life. Its cells secrete the catecholamines, adrenaline, noradrenaline and dopamine; its function will not be discussed further.

The adrenal cortex is divided into three roughly concentric zones based on the light microscopic appearances of the cells (Arnold, 1866). The outer zone, the zona glomerulosa constitutes 15% of the cortex and consists of poorly demarcated islands of cells lying subjacent to the capsule of the gland. In the majority of

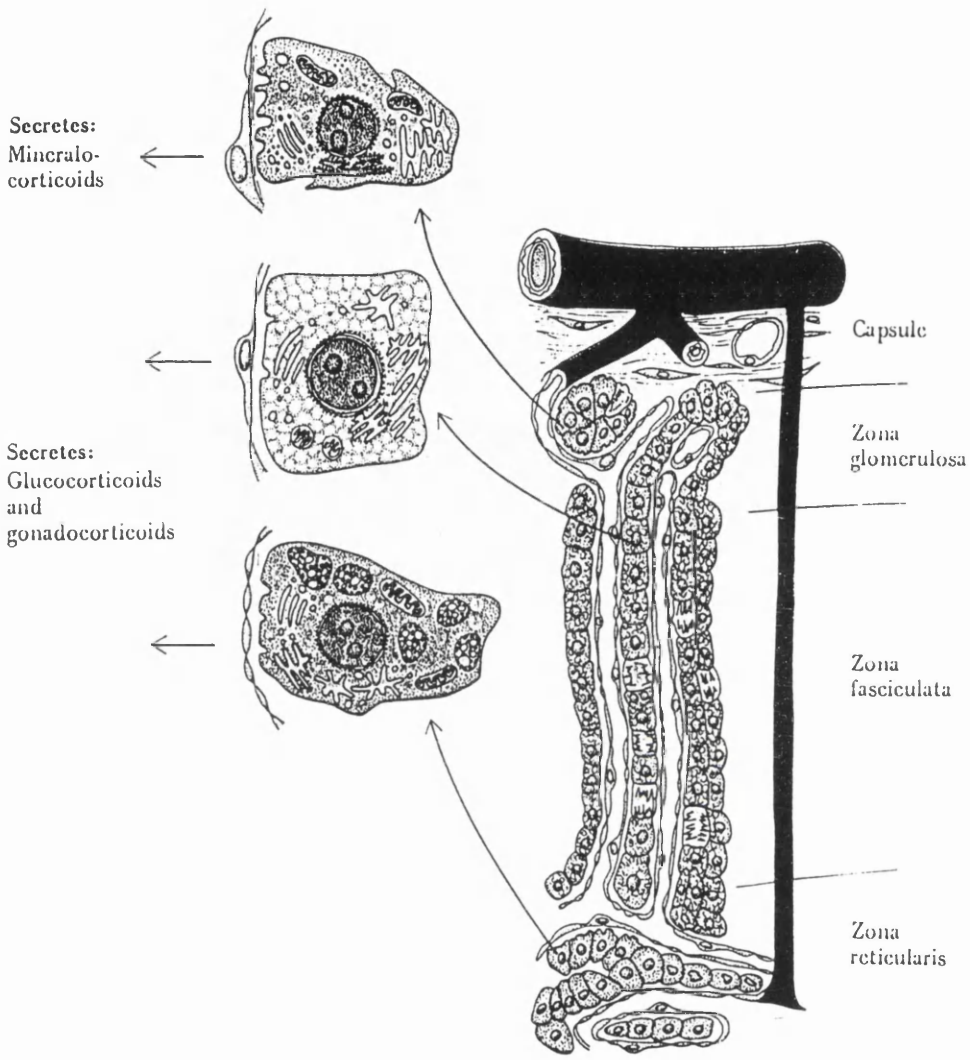



**Figure 1.5 a Microscopic anatomy of the Adrenal Cortex.**
**After Gray's Anatomy, 36 Ed.**

instances these islands do not form a continuous layer. The glomerulosa cells are small and have a low cytoplasm:nuclear ratio and an intermediate number of lipid inclusions. The adjacent zone is the zona fasciculata which constitutes 75% of the cortex. It is not well demarcated from the glomerulosa and cells from the fasciculata may penetrate the glomerulosa to contact the capsule. Cells in this zone have a high cytoplasm:nuclear ratio, are large and have a foamy, vacuolated cytoplasm because of the numerous lipid deposits. The innermost zone of the cortex is the zona reticularis, and is sharply demarcated from the fasciculata and medulla. Cells in this zone have a compact, lipid-poor cytoplasm and lie in anastamosing cords separated by sinusoidal spaces (Figure 1.5 a) (Orth et al., 1992).

### 1.5.2 Steroid Synthesis in the Adrenal Cortex

The adrenal cortex is the site of synthesis of a number of important steroid hormones in man. Steroid hormones are derived from the cyclopentanophenanthrene ring structure consisting three cyclohexane rings and one cyclopentane ring (Figure 1.5 b).

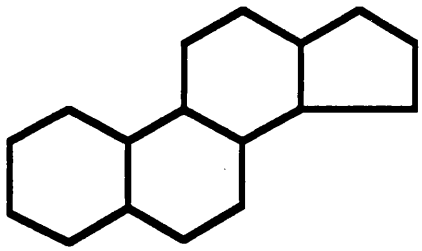


**Figure 1.5b Cyclopentanophenanthrene ring structure**

The starting point for the synthesis of all adrenal corticosteroid hormones in man is cholesterol. Cholesterol can be synthesised *de novo* from acetate, mobilised from intracellular cholesteryl ester pools or imported from plasma lipoproteins by steroidogenic tissues. About 80% of cholesterol is provided by circulating lipoproteins, principally low density lipoprotein (LDL) which acts via a cell surface receptor (Gwynne and Strauss, 1982). The LDL particle undergoes receptor-mediated endocytosis to be internalised by the cell and cholesteryl esters are liberated for use as steroidogenic substrates (Goldstein et al., 1979). Under normal

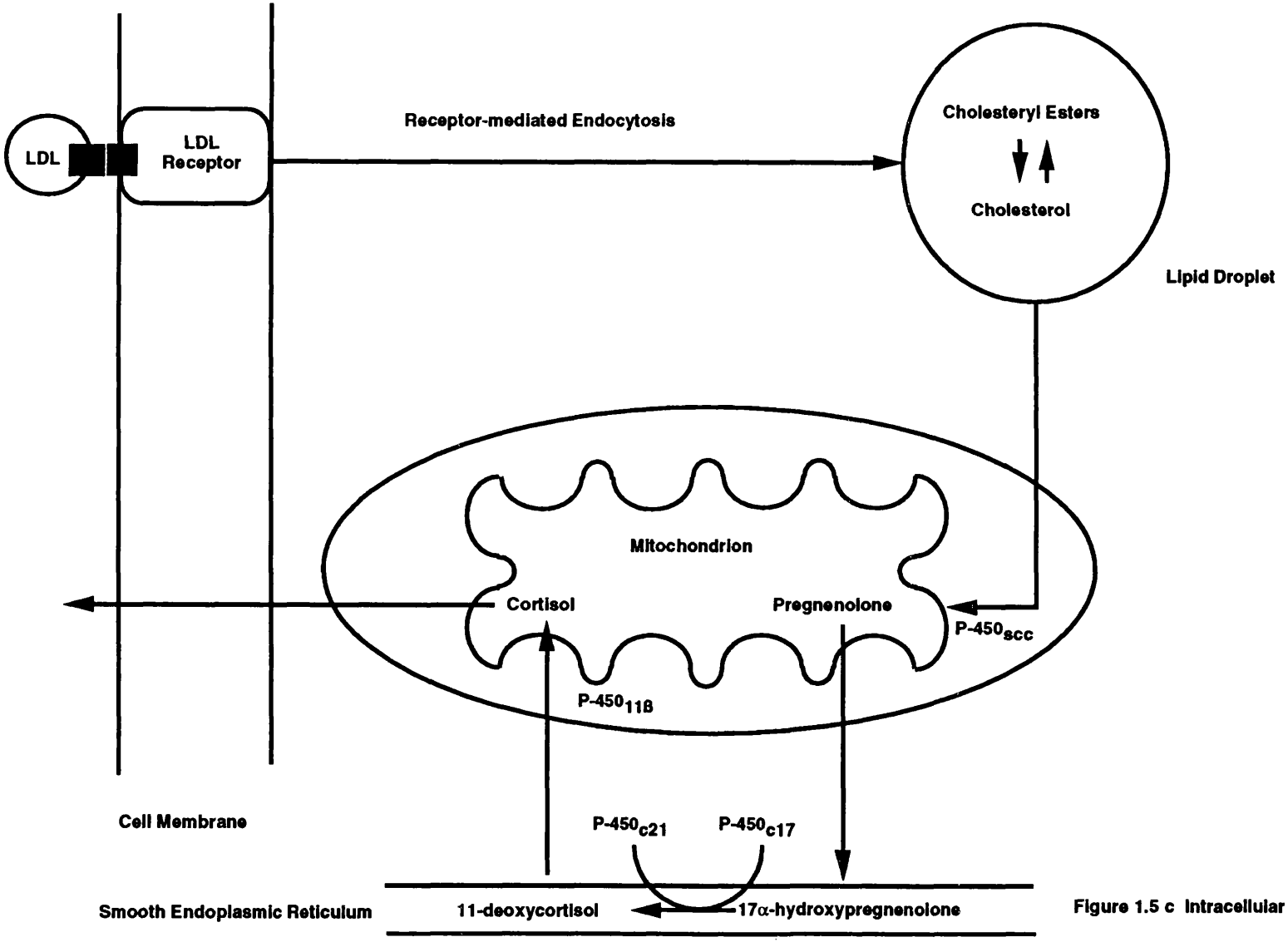



Figure 1.5 c Intracellular localisation of steroidogenic enzymes

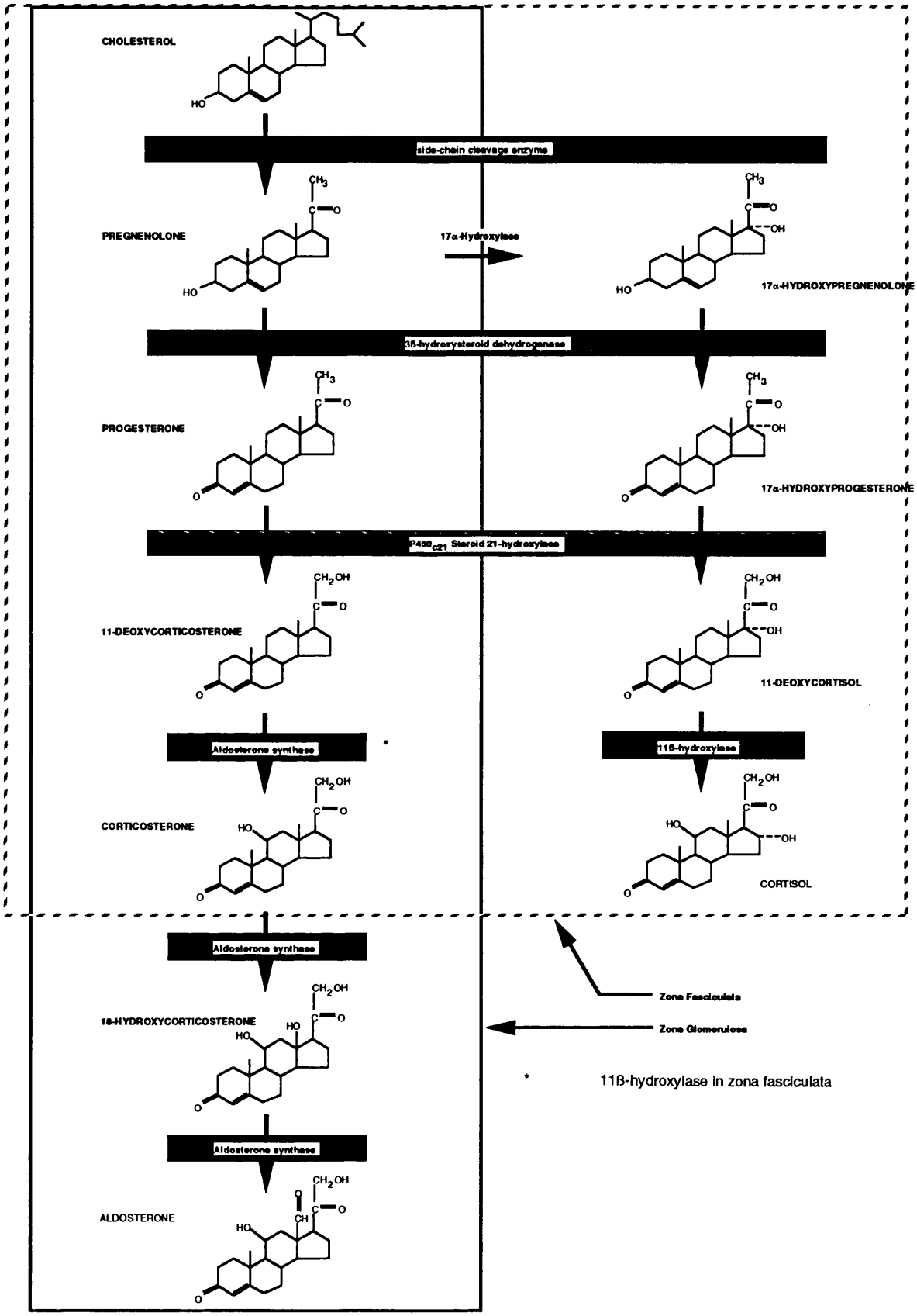



**Figure 1.5 d Synthesis of aldosterone and cortisol in the adrenal cortex**

conditions, a further 20% of cholesterol is synthesised *de novo* from acetyl coenzyme A (Gwynne and Strauss, 1982).

Three major groups of steroid hormones are synthesised in the adrenal cortex in man. The outer zona glomerulosa is the exclusive site of synthesis of aldosterone whilst the zonae fasciculata and reticularis synthesise cortisol and the adrenal androgens dehydroepiandrosterone (DHEA) and androstenedione. The most abundant product of the adrenal cortex, dehydroepiandrosterone sulphate (DHEAS), is formed exclusively in the zona reticularis (Orth et al, 1992). Synthesis of the various adrenal corticosteroids is compartmentalised within the cells of the adrenal cortex by virtue of the subcellular location of the enzymes involved (Figure 1.5 c). The biosynthetic pathways for conversion of cholesterol to aldosterone, cortisol and the adrenal androgens have been determined and are shown in Figure 1.5 d. The aldosterone biosynthetic pathway is discussed in detail in section 1.7.

The synthetic action of the steroidogenic enzymes is made possible by the existence of a coupled enzyme system which transfers electrons to the P-450 enzymes as reducing equivalents and allows the final hydroxylation step to occur. Two such systems exist in man, coupled to the action of the steroidogenic P-450 enzymes. The first is based on adrenodoxin, a non-haem iron-binding protein that exists in a soluble form in the mitochondrial matrix and in man is linked to the activity of $P\text{-}450_{SCC}$, $P\text{-}450_{11\beta}$ and $P\text{-}450_{aldo}$ (Kimura and Suzuki, 1967). Man has a single gene for adrenodoxin and two pseudogenes (Morel et al., 1988). Linked to adrenodoxin is the enzyme adrenodoxin reductase which accepts electrons from NADPH in the first step of the reduction cascade. In man there is a single gene for adrenodoxin reductase but two forms of the enzyme are produced by alternative splicing of the DNA (Solish et al., 1988). This so-called 'electron shuttle' system is illustrated in Figure 1.5 e. NADPH donates electrons to an adrenodoxin reductase and converts it to its reduced state. This then acts to convert adrenodoxin to a reduced state which in turn transfers reducing equivalents to the P-450 enzyme.

A second system exists coupled to the activity of $P\text{-}450_{c17}$ and $P\text{-}450_{c21}$ (Kominami et al., 1980). This system utilises a flavoprotein distinct from adrenodoxin reductase. This enzyme, P-450 reductase, transfers two electrons from NADPH to $P\text{-}450_{c17}$ and $P\text{-}450_{c21}$. In addition, cytochrome $b_5$ may also donate electrons to $P\text{-}450_{c17}$ and $P\text{-}450_{c21}$ (Yanagibashi and Hall, 1986) (Figure 1.5 f).

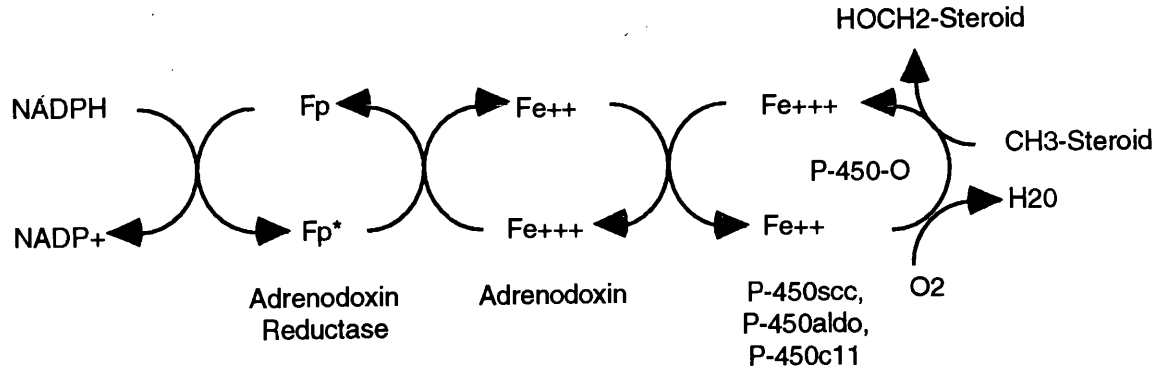



**Figure 1.5 e Electron shuttle system for P-450scc, P-450aldo and P-450c11**

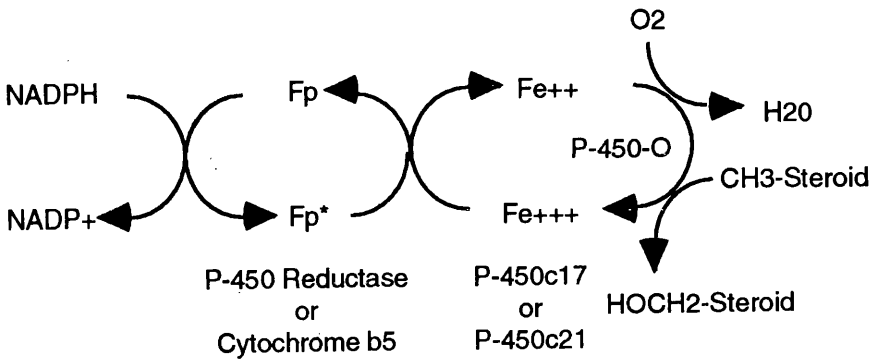



**Figure 1.5 f Electron shuttle system for P-450c17 and P-450c21**

Cholesterol is converted to pregnenolone by the enzyme $P\text{-}450_{SCC}$ located on the inner mitochondrial membrane. This step is the rate-limiting step in steroidogenesis and involves two oxidations at the C20 and C22 positions followed by the removal of isocaproic acid from the C-20 position (Strott, 1990). This enzyme, the so-called 'Side-Chain Cleavage' enzyme is encoded by a single gene found on chromosome 15 and is found in all steroidogenic tissues (Chung et al., 1986a). The newly synthesised pregnenolone is returned to the cytosolic compartment where a series of microsomal enzymes convert it to 11-deoxycortisol. Pregnenolone is converted to progesterone by the enzyme 3ß-hydroxysteroid dehydrogenase/$\Delta^5$ -$\Delta^4$ isomerase, a non P-450 enzyme encoded by a gene on chromosome 1 (Bérubé et al., 1989). A number of isoforms of this enzyme have been described with tissue-specific expression (Lachance et al., 1991).

The enzyme $P\text{-}450_{c17}$ is encoded by a single gene, CYP17A, on chromosome 10 (Matteson et al., 1986) which transcribes a single mRNA message and protein

product (Chung et al., 1986b, Kagimoto et al., 1988). In the zonae fasciculata and reticularis both pregnenolone and progesterone may undergo hydroxylation at the C-17 position in addition to the cleavage of the residual two-carbon chain at C-17 although this depends on which biosynthetic pathway they enter.

The degree of P-$450_{c17}$ activity to some extent determines which pathway the reaction products take. In the zona glomerulosa P-$450_{c17}$ activity is not present and therefore pregnenolone is converted to progesterone as a precursor in the formation of aldosterone. In the zona fasciculata pregnenolone undergoes hydroxylation of the C-17 position and the product, 17α-hydroxyprogesterone undergoes subsequent hydroxylation steps to become cortisol. However, some pregnenolone may be converted to progesterone before being converted to 17α-hydroxyprogesterone and subsequently cortisol. If the action of the enzyme is to cleave the two-carbon chain at C-21 in addition to the hydroxylation of pregnenolone (i.e. 17α-hydroxylase 17,20 lyase) the product is dehydroepiandrosterone a major adrenal androgen and the precursor of other adrenal androgens (Orth et al, 1992).

Whether 17-hydroxycorticosteroids are formed by this enzyme system is determined by the relative electron supply from the P-450 reductase system. This system donates electrons to P-$450_{c17}$ in competition with P-$450_{c21}$ and the relative excess or deficit of donated electrons can determine the degree of P-$450_{c17}$ activity (Miller, 1988). In the zona glomerulosa there is no P-$450_{c17}$ activity.

The next step in the sequence is the conversion of 17α-hydroxyprogesterone to 11-deoxycortisol by the enzyme P-$450_{c21}$. This enzyme resides in the smooth endoplasmic reticulum of the cell and is encoded by the gene CYP21A which lies on chromosome 6 in the midst of the Major Histocompatibility Complex region. It lies in tandem with ia highly homologous pseudogene, CYP21P, which has no known function and can participate in gene conversion events to cause the clinical syndromes of steroid 21-hydroxylase deficiency (White et al., 1984, White et al., 1984).

Once formed, 11-deoxycortisol is transported to the inner mitochondrial membrane where the final hydroxylation step to form cortisol occurs. This step is catalysed by the enzyme P-$450_{11β}$ whose gene, CYP11B1, is found on the long arm of chromosome 8 (8q22) (Mornet et al., 1989). In addition to the formation of cortisol, P-$450_{11β}$ is also thought to catalyse the formation of corticosterone, 18-hydroxy-11-deoxycorticosterone and 19-hydroxy-11-deoxycorticosterone from 11-deoxycorticosterone in the zona fasciculata, underscoring the close relationship

between this enzyme and P-$450_{aldo}$ in their catalytic properties (Shizuta et al., 1992).

The gene for P-$450_{11\beta}$ and its closely related homologue, CYP11B2 encoding P-$450_{aldo}$, are of central importance in the biosynthesis of aldosterone and in the pathogenesis of hypertensive disorders of adrenal metabolism and are discussed in detail in section 1.7.

### 1.5.3 Regulation of Steroid Secretion

The zonae fasciculata and reticularis are the site of synthesis of cortisol and the adrenal androgens in man. The control of the rate of synthesis of cortisol and the other secretory products of these zones is determined by factors outwith the adrenal gland, namely interactions between the hypothalamus, the anterior pituitary, neural and other influences such as stress. Figure 1.5 g outlines the normal regulation of cortisol secretion in man.

### 1.5.4 Adrenocorticotrophin

The principal secretory stimulus to the cortisol-producing cells of the adrenal cortex is ACTH. ACTH is synthesised as a part of a large precursor molecule (241 amino acids), pro-opiomelanocorticotropin (POMC), which also contains other peptides, including melanocyte-stimulating hormone. The gene for POMC is situated on chromosome 2 (Owerbach et al., 1982, Whitfield et al., 1982). The gene product of the POMC gene undergoes extensive post-translational processing, including cleavage to produce multiple peptides. In the anterior pituitary the products of this enzymatic cleavage are ACTH, ß-lipotrophin and the joining (J) peptide. ACTH is a 39 amino acid peptide, the first 24 of which are conserved in all species studied, and the first 18 $NH_2$-terminal amino acids of this peptide confer its biological activity (Smith and Funder, 1988) (Figure 1.5 h).

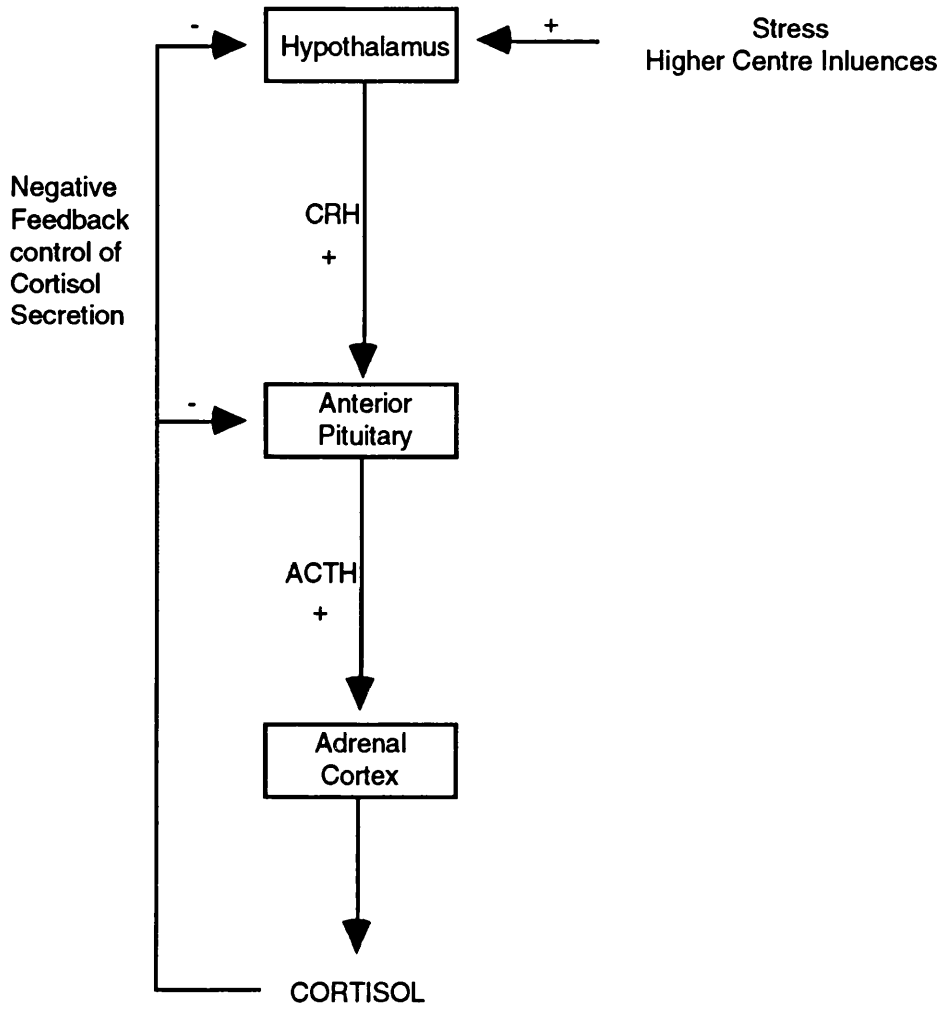



**Figure 1.5 g Control of Cortisol secretion in man**

The ACTH peptide also contains the precursor molecule for one of the melanocyte-stimulating hormones (α-MSH) whose effect can be seen when ACTH is present in gross excess in Nelson's syndrome, i.e. skin pigmentation secondary to chronically elevated plasma ACTH levels from an ACTH-secreting pituitary adenoma

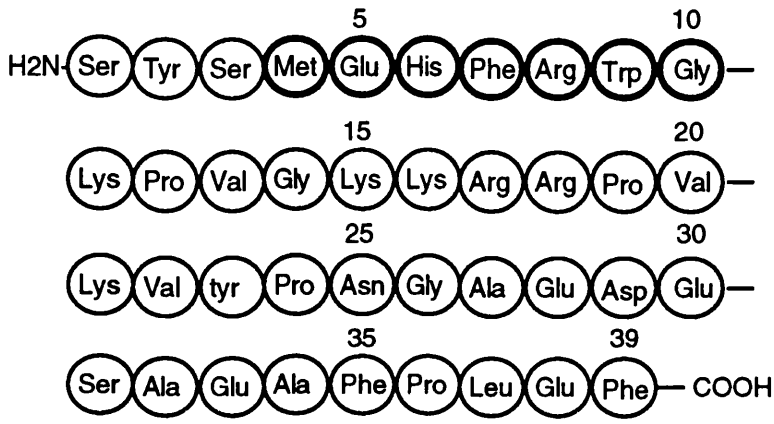



**Figure 1.5 h Amino acid sequence of human ACTH. Bold residues indicate those common to ACTH, alpha and beta MSH**

ACTH release from the pituitary gland is pulsatile, i.e. brief episodic bursts of ACTH release occur throughout the 24 hour period. In the early part of the day, the release of ACTH is greater due to pulses of greater amplitude (Veldhuis et al., 1990) . The increase in amplitude of the pulses occurs after 3-5 hours of sleep and reaches a maximum prior to waking and for 1 hour after waking, before declining during the day (Veldhuis et al, 1990). Plasma cortisol levels mirror these changes in ACTH secretion but the longer clearance time for cortisol results in a slower decline in plasma cortisol relative to plasma ACTH (Krieger et al., 1971). This so-called 'nyctothemeral' or 'diurnal' rhythm is maintained in the physiological state but is lost in disease states where cortisol or ACTH secretion becomes autonomous, e.g. Cushing's syndrome (ACTH-secreting pituitary adenoma) or a cortisol-secreting adrenal adenoma/carcinoma.

Under normal circumstances ACTH and cortisol regulate their own secretion by means of a negative feedback loop whereby high levels of cortisol inhibit the secretion of ACTH and corticotrophin releasing hormone (which regulates ACTH secretion). However, ACTH secretion and consequently plasma cortisol levels can be influenced by inputs from 'higher' centres, i.e. hypoglycaemia, fever and mental stress all raise ACTH and cortisol levels and are important sources of variation in day-to-day measurement of plasma levels.

The primary action of ACTH on the cells of the zona fasciculata is to increase cortisol secretion by increasing its synthesis; intra-adrenal storage of cortisol is minimal (Dickerman et al., 1984, Hall, 1985). ACTH depletes adrenal cholesterol content to an extent which corresponds with increased steroid synthesis (Long, 1985, Péron and Koritz, 1960). ACTH acts by binding to a high-affinity cell-surface

receptor , and each adrenocortical cell is said to possess approximately 3600 ACTH-binding sites (Orth et al, 1992). ACTH binding promotes the generation of cyclic AMP (cAMP) via adenylate cyclase, which in turn activates protein kinase A and leads to the phosphorylation of a number of proteins and increase in nuclear mRNA production (Simpson and Waterman, 1988).

The effects of ACTH on steroidogenesis can be divided into acute (within minutes) and chronic (after several hours) effects. The acute effect of ACTH is to increase conversion of cholesterol to pregnenolone, the rate-limiting step of cortisol synthesis, by activation of $P450_{SCC}$ (Simpson and Waterman, 1988). Using polyclonal antibodies directed against the various steroidogenic enzymes it has been demonstrated that ACTH treatment of bovine adrenal cortex cells results in a 4-5-fold increase in the rate of synthesis of $P\text{-}450_{SCC}$, $P\text{-}450_{11\beta}$, $P\text{-}450_{C21}$, $P\text{-}450_{17\alpha}$ and adrenodoxin some 24-36 hours after ACTH treatment. ACTH and cAMP treatment lead to similar increases in the levels of translatable mRNA of all of these enzymes (Simpson and Waterman, 1988), thought to be due to an increase in transcription of the corresponding genes. These increases in mRNA synthesis can be detected as little as 4 hours after ACTH treatment. Similar results have been found when human fetal adrenal cells were studied (John et al., 1986).

The chronic effects of ACTH involve increased synthesis of most of the enzymes of the steroidogenic pathway as well as actions on cell growth (Hall, 1985, Simpson and Waterman, 1988). When there is prolonged deficiency of ACTH, such as following hypophysectomy, the level of steroidogenic enzymes and RNA synthesis fall in association with atrophy of the adrenal cortex. These changes are reversed by ACTH administration but may require several days to return to normal (Orth et al, 1992). Using nuclear run-on assays, it has been demonstrated that ACTH administration results in increased transcription of the various steroidogenic enzyme genes, and that the increased mRNA synthesis leads to chronically enhanced enzyme production (John et al, 1986). Also, the addition of cycloheximide, an inhibitor of RNA translation, to ACTH-stimulated bovine adrenocortical cells inhibits production of steroidogenic enzyme RNA species, but total mRNA levels remain normal. Therefore, it would appear that ACTH acting via cAMP generation, generates short-lived protein factor(s) which may interact directly with the steroidogenic genes to influence their expression. The factors responsible for this direct effect on gene expression have not been fully identified but are discussed more fully in 1.7.

### 1.5.5 Angiotensin II and Potassium

The zona glomerulosa of the adrenal cortex is the sole site of aldosterone synthesis and secretion in man. Recently however, aldosterone synthase gene expression has been detected in vascular endothelium and vascular smooth muscle cells in association with mineralocorticoid receptor gene expression by RT-PCR (Hatakeyama et al., 1994). In addition, local extra-adrenal aldosterone synthesis was demonstrated. Although the degree of aldosterone synthase gene expression was low (1/50 th that of the adrenal) this suggests that vascular aldosterone synthesis may be important in the control of vascular development.

Three major stimulants of aldosterone secretion have been identified in man, angiotensin II (Ang II), plasma [$K^+$] and ACTH. Ang II is an octapeptide hormone generated by the renin-angiotensin system. This system consists of one precursor protein, angiotensinogen produced by the liver, and two enzymes, renin first described in the juxtaglomerular cells of the kidney, and angiotensin-I converting enzyme (ACE), widely distributed but found in large amounts in the pulmonary vascular endothelium. The components of this system are linked in the 'classical' renin-angiotensin system (RAS) (Figure 1.5 i).

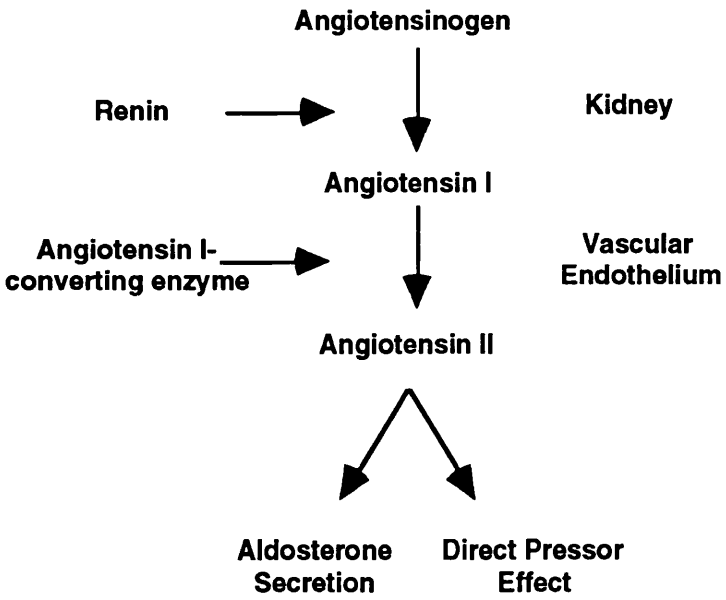



**Figure 1.5 i Classical renin-angiotensin system**

Renin (EC 3.4.23.15) is an aspartyl protease secreted by the juxtaglomerular cells of the kidney. It has only one substrate *in vivo*, angiotensinogen, and cleaves this protein to release angiotensin I (Ang I). Ang I is the converted into the octapeptide Ang II in the pulmonary circulation by ACE. Thus formed, Ang II has two major effects, firstly to stimulate aldosterone secretion by the adrenal cortex and secondly to produce a direct pressor response through increased resistance vessel tone (Swales, 1994). This classical pathway is important in acute cardiovascular homeostasis, i.e. the response to head-up tilt or blood loss (Dzau and Pratt, 1986) and in the regulation of aldosterone secretion in response to salt depletion (Zoccali et al, 1983).

In addition to the classical pathway, there is evidence that a paracrine renin-angiotensin system exists where all the components of the RAS are found or generated. Tissues such as vascular endothelial and smooth muscle cells, the brain and the adrenal cortex have all been shown to possess the elements necessary for *in situ* generation of Ang II (Ehlers and Riordan, 1990, Gould et al., 1964, Racz et al., 1992, Samani, 1994) (Table 1.5 a).

| Tissue | Renin | Angiotensinogen |
|---|---|---|
| Adrenal | +++ | + |
| Kidney | +++++ | ++ |
| Liver | +(+) | +++++ |
| Brain | +++ | +++ |
| Aorta | + | + |
| Heart | + | ++ |
| Testis | ++ | - |

**Table 1.5 a Distribution of renin and angiotensinogen mRNA in tissues (Samani, 1994)**

Ang II is thought to produce its effect on aldosterone secretion via the type I angiotensin II receptor (ATI). On binding with the ATI receptor which is coupled to a G-protein, a number of post-receptor events occur. Phospholipase C activation occurs which leads to the hydrolysis of phosphatidyl inositol biphosphate , producing inositol triphosphate (IP3) and diacylglycerol (DAG) which leads to a rapid rise in intracellular calcium and activation of aldosterone synthesis (Farese et al., 1984). In tandem with this there is a decrease in membrane potassium permeability and resultant rapid depolarisation of the cell (Shepherd, 1989). Voltage-dependent calcium channels open and there is entry of extracellular calcium, a process which can be blocked by the dihydropyridine class of calcium

channel blocking drugs (Kojima et al., 1985a). The rise in intracellular [$Ca^{2+}$] results in opening of calcium-dependent potassium channels and repolarization of the membrane. This rise in intracellular [$Ca^{2+}$] correlates well with aldosterone production (Braley et al., 1986). In addition, DAG activates protein kinase C which then acts on cellular proteins to alter gene expression, principally at the side-chain cleavage step (Aguilera and Catt, 1978) and the aldosterone synthase step (Curnow et al., 1991).

In man, studies have defined the sensitivity threshold to intravenous Ang II infusion (Oelkers et al, 1974). The threshold varies from 0.3 to 1.0 ng AngII/kg body weight $min^{-1}$ in individuals consuming 100-200mEq of sodium. A low sodium diet increases this sensitivity and the magnitude of the response to Ang II rises by up to threefold. The threshold sensitivity is often reduced to 0.3 AngII/kg body weight $min^{-1}$, an infusion rate that produces no measureable change in circulating Ang II levels (Hollenberg et al, 1974). Potassium loading also increases the maximum aldosterone response to Ang II infusion, but by a factor of 1/3 of those caused by changes in sodium restriction (Hollenberg et al., 1975). With prolonged Ang II infusion, aldosterone secretion decreases, perhaps secondary to whole body potassium depletion (Ames et al., 1965).

Potassium stimulates aldosterone secretion which in turn facilitate renal potassium excretion thereby forming a means of regulating body potassium balance. In man, a 25% increase in aldosterone secretion can be detected after infusion of 10 mEq of potassium, an amount which does not increase plasma [$K^{+}$] (Hollenberg et al, 1975). Changes in dietary potassium intake for as little as 24 hours can substantially modify the adrenal response to acute potassium administration: high dietary potassium intake enhances responsiveness, while low potassium reduces it (Dluhy et al., 1972). No such changes have been observed when dietary sodium intake is varied (Dluhy et al, 1972).

It is thought that potassium acts at the plasma membrane by modifying the membrane potential and that the zona glomerulosa cell is peculiarly sensitive to potassium-induced membrane depolarisation. Increasing potassium concentration leads to depolarisation of the cell membrane and activation of voltage-dependent calcium channels in the plasma membrane as the initial step (Kojima et al., 1985b). Calcium influx leads to a sustained rise in cytosolic [$Ca^{2+}$], present for the duration of the stimulus, which correlates with aldosterone production (Braley et al, 1986).

ACTH may also increase aldosterone secretion but this is only temporary and after 24 hours of continuous ACTH administration, the plasma aldosterone falls to

normal or sub-normal levels in normal individuals (Connell et al., 1987). Some of this effect would appear to be due to the rapid sodium retention, and thus suppression of Ang II formation, caused by the ACTH-mediated elevation in plasma concentrations of intermediates such as 11-deoxycorticosterone, corticosterone and cortisol itself (Connell et al, 1987). However, this is not the whole reason as sodium restricted individuals given ACTH exhibit the same rise and fall in plasma aldosterone despite minimal sodium retention (Connell et al., 1988). The mechanisms whereby ACTH increases aldosterone secretion are thought to be similar to those in the zona fasciculata (Aguilera and Catt, 1978). Of interest is the observation that removal of the pituitary gland does not result in any immediate change in aldosterone secretion (Orth et al, 1992).

It is clear that Ang II and $K^+$ have important effects on aldosterone secretion and that changes in intracellular calcium are central to the action of both stimuli. Ang II has added effects mediated through the generation of IP3, which alters intracellular [$Ca^{2+}$], and DAG. ACTH has a temporary effect on aldosterone secretion in normal man. How these changes might effect the synthesis of aldosterone via alterations in the expression of the steroidogenic enzymes involved in aldosterone secretion is not clear but is discussed further in 1.7.

There are a number of other factors which have been shown to influence physiological control of aldosterone secretion, namely atrial natriuretic peptide, dopamine and serotonin (Ferriss, 1992). These compounds play a minor role in the control of aldosterone secretion in man compared with Ang II, potassium and ACTH.

### 1.5.6 Glucocorticoids versus mineralocorticoids

The adrenal corticosteroids which play a major role in the control of blood pressure are divided into two classes, glucocorticoids and mineralocorticoids, on the basis of their major physiological properties.

Mineralocorticoids stimulate unidirectional transepithelial sodium transport mainly in exchange for potassium (Crabbe, 1961). The principal human physiological mineralocorticoid is aldosterone, although other steroids, in particular DOC have mineralocorticoid potency. Mineralocorticoids promote the active reabsorption of sodium in exchange for potassium in the kidney mediated via the action of $Na^+K^+$ ATPase. Mineralocorticoids also mediate hydrogen ion secretion in the kidney by an apparently independent mechanism involving an ATP-dependent hydrogen ion pump.

The classification of glucocorticoids was based on the principal physiological effect of steroids on intermediary carbohydrate metabolism. This definition is perhaps not ideal as it really only refers to one of the more easily observed effects of cortisol despite the fact that glucocorticoids have potent effects on lipid metabolism, the modulation of immune responses and inflammatory processes, tissue development, also sodium: hydrogen exchange and glomerular filtration rate.

Another perhaps more appropriate way to classify corticosteroids is to look at their binding properties to nuclear receptors. Nuclear steroid receptors form part of a larger nuclear receptor family encompassing steroid, thyroid, retinoic acid, vitamin D and other orphan receptors. Two receptors of direct importance to this discussion are the human glucocorticoid receptor (hGR or GR) and the human mineralocorticoid (hMR or MR) receptor.

The cDNA for hGR was first cloned from lymphocytes (Hollenberg et al., 1985). Subsequently, the human mineralocorticoid receptor was cloned and sequenced from a kidney cDNA (Arriza et al., 1987). The DNA binding regions of the two receptors was 94% identical and the C-terminal ligand binding domains are 57% identical whilst the N-terminal region of the receptors is < 15% homologous (Figure 1.5. j).

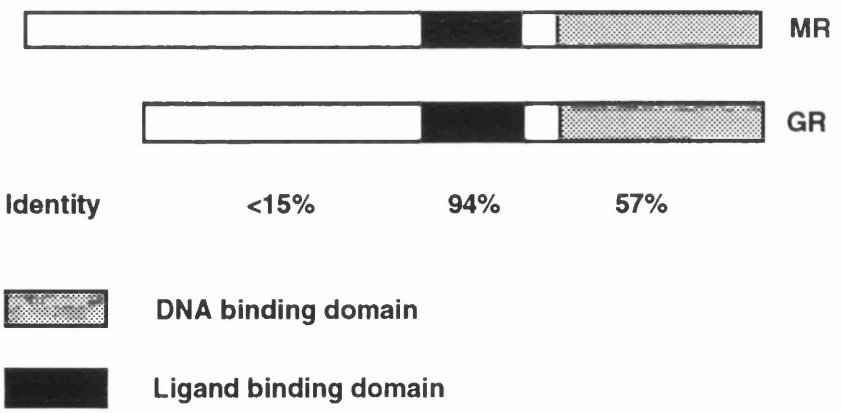



**Figure 1.5 j Schematic representation of human mineralocorticoid (MR) and glucocorticoid (GR) receptors**

Binding of steroids to steroid hormone receptors leads to the activation of *cis*-acting elements located in or near hormone responsive genes (Beato, 1989). On

binding steroids the receptor and its associated molecules ('chaperonins' (Bodine and Litwack, 1988)) dissociate and the steroid-receptor complex can travel to the cell nucleus. Dissociated from the satellite proteins, the steroid-receptor complex can then bind as a dimer to a specific region of DNA termed the hormone response element (HRE). HRE are present in many genes and fall into three groups, the glucocorticoid/progesterone-responsive element (GRE/PRE) which also serves as the mineralocorticoid-responsive element, the estrogen responsive element (ERE), and the thyroid responsive element (TRE) (Table 1.5 b). Binding to these sites is through the highly conserved 'zinc finger' region of the receptor which, if altered, leads to depression of DNA binding (Freedman, 1992). Thus bound, the steroid-receptor complex leads to transcriptional activation of the bound gene by switching on enhancer regions of the gene (Carson-Jurica et al., 1990), a process which may be modified by other factors, e.g. insulin and binding of activated glucocorticoid receptor to DNA in the liver (Tsawdaroglou et al., 1989). It is through this series of events that steroid hormones exert their effects in man. In the case of MR, the exact mechanism is not known but it is likely to be similar to that of GR (Carson-Jurica et al, 1990).

| Hormone-responsive element | Consensus sequences |
|---|---|
| GRE - MRE - ARE | 5'-AGGACANNNTGTACC-3' |
| ERE | 5'-NGGTCANNNTGACCN-3' |
| VDRE | 5'-TTGGTGACTCACCGGGTGAAC-3' |
| TRE | 5'-GGGTCATGACAG-3' |
| RARE | 5'-AGGACATGACCT-3' |

GRE - glucocorticoid-responsive element, MRE - mineralocorticoid-responsive element, ARE - androgen-responsive element, ERE - estrogen-responsive element, VDRE - vitamin D-responsive element, TRE - thyroid-responsive element, RARE - retinoic acid-responsive element. N - any nucleotide.

**Table 1.5 b Consensus sequences of gene hormone-responsive elements**

Studies of the *in vitro* receptor affinity of GR and MR have revealed that the affinity of GR for steroids mirrors their *in vivo* therapeutic potency whilst MR has equal affinity for aldosterone, cortisol and corticosterone in both adrenal tissue and recombinant MR studies (Arriza et al, 1987, Krozowski and Funder, 1983) This does not mirror the *in vivo* observations of the effects of aldosterone and cortisol in classic mineralocorticoid target tissues (Sheppard and Funder, 1987). In normal subjects cortisol concentrations are 100-1000 times that of aldosterone and would thus be expected to exert important mineralocorticoid effects well in excess of aldosterone at normal plasma concentrations which is clearly not the case *in vivo*.

It has been shown that cortisol undergoes dehydrogenation to form cortisone, an inactive metabolite in man. The enzyme which performs this reaction, 11ß-hydroxysteroid dehydrogenase (11ß-OHSD), also catalyses the opposite 11-oxoreductase step in man (Figure 1.5. k) (Edwards et al., 1988).

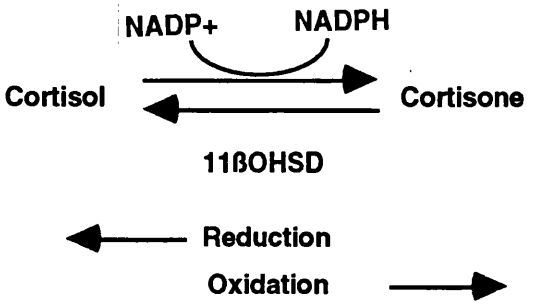


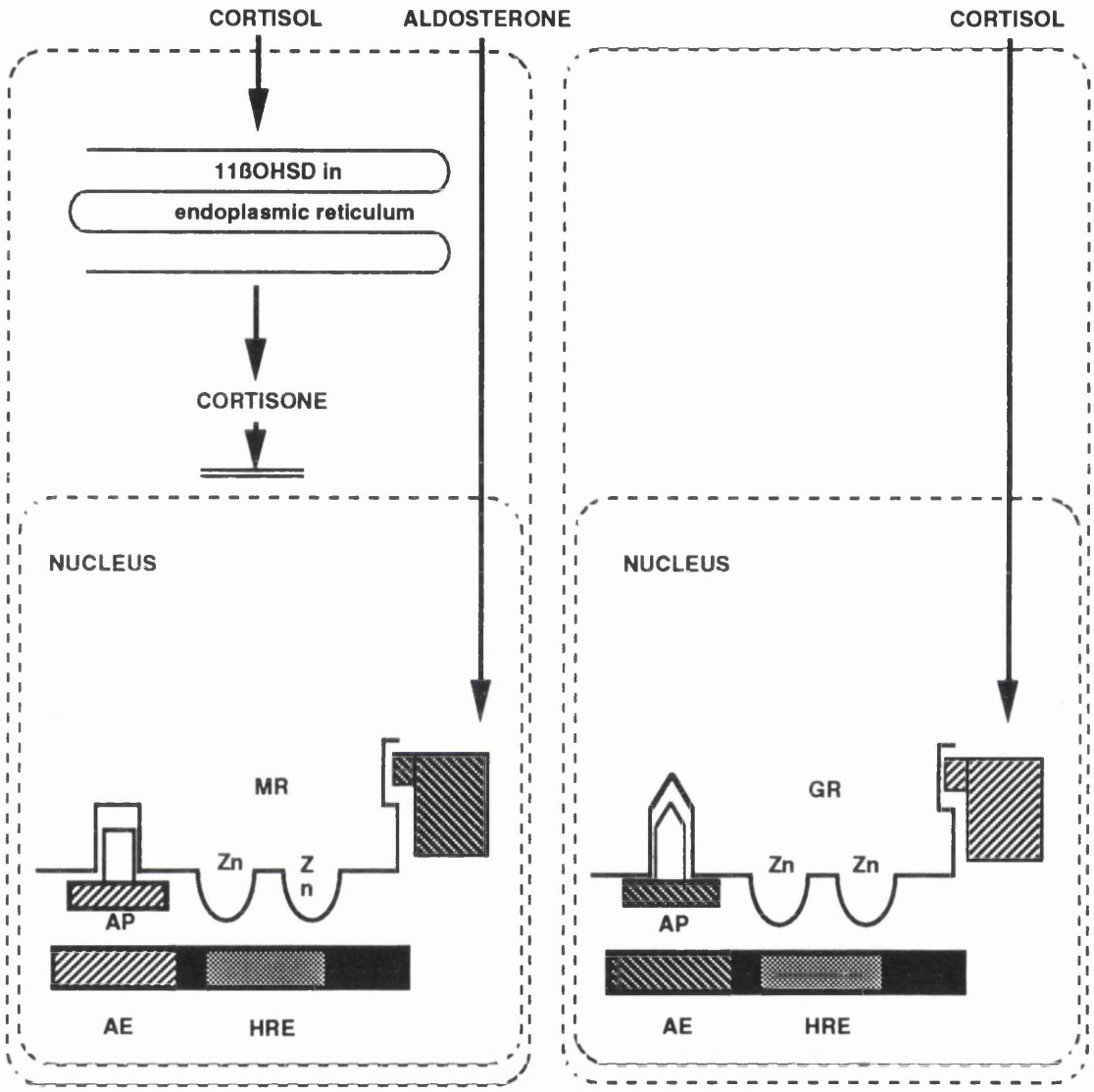

**Figure 1.5 k 11ß-hydroxysteroid dehydrogenase (11ßOHSD) activity**

A single gene on chromosome 1 encodes an 11ß-OHSD enzyme (Tannin et al., 1991) and which is expressed in mineralocorticoid target tissues such as the kidney and salivary glands (Edwards et al, 1988, Tannin et al, 1991).

It was hypothesised that 11ß-OHSD protects MR from the effects of cortisol in target tissues by converting it to cortisone, which does not bind to MR (Oberfield et al., 1983) and thus allows only aldosterone to exert a physiological effect at the MR (Oberfield et al, 1983, Stewart et al., 1987). Studies of glycyrrhetinic acid, the active derivative of liquorice, have shown that liquorice-induced hypertension occurs only in the presence of cortisol or ACTH (Hoefnagels and Kloppenborg, 1983), thus ruling out the possibility of a direct effect of glycyrrhetinic acid on the MR in the kidney producing hypertension. Therefore, it would appear that if 11ßOHSD was inactivated, binding of cortisol, or corticosterone in the rat, would occur and induce mineralocorticoid effects. The administration of inhibitors of 11ßOHSD (liquorice and carbenoxolone ) to rats (Edwards et al, 1988, Funder et al., 1988) given [$^3$H]-corticosterone was shown to result in binding of both [$^3$H]-corticosterone and [$^3$H]-aldosterone to MR target tissues confirming that 11ßOHSD confers protection on the MR from cortisol in man or corticosterone in the rat.

Figure 1.5 l outlines the mechanism whereby aldosterone and cortisol interact with their respective receptors and the role of 11ß-OHSD in modulating the effect of cortisol in aldosterone target tissues.

Mineralocorticoid effects are therefore caused by binding of a corticosteroid to the MR and the nature of the steroid itself is may not be of principal importance. The




AE - Accessory element, AP - Accessory Proteins, e.g. cFos, cJun

HRE - Hormone-response element

GR - Glucocorticoid Receptor, MR - Mineralocorticoid Receptor

Zn - Zinc finger DNA-binding region of receptor

11ß-OHSD - 11ß-hydroxysteroid dehydrogenase enzyme

**Figure1.5 I Mechanism of enzyme-mediated protection of mineralocorticoid receptor**

effects of mineralocorticoids on target tissues are discussed in 1.6 whilst the effects of abnormalities in 11ßOHSD activity are reviewed in 1.8.4.

## 1.6 Aldosterone and its Effects in Man

The principal physiological effect of aldosterone is to regulate electrolyte transport across epithelial surfaces. The principal physiological target organ for aldosterone is the kidney, but the salivary gland and the colon also demonstrate aldosterone-sensitive regulation of electrolyte transport (Orth et al, 1992). This occurs through binding to high-affinity receptors, MR, as described in 1.5.6. In addition, MR is present in liver, hippocampus, pituitary, and peripheral blood leucocytes (Orth et al, 1992). The specificity of the human MR for cortisol and aldosterone is identical and it is the enzyme 11ßOHSD which confers enzyme-mediated receptor protection for the MR in situations where cortisol is present in a large excess over aldosterone.

Although the classical MR is a member of the nuclear receptor superfamily, recent work has suggested that membrane receptors for aldosterone exist and may mediate rapid non-genomic effects in human mononuclear leucocytes .

### 1.6.1 Aldosterone and the renal tubule

Receptors in the kidney bind aldosterone and effect a net increase in sodium resorption at the expense of potassium and hydrogen ions (Figure 1.6 a). The major sites of aldosterone-induced sodium and potassium transport are the cortical collecting tubules, and the distal convoluted tubule and it is the luminal cells which appear to mediate sodium:potassium transport. The basolateral membrane of these cells is lined by a $Mg^{2+}$-dependent $Na^{+}K^{+}$-activated ATPase. This ubiquitous transmembrane protein maintains the sodium and potassium electrochemical gradient across the cell membrane. During chronic aldosterone administration, these cells undergo characteristic ultrastructural changes with a marked increase in basolateral membrane surface area, and a high degree of expression of the genes for the $\alpha$- and ß- subunits of the $Na^{+}K^{+}$-ATPase (Komesaroff et al., 1994). Aldosterone acts to promote sodium retention at the expense of potassium by this and other mechanisms (See 1.6.3). Approximately 95% of all filtered sodium is reabsorbed prior to arriving at the site of aldosterone-sensitive sodium reabsorption by the loop of Henlé. However, the remaining 5% still constitutes some 80 grams of sodium daily, 10-20 times the normal amount ingested daily, and regulation of the reabsorption is clearly important in regulating blood pressure.

Aldosterone also regulates hydrogen ion excretion by the kidney in the distal nephron. The mechanisms are different, however, from those of sodium and potassium. Aldosterone-mediated hydrogen ion secretion occurs in the intercalated

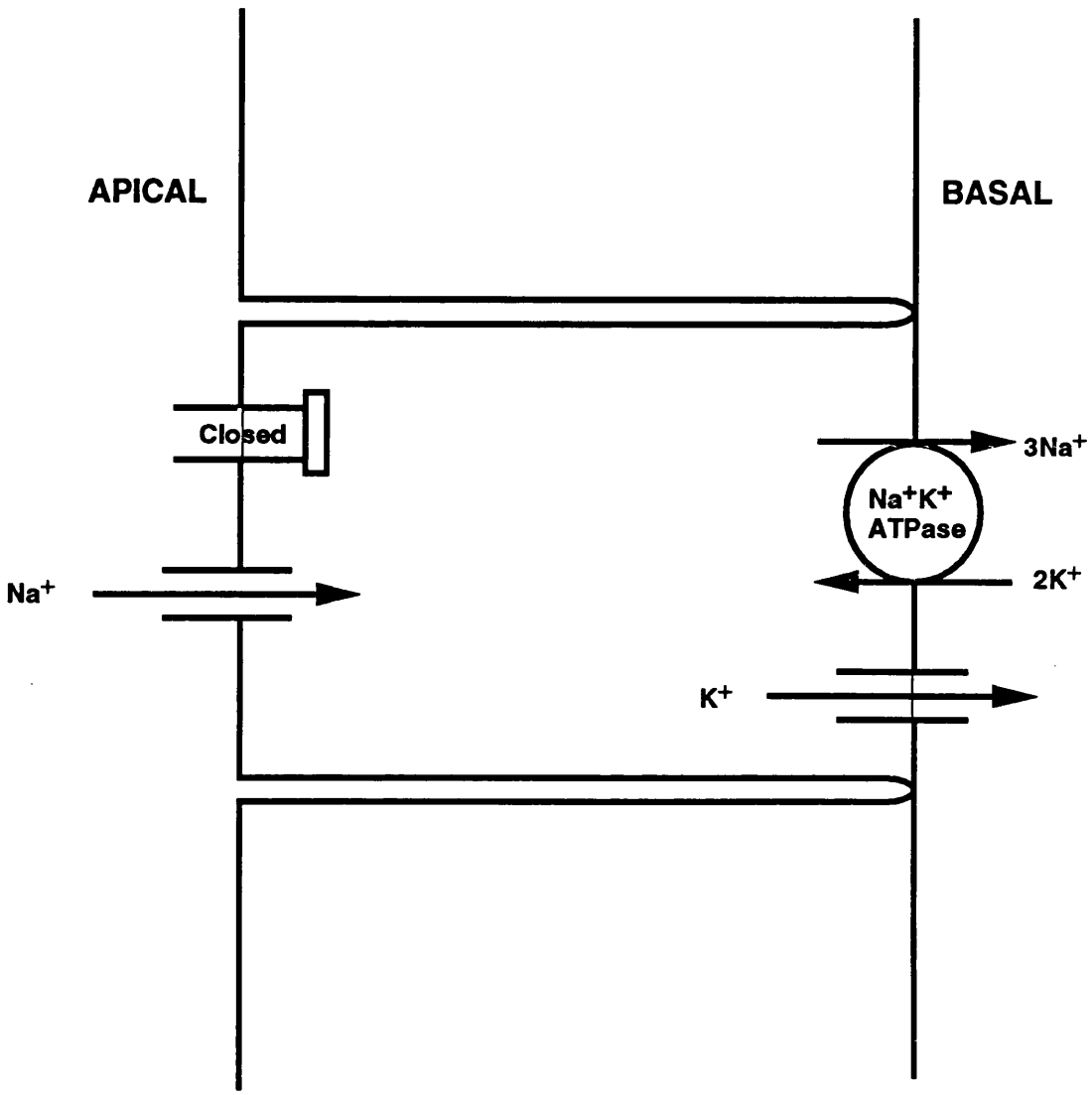



**Figure 1.6 a Sodium transport across the renal tubular epithelium**

cells of the collecting tubule. These cells along with the principal cells of the tubule arise from a common precursor, the ß-intercalated cell (Komesaroff et al, 1994).

Hydrogen ion secretion is through a sodium-insensitive route; given that this segment of the nephron exhibits little or no aldosterone-induced sodium transport, aldosterone-induced natriuresis and hydrogen ion secretion appear to be independent events. This effect appears to be mediated via an effect of aldosterone on the activity of the ATP-dependent apical hydrogen ion pump and parallel regulation of the basolateral membrane $Cl^-/HCO_3^-$ exchanger (Hays, 1992).

The net effect of aldosterone on the renal tubule is therefore to promote sodium retention at the expense of potassium and also to promote hydrogen ion excretion by the kidney. This explains the clinical features observed in cases of primary aldosterone excess, i.e. plasma hypokalaemia, alkalosis, a raised exchangeable sodium content and low total body potassium (Ferriss et al., 1983, Kremer et al., 1977).

### 1.6.2 Aldosterone and the heart

Although the classical effects of aldosterone are those related to the control of electrolyte balances across epithelia, a number of non-epithelial actions of aldosterone on blood pressure, ß-adrenoceptor density and salt appetite have been noted (Funder, 1992). Another important non-epithelial effect of aldosterone reported is the promotion of perivascular and interstitial cardiac fibrosis when aldosterone levels are inappropriately high in relation to sodium status. That is, in sodium-loaded rats with aldosterone excess due to infusion there is a marked accumulation of fibrillar type I collagen around the intracoronary arteries and extending into the neighbouring interstitial spaces (Weber et al., 1992). In these models, hypertension is present and left ventricular hypertrophy develops. However, fibrosis can develop in the absence of hypertension or hypertrophy in animals with aldosterone excess (Weber et al, 1992), and pre-treatment of animals with the aldosterone antagonist spironolactone, inhibits the accumulation of cardiac collagen (Brilla et al., 1993b). Comparable studies using infrarenal aortic banding to produce aldosterone-independent hypertension and ventricular hypertrophy to a similar degree have shown that in these models there is no appreciable increase in myocardial collagen (Weber et al, 1992).

Thus it appears that in animal models of aldosterone-dependent hypertension myocardial fibrosis may result from non-epithelial actions of aldosterone. Nuclear MR are present in all four chambers of the heart but are not protected by the

presence of 11ßOHSD and are thus in effect high-affinity glucocorticoid receptors given the disparity in plasma concentrations of aldosterone and cortisol (Funder et al, 1988). This raises the possibility that aldosterone may be acting to produce myocardial fibrosis via mechanisms other than the classic nuclear receptor despite the fact that spironolactone can inhibit the fibrotic reaction,. The description of high-affinity aldosterone receptors on leucocyte membranes with low affinity for spironolactone or cortisol may provide an alternative mechanism (Wehling et al., 1992). Cardiac fibroblasts can produce collagen *in vitro* in response to low doses of aldosterone which is not inhibited by spironolactone, an observation which supports a role for the membrane bound aldosterone receptor (Funder, 1992).

There are few data about such a role in man but one post-mortem study of 5 patients with aldosterone-secreting adenomata revealed the presence of perivascular fibrosis of the intramural cardiac and systemic arteries in a similar distribution to that seen in rats with mineralocorticoid excess (Campbell et al., 1992). Thus non-classical effects of aldosterone may be important in modulating blood pressure by altering vascular compliance or influencing the progression of end-organ damage such as left ventricular hypertrophy by interaction with membrane bound or classical MR.

### 1.6.3 Mechanism of action of aldosterone

The mechanism of action of aldosterone is not fully understood at present. Obviously its actions may differ depending on which receptor is involved, i.e. nuclear or membrane. In tissues such as the mononuclear leucocyte, the effects are thought to be non-genomic and mediated through interaction of the membrane bound aldosterone-receptor complex with a second messenger system which increases intracellular levels of IP3 and results in activation of $Na^+/H^+$ antiporters (Wehling et al, 1992).

The slower, genomic effects are produced by a series of steps following binding of aldosterone to its receptor and take a number hours to days to reach a maximum and involves the synthesis of mRNA and proteins . When MR binds aldosterone it migrates to the cell nucleus where it binds to an HRE thought to be similar to that of the human GR (Beato, 1989). Following this transcriptional activation occurs and during this latent period DNA transcription and protein translation occurs. The resultant gene product(s), termed 'Aldosterone-induced protein (AIP), is then thought to promote sodium reabsorption and excretion of potassium and hydrogen ions by one of three mechanisms (Figure 1.6 b) (Komesaroff et al, 1994).

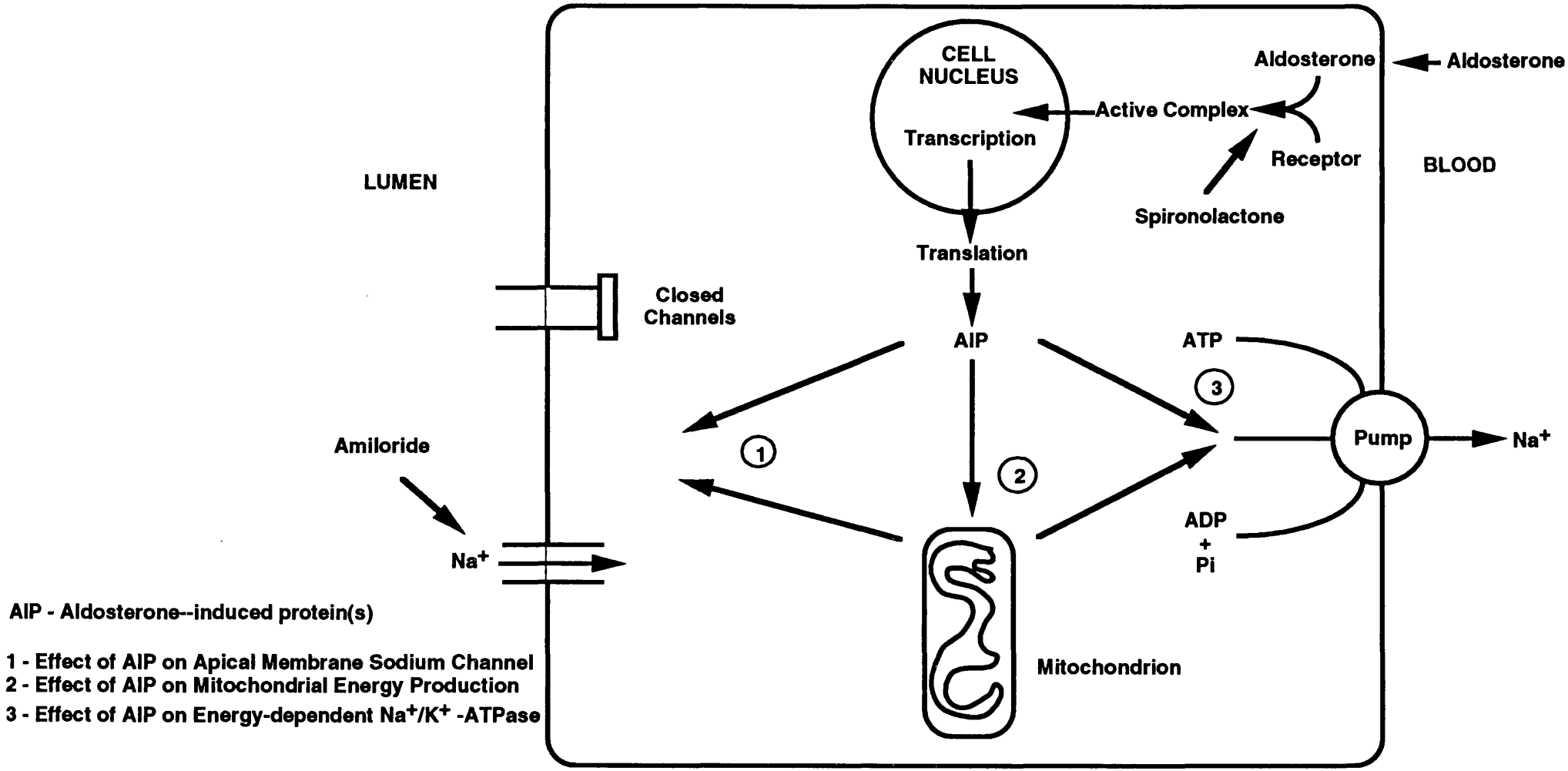



Figure 1.6 b Intracellular effects of aldosterone

Sodium enters the tubular cell via amiloride-sensitive sodium channels in the apico-luminal membranes of the cell by passive diffusion down a concentration gradient; its exit from the cell across the basolateral membrane in exchange for potassium is mediated by the energy dependent $Na^+K^+$-ATPase. AIP may have effects on the apical membrane, cellular energy production, and/or the sodium pump. It would appear that the initial response is an increase in apical sodium conductance and an increase in $Na^+K^+$-ATPase activity subsequent upon the rise in intracellular [$Na^+$] with chronic aldosterone administration leading to an increase in the synthesis of pump proteins (Komesaroff et al, 1994, Schafer and Hawk, 1992). Similarly the synthesis of citrate synthetase and other Krebs' cycle enzymes increases following the acute response (Komesaroff et al, 1994, Schafer and Hawk, 1992).

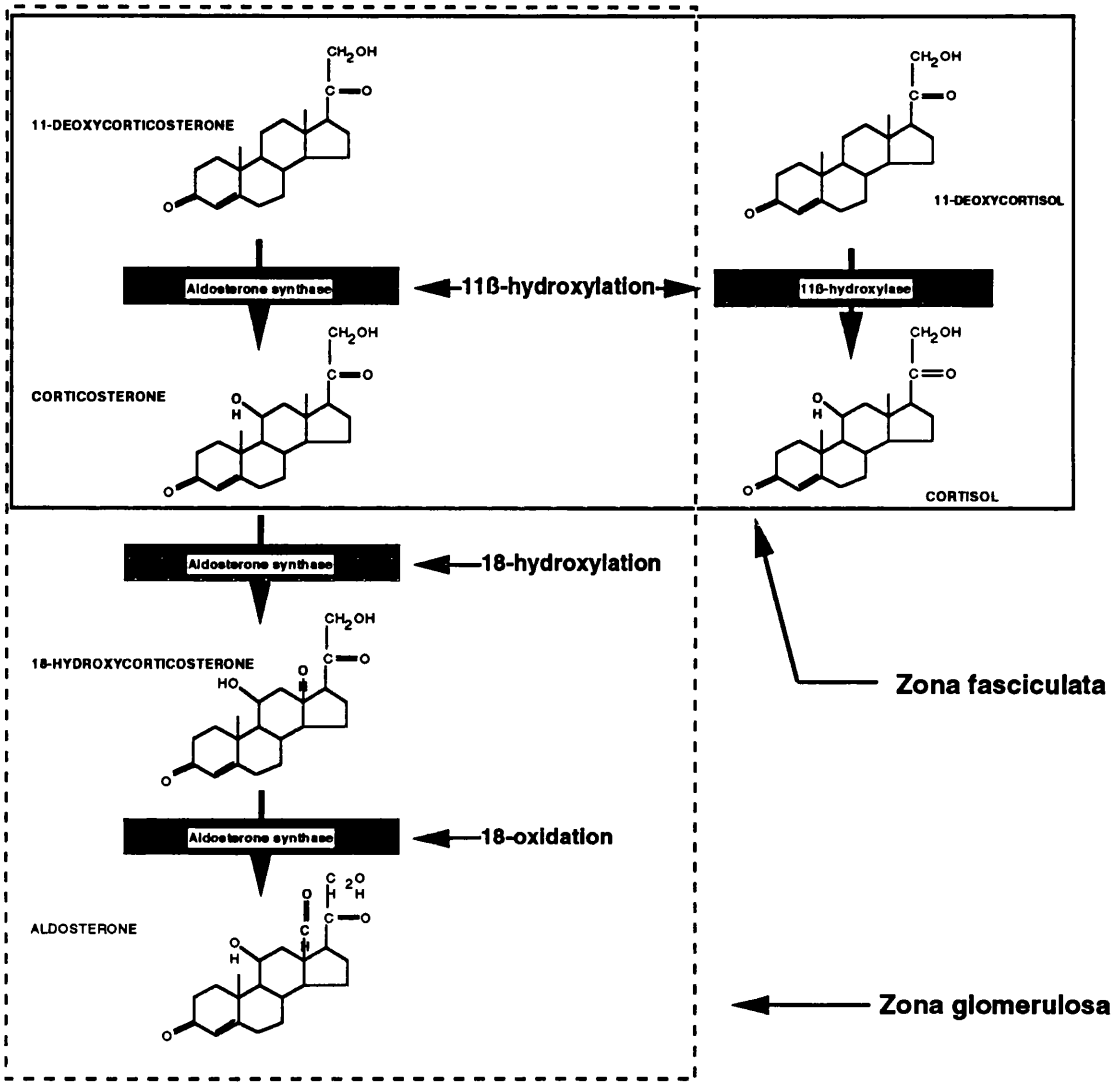
The effect of aldosterone on apical sodium channels appears not to be an increase in synthesis of sodium channels. There is evidence however that aldosterone increases the number of open channels in the membrane by some post translational effect, either by methylation of a channel subunit by enzymes which produce a concomitant rise in *s*-adenosylmethionine levels, or by altering some modulatory subunit of the channel (Komesaroff et al, 1994).

## 1.7 Molecular Biology of Aldosterone Synthesis

Aldosterone is synthesised exclusively in the zona glomerulosa in man and is the principal human mineralocorticoid. Aldosterone is the end-product of a series of reactions starting with the conversion of cholesterol to pregnenolone by P-450$_{SCC}$ in the inner mitochondrial membrane. Pregnenolone is then converted to progesterone by 3ß-hydroxysteroid dehydrogenase/$\Delta^5$ -$\Delta^4$ isomerase and progesterone converted to 11-deoxycorticosterone by P-450$_{c21}$ in the smooth endoplasmic reticulum of the cell. In the zona glomerulosa there is no significant expression of P-450$_{c17}$ (Curnow et al, 1991) and the result is the production of 17-deoxycorticosteroids, i.e. 11-deoxycorticosterone (DOC). In the zona fasciculata not all progesterone undergoes 17α-hydroxylation by P-450$_{c17}$ (White et al., 1994) and as a result both DOC and 11-deoxycortisol are formed. It is at this point that the major difference between zona glomerulosa and zona fasciculata cells becomes apparent. In man, both the zona fasciculata and the zona glomerulosa can convert DOC to corticosterone. Indeed, in man the major quantitative source of corticosterone is the zona fasciculata. However, it is the formation of aldosterone from DOC through corticosterone which is unique to the cells of the zona glomerulosa.

### 1.7.1 11ß-hydroxylation in the adrenal cortex

The steps involved in the conversion of DOC to aldosterone and 11-deoxycortisol to cortisol are often termed the 'late reactions' or 'late pathways' in adrenal steroid synthesis. The conversion of 11-deoxycortisol to cortisol and DOC to corticosterone requires the addition of an 11ß-OH group at the C11 position of both DOC and 11-deoxycortisol (Figure 1.7 a). The 11ß-hydroxylase step is catalysed by the mitochondrial enzyme P-450$_{11ß}$ in man. This enzyme is able to 11-hydroxylate both substrates in man and is encoded by a single gene on chromosome 8, CYP11B1 (Chua et al., 1987, Mornet et al, 1989). Analysis of a panel of human somatic cell hybrids coupled with *in situ* hybridisation to metaphase chromosomes has localised this gene to chromosome 8q22 (Wagner et al, 1991). *In vitro* expression of human P-450$_{11ß}$ cDNA in COS-7 cells has shown that the conversion of DOC to aldosterone by human P-450$_{11ß}$ is negligible although it can form corticosterone and a little 18-hydroxycorticosterone (Table 1.7 a) (Kawamoto et al., 1992). Thus we have good evidence that in man P-450$_{11ß}$ is responsible for the formation of cortisol and corticosterone in the zona fasciculata but is unable to perform the necessary reactions for the conversion of DOC to aldosterone.




**Conversion of 11-deoxycorticosterone to aldosterone is performed by a single enzyme, aldosterone synthase,without the release of intermediates.**

**Conversion of 11-deoxycorticosterone to corticosterone is carried out in both the glomerulosa and the fasciculata zones. However, 11ß-hydroxylase in the fasciculata cannot catalyse the formation of aldosterone.**

**Figure 1.7 a 'Late reactions' of adrenal corticosteroidogenesis**

| Substrate | Product | Hydroxylase Activity | |
|---|---|---|---|
| | | $P\text{-}450_{11\beta}$ | $P\text{-}450_{aldo}$ |
| DOC | Corticosterone | 482 | 438 |
| | 18-OH-Corticosterone | 2.7 | 14 |
| | Aldosterone | <0.02 | 2.0 |
| 11-deoxycortisol | Cortisol | 411 | 393 |
| | 18-OH-cortisol | 1.1 | 7.0 |
| | 18-oxocortisol | <0.02 | 1.8 |
| Corticosterone | 18-OH-Corticosterone | 1.5 | 10.6 |
| | Aldosterone | <0.02 | 0.9 |
| Cortisol | 18-OH-cortisol | 0.7 | 4.0 |
| | 18-oxocortisol | <0.02 | 0.4 |

**Table 1.7 a Hydroxylase activity of P450s expressed in COS-7 cells. Results are expressed as pmol/mg of protein. Adapted from (Kawamoto et al, 1992).**

The bovine adrenal gland may be different from the human gland. Bovine $P\text{-}450_{11\beta}$ can convert corticosterone to aldosterone in addition to the 11ß-hydroxylation of DOC (Yanagibashi et al., 1986, Yanagibashi et al., 1988). Further characterisation of the bovine enzyme suggested that not one but two isozymes existed; both had 11ß-hydroxylase and 18-hydroxylase activity and could convert DOC to aldosterone (Ogishima et al., 1989b) This suggested that two isozymes of bovine $P\text{-}450_{11\beta}$ existed in the adrenal cortex with differing substrate specificities relative to their location within the adrenal cortex. In the rat two distinct forms of $P\text{-}450_{11\beta}$ have been identified with differing catalytic activities. One has a molecular mass of 51 kDa and can convert DOC to corticosterone but has minimal ability to convert DOC to aldosterone. The other has a molecular mass of 49 kDa but is highly active in converting DOC to aldosterone (Ogishima et al., 1989a).

Therefore, there is evidence from studies of rat adrenal corticosteroidogenesis that aldosterone synthesis from DOC can be performed by an enzyme distinct from $P\text{-}450_{11\beta}$. Thus, in the human zona glomerulosa aldosterone synthesis could also be performed by an enzyme similar to but distinct from $P\text{-}450_{11\beta}$.

### 1.7.2 Aldosterone synthase activity in the adrenal cortex

In addition to describing the presence of a gene for $P\text{-}450_{11\beta}$ in man, Mornet described the presence of a second gene, CYP11B2, with a high degree of homology to CYP11B1 in human genomic clones (Mornet et al, 1989). The nucleotide sequence of CYP11B1 and CYP11B2 are 95% identical in coding

regions, and 90% identical in the intronic regions. The predicted amino acid sequence of the gene products of CYP11B1 and CYP11B2 are 93% identical. However, analysis of mRNA from human adrenal glands showed that there was strong hybridisation of a CYP11B1-specific probe to the adrenal mRNA but no detectable hybridisation of a CYP11B2-specific probe, suggesting that the gene CYP11B2 was a pseudogene with no function or was expressed at very low levels in normal adrenals in man. Later, a cDNA clone was isolated from tissue from a patient with idiopathic hyperaldosteronism which was shown to be the transcript of CYP11B2 and to possess the ability to convert DOC to aldosterone (Kawamoto et al., 1990a). This was the first evidence that CYP11B2 may encode a functionally important gene product with aldosterone synthase activity, i.e. $P\text{-}450_{aldo}$. A similar report of $P\text{-}450_{aldo}$ expression in aldosterone-producing adenoma tissue showed that $P\text{-}450_{aldo}$ did indeed catalyse aldosterone synthesis from DOC and the protein product had a molecular mass of 48.5 kDa compared with 50 kDa for $P\text{-}450_{11\beta}$ (Ogishima et al., 1991).

The final confirmation that $P\text{-}450_{aldo}$ was entirely responsible for aldosterone synthesis in the human zona glomerulosa came from Curnow (Curnow et al, 1991). She showed that although Northern blot analysis did not reveal the presence of CYP11B2 in human adrenal tissue, a more sensitive method using a reverse transcriptase-PCR (RT-PCR) assay did reveal CYP11B2 transcripts in normal adrenal glands. The level of transcription in normal human adrenal was low, both compared to CYP11B1 transcripts in normal adrenals and the level of transcription of CYP11B2 seen in aldosterone-secreting tumours. The entire coding sequences of CYP11B1 and CYP11B2 were then amplified from aldosterone-secreting tumour tissue and expressed in COS-1 cells along with pCD-Adx (a plasmid containing the cDNA for adrenodoxin which is inadequately expressed in COS-1 cells (Zuber et al., 1988)). These cells were incubated with [$^{14}$C]DOC or [$^{3}$H]11-deoxycortisol. Cells transfected with CYP11B1 converted 11-deoxycortisol almost entirely to cortisol although a little cortisone was formed by the action of the intrinsic 11ß-hydroxysteroid dehydrogenase activity of the COS-1 cells. DOC was converted to corticosterone and 11-dehydrocorticosterone but no aldosterone was detected. Cells transfected with CYP11B2 produced substantial amounts of corticosterone and 18-hydroxycorticosterone from DOC and in addition, aldosterone was detected. With 11-deoxycortisol as the substrate, CYP11B2 transcripts produced cortisol, cortisone, and a third steroid 18-hydroxycortisol. Unpublished observations from this group (PC White, personal communication) revealed that the conversion of DOC to aldosterone by $P\text{-}450_{aldo}$ is similar to that for the conversion of cholesterol to pregnenolone, i.e. the three stages of the reaction occur without the

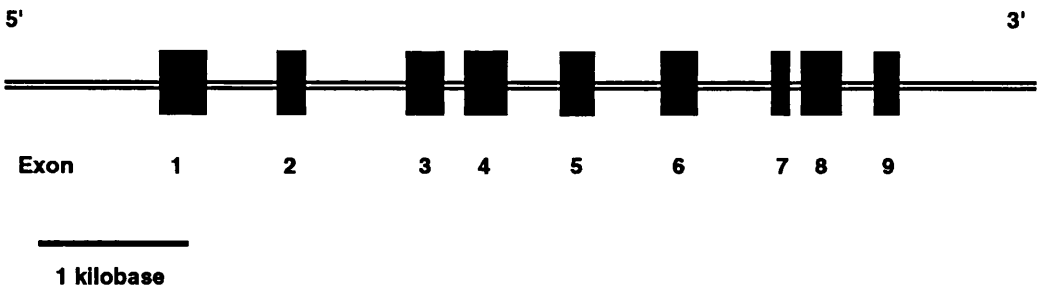
release of intermediate products (Lambeth et al., 1982). White has shown that both corticosterone and 18-hydroxycorticosterone are much less good as substrates for P-450$_{aldo}$ than DOC and that addition of both corticosterone and 18-hydroxycorticosterone does not inhibit aldosterone synthesis from DOC.

### 1.7.3 Molecular genetics of CYP11B1 and CYP11B2

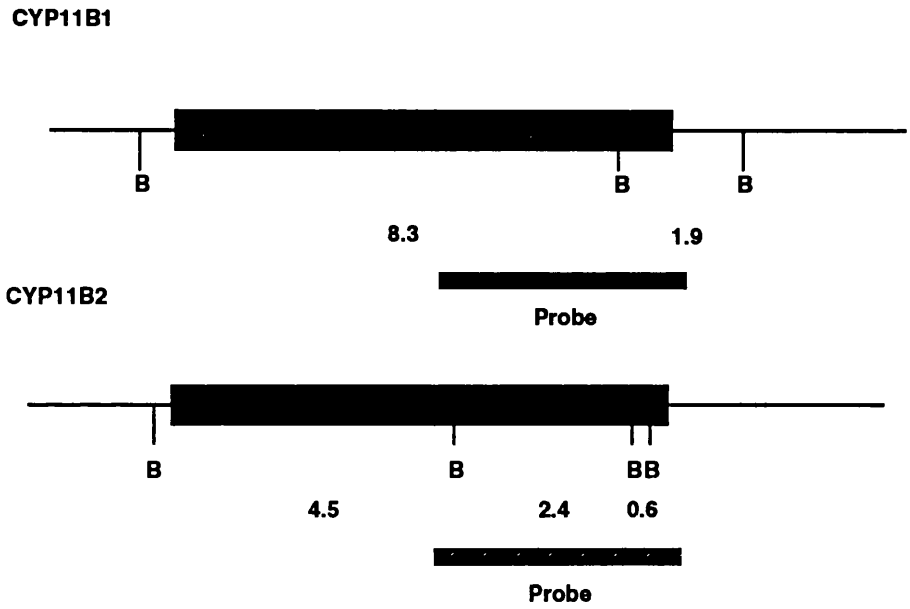
The genes CYP11B1 and CYP11B2 are very similar in many respects. Both lie on chromosome 8q21-22 (Chua et al, 1987, Mornet et al, 1989, Wagner et al, 1991). The organisation of introns and exons in each gene is similar; each gene consists nine exons with the coding regions of each gene spread over approximately 7 kilobases of DNA (Figure 1.7 b). The nucleotide sequence of the exons is 95% identical and that of the introns approximately 90%. The putative proteins encoded by CYP11B1 and B2 each contain 503 amino acids including a 24-residue signal peptide. These sequences are 93% similar to each other and 75% similar to the predicted sequence of bovine P-450$_{11\beta}$ and 36% identical to that of CYP11A. The positions of the introns of CYP11B1 and B2 are identical to those of CYP11A (gene for P-450$_{SCC}$) (Morohashi et al., 1987). For these reasons these genes are grouped into a single family within the cytochrome P-450 gene superfamily (Nelson et al., 1993).

Both CYP11B1 and B2 possess a TATA box variant at position -35 to -29 (GATAAAA) relative to the transcription initiation site. Both possess a palindromic sequence identical to sequences seen in cAMP-responsive genes (TGACGTA), i.e. a cAMP response element (CRE), which binds to cAMP-responsive element binding protein (CREB), downstream from the TATA box. Factors that bind to TATA boxes (Kao et al., 1990) and cAMP response elements (Meyer and Habener, 1993) have been identified. Clinical and biochemical observations of *in vitro* responses to ACTH suggest that cAMP is responsible for the regulation of steroidogenic enzyme synthesis (Simpson and Waterman, 1988) through increased transcription of steroidogenic enzyme cDNA (John et al, 1986), and expression of cDNA constructs *in vitro* has shown that cAMP regulates the transcription of CYP11B1 (Kawamoto et al., 1990b). It is thought that the generation of cAMP by adenylate cyclase leads to the activation of cAMP-dependent kinase and subsequent phosphorylation of CREB which binds to the CRE with resultant increased gene expression.

Cell-specific expression of steroidogenic enzymes may arise due to sharing of a specific promoter element in these genes. In support of this was the discovery of a AGGTCA motif in the promoter regions of mouse P450$_{SCC}$, P450$_{C21}$, P450$_{aldo}$, and




i. Structure of human CYP11B genes




ii. BamHI restriction sites of CYP11B1 and CYP11B2

Figure 1.7 b Organisation of human CYPB 11genes

bovine $P450_{11\beta}$, which binds a protein alternatively designated steroidogenic factor I (SF-I) (Rice et al., 1991), or adrenal-4-binding protein (Morohashi et al., 1992). Several copies of this sequence appear in the regulatory regions of all steroid hydroxylase genes expressed in the adrenal cortex and the gonads and are found in the 5' flanking regions of CYP11B1 and B2.

Isolation of the cDNA for SF-I from a bovine adrenal library and study of the nucleotide sequence revealed that the nucleotide sequence and organisation of the SF-I gene was similar to that of members of the nuclear receptor family, i.e. thyroid, steroid, retinoic acid and vitamin D receptors (Morohashi et al, 1992). However, the nature of the *in vivo* ligand of this receptor is not yet known (Honda et al., 1993). In addition, SF-I is expressed only in steroidogenic tissues (Lala et al., 1992) and its cDNA bears considerable homology to two unusual proteins. Firstly, SF-I cDNA resembles that of embryonal long-terminal repeat binding protein (ELP), which silences retroviral expression in mouse embryonal carcinoma cells (Tsukiyama et al., 1992). It has been shown that both ELF and SF-I are encoded by the same gene but that transcripts differ due to alternative processing of the gene at the time of transcription depending on the developmental stage of the animal (Tsukiyama et al, 1992). Secondly, it resembles the cDNA for encoding a protein that regulates *fushi tarazu* (*ftz*) homeobox gene in *Drosophila*, termed FTZ-F1 (Tsukiyama et al, 1992). Two different forms of FTZ-Z1 exist, early and late, expressed at different stages in development, but with similar DNA binding properties (Ueda et al., 1990). In summary, there is some evidence to suggest that the FTZ-F1 gene in *Drosophila* and mouse encode developmentally specific proteins that provide alternative functions: ELP/early FTZ-F1, which are essential in early embryonic development, and SF-I/late FTZ-F1 which are expressed at a later stage and persist in the adult animal. These proteins have important factors in gene regulation. In particular SF-I can bind to and increase the transcription of steroidogenic enzymes in the adrenal gland. Finally, recent work has shown that by using gene targeting to disrupt the SF-1 gene in mice, SF-1 deficient mice do not survive the neonatal period and do not develop adrenal glands or gonads (Ikeda et al., 1994). Therefore, SF-1 is essential to the post-embryonic development and SF-1 may be an important global regulator of steroid hydroxylase gene expression.

Aldosterone synthase activity is confined to the zona glomerulosa cells and therefore expression of CYP11B2 must be regulated in a manner different from that of CYP11B1 (1.7.2). In primary cultures of human zona glomerulosa cells, Ang II markedly increases the level of both CYP11B1 and B2 transcripts (Curnow et al, 1991). ACTH increases CYP11B1 mRNA levels but has no effect on CYP11B2

transcription (Curnow et al, 1991). In NCI-H295 human adrenocortical carcinoma cells, which synthesise both cortisol and aldosterone, Ang II increases CYP11B2 mRNA levels and aldosterone synthesis (Bird et al., 1993). These cells are unresponsive to ACTH, but both CYP11B1 and B2 mRNA levels are increased by 8-bromo cAMP administration, i.e. the ACTH second messenger (Staels et al., 1993).

Differences in gene transcription may result from important divergence's in the structure of the relative promoter regions of the two genes. In the 5' untranslated region of both genes, outwith the immediate vicinity of the transcription initiation site, the nucleotide sequence of the two genes diverges quite considerably. These regions are only 48% identical, the difference arising principally due to the presence of a long palindromic sequence in CYP11B2 from -1734 to -1001 (Kawamoto et al, 1992). For CYP11B1 the 5' flanking region up to -1093 displays 8-bromo cAMP-responsive promoter ability. Deletion of the promoter to -760 or -505 results in a diminution of promoter activity and deletion to -294 virtually abolishes promoter activity. Deletion to -105 or -47 also abolishes promoter activity. CYP11B2 is different. Expression of constructs containing up to -2015 of the 5' flanking region had no promoter activity in response to 8-bromo cAMP. Deletion of the region to -1490, -654, -373 also destroyed promoter activity. A weak, presumably basal promoter response, however, was detected when the flanking region was deleted to -64, i.e. the region -2015 to -65 in CYP11B2 inhibits the promoter activity of CYP11B2 *in vitro* (Figure 1.7 c) (Kawamoto et al, 1992).

These differences in the promoter regions between genes clearly result in important alterations in promoter-dependent regulation of gene expression, particularly the differential response to cAMP effects, and may also have a role in determining the site-specific expression of the genes.

Finally, in the mouse, CYP11B1 and CYP11B2 lie in tandem with CYP11B2 on the left, if the genes are pictured as being transcribed right to left (Domalik et al., 1991). As yet no genomic clones have been identified which link these genes in man but evidence from studies of patients with congenital hypoaldosteronism due to aldosterone synthase deficiency (corticosterone methyloxidase II deficiency) (Pascoe et al., 1992b) and glucocorticoid-suppressible hyperaldosteronism (1.9) suggest that the same arrangement exists in man. Study of large restriction fragments generated by rare cutting restriction enzymes and separated by pulsed field gel electrophoresis suggests that the two genes are located in close proximity, approximately 40 kilobases apart (Lifton et al., 1992, Pascoe et al., 1992a).

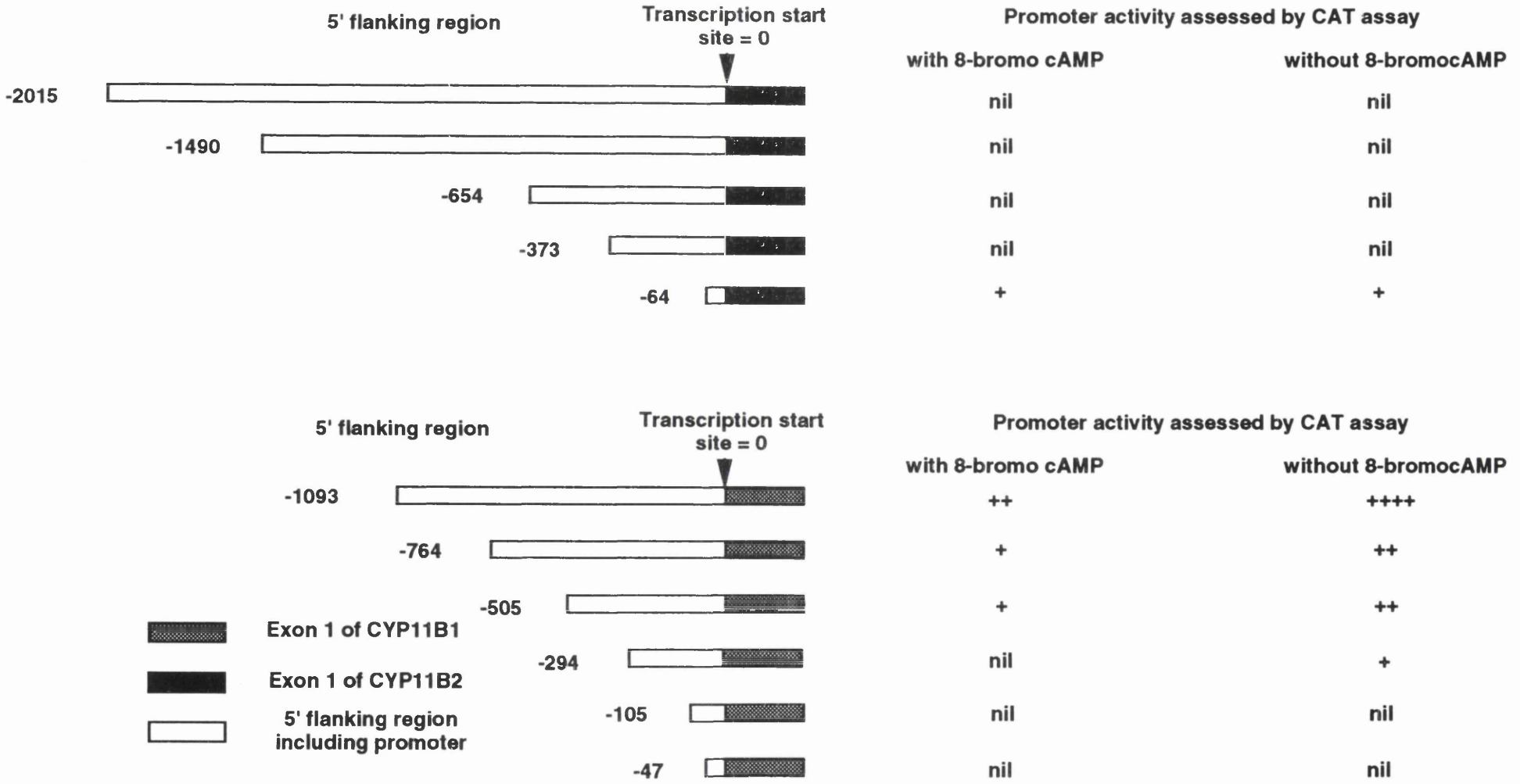



**Figure 1.7 c Promoter constructs of CYP11B1 and CYP11B2: effects of structuralvariation on cAMP-induced activity**

In summary, in man two highly homologous genes encoding steroidogenic enzymes lie together on chromosome 8q21-22 approximately 40 kilobases apart. One gene, CYP11B1 encodes the enzyme P-$450_{11\beta}$ which catalyses the formation of cortisol and corticosterone in the zona glomerulosa. Its transcription is regulated by ACTH via cAMP and it is unable to catalyse the formation of aldosterone. The other, CYP11B2 is expressed only in the zona glomerulosa in normal human adrenal tissue and catalyses the synthesis of aldosterone from DOC. CYP11B2 is regulated *in vitro* by Ang II and its promoter region differs considerably from that of CYP11B1, a difference which may reduce responsiveness to cAMP. These genes are clearly of central importance in the synthesis of aldosterone and cortisol and mutations altering the activity of their gene products may have important effects on blood pressure in man.

The entire sequence of both genes is listed in Appendix IV.

## 1.8 Mineralocorticoid-hypertension in Man

Mineralocorticoid-dependent hypertension is uncommon in man (1.3.1). However, there are a number of important clinical syndromes in which either aldosterone or other mineralocorticoids are secreted in excess. Several of these have a genetic basis and may give an insight into the nature of the genetic component of essential hypertension.

### 1.8.1 Syndromes of Primary Aldosterone Excess

Excessive aldosterone production occurs in three principal situations - Aldosterone-secreting adenoma or carcinoma, idiopathic hyperaldosteronism due to bilateral adrenal hyperplasia, and glucocorticoid-suppressible hyperaldosteronism. Hyperaldosteronism is an uncommon cause of hypertension with a prevalence reported between 2-12% in some series of patients with hypertension (Grim et al., 1977, Streeten et al., 1979), although in most series of screened populations the prevalence is considerably lower (Lewin et al, 1985, Sinclair et al, 1987). However, given the high prevalence of hypertension in western populations, in the order of 20% at some time in the lifetime of an individual, as many as 1 in 10000 of the population could be expected from these estimates to have primary hyperaldosteronism as the cause of their hypertension although this is likely to be an over estimate.

Aldosterone-secreting adrenal adenoma was first described in 1955 (Conn, 1955), less than 3 years after the discovery of aldosterone (Simpson et al., 1953). The case report detailed a patient with hypertension and hypokalaemia with aldosterone excess. The patient had a 4cm adenoma arising from the cortex of the right adrenal gland which had detectable aldosterone activity some 75-150 times that of normal adrenal tissue. Following this report numerous other descriptions of aldosterone-secreting adenomas were published along with studies of the abnormal physiology in such patients (Brown et al., 1972b).

Aldosterone-secreting adenomas account for approximately 75% of patients with primary hyperaldosteronism (Biglieri et al., 1989). The majority of cases occur in individuals aged 30-50 years and in more females than males (Ferriss et al, 1983). The majority of such patients are asymptomatic although some may manifest symptoms of hypokalaemia such as polyuria, muscle weakness and parasthesiae (Conn et al., 1964). Hypertension is a virtually invariable outcome of aldosterone-secreting adenomas and can result, albeit rarely, in malignant phase hypertension

(Brown et al., 1965). Excessive aldosterone secretion leads to sodium retention and renal potassium excretion which may be reflected by plasma hypernatremia and hypokalaemic alkalosis. Exchangeable sodium levels are elevated in proportional to the degree of hypertension (Davies et al., 1979), and total body potassium levels low. Sodium-induced plasma volume expansion leads to suppression of plasma renin activity and plasma Ang II but has no effect on plasma aldosterone concentrations as this is produced autonomously by the adrenal adenoma.

Plasma aldosterone concentrations in patients with primary hyperaldosteronism with adenoma (Conn's syndrome) may vary throughout the day, with an ACTH-like diurnal rhythm, and at times may lie within the normal range (Weinberger et al., 1979) and occasionally other measurements are require to confirm the diagnosis. Measurement of plasma concentrations of precursors of aldosterone, in particular 18-hydroxycorticosterone (raised in virtually all cases (Biglieri and Schambelan, 1979, Fraser et al., 1981)) and DOC (raised in 50% of cases (Biglieri and Schambelan, 1979)), may be helpful in some situation. In patients with marked hypokalaemia, conversion of 18-hydroxycorticosterone to aldosterone may be impaired and measurement of 18-hydroxycorticosterone levels may be of greater value than aldosterone alone (Biglieri and Schambelan, 1979). In normal subjects, assuming an upright posture stimulates renin secretion and a rise in Ang II and subsequently plasma aldosterone. In subjects with Conn's syndrome plasma renin and Ang II are suppressed and on assuming an upright posture there is usually a fall or no change in plasma aldosterone concentrations . Similarly, infusion of normal saline or administration of oral 9$\alpha$-fludrocortisone (a synthetic mineralocorticoid) in normal subjects results in sodium retention and suppression of the renin angiotensin system and a fall in plasma aldosterone concentration. Individuals with Conn's syndrome fail to suppress plasma aldosterone in such circumstances (Streeten et al, 1979, Weinberger et al, 1979). Another features of altered aldosterone regulation in Conn's syndrome is the absence of aldosterone secretory response to Ang II infusion (Fraser et al, 1981) and an augmented response to ACTH infusion. However, some authors have reported a sub-group of aldosterone-secreting adenomas which are Ang II sensitive which highlight the importance of not relying entirely on physiological testing to determine the pathological diagnosis of hyperaldosteronism (Gordon et al., 1987).

Diagnosis of Conn's syndrome is made by documenting primary hyperaldosteronism and identifying an adrenal tumour as a source of the excess aldosterone secretion. In addition, clinical observations of the response of blood

pressure and plasma electrolytes to therapy with spironolactone or amiloride can give an indication of both the presence of an adenoma and the likelihood that surgical removal will lead to resolution of the hypertension (Brown et al., 1972, Kremer et al, 1977). That is, resolution of hypertension and plasma electrolyte abnormalities with spironolactone or amiloride indicates a high chance of complete cure following surgery. Tumours can be detected by computed tomography (CT) scanning, ultrasound, magnetic resonance imaging and adrenal vein sampling to measure aldosterone concentrations. CT scanning can detect up to 90% of adenomas (Davies et al, 1979) whilst the majority of the rest can be localised to one adrenal by analysis of adrenal vein blood (McAreavey et al., 1981).

Idiopathic hyperaldosteronism (IHA) is less common than true Conn's syndrome and accounts for approximately 25 % of cases of hyperaldosteronism (Ferriss, 1992). Subjects with IHA also display many of the classical features of hyperaldosteronism although in general the degree of aldosterone excess is less than in patients with Conn's syndrome (Ferriss et al., 1978b). In particular serum potassium levels are more likely to be within the normal range, plasma aldosterone concentrations only modestly elevated and blood pressure levels lower than in Conn's syndrome patients (Ferriss et al, 1978b). In contrast to patients with Conn's syndrome, patients with IHA display a normal aldosterone postural response, i.e. aldosterone rises on assuming an upright posture (Ganguly et al., 1973, Schambelan et al., 1976). A similar response is seen in plasma levels of 18-hydroxycorticosterone, the levels of which are generally much lower than in Conn's syndrome (Fraser et al, 1981). In contrast to patients with adenomas, IHA patients display an increased adrenal sensitivity to Ang II infusion (Fraser et al, 1981). Another feature often observed, although not constantly present, in patients with IHA is the circadian rhythm of plasma aldosterone in parallel with cortisol (Ganguly et al, 1973, Schambelan et al, 1976). Features of IHA overlap those of low renin essential hypertension and its validity as a separate syndrome has been questioned.

In patients with IHA there is no lateralisation of aldosterone secretion and there is no discrete adrenal adenoma to account for the aldosterone excess although various forms of bilateral, micronodular hyperplasia have been described. This has also been described in patients with essential hypertension and normal post mortem specimens. Such patients should receive specific anti-mineralocorticoid therapy with spironolactone or amiloride to control blood pressure. Surgical removal of adrenal tissue is non-curative and can lead to recurrence and

exacerbation of hypertension possibly via the adrenal regeneration hypertension mechanism (Ferriss et al., 1978a, Kenyon, 1994).

Glucocorticoid-suppressible hyperaldosteronism is discussed in 1.9.

### 1.8.2 Deficiency of 11ß-hydroxylase

Clinical deficiency of 11ß-hydroxylase was first noted in 1951 (Shepard and Clausen, 1951). Although the majority of patients with congenital adrenal hyperplasia lose salt, it was noted that a small proportion developed hypertension in the face of impaired cortisol synthesis (White et al, 1994). Although there is impaired 11ß-hydroxylase activity, aldosterone synthesis is unimpaired although plasma aldosterone may be low due to suppression of renin synthesis (Levine et al., 1980). In these patients hypertension is relieved by the administration of glucocorticoids. Other features of clinical 11ß-hydroxylase are those attributable to adrenal androgen excess, i.e. masculinization of the female genitalia, premature growth spurt, early closure of bony epiphyses with subsequent short stature. Figure 1.8 a shows the effects of a block in cortisol synthesis at the level of 11ß-hydroxylase. The build up of precursors of cortisol and aldosterone such as DOC and 11-deoxycortisol, and perhaps 18-hydroxy-DOC and 19-nor metabolites of DOC, results in mineralocorticoid effects, i.e. volume-dependent hypertension, in addition to a shunting of the excess precursors into the androgen biosynthetic pathways. Deficiency of cortisol results in a lack of feedback inhibition of ACTH secretion and this leads to a rise in ACTH secretion and a subsequent rise in the production of DOC and adrenal androgens. The build-up of DOC leads to suppression of renin activity. Administration of glucocorticoids, dexamethasone, prednisolone or ideally cortisol to replace absent physiological cortisol, suppresses ACTH secretion which in turn switches of the excessive formation of DOC and adrenal androgens and also provides replacement for deficient cortisol production. Alleviation of the mineralocorticoid excess leads to renal sodium loss, correction of hypokalaemia, resolution of hypertension and a rise in plasma renin activity.

The incidence of classic 11ß-hydroxylase deficiency is about 1/100000 births in the general Caucasian population (Zachmann et al., 1983). In other populations, particularly Jews of Moroccan descent living in Israel, the incidence is considerably higher, 1/5000 - 1/7000 births with a gene frequency of 1.2-1.4% (Rösler et al., 1992). The condition demonstrates an autosomal recessive form of inheritance and thus it is not surprising that the incidence of the condition is higher in populations which are relatively inbred.

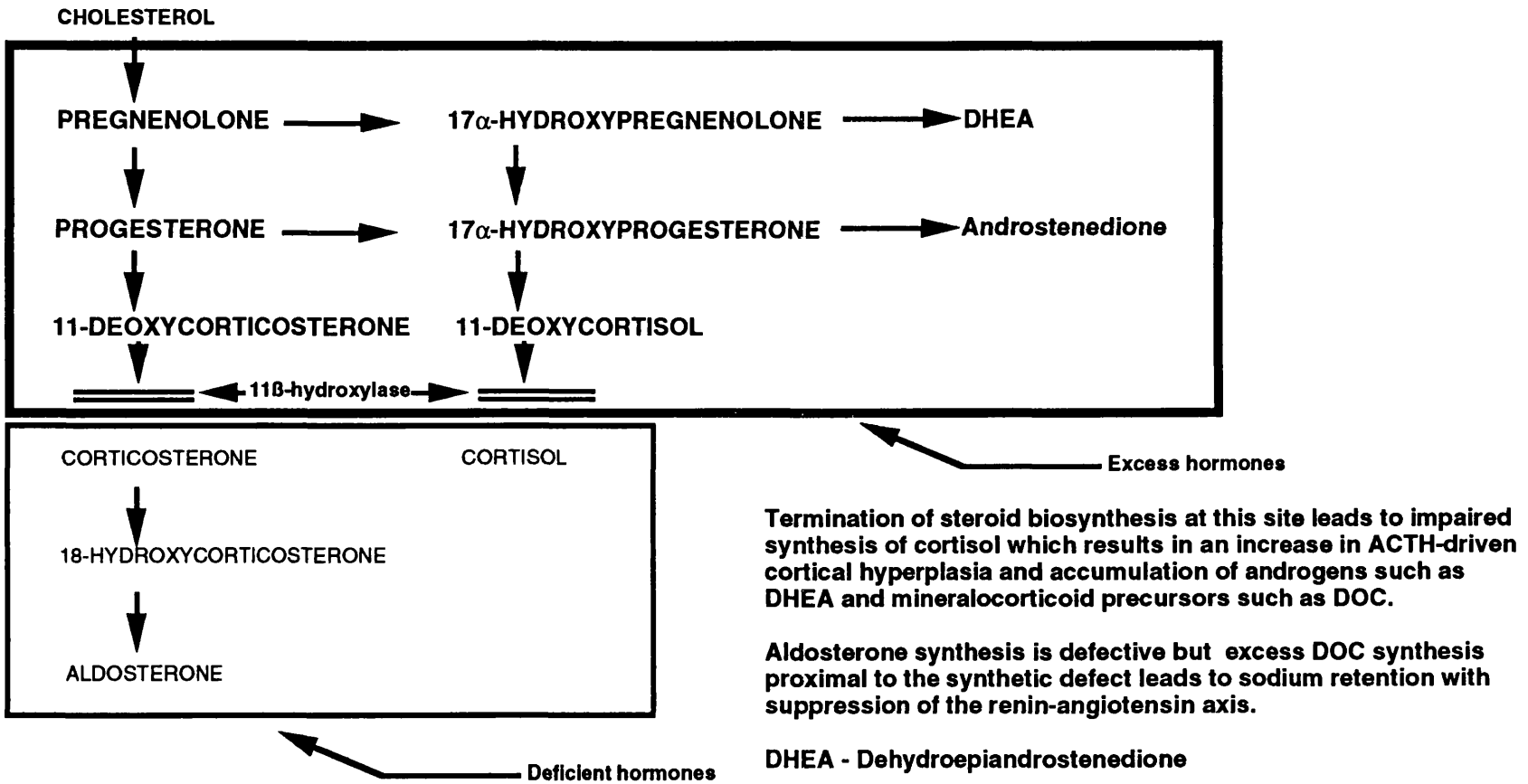



**Figure 1.8 a Deficiency of steroid 11ß-hydroxylase in man**

Deficiency of 11ß-hydroxylase results from mutations in CYP11B1 (Table 1.8 a).

| Mutation | Exon | Activity (%normal) | Effect | Clinical Severity | Ethnic Group |
|---|---|---|---|---|---|
| P32Δc | 1 | 0 | Frameshift | +++ | White |
| W116X | 2 | 0 | Nonsense | +++ | Japanese |
| K174X | 3 | 0 | Nonsense | +++ | White |
| T318M | 5 | 0 | ?Alters Proton Transfer | +++ | Yemenite |
| Q338X | 6 | 0 | Nonsense | +++ | Sikh |
| Q356X | 6 | 0 | Nonsense | +++ | Afro-American |
| R374Q | 6 | 0 | Adrenodoxin Interaction | +++ | Lebanese |
| R384Q | 7 | 0 | ?Substrate Binding | +++ | White |
| N394 + 2nt | 7 | 0 | Frameshift | +++ | Turkish |
| V441G | 8 | 0 | ?Alters $2^0$ structure | +++ | White |
| R448H | 8 | 0 | ?Affects Haem Binding | +++ | Moroccan Jews |

**Table 1.8 a Mutations in CYP11B1 causing clinical 11ß-hydroxylase deficiency**

In Moroccan Jews, almost all affected subjects have the same disease allele, R448H (White et al., 1991). R448 is adjacent to C450 which is presumed to be a ligand of the haem iron atom in the P-450 complex, and is highly conserved in all eukaryotic P-450 enzymes examined thus far. This would suggest that C450 is of great importance to the function of $P450_{11ß}$ and mutation of this amino acid abolishes enzyme activity (Curnow et al., 1993). So far, 11 additional mutations have been identified in patients with classic 11ß-hydroxylase deficiency (Curnow et al, 1993, Helmberg et al., 1992, Skinner et al., 1993, White et al, 1991) (Figure 1.8 b). These mutations are of two types; frameshift mutations in codons P32 (1 nucleotide) and N394 (2 nucleotides) and nonsense mutations W116X, K174X, T318M, Q338X, Q356X, R374Q, R378Q, V441G and R448H. All of these mutations abolish enzyme activity and all missense mutations occur in regions of functional importance to $P450_{11ß}$ (Curnow et al, 1993). In particular, the T318M mutation modifies an absolutely conserved residue that is thought to be critical for proton transfer to the bound oxygen molecule on the P-450 enzyme (Ravichandran et al., 1993). In addition, R374Q mutates a completely conserved residue which may affect adrenodoxin binding, whilst R384Q is in a region which may form part of the substrate binding pocket (Ravichandran et al, 1993). Finally, V441G is adjacent

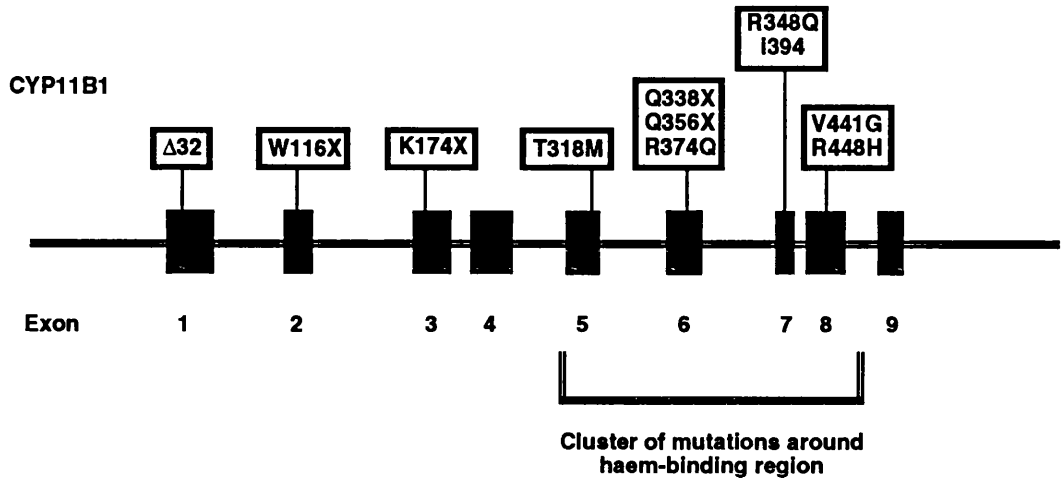



**Figure 1.8 b Distribution of mutations in CYP11B1 causing steroid 11ß-hydroxylase deficiency in man**

to the highly conserved haem binding region, and this mutation may alter the secondary structure of P-$450_{11\beta}$ in this region.

The phenotype correlation in patients with 11ß-hydroxylase deficiency are often at variance with the genetic features of the mutation in the affected individuals. Mutations which result in complete absence of enzymatic activity *in vitro* may lead to clinical features which are mild in comparison to other mutations which would be expected to result in less disruption of the gene product. For example, R448H leads to a wide variety of observed phenotypes, both clinical and biochemical, e.g. plasma levels of 11-deoxycortisol and DOC, in Moroccan Jews. However, nonsense mutations encoding a truncated non-functional enzyme (P32n) result in only mild virilistion of affected females (Curnow et al, 1993, Rösler et al, 1992, White et al, 1991). This disparity between clinical and biochemical phenotype and the enzymatic activity of expected the mutated gene product suggests that unknown factors outwith the CYP11B1 locus may determine phenotypic differences in 11ß-hydroxylase deficiency.

### 1.8.3 Deficiency of 17α-hydroxylase

First described in 1966, 17α-hydroxylase deficiency is an uncommon cause of mineralocorticoid-dependent hypertension (Biglieri et al., 1966). $P450_{C17}$ is active in the zona fasciculata where it is a key enzyme involved in the synthesis of cortisol and the adrenal androgens. In the gonad, P-$450_{C17}$ catalyses the formation of testosterone and oestrogens because of its combined 17α-hydroxylase/17, 20 lyase activity. In man, P-$450_{C17}$ is not expressed in the zona glomerulosa. Absence of 17α-hydroxylase activity leads to impaired synthesis of 11-deoxycortisol and cortisol in the adrenal, with resultant increased ACTH secretion from the pituitary (Figure 1.8 c). Synthesis of estradiol and testosterone is similarly impaired with a resulting rise in LH and FSH secretion. The combined effects of excess ACTH and gonadotrophin secretion are two-fold. First, there is a build up of immediate precursors of P-$450_{C17}$ such as progesterone and pregnenolone. Secondly, increased ACTH secretion results in activation of conversion of DOC to corticosterone and 18-hydroxyDOC in the zona glomerulosa. DOC, and to a lesser extent, 18-hydroxyDOC have sodium retaining properties which suppress renin secretion and as a result plasma aldosterone levels are suppressed (Table 1.8 b). Signs of glucocorticoid deficiency are usually absent and the very high levels of corticosterone probably compensate for the lack of cortisol (Fraser et al., 1987).

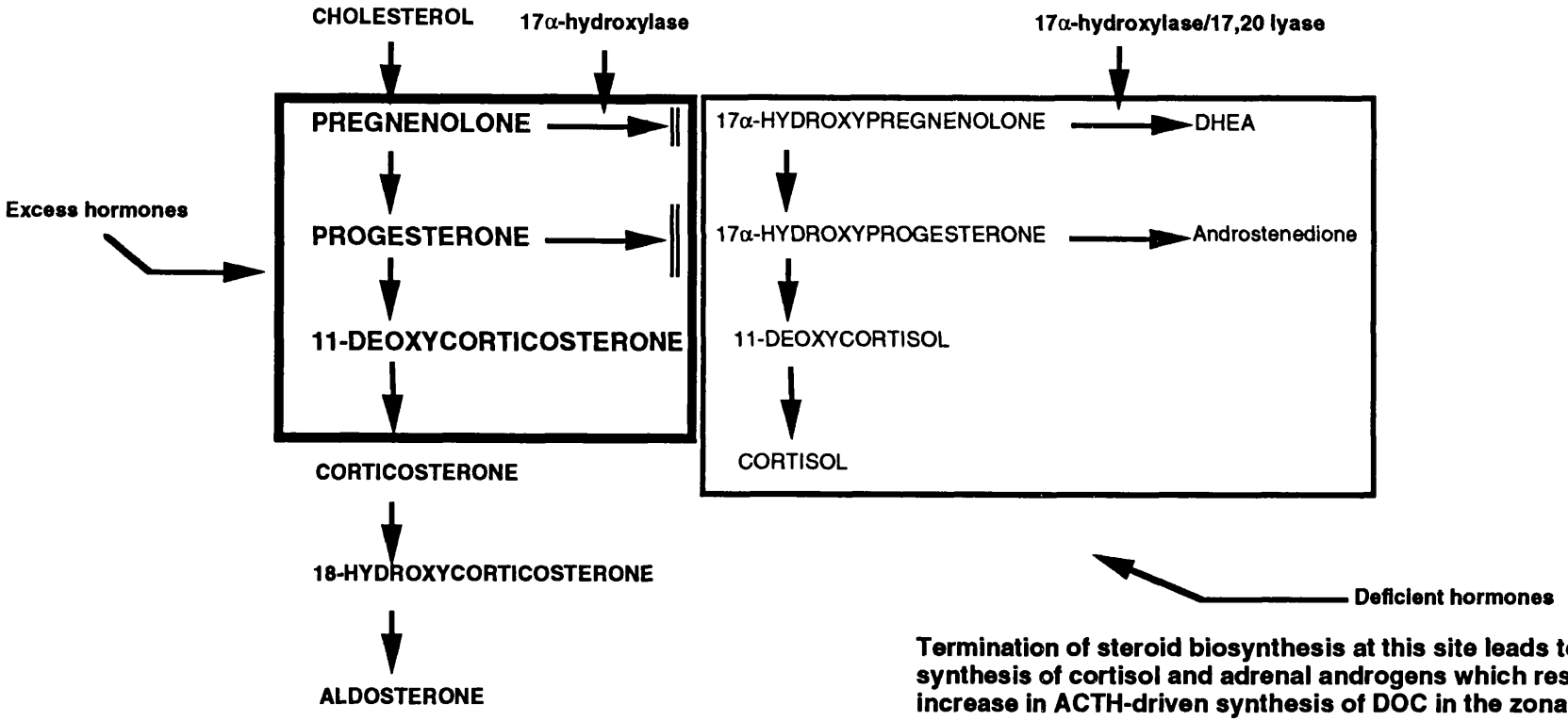



**Figure 1.8 c Deficiency of steroid 17α-hydroxylase in man**

**Termination of steroid biosynthesis at this site leads to impaired synthesis of cortisol and adrenal androgens which results in an increase in ACTH-driven synthesis of DOC in the zona fasciculata.**

**Gonadal steroidogenesis is also impaired and secondary sex characteristics fail to develop.**

**Aldosterone synthesis is normal because 17α-hydroxylase is not expressed in the zona glomerulosa but excess DOC synthesis in the fasciculata leads to sodium retention with suppression of the renin-angiotensin axis and the development of hypertension.**

**DHEA - Dehydroepiandrostenedione**

| | Before | After | Normal |
|---|---|---|---|
| **Cortisol** | 4 | 0.06 | **6-26 (μg/100ml)** |
| **11-deoxycortisol** | 3 | 15 | **40-400 (ng/100ml)** |
| **DOC** | 154 | 13 | **3-16 (ng/100ml)** |
| **18-OH-DOC** | 438 | 41 | **10-50 (ng/100ml)** |
| **Corticosterone** | 29 | 0.7 | **0.08-0.8 (μg/100ml)** |
| **18-OH-corticosterone** | 104 | 6.7 | **3-25 (ng/100ml)** |
| **Aldosterone** | 2 | 12 | **4-18 (ng/100ml)** |
| **ACTH** | 154 | <20 | **18-60 (pg/ml)** |
| **Renin** | 3 | 18 | **4-20 (U/l)** |
| **Angiotensin II** | 9 | 36 | **5-35 (pg/ml)** |
| **Testosterone** | 1.3 | not detectable | **2.0±0.4 (nmol/l)** |
| **Estradiol** | 73 | not detectable | **180±1470 (pmol/l)** |
| **Progesterone** | 12.5 | 1.3 | - |
| **17α-hydroxy-progesterone** | 1.0 | - | - |
| **FSH** | 79.1 | 96.9 | **- (U/l)** |
| **Blood Pressure** | 224/153 | 134/82 | |

**Table 1.8 b Blood pressure and hormone data in a 46XX female with 17α-hydroxylase deficiency before and after dexamethasone therapy. Adapted from (Fraser et al, 1987).**

The clinical features of 17α-hydroxylase deficiency are as follows: hypertension, absence of secondary sex characteristics such as breast development or pubic hair; in XY genetically male fetuses there is a variable degree of pseudohermaphroditism, secondary sex characteristics do not develop at puberty and affected males are often raised as females (Fraser et al, 1987, Goldsmith et al., 1967).

P-450$_{c17}$ is encoded by a single gene on chromosome 10q24-25 (Matteson et al, 1986) and a number of mutations leading to clinical 17α-hydroxylase deficiency have been described. Of these cases three were due to nonsense mutations (Ahlgren et al., 1992, Yanase et al., 1988, Yanase et al., 1992), three were deletions (Biason et al., 1991, Fardella et al., 1993, Yanase et al., 1989), and three were duplications (Imai et al., 1993, Imai et al., 1992, Yanase et al., 1990). Six were due to point mutations leading to single amino acid changes in P-450$_{c17}$ (Ahlgren et al, 1992, Fardella et al., 1994a, Imai et al, 1993, Lin et al., 1991, Monno et al., 1993, Yanase et al, 1992).

It is of interest that in a number of cases of 17α-hydroxylase deficiency, affected individuals are not homozygous for a single mutant allele (Ahlgren et al, 1992, Yanase et al, 1992). In these cases both alleles of P450$_{c17}$ are sufficient to abolish 17α-hydroxylase activity but this is in contrast to the known cases of 11ß-

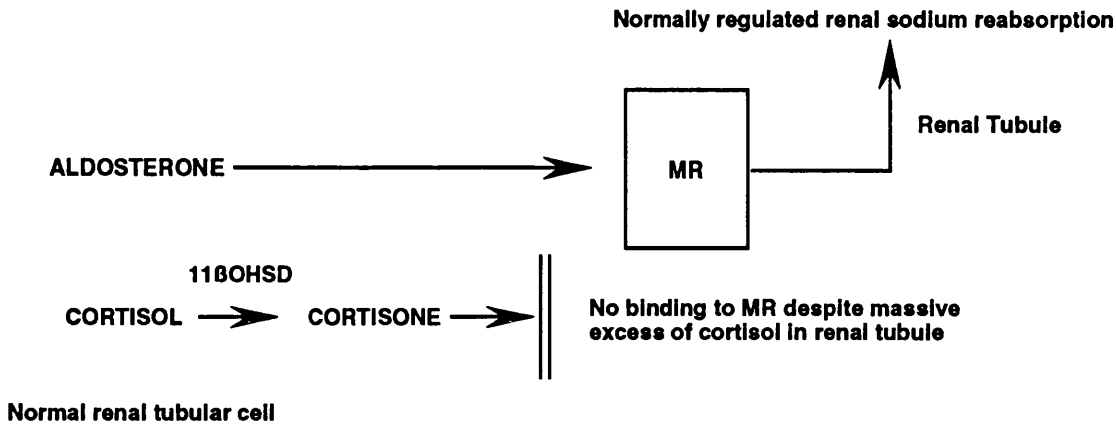
hydroxylase deficiency where all reported mutations are homozygous for the disease allele. This may suggest that mutations in CYP11B1 occur less frequently but have occurred in populations where consanguineous relationships are more frequent, whereas mutation in CYP17A are more common but have occurred in populations which limit consanguineous relationships and thus the possibility of 17α-hydroxylase deficiency is less.

### 1.8.4 Deficiency of 11ß-hydroxysteroid dehydrogenase

11ß-OHSD plays an important role in modulating the mineralocorticoid effect of cortisol in tissues such as the kidney (1.5.6). In man, three situations arise where disordered 11ß-OHSD action leads to hypertension; firstly ingestion of liquorice, secondly ingestion of carbenoxolone for the treatment of peptic ulcer disease, and finally in the uncommon condition of apparent mineralocorticoid excess.

Ingestion of liquorice has long been known to cause hypertension (Nicholls and Espiner, 1983). Glycyrrhetinic acid has been shown to be the active component of liquorice causing hypertension (Stewart et al, 1987). Glycyrrhetinic acid inhibits 11ß-OHSD in man which results in so-called 'deprotection' of renal mineralocorticoid receptors (Stewart et al, 1987). Absence of 11ß-OHSD activity in the renal tubule allows cortisol, normally converted to inactive cortisone, to bind to mineralocorticoid receptors to promote sodium retention and subsequent sodium-dependent hypertension (Stewart et al, 1987) (Figure 1.8.d). In addition, there is some evidence to suggest that glycyrrhetinic acid inhibits 5α- and 5ß-reductase pathways in the metabolism of aldosterone (Latif and Morris, 1989). Carbenoxolone is a semisynthetic hemisuccinate derivative of glycyrrhetinic acid and has its effect through a mechanism analogous to that of liquorice (Funder et al, 1988).

Apparent mineralocorticoid excess (AME) is a rare syndrome of hypertension and hypokalaemia associated with suppression of plasma renin activity and low plasma concentrations of aldosterone and other known mineralocorticoids (Ulick et al., 1979). Studies of a number of these patients revealed that the hypertension in this disorder is sodium-dependent but that there is no detectable excess secretion of mineralocorticoids (Ulick et al, 1979). However, administration of 11-[$H^3$]-cortisol to patients with AME results in impaired excretion of [$H^3$]-water suggesting that the problem is impaired conversion of cortisol to cortisone (Stewart et al., 1988, Ulick et al, 1979), i.e. defective dehydrogenase activity of 11ß-OHSD. This suggestion has been supported by observations on the urinary excretion of metabolites of cortisol (tetrahydrocortisol, THF) and cortisone (tetrahydrocortison, THE) with AME



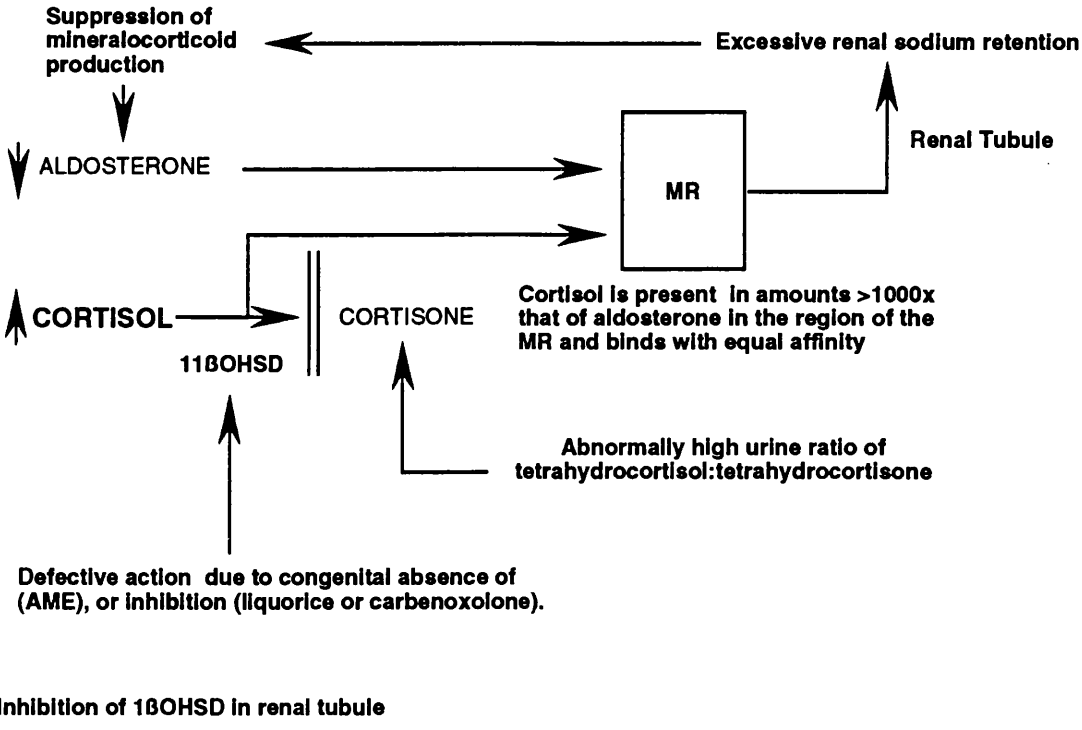




**Figure 1.8 d Development of hypertension following inhibition of 11ß-hydroxysteroid dehydrogenase in man**

patients noted to have abnormally low urinary THE:THF ratios (<1) (Stewart et al, 1988). Administration of dexamethasone causes reversal of the clinical features (by suppression of ACTH-dependent cortisol secretion), which are reproduced by administration of physiological doses of cortisol (Stewart et al, 1988). A variant of the syndrome has been described where the urinary excretion of THF and THE is normal (Ulick et al., 1990), suggesting that in some cases both oxidation and reduction defects may be present.

Following cloning of the human cDNA for 11ßOHSD it was hoped that study of this gene in patients with AME would reveal the molecular defect underlying AME in these patients. However, detailed molecular studies of the 11ßOHSD gene in patients with AME have not detected any mutations to explain the clinical syndrome (Nikkila et al., 1993). Recently, however, a cDNA for a second 11ß-OHSD transcript has been described in the human kidney (Albiston et al., 1994). This transcript is not detected in the liver, lung or heart, is exclusively NAD-dependent and is inhibited by carbenoxolone. Thus it appears that this transcript encodes a kidney-specific isoform of 11ßOHSD and analysis of this gene in patients with AME may shed light on the pathogenesis of AME.

### 1.8.5 Miscellaneous forms of mineralocorticoid excess

Adrenal carcinoma may arise from the adrenal cortex and give rise to either excessive aldosterone or DOC secretion (Ferriss, 1992, Gordon et al., 1994). They account for approximately 1% of mineralocorticoid-secreting tumours and are often associated with a greater degree of aldosterone excess than benign tumours. They are generally larger than adenomas and display similar alterations in aldosterone secretion in relation to orthostasis or circadian variations (Ferriss, 1992, Gordon et al, 1994).

Liddle's syndrome is a congenital syndrome of hypertension, hypokalaemia and suppressed plasma renin and aldosterone levels (Gordon et al., 1992, Liddle et al., 1963). The pathogenesis of this condition is unclear but may be caused in part by increased renal tubular sodium permeability which leads to increased renal sodium retention and suppression of the RAS. Renal potassium loss is also a feature of the syndrome suggesting that the distal renal tubule reabsorbs sodium in excess and at the expense of potassium and hydrogen ions (Gordon et al, 1992). Treatment with triamterene (a drug to inhibit renal sodium resorbtion independent of aldosterone) is only partially effective and requires the addition of dietary sodium restriction to control blood pressure (Gordon et al, 1992, Liddle et al, 1963).

Similarly spironolactone is ineffective in this condition because aldosterone levels are often undetectable (Liddle et al, 1963).

## 1.9 Glucocorticoid-Suppressible Hyperaldosteronism

Glucocorticoid-suppressible hyperaldosteronism (GSH), also known as dexamethasone-suppressible hyperaldosteronism and glucocorticoid-remediable aldosteronism, is an uncommon cause of secondary hypertension in man. It was first described in the medical literature by a Canadian group (Sutherland et al., 1966), who investigated a father and son with hypertension. Both patients had elevated blood pressure, hypokalaemia, increased aldosterone secretion rate and suppressed plasma renin activity. In neither case was there an adrenal adenoma as a source of aldosterone hypersecretion, and removal of one adrenal gland from the father had no effect on blood pressure although his aldosterone secretion rate halved. However, following the administration of dexamethasone, a synthetic glucocorticoid with no *in vitro* mineralocorticoid activity, to both patients blood pressure, aldosterone secretion rate and serum [$Na^+$] fell, and plasma renin activity and serum [$K^+$] rose. An associated fall in urinary potassium excretion and a rise in urinary sodium excretion was observed. Throughout the period of investigation no abnormality in cortisol metabolism was found.
The features observed in the initial report outline the key clinical and biochemical features observed in affected individuals with GSH:

| **Hyperaldosteronism with suppression of plasma renin activity** |
|---|
| **A dominant mode of inheritance** |
| **Hypertension** |
| **Hypokalaemia** |

Following the initial description of the condition, a number of other kindreds with the disorder were identified and reported in the medical literature (Connell et al., 1986, Davis et al., 1988, Ganguly et al., 1981, Giebink et al., 1973, Grim and Weinberger, 1980, Jamieson et al., 1994, Lifton et al, 1992, Miura et al., 1968, New and Peterson, 1967, Salti et al., 1969, Stockigt and Scoggins, 1987, Woodland et al., 1985). Study of the GSH patients described in these reports has resulted in the identification of a number of important biochemical and physiological abnormalities in adrenal steroid metabolism in GSH.

### 1.9.1 Clinical Features of GSH

The most obvious clinical abnormality noted in patients with GSH is the presence of hypertension. The initial case report noted that the son of the index cases was hypertensive, BP 180/120 mmHg, at age 16 years (Sutherland et al, 1966). The discovery of hypertension in young patients has been a key feature in cases of GSH, with hypertension detected in children as young as 2.5 years (New and Peterson, 1967). In the study of one large North American kindred, hypertension was present in all affected individuals with GSH. In addition, adult patients were all diagnosed as hypertensive before the age of 21 years (mean 16.1+/-3.4) (Rich et al., 1992). In general, the majority of patients will develop hypertension at some time during their life although the age of onset may be quite variable (Chapter 4) (Jamieson et al, 1994).

A second feature of GSH which was initially described by Sutherland (Sutherland et al, 1966) was hypokalaemia. In both the index case and his son plasma [$K^+$] varied between 2.5-3.5 mmol/l (normal 3.6-5.2 mmol/l). Subsequent reports varied as to the level of plasma [$K^+$] in affected individuals, although in a significant number it may be normal (Fallo et al., 1985, Grim and Weinberger, 1980, New and Peterson, 1967, O'Mahony et al., 1989, Stockigt and Scoggins, 1987). The serum [$K^+$] in the study by Rich (Rich et al, 1992) was found to be normal in all subjects screened and serum [$K^+$] was no different in affected and unaffected relatives. However, they did note that development of severe hypokalaemia in affected individuals given thiazide diuretics was characteristic of GSH patients. Indeed, cardiac arrest secondary to hypokalaemia in a patient with GSH given a thiazide has been reported (Gill and Bartter, 1981).

As noted previously, hypertension is an important risk factor for stroke. A study of the pedigrees of most kindreds with GSH reveals a considerable excess mortality from stroke, in particular stroke before the age of 50 years (Connell et al, 1986, Rich et al, 1992, Sutherland et al, 1966). The reason for this is unclear as the majority of individuals with GSH have only moderate degrees of aldosterone-excess and moderate hypertension. However, this elevation of blood pressure is present from an early age and this may be an important factor in the genesis of 'premature' stroke in these patients.

In addition to stroke, GSH has been associated with the development of a syndrome indistinguishable from acute glomerulonephritis with evidence of nephrosclerosis on renal biopsy (Lee et al., 1982).

Finally, the presence of an autosomal dominant mode of inheritance of the condition is universal. This is often most revealing when the cause of death of patients not known to have hypertension is established. If a relative of a patient suspected as having GSH died under the age of 50 from a stroke or renal failure then it is highly likely that they also had GSH.

Treatment of GSH with dexamethasone is clearly effective. In addition, amiloride and spironolactone, an aldosterone antagonist, have been used effectively to control blood pressure in these patients (Connell et al., 1986, Jamieson et al, 1994).

### 1.9.2 Zona Glomerulosa Function in GSH

In normal man, the diurnal rhythm of cortisol is mirrored by that of the ACTH-dependent precursors of aldosterone, DOC and corticosterone. Diurnal variation in aldosterone secretion is less marked, presumably due to the important superimposed effects of the renin-angiotensin system and plasma [$K^+$]. However, in patients with GSH the diurnal variation in plasma cortisol correlates strongly with plasma aldosterone concentrations (Connell et al, 1986). Similarly, the plasma levels of DOC also correlate strongly with plasma aldosterone concentration (Connell et al, 1986). In GSH patients, long-term dexamethasone administration abolishes the diurnal rhythm of both cortisol and aldosterone, although plasma aldosterone concentrations, after initially being undetectable rise to within the normal range (Connell et al, 1986). This maintenance of aldosterone secretion after suppression of ACTH reflects a secondary rise in renin levels, permitting a return to normal regulation of aldosterone by the RAS.

In patients untreated with GSH, administration of ACTH, either as an intravenous infusion, an intramuscular injection or topically as a gel, results in an exaggerated rise in plasma aldosterone and plasma 18-hydroxycorticosterone concentrations (Connell et al, 1986, Ganguly et al., 1984). In addition, there is an associated rise in blood pressure and exaggeration of the aldosterone-dependent effects, i.e. hypokalaemia, suppression of plasma renin activity and associated urinary electrolyte excretion patterns (Connell et al, 1986, Ganguly et al, 1984). When aminoglutethimide, an inhibitor of the conversion of cholesterol to pregnenolone, is given to GSH patients receiving ACTH there is a reduction in blood pressure and aldosterone secretion and a rise in plasma [$K^+$] and renin activity which reversed on cessation of aminoglutethimide (Gill and Bartter, 1981). Conversely, when patients receive metyrapone, an inhibitor of P-$450_{11\beta}$, plasma levels of cortisol and aldosterone fall dramatically. This is associated with a marked rise in blood

pressure, probably due to ACTH-driven DOC production (New and Peterson, 1967). Plasma 11-deoxycortisol concentrations are aldo raised. After 7-10 days of metyrapone therapy there is an 'escape' in cortisol and aldosterone secretion, i.e. plasma cortisol and aldosterone levels rise despite continued metyrapone administration. The reason for this is unknown but may relate to ACTH-induced hyperexpression of the gene causing GSH, which can synthesise cortisol *in vitro*, with resultant recovery of cortisol secretion (Pascoe et al, 1992a).

In contrast to the exaggerated aldosterone-responsiveness of GSH patients to ACTH, Ang II has relatively little effect on aldosterone secretion. If untreated patients with GSH are subjected to dietary sodium restriction (Oberfield et al., 1981), frusemide administration (Ganguly et al, 1981), or head-up tilt (Ganguly et al, 1981), in an attempt to enhance endogenous Ang II production via the RAS, there is a much diminished plasma aldosterone response. Similarly, intravenous infusion of Ang II to such patients, up to doses of 4 ng/kg/min results in little change in plasma aldosterone concentration (Connell et al, 1986, Fallo et al, 1985). Normal individuals subjected to such interventions would expect to experience a 2 to 3-fold increase in plasma aldosterone concentrations (Zoccali et al, 1983).

The effect of dexamethasone on the control of aldosterone secretion in GSH is dramatic. Sodium depletion in dexamethasone-treated patients with GSH induces a normal rise in plasma aldosterone and 18-hydroxycorticosterone concentrations and a rise in plasma renin activity (Oberfield et al, 1981). Similarly, head-up tilt and frusemide administration also increase plasma renin and aldosterone concentrations in dexamethasone treated patients (Ganguly et al, 1981). The normal plasma aldosterone response to intravenous Ang II infusion in dexamethasone-treated patients is also restored (Connell et al, 1986, Fallo et al, 1985).

Similarly, ACTH infusion in GSH patients taking dexamethasone show that the previously exaggerated rises in blood pressure and plasma aldosterone concentration is diminished (Connell et al, 1986). However, it has been noted that prolonged high dose ACTH administration (Oberfield et al, 1981) and psychological stress and trauma (N Benjamin, personal communication) can result in excessive rises in blood pressure and plasma aldosterone concentration in patients with GSH taking dexamethasone.

These findings suggest that ACTH has characteristic effects on aldosterone secretion in GSH:

1) a direct effect on aldosterone secretion, principally by increasing aldosterone synthase activity and increasing the conversion of corticosterone to aldosterone

2) the normal regulation of aldosterone secretion by Ang II is disturbed and aldosterone secretion is controlled by ACTH

3) the removal of the influence of ACTH, by suppression of ACTH with exogenous glucocorticoids, restores the regulation of aldosterone secretion to normal.

In summary, individuals with GSH clearly exhibit aberrant responses to physiological stimuli such as ACTH, and Ang II. In addition, the control of aldosterone secretion in patients with GSH is upset. Observations in patients with GSH have shown that the abnormality in GSH centres around increased aldosterone secretion which appears to ACTH-dependent.

### 1.9.3 Unusual Biochemical Features of GSH

Although hypertension in GSH could easily be ascribed to the presence of elevated plasma aldosterone concentrations, early work suggested that it might not be the sole cause of the hypertension in this condition. A series of elegant investigations in a subject with GSH suggested the possibility that an ACTH-induced steroid, not aldosterone, 18-hydroxy-DOC or DOC, was responsible for some of the pressor effects of ACTH (New et al., 1976). In particular, these workers showed that infusion of aldosterone, 18-hydroxy-DOC or DOC to a dexamethasone-treated GSH patient did not produce the same effect on blood pressure as stopping dexamethasone. In addition, metyrapone caused hypertension and sodium retention but the addition of aminoglutethimide resulted in a considerable fall in blood pressure, natriuresis and reduction in aldosterone secretion. Administration of metyrapone to normal subjects results in impairment of cortisol and corticosterone synthesis by inhibiting P-$450_{11\beta}$. A compensatory rise in ACTH secretion occurs with an overproduction of precursors, predominantly DOC and 11-deoxycortisol, sufficient to maintain blood pressure within the normal range or even elevate it slightly (Orth et al, 1992). Aminoglutethimide has similar effects in normal individuals as those observed in patients with GSH (Orth et al, 1992). Finally the patient received intravenous ACTH and oral dexamethasone. The results showed that dexamethasone reduces blood pressure and aldosterone secretion by suppressing ACTH secretion rather than by any direct effect of dexamethasone. One caveat in the interpretation of these studies is the absence of cortisol following many of the pharmacological manipulations, i.e. dexamethasone, metyrapone and aminoglutethimide administration. This may remove any effect cortisol might have

on maintaining blood pressure in patients with GSH if aldosterone secretion were to be lowered independently.

This series of studies did however suggest the possibility that other ACTH-regulated steroids with blood pressure raising effects were being produced by the adrenal cortex in GSH.

Workers looking at the secretory products of aldosterone-secreting adenomas had postulated that although the adenoma was the source of the hypertension, the degree of hypertension was often disproportionately high in comparison to the degree of hyperaldosteronism. When the urine of patients with aldosterone-secreting adenomas was studied using gas chromatography/ mass spectrometry, it was found that in the unconjugated fraction of the samples a single steroid molecule accounted for more than half of the mass of the extract. The compound was identified as the 20,18,-hemiketal form of 11ß, 17α, 18, 21-tetrahydroxy-4-pregnen-3, 20-dione and the trivial name 18-hydroxycortisol was proposed (Chu and Ulick, 1982). Further study using aldosterone-secreting adenoma tissue and bovine adrenal slices and tritiated cortisol showed that cortisol was the immediate precursor of 18-hydroxycortisol, and that corticosterone could not be converted to 18-hydroxycortisol (Chu and Ulick, 1982). Study of patients with aldosterone-secreting adenomas demonstrated that they excreted between 0.3-2.0 mg/ 24 hours of 18-hydroxycortisol in contrast to normal individuals who secrete < 0.1 mg/ 24 hours (Ulick and Chu, 1982).The only other patient group shown to excrete appreciable amounts of 18-hydroxycortisol were GSH patients (Ulick and Chu, 1982).

Further studies of aldosterone-producing adrenal tissue showed that cortisol is also readily converted into a second substance 18-oxocortisol (Ulick et al., 1983a), resulting from further 18-hydroxylation and dehydration of 18-hydroxycortisol. An alternative trivial name for this steroid is 17α-hydroxyaldosterone.

Study of the biological properties of both 18-hydroxycortisol and 18-oxocortisol show that both have relatively low affinity for steroid hormone receptors (Table 1.9 a).

In particular, 18-hydroxycortisol has an extremely low affinity for both glucocorticoid and mineralocorticoid receptors (Gomez-Sanchez et al., 1984, Ulick et al, 1983b), whilst 18-oxocortisol has greater affinity than 18-hydroxycortisol for both receptors. Even so, the affinity of 18-oxocortisol for the mineralocorticoid receptor was only

1% of that of aldosterone and 3% of that of dexamethasone for the glucocorticoid receptor (Gomez-Sanchez et al., 1985, Ulick et al, 1983b).

| | | Relative binding % | |
|---|---|---|---|
| Steroid | | Receptor | |
| | GR[a] | MR[b] | CBG[c] |
| Dexamethasone | 100 | 8 | <0.01 |
| Cortisol | 32 | 17 | 100 |
| Aldosterone | - | 100 | <1 |
| 18-hydroxycortisol | <0.3 | <0.01 | <1 |
| 18-oxocortisol | 2.4 | 1.7 | 8 |

CBG - cortisol binding globulin, GR - glucocorticoid receptor, MR - mineralocorticoid receptor
a - relative to dexamethasone, b - relative to aldosterone, c - relative to cortisol

**Table 1.9 a Relative binding of C18-oxygenated and related steroid hormones for corticosteroid hormone receptors and binding globulin (Ulick et al., 1983b)**

Studies of normal subjects have revealed that 18-hydroxycortisol is secreted in small amounts and that its secretion is enhanced by ACTH and salt restriction, but not by Ang II infusion (Corrie et al., 1985). In normal subjects, ACTH raises urinary 18-hydroxycortisol excretion in parallel with that of cortisol and 18-oxocortisol (Gomez-Sanchez et al., 1988, Gomez-Sanchez et al., 1987). As expected dexamethasone leads to a suppression of urinary 18-hydroxycortisol excretion (Gomez-Sanchez et al, 1987). Sodium depletion (10mEq/day restriction) also has an effect on 18-hydroxycortisol secretion, causing a 2-3 fold increase in urinary 18-hydroxycortisol excretion (Gomez-Sanchez et al, 1987).

In patients with GSH, urinary excretion rates of both 18-hydroxy- and 18-oxocortisol are raised (Connell et al, 1986, Gomez-Sanchez et al., 1984, Ulick and Chu, 1982). Studies of untreated patients with GSH have shown that 18-oxocortisol is excreted in the urine in large amounts (25-55 μg/24hrs) compared to normal man (0.8-7.0 μg/24hrs) (Gomez-Sanchez et al, 1984). Treatment with dexamethasone suppresses urinary 18-hydroxycortisol and 18-oxocortisol excretion rates to normal (Connell et al, 1986, Rich et al, 1992, Ulick et al., 1990). Plasma 18-hydroxycortisol concentration in GSH patients follows the circadian rhythm of cortisol and ACTH administration leads to an exaggerated rise in plasma concentration (Connell et al, 1986).

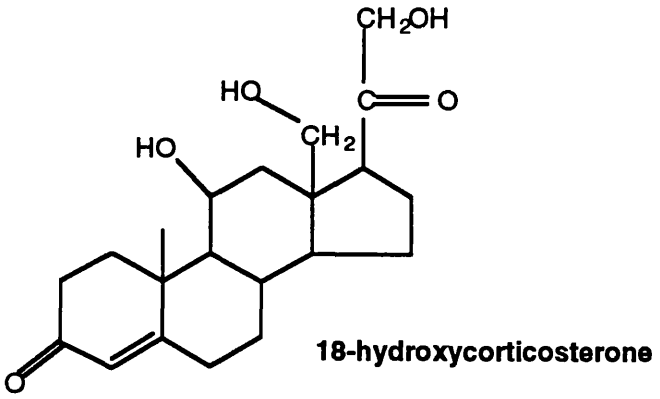
Comparison of the ratio of urinary 18-hydroxycortisol or 18-oxotetrahydrocortisol (18-oxoTHF), the principal urinary metabolite of 18-oxocortisol, and tetrahydroaldosterone (THAldo), the principal urinary metabolite of aldosterone, has been used to aid diagnosis of the condition (Rich et al, 1992, Ulick et al, 1990). In addition to grossly elevated basal urinary excretion of C18-oxygenated cortisol derivatives, urine ratios of 18-oxoTHF:THAldo >3 and 18-hydroxycortisol:THAldo >5 are considered diagnostic of GSH (Rich et al, 1992, Ulick et al, 1990).

Both 18-hydroxycortisol and 18-oxocortisol bear striking structural similarities to 18-hydroxycorticosterone and aldosterone, their 17deoxy- equivalents (Figure 1.9 a). These steroids are derived from cortisol, the obligate precursors of both, following 18-hydroxylation and subsequent dehydration. These steps are identical to those performed by aldosterone synthase in the conversion of corticosterone to aldosterone. Cortisol, however, is synthesised by the zona fasciculata in man, a tissue where aldosterone synthase is not expressed (Curnow et al, 1991). Therefore for synthesis of 18-hydroxycortisol and 18-oxocortisol to occur, cortisol must be formed in cells which also express aldosterone synthase, i.e. ectopic aldosterone synthase gene expression in the human zona fasciculata.

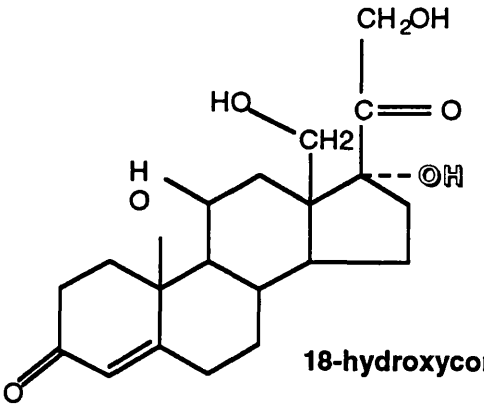
In summary, patients with GSH show excessive secretion of secretion of 18-hydroxycortisol and 18-oxocortisol which is ACTH-responsive. This situation is similar to that of aldosterone secretion in GSH, suggesting that there is ACTH-responsive aldosterone synthase activity in the zona glomerulosa and also in the zona fasciculata.

### 1.9.4 Molecular genetics of GSH

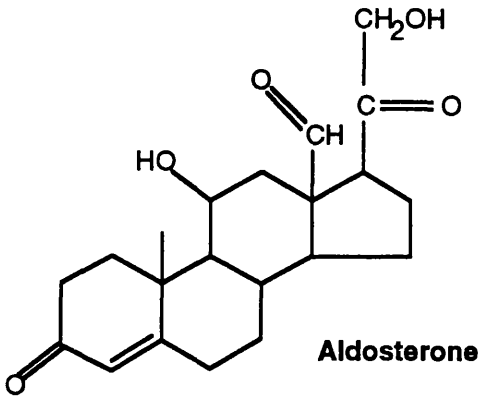
The biochemical and physiological studies of aldosterone secretion in GSH suggest that aldosterone secretion and by inference, aldosterone synthase activity and gene expression, are regulated by ACTH in a manner different to that seen in normal individuals. In addition, the finding of 18-hydroxylated derivatives of cortisol, a zona fasciculata-derived steroid, suggests that ectopic aldosterone synthase activity in the zona fasciculata may be responsible for their formation. Molecular genetic studies have shown that the genes CYP11B1 and CYP11B2 lie in tandem together on the long arm of chromosome 8 (8q22) and that both genes are highly homologous, particularly in their exons. CYP11B1 is regulated *in vitro* by ACTH, has the ability to convert DOC to corticosterone and 11-deoxycortisol to cortisol and is expressed in the zona fasciculata. CYP11B2 is expressed solely in the zona glomerulosa, is regulated by Ang II *in vitro* and performs the conversion of DOC to aldosterone. Thus a mutation involving the control of CYP11B1 and CYP11B2



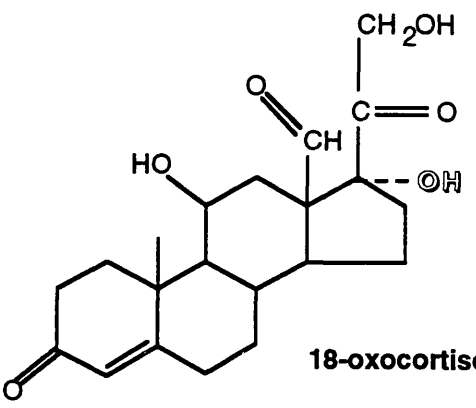

18-hydroxycorticosterone




18-hydroxycortisol




Aldosterone




18-oxocortisol

**Figure 1.9 a Comparison of 17-desoxy-18-hydroxy- and 17-oxy-18-hydroxy-corticosteroids**

expression could give rise to the biochemical and physiological abnormalities in steroid metabolism noted in GSH.

Lifton (Lifton et al, 1992) showed that a mutation involving these genes was indeed the basis for the steroid abnormalities found in GSH. Subjects from a single North American kindred with GSH had hypertension and biochemical abnormalities appropriate to GSH. DNA from these individuals was digested with the restriction enzyme BamHI, and transferred to a nylon membrane by the Southern technique. The membrane was probed with a probe containing exons 3-4 of CYP11B1 and individuals with GSH were shown to possess a novel 6.3kb hybridising species in addition to the two expected normal species (8.5kb - CYP11B1, 4.5kb - CYP11B2). A linkage study in the kindred yielded a maximum lod score of 5.23 for complete linkage of the 6.3kb band and GSH. A densitometry study of the 6.3kb band showed that it had an intensity approximately 50% of that of the bands for CYP11B1 and CYP11B2 suggesting that individuals with GSH have one copy of an additional CYP11B1/B2-like gene in addition to their normal complement of CYP11B1/B2 genes. These findings were explained by postulating that an unequal crossover event between homologous regions of CYP11B1 and CYP11B2, resulting in a chimeric gene with the 5' sequence, including the ACTH-responsive element, of CYP11B1 fused to a coding region from CYP11B2 conferring aldosterone synthase activity on the resultant gene product (Figure 1.9 b, c). Such a gene would be regulated *in vivo* by ACTH and have aldosterone synthase activity. If expressed in the zona glomerulosa, the result would be aldosterone secretion which follows the diurnal rhythm of cortisol and which is sensitive to dexamethasone. The output of aldosterone would be high as ACTH secretion is not suppressed by aldosterone. Thus mineralocorticoid excess would supervene and result in hypertension and the other features of the syndrome. If expressed in the zona fasciculata, cortisol could be converted to 18-hydroxycortisol and 18-oxocortisol in an ACTH-dependent process.

The findings of Lifton were confirmed by Pascoe (Pascoe et al, 1992a) in subjects from 4 unrelated kindreds. Furthermore, Pascoe selectively amplified the chimeric genes of the 4 subjects by a polymerase chain reaction (PCR) method using oligonucleotide primers specific to CYP11B1 and CYP11B2. The region of the suspected crossover was sequenced in the 4 subjects and localised to a region encompassing intron 2 to intron 4 of the chimeric gene. Kindreds had discrete crossover regions although the exact site of the crossovers were not reported. Subsequent findings from other groups have confirmed that all chimeric genes causing GSH so far described have crossover regions lying within the intron 2 to

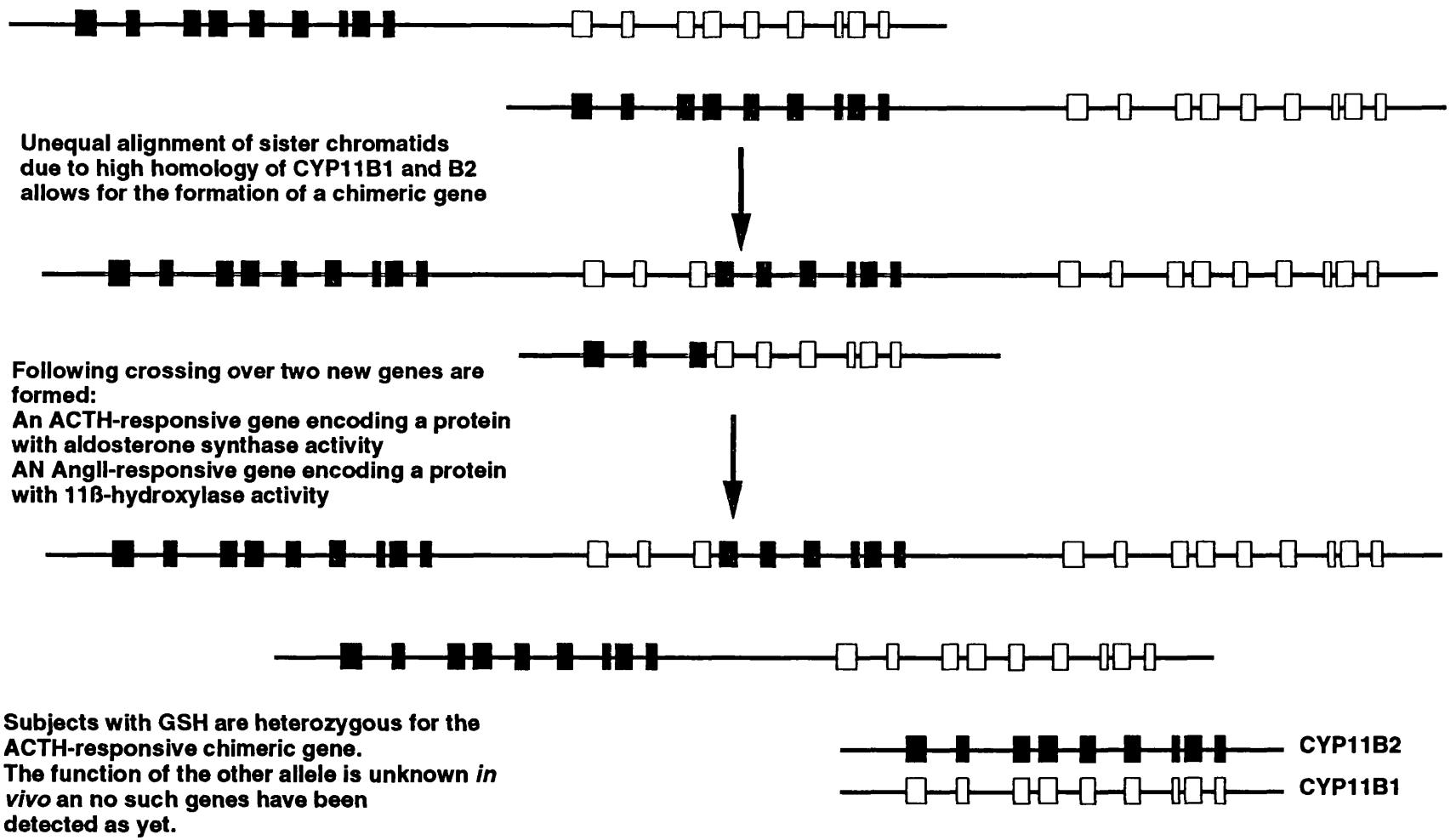



**Figure 1.9 b Formation of a chimeric CYP11B1/B2 gene by unequal crossing over of sister chromatids at meiosis**

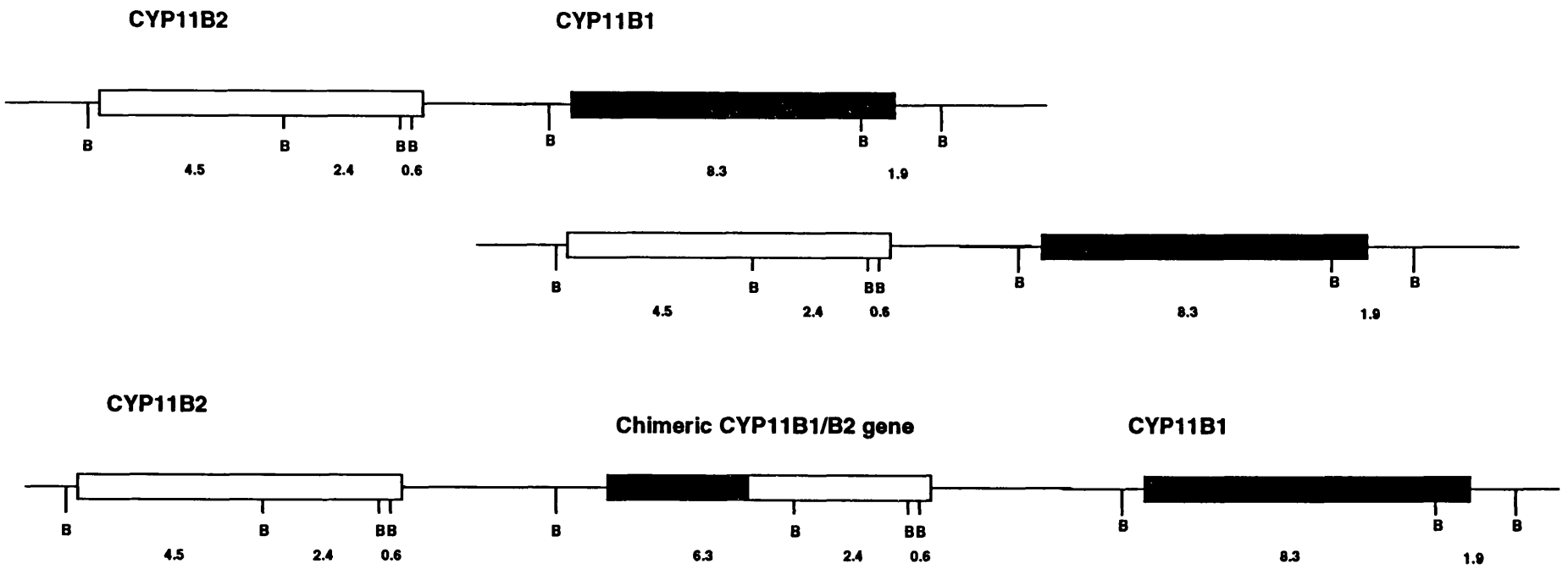


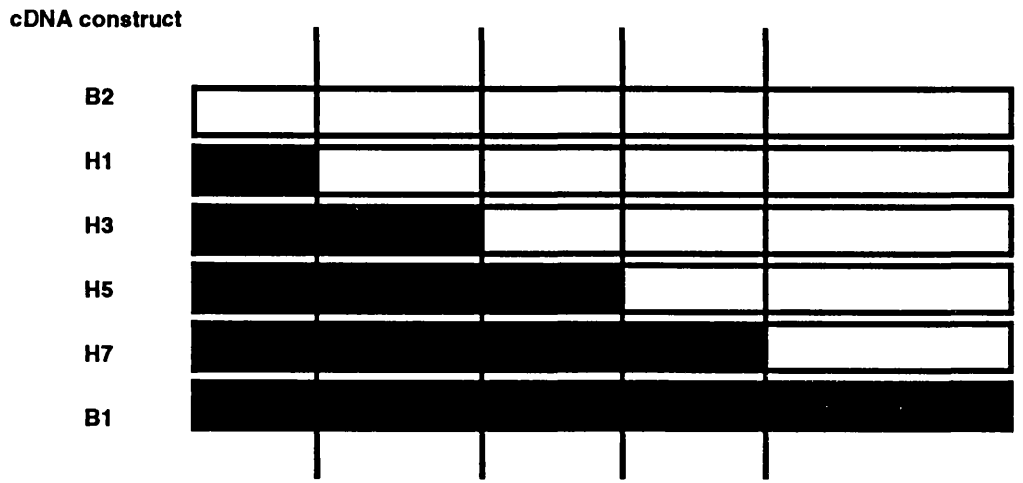

**Figure 1.9 c Origin of restriction fragment length polymorphism during formation of chimeric CYP11B1/B2 gene**

intron 4 region of the chimeric gene and have given details of the site of the crossovers based on direct sequencing (Lifton et al, 1992, Miyahara et al., 1992).

Thus, in all kindred so far identified with GSH, the cause of the condition is the inheritance of a copy of a chimeric 11ß-hydroxylase/aldosterone synthase gene from either parent. No *de novo* mutations have been detected so far and all of the chimeric genes are of similar structure, i.e. they consist of a 5' region of CYP11B1 comprising the ACTH-responsive promoter region an up to and including the first 4 exons coupled to a variable length of CYP11B2 but always including exon 5. This observation has been complemented by *in vitro* studies of the steroidogenic capacity of cDNA constructs in COS cells. These constructs comprised variable proportions of the CYP11B1 and CYP11B2 exonic DNA formed in a manner similar to those found in GSH (Pascoe et al, 1992a). Constructs comprising exons 1-3 of CYP11B1 coupled to the remainder from CYP11B2 possessed aldosterone synthase activity (Figure 1.9 d). Constructs possessing exons 1-5 or more of CYP11B1 had no detectable aldosterone synthase activity, again suggesting that exon 5 of CYP11B1 has residues important for conferring aldosterone synthase activity on the resultant gene product. Steroidogenic ability of cDNA's of CYP11B1 in COS cells can be altered by site-directed mutagenesis. Alteration of a few nucleotides in the CYP11B1 cDNA to change the sequence of exon 5 of CYP11B1 to that of CYP11B2 confers aldosterone synthase activity on the resultant cDNA (Pascoe and Curnow, 1994).

It is not surprising therefore, that GSH is caused by chimeric genes resulting from crossovers limited to the first 4 exons of CYP11B1, given the results of these *in vitro* observations. Why this region of the gene should be so important in controlling the nature of the gene products catalytic powers is not clear. Mutations involving CYP11B1 may result in clinical 11ß-hydroxylase deficiency (see 1.8.2) and only one mutation involving exon 5 of CYP11B1, T318M, has been described (Curnow et al, 1993). However, this mutation modifies an absolutely conserved residue that is thought to be critical for proton transfer to the bound oxygen molecule on the P-450 enzyme (Ravichandran et al, 1993) and results in profound loss of 11ß-hydroxylase activity with no evidence of aberrant aldosterone synthase activity (Curnow et al, 1993).

Another striking observation is the unusual preponderance of GSH kindreds with a Celtic origin. Lifton has reported that of 12 kindreds identified with GSH, 8 had definite or likely Irish ancestry (Lifton et al, 1992). Of the kindreds known throughout the United Kingdom, 6 out of 7 are of Scottish origin and a seventh is




| | % conversion from 11-DOC | | |
|---|---|---|---|
| | B | 18-OHB | Aldo |
| B2 | 30 | 22 | 11 |
| H1 | 21 | 7 | 5 |
| H3 | 28 | 9 | 6 |
| H5 | 8 | 0 | 0 |
| H7 | 13 | 0 | 0 |
| B1 | 74 | 0 | 0 |

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

**Figure 1.9 d Aldosterone synthase activity of chimeric CYP11B1/B2 cDNA constructs *in vitro***

from Northern Ireland. Thus, of 21 kindreds with definite GSH, 15 have a Celtic background, i.e. 71%. One attempt to explain this unexpected susceptibility of Celts was to ascribe it to a founder gene effect. However, detailed sequence analysis has shown that there are at least 7 different chimeric genes n these kindreds, suggesting that a founder effect is unlikely (Lifton et al, 1992) (and Chapter 5). Alternatively, there may be an allele in these populations which favours the formation of chimeric genes (Lifton et al, 1992). A Single-Strand Conformational Polymorphism (SSCP) identified by Lifton, AldoX9, in exon 9 of CYP11B2 was used to test such a hypothesis (Lifton et al, 1992). In 9 pedigrees of Irish/English origin, all possessed allele 1 of the SSCP in the aldosterone synthase gene of the chromosome carrying the chimeric gene, a significant deviation from that expected by chance; 8 out of 9 had allele 1 present in the chimeric gene. Among these 9 kindreds, 4 different crossover sites have been identified and three different haplotypes have been seen, indicating that within this subgroup independent duplications had occurred (Lifton et al, 1992). Although the importance of this finding is unclear in the genesis of the chimeric genes causing GSH it does underscore the importance that the discovery of allelic variation may have on determining the mechanisms responsible for the inheritance of GSH.

## 1.10 Aims of Investigation

Genetic factors are important in the development of hypertension in man. What these factor may be is not yet clear but abnormalities of steroid hormone metabolism may be important. The underlying molecular pathology of GSH has been defined in a number of affected individuals and suggestions regarding the possible pathogenesis of the disorder have been made. In addition, abnormalities of steroid secretion in essential hypertension have been detected which may be important in the aetiology of hypertension and could have a genetic basis.

### 1.10.1 Glucocorticoid-Suppressible Hyperaldosteronism

In Glasgow a number of kindreds with GSH have been identified over the last 15 years. This has been achieved by a combination of clinical observation and biochemical testing.

The purpose of the work described in this thesis was:

1) To assess the viability of using a genetic test to screen kindreds with GSH to detect milder and perhaps asymptomatic cases

2) To determine the precise molecular structure of the chimeric 11ß-hydroxylase/aldosterone synthase gene in these kindreds

3) To assess the role, if any, of a polymorphism in the promoter region of CYP11B2 on the inheritance of GSH

4) To define the biochemical and clinical features, i.e. phenotype, of GSH in these kindreds and relate this to genotypic differences

4) To assess the effect of prolonged hyperaldosteronism on left ventricular mass index in patients with GSH

### 1.10.2 Adrenal Steroid Abnormalities in Hypertension

The aims of this part of the thesis were:

To measure the effect of ACTH-stimulation on steroid secretory responses in normal subjects and patients with essential hypertension, GSH and Conn's syndrome

The findings of the studies are presented in Chapters 3-7 and the conclusions drawn in Chapter 8.

## Chapter 2

## MATERIALS AND METHODS

### 2.1 Materials

All reagents employed were of the highest quality available and were obtained from the following suppliers:

#### 2.1.1 General reagents

The following reagents were obtained from Sigma Chemical Company Ltd., Poole, Dorset, U.K.: type V high gelling temperature agarose, urea, ethidium bromide, mineral oil, dried milk powder, N,N'-methylene-bis-acrylamide, N,N,N,N'-tetramethylethylenediamine (TEMED), Sephadex-G50, mineral oil.

The following reagents were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.: sodium tetradecyl sulphate (SDS), ammonium persulphate, acetic acid (glacial), glycerol, bromophenol blue, Triton X-100.

The following reagents were obtained from FSA Laboratory Supplies, Loughborough, United Kingdom: sucrose, diaminoethanetetra-acetic acid disodium salt (EDTA), Tris hydrochloride, sodium hydroxide, sodium citrate, ammonium acetate, sodium chloride, potassium chloride, magnesium chloride.

The following reagents were obtained from University of Glasgow Chemistry Store: absolute ethanol, absolute methanol, chloroform, hydrochloric acid.

Rathburn Chemicals, Westburn, United Kingdom supplied phenol.

Ciba Laboratories (Horsham, United Kingdom) supplied tetracosactin, 250µg/ml (Synacthen).

Baxter Healthcare Ltd. (Norfolk, United Kingdom) supplied 0.9% sodium chloride.

#### 2.1.2 Molecular Genetic Reagents

The following reagents were obtained from Gibco BRL-Life Technologies, Paisley, United Kingdom: the restriction enzymes BamHI and HaeIII, React1 and React2 buffers, random primer labelling kit (18187-013).

The following reagents were obtained from Promega, Madison, WI, U.S.A.: proteinase K, Taq DNA polymerase, deoxyribonucleotides, T4 polynucleotide kinase, 10x reaction buffers.

Epicentre Technologies, Madison, WI, U.S.A. supplied GELase enzyme preparation.

Sequenase DNA sequencing Kit was supplied by United States Biochemicals, Ohio, U.S.A.

Bethesda Research Laboratories-Life Technologies, Inc, Paisley, United Kingdom supplied: double stranded DNA molecular size standard.

NuSieve GTG Metaphor agarose was obtained from Flowgen, Instruments Ltd, Sittingbourne, UK.

Oswel DNA Service, University of Edinburgh, United Kingdom, supplied oligonucleotides for polymerase chain reactions and DNA sequencing.

Amersham Ltd, U.K. supplied Hybond N+ nylon membrane.

### 2.1.3 Radiochemicals

The following radioisotopes and products were purchased from Amersham International plc, Buckinghamshire, United Kingdom: [$\gamma^{32}$P] adenosine 5'-triphosphate, $^{32}$P deoxycytidine 5' triphosphate, hypercassette (18x24 cm), and hyperscreen intensifying screens (18x24 cm).
New England Nuclear, U.S.A. supplied $^{35}$S - adenosine 5'-triphosphate.

### 2.1.4 Photographic Equipment

Photographs of agarose gels were obtained using polaroid type 667 film (Sigma Chemical Company Ltd., Poole, Dorset, U.K.) in a Polaroid DS34 Instant Print

camera fitted with a Kodak 22A Wratten filter. Autoradiographs were exposed on Hyperfilm NP (Amersham, U.K.)

## 2.2 Blood Samples

Blood samples were taken using Vacutainer needles and bottles supplied by Becton Dickson, Vacutainer Systems Europe, Meylan, Cedex-France.

## 2.3 Apparatus

The following apparutus were obtained as listed:
Laminar flow hood (MDH, Andover, Hampshire, United Kingdom), 11x64mm polystyrene tubes, sterile polyethylene eppendorfs, universals and plastic Pasteur pipettes (standard and fine tipped) (Alpha Laboratories Ltd., Hants., United Kingdom), GNA-100 electrophoresis tanks, teflon combs, glass plates, electrophoresis constant power supply ECPS 3000/150 (Pharmacia Biosystems Ltd., Milton Keynes, United Kingdom), Model S2 sequencing gel apparatus, spacers (Life Technologies Ltd., Paisley, United Kingdom), Beckman DU-70 spectrophotometer (Beckman, Buckinghamshire, United Kingdom), UV Stratalinker 1800 (Statagene Industries Inc., U.S.A.), 316nm UV transilluminator (UVP, United Kingdom), IMED 940A volumetric infusion pump (Imed Corp., California, U.S.A.), Hybaid OmniGene Thermal Cycler (Hybaid Ltd., Teddington, United Kingdom), Maxidry Gel Dryer (Watman Ltd., United Kingdom), Hybridisation oven (Technie S.A., France), mercury sphygmomanometer (Accuson Ltd., United Kingdom).

## 2.4 General Procedures

### 2.4.1 Glassware

All items of glassware were washed in solutions of the detergent decon 75 (Decon Laboratories Ltd., Hove, United Kingdom), rinsed thoroughly with tap water, then distilled water ($dH_2O$) and dried in an oven at $37^0C$.

### 2.4.2 Micropipetting

Solution volumes in the range 0.1-25.00µl were transferred accurately using Rainin EDP-plus electronic pipettes (Rainin Instrument Co., Inc., Woburn, USA). Volumes

in the range 25-1000µl were transferred reproducibly using Finnpipettes (Labsystems, Helsinki, Finland).

### 2.4.3 pH measurement

Measurements of pH were performed using a digital pH/temperature meter obtained from Electronic Instruments Ltd. (United Kingdom). The apparatus was regularly standardised with a solution of pH 7 prepared from buffer tablets.

### 2.4.4 Centrifugation

Forces of up to 3500 r.p.m. (1500 g) were attained using a Damon/IEC division DPR-6000 centrifuge purchased from International Equipment Company (Bedfordshire, United Kingdom). Microcentrifugation at 13000 r.p.m. (100 g) was performed using a Microcentaur centrifuge (MSE Ltd., United Kingdom).

## 2.5 Clinical Methods

### 2.5.1 Blood Pressure Measurement

Blood pressure was measured in supine, rested individuals. Three measurements were taken over 30 minutes and the mean taken. Blood pressure was measured using a standard mercury sphygmomanometer and inflatable bladder and cuff, sized appropriately for each patient. Korotkoff phase V was taken as the diastolic pressure.

### 2.5.2 Blood sampling

Blood samples were obtained via direct puncture of a forearm vein in the absence of an occlusive tourniquet using the *Vacutainer* system. Blood for renin estimation and DNA extraction was taken into chilled 4.5ml bottles containing potassium EDTA (0.054ml of 0.34M EDTA $K_3$) as anticoagulant. Blood for aldosterone, potassium and steroid assays was taken into chilled 7ml bottles containing lithium heparin (143 USP units) as anticoagulant. Plasma was separated and stored at -20$^0$C until assayed.

### 2.5.3 Basal State Sampling

Basal blood samples were obtained via an indwelling intravenous cannula (23G intavenous cannula, Becton Dickson, Meylan, Cedex-France) using the *Vacutainer* system. Subjects forearm veins were cannulated 14 hours before sampling and the subjects lay supine overnight prior to sampling at 9.00am.

### 2.5.4 Sampling During Continuous Intravenous Infusion

During a continuous intravenous infusion, blood was sampled from the arm contralateral to the infusion at 15 minute intervals from 30 minutes prior to commencing the infusion until the completion of the infusion.

### 2.5.5 Intravenous ACTH Infusion

A graded dose intravenous infusion of ACTH was administered to individuals in a quiet environment at 9.00am following an overnight fast and recumbency. An intravenous cannula was placed into a vein in both forearms and basal blood samples taken at -30, -15 and 0 minutes. Blood pressure and heart rate were measured at the time of blood sampling. At time 0 minutes, determined by a settled heart rate < 75 beats/minute, an intravenous infusion of ACTH was commenced. The infusion solution was prepared in the following way:

| | |
|---|---|
| 2ml of ACTH (250μg/ml) added to 498ml 0.9% NaCl | Solution A |
| 50ml of Solution A added to 450 ml of 0.9% NaCl | Solution B\ |

Solution B (0.1μg/ml ACTH) was administered by continuous intravenous infusion using an IMED 800 pump in incremental doses: 0.1μg/kg/min, 1.0μg/kg/min, 10.0μg/kg/min, for 30 minutes at each dose. Blood was taken and blood pressure and heart rate measured at 15 minute intervals until the end of the 90 minute infusion.

### 2.5.6 Echocardiographic Measurements

Echocardiograms were performed by a single operator using an Accuson 128 PX ultrasound machine with a 3.75MHz transducer (Accuson Ltd, USA). Patients were placed in the partial ($45^0$) left lateral decubitus position with $30^0$ of upright tilt.

Echocardiographic measurements for the calculation of left ventricular mass were taken from standard M-mode echocardiograms obtained from the left parasternal short-axis view.
Left ventricular mass was calculated using the Penn convention formula and corrected for body surface area to deduce the left ventricular mass index (LVMI) for each individual (Devereux and Reichek, 1977).
Body surface area was calculated from the formula of Du Bois and Du Bois (Du Bois and Du Bois, 1916).

## 2.6 Biochemical Assays

### 2.6.1 Aldosterone

Plasma aldosterone concentrations were measured using a solid phase (coated tube) radioimmunoassay (Diagnostic Products (UK) Ltd). the coefficient of variation is <8.3% and the limit of detection 1ng/dl.

### 2.6.2 Renin

Plasma active renin concentration was determined using an antibody trapping technique developed in the MRC Blood Pressure Unit, Western Infirmary, Glasgow (Millar et al., 1980). The coefficient of variation is 3.4% and the limit of detection < 0.5μU/ml.

### 2.6.3 Potassium

Plasma potassium samples were assayed in the routine clinical biochemistry laboratory, Gartnavel General Hospital, Glasgow, using an indirect ion selective electrode on an Olympus AU5200 Analyser (Olympus Optical Co. (UK) Ltd., Eastleigh, United Kingdom).

### 2.6.4 Steroid assays

Plasma cortisol was measured in the routine clinical biochemistry laboratory, Gartnavel General Hospital, Glasgow, using a coated tube radioimmunoassay (Diagnostic Products (UK) Ltd).

Plasma concentrations of 11-deoxycorticosterone (DOC), corticosterone, 11-deoxycortisol and 18-hydroxycorticosterone were measured in the Steroid Laboratory of the MRC Blood Pressure Unit, Western Infirmary, Glasgow by radioimmunoassay after extraction and partial purification by paper chromatography (Belkien et al., 1980, Fraser et al., 1975). Samples (2ml) to which had been added $^{3}H$-steroid standards had been added were extracted with freshly distilled dichloromethane and the extract evaporated to dryness under nitrogen at 30$^{0}$C. The residues were chromatographed on paper (Whatman 2) using a volatile system and the steroid regions located by isotope scanning and eluted in methanol. Aliquots were assayed using $^{3}H$-steroids and antisera raised in rabbits to corticosterone-3-carboxymethyloxine conjugated to bovine serum albumin. Bound and free steroid were separated using the dextran-coated charcoal method (Fraser et al, 1975).

## 2.7 Genomic DNA extraction

### 2.7.1 DNA extraction from blood

Genomic DNA was extracted from peripheral blood samples (fresh and frozen) using a variation of the method of Sambrook (Sambrook et al., 1989).
Ten millilitres of EDTA-preserved whole blood was placed in a Universal tube and 40 ml of cell lysis mix (Appendix I) added. Tubes were left on ice for 10 minutes before centrifuging at 2800 r.p.m. for 10 minutes at 4$^{o}$C. The resulting pellet was resuspended in 3 ml of nuclei lysis mix (Appendix I), and 200µl 10% SDS and 100µl proteinase K (10mg/ml) were added. Tubes were incubated overnight at 37$^{o}$C. After incubation, 1 ml of 6M NaCl was added with vigorous shaking, then 5 ml of phenol:chloroform:isoamyl alcohol (25:24:1), pH aqueous phase >7.6, was added and the tubes spun at 2800 r.p.m. for 20 minutes at 4$^{o}$C. The upper aqueous phase of the supernatant was then transferred to a fresh universal container and two volumes of absolute 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$^{o}$C.

### 2.7.2 Determination of DNA concentration

The concentration of each DNA sample was determined by measuring the optical density of samples at 260nm. One microlitre of the DNA solution was added to 1ml $dH_20$ in a quartz cuvette and the $OD_{260}$ measured in a dual beam

spectrophotometer with a deuterium lamp. An optical density of 1 corresponds to 50mg/ml of DNA.

## 2.8 Southern Blot Methods

### 2.8.1 Preparation of Agarose Gels

Agarose (0.8-5% w/v) was added to 100 ml EB buffer, shaken and heated in a 650W microwave oven for 120 seconds. Whilst cooling, 1µl of ethidium bromide was added to the agarose, the agarose shaken and poured into gel moulds with teflon combs. The gel was allowed to set for 1 hour at 4°C before the combs were removed.
Gels were placed in standard electrophoresis tanks containing 100ml EB buffer and connected to a constant power source.

### 2.8.2 Restriction enzyme digestion of genomic DNA

Ten micrograms of DNA was placed in a 1.5ml Eppendorf tube with 1µl of high concentration BamHI restriction enzyme (50U/ml) and 1µl of 20X buffer (Appendix II). $dH_2O$ was added to make the final reaction volume 20µl. Digests were incubated at 37°C for 2 hours and a further 2µl of high concentration enzyme were added and the mixture incubated overnight. The completeness of each digestion was determined by electrophoresis of 10µl of each reaction mix in a 1.0% agarose gel.

### 2.8.3 Restriction enzyme digestion of PCR products

Eighteen microlitres of each PCR product were placed in a sterile 1.5ml Eppendorf tube together with 1µl of 10x buffer (React 2) and 1µl of HaeIII enzyme (10U/µl) and the reaction mixture incubated for 4 hours at 37°C. The completeness of each digestion was determined by electrophoresis of 10µl of each reaction mix in a 3.0% Metaphor agarose gel.

### 2.8.4 Agarose gel electrophoresis for Southern Blotting

DNA was resolved on agarose gels containing 0.05% ethidium bromide (10mg/ml). Ten microlitres of digest was added to 5µl of loading mix (Appendix II) in a sterile Eppendorf tube and then placed into wells by pipetting and electrophoresed at

200mA (200V) for 5 hours until the digest was fully resolved. One well of the gel contained 10µl of DNA size marker with 5µl of loading mix. Gels were visualised on an UV transilluminator (316nm) and photographed.

### 2.8.5 Southern Blotting

Southern blotting was carried out using a modification of the method of Southern (Southern, 1975). Gels were placed in a bath containing 0.25M HCl for 45 minutes to allow depurination to occur. Completion of this step was gauged by the colour change of the marker dye from blue to yellow. Next the gel was rinsed with $dH_2O$ and placed in 0.4M NaOH for 45 minutes to denature the digested DNA. Denaturation was deemed complete when the gel dye marker changed from yellow to blue. The gel was then transferred to the blotting apparatus.
A wick made from 0.4M NaOH-soaked 3mm Whatman paper was placed on top of a sheet of glass with its ends reaching the surface of an underlying tank containing 0.4M NaOH. The gel was then placed on top of the Whatman paper base up, rinsed with 0.4M NaOH, and a precut piece of Hybond N+ hybridisation membrane placed on the surface of the gel. Care was taken to remove all air bubbles from the gel-membrane interface. Two pieces of 3mm Whatman paper cut to the same size as the membrane and soaked in 0.4M NaOH solution were placed on the surface of the membrane. Paper towels to height of 15cm were placed on top of the Whatman paper, and a glass plate with a 1kg weight placed on top of the towels. The gel was left for 16-24 hours to allow full transfer of DNA to the membrane. When DNA transfer was complete, the membrane was removed, rinsed in 2X SSC and exposed to short wave ultraviolet light (340 nm) in a UV Stratalinker for 30 seconds to allow fixation of the DNA to the filter.

### 2.8.6 Blotting of low molecular weight DNA

The technique was the same as that for the transfer of high molecular weight DNA except that transfer took place using 6XSSC/1%SDS as the transfer buffer (Curnow et al, 1993).

### 2.8.7 Prehybridisation of membranes

Membranes were placed in a glass hybridisation cylinder with 10 ml of pre-hybridisation solution: 6XSSC/1%SDS/Dried milk powder 40mg/100mg heat denatured salmon sperm DNA, and placed in a rotatory oven at 65$^0$C for one hour

to prevent non-specific hybridisation of the probe to the membrane. At the end of the pre-hybridisation period the pre-hybridisation solution was removed from the glass cylinder and replaced with 15 ml hybridisation solution and the radiolabelled probe.
For transfer of low molecular weight DNA molecules to membranes no pre-hybridisation step was performed as the subsequent washing stages were so stringent that non-specific hybridisation was eliminated (Curnow et al, 1993).

### 2.8.8 Radioactive labelling of DNA probes

Labelling of DNA probes for hybridisation to membranes was performed using the random primer method for large probes, i.e. greater than 500 basepairs, or end labelling of synthetic oligonucleotides using polyadenylate kinase.
Random primer labelling of probes was carried out using a standard kit (Random Primer Labelling System, Gibco BRL) in a regulation radioactivity hood. The method relies on the ability of the hexanucleotide random primers supplied with the kit to hybridise with a complimentary sequence in the probe DNA. Following hybridisation the Klenow fragment of DNA polymerase uses the available dNTP's, including, $^{32}$P-dCTP to extend the oligonucleotide and create a new molecule of the template DNA with $^{32}$P labelled dCTP incorporated into it.
Probe was prepared by PCR amplification of genomic DNA using oligonucleotide primers 3 and 5 as described in 2.9.2.
The PCR product was run on a 0.8% agarose gel to confirm the presence of the desired fragment, and then cut out of the gel using a sterile scalpel blade. The gel fragment was weighed and placed in a 1.5ml Eppendorf tube with 2 volumes of $dH_2O$ and boiled for 20 minutes to ensure both the adequate solubilisation of the agarose and that the probe DNA was in the single stranded state. The optical density of the probe DNA was determined and then 25 ng of probe DNA (volume approximately 20µl) was added to an Eppendorf tube containing:

2µl each of dATP, dGTP, dTTP (0.5 mM)
5µl $^{32}$P labelled dCTP (0.5 mM strength, 1.85 MBq)
1µl Klenow fragment of DNA polymerase (3 U/µl)
15µl of random primer buffer mix
3µl $dH_2O$ to make the total reaction volume 50 µl.

The mixture was incubated at 37$^0$C for one hour. The solution containing the labelled probe was then passed through a Sephadex G50 column primed with

1XSSC/0.1%SDS and the eluate collected in eppendorfs. When the count of the column eluate rose above 1000 cpm, the eluate was collected until the count fell back below 1000 cpm. This eluate contained probe with the highest specific activity and was used for hybridisation.

### 2.8.9 Preparation of end-labelled oligonucleotides

Oligonucleotide probes were prepared by end-labelling them with [$\gamma^{32}$P] ATP. The reaction utilises the enzyme T4 polyadenylate kinase to covalently bond the $\gamma^{32}$-phosphate from ATP to the 5'-terminus of polynucleotides bearing a 3'-phosphate group.
The labelling reaction was carried out as follows:

4µl of oligonucleotide (10pmol/ml) was added to 25µl of $dH_2O$ and heated to 80°C for 2 minutes. Five microlitres of reaction buffer and 15µl of $\gamma^{32}$PATP are added with 1.5µl of T4 polyadenylate kinase and the reaction mix heated to 37°C for 30 minutes and then to 65°C for 10 minutes. The end-labelled oligonucleotide is stored at -20°C for up to one week prior to use.

### 2.8.10 Probe hybridisation

Labelled probe was added to the glass hybridisation cylinder containing the membrane, 20ml of hybridisation solution (6XSSC/1%SDS/0.25% fat-free skimmed dried milk) and 20µl of labelled probe and placed in a rotary oven at 65°C. Genomic DNA blot hybridisation took 18 hours, whilst blots of PCR products required only 4 hours at 42°C.

### 2.8.11 Filter washing and autoradiography

After hybridisation, genomic DNA filters were washed for 10 minutes in 2XSSC/0.1%SDS at room temperature, 10 minutes in 1XSSC/0.1%SDS at room temperature, 10 minutes in 0.5XSSC/0.1%SDS at 65°C, and in 0.1XSSC/0.1%SDS at 65°C for 10 minutes or until the background radioactivity of the membrane was 10 cpm.
Membranes blotted with PCR products were washed in 6XSSC/1%SDS for 40 minutes at 58°C, ASO - 2360, or 56°C, ASO - 2361. The substitution of C/G (2360) for a T/A (2361) leads to a 2°C reduction in the melting temperature of the ASO. This meant that at 58°C 2361 is stripped from the filter whilst 2360 will still

remain hybridised and thus allow alleles to be differentiated on this basis (5.2.1) (Curnow et al, 1993, Kogan et al., 1987, White et al, 1991).
Membranes were then wrapped in clingfilm, placed in a X-ray cassette with Hyperfilm MP and left at -70$^0$C for 48 hours. Autoradiographs were developed in a Kodak X-Omat automated developer.

## 2.9 Polymerase Chain Reaction amplification of DNA

### 2.9.1 Preparation of synthetic oligonucleotides

Synthetic oligonucleotides were designed from the published sequences of CYP11B1 and CYP11B2 and obtained from a commercial source (2.1.2).

### 2.9.2 Polymerase chain reaction amplification of genomic DNA

Amplification of genomic DNA to provide probes for Southern Blot procedures or amplification of specific chimeric sequences to allow determination of DNA sequences was performed using the polymerase chain reaction (PCR) method. Genomic DNA extracted from the blood of affected individuals or DNA extracted from the placenta of one affected individual was used as the reaction template. Reactions were carried out in sterile polypropylene Eppendorf tubes. A standard reaction protocol was used for amplification reactions although the template and oligonucleotide primers were altered according to the requirements of the amplification.
A standard reaction mix was prepared as follows:

A 1µl (approximately 50ng/µl) aliquot of the 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:

| | |
|---|---|
| $dH_2O$ : | 34.5µl |
| dNTP's: | 1µl each of dATP, dCTP, dGTP, dTTP (10mM strength) |
| Primer No. 1: | 2.5µl (10pM/µl) |
| Primer No. 2: | 2.5µl (10pM/µl) |
| 10X Buffer : | 5µl |
| Taq Polymerase: | 0.5µl (5U/µl) |

Taq Polymerase, a thermostable DNA polymerase, was used to perform the amplification reactions and was added last to the pre-mix directly from storage at -20°C, immediately prior to adding an aliquot of the pre-mix to the DNA. Reaction mixtures were overlain by 50µl of mineral oil , and placed in a thermal cycler. The heating block temperature was raised to 94°C for 60 seconds, to allow complete denaturation of the DNA template to occur prior to carrying out the PCR protocol. The amplification protocol used was as follows:

Step 1: 94°C for 60 seconds
Step 2: 65°C for 60 seconds - primer annealing
Step 3: 72°C for 120 seconds,
increasing by 5 seconds for each
subsequent cycle -primer extension

Steps 1 to 3 were repeated for 35 cycles, and followed by a final extension step of 72°C for 15 minutes.
The total reaction time was approximately 4 hours 30 minutes.

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

### 2.9.3 Purification of PCR products from agarose gels

To use the PCR product for DNA sequencing, it must be obtained in a pure state free from any other DNA or oligonucleotides. PCR products were purified using the GELase system.
PCR products were loaded onto an ethidium bromide stained 1% low melting point agarose gel (SeaPlaque GTG agarose, FMC Bioproducts, U.S.A.) and electrophoresed for 3 hours at a low current (60mA) to allow for adequate separation of the PCR product from all other reaction constituents. Bands were visualised using a long wave ultraviolet light source and removed from the gel using a sterile scalpel and forceps. Gel slabs were then placed in sterile Eppendorf tubes, weighed and 3 volumes of GELase buffer added to the tube. The mixture was left at room temperature for 1 hour and the buffer then removed by pipetting. The tube containing the gel was then placed in a water bath at 70°C for 20 minutes until molten, spun briefly in the microcentrifuge and then placed in a second water bath at 45°C and the GELase enzyme added.

600mg of agarose was added to 1U of enzyme (on average each gel slice weighed 150mg) and the reaction incubated at 45°C for 1 hour. Following incubation, the volume of the mixture was determined by pipetting, 1 volumes of 5M ammonium acetate and 2 volumes of absolute ethanol added to precipitate the DNA and the total mixture was centrifuged for 35 minutes at 3500 r.p.m. and 4°C. The supernatant was removed by pipette, 500µl of 70% ethanol was added and the tube centrifuged for a further 4 minutes. The 70% ethanol was carefully removed and the sample air dried. The DNA pellet was re-suspended in 20µl of 1X TE buffer ready for use in sequencing reactions.

## 2.10 Direct DNA sequencing from PCR products

### 2.10.1 DNA sequencing using the Sequenase System

Determination of nucleotide sequences was performed on PCR-amplified DNA by a modification of the chain termination method using the commercially available Sequenase Kit (United States Biochemicals, Ohio, U.S.A.). Dimethyl sulphoxide (DMSO) was included to enhance the sequencing reaction by improving hybridisation of the primer to the template DNA (Winship, 1989). Details of buffers are in Appendix II.

The chain termination method of DNA sequencing involves the synthesis of a DNA strand by a DNA polymerase *in vitro* using a single-stranded DNA template. Synthesis is initiated at the site of hybridisation of an oligonucleotide of known sequence, the so-called "sequencing primer". The sequencing primer 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 support further elongation. Such analogues include the 2',3'-dideoxynucleoside 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 the labelled chains of varying length can be visualised by autoradiography after high resolution electrophoresis.

### 2.10.2 Sequencing Protocol: A) Annealing

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

a) 4 tubes containing 2.5µl of each of the four ddNTP's in a termination mix were placed in a bath at 37°C (for 2.10.3, b),

b) 4 tubes were prepared containing the following:

| | | |
|---|---|---|
| DNA | 5µl | (approximately 1µg) |
| $H_2O$ | 2µl | |
| DMSO 25% | 1µl | |
| Sequencing Primer | 1µl | |
| Total Volume | 10µl | |

All four were placed in a water bath at 98°C for 120 seconds, and then spun briefly. Tubes were then allowed to cool to room temperature over 10 minutes, 2µl of Reaction Buffer was added and the tubes spun and left at room temperature.

2.10.3 Sequencing Protocol: B) Labelling and Termination Reactions

The labelling reaction was perfomed as follows:

a) To 10µl of annealed DNA mixture was added:

| | |
|---|---|
| DTT, 0.1M | 1µl |
| Diluted labelling mix | 2µl |
| [$^{35}$S] dATP | 1µl |
| Diluted Sequenase | 2µl |

The reaction mixture was spun briefly and left at room temperature for 5 minutes.

b) After incubation at room temperature, 3.5µl of labelling reaction mixture was added to each tube containing the termination mix and incubated at 37°C for 5 minutes. 4µl of Stop Solution were then added to each tube and the mixture kept at -20°C until run on a polyacrylamide sequencing gel.

2.10.4 Polyacrylamide gel preparation

Polyacrylamide gels were prepared using a modification of a standard protocol. Two glass plates were siliconised and left to dry in air with the teflon spacers in place. A mixture for a Polyacrylamdide : Urea 6% gel was prepared as follows:

| | |
|---|---|
| Acrylamide:bisacrylamide (19:1) | 6g |
| Urea | 62.8g |
| 10xTE buffer | 13.7ml |

were mixed together and the volume adjusted to 100ml with $dH_20$ and filtered through 3mm Whatman paper. To form a seal at the base of the gel, 3ml of the acrylamide:urea mix were removed and 30µl of 10% ammonium persulphate and 1µl of TEMED added. This mixture was then poured into the gel plates and allowed

to set. The remainder of the acrylamide:urea mix was polymerised by adding 1ml of 10% ammonium persulphate and 25µl of TEMED, and poured into the plates. Air bubbles were removed, the well combs inserted into the top of the gel and the gel allowed to set for approximately 3 hours at a $10^0$ angle to the horizontal.

### 2.10.5 Polyacrylamide gel electrophoresis

Once the sequencing gel was ready for use, it was placed on the electrophoresis apparatus and secured by side clamps. The lane combs and spacers were removed and the gel heated to 45°C. The lanes were cleared of bubbles by a fine-tipped Pasteur pipette and the loading combs replaced.

For each lane of the gel, 3.5µl of the terminated sequencing mix were placed in a well. Four reactions were run in parallel on the gel for each sequencing primer in the order: GATC allowing the complete sequence of the DNA to be determined. Gels were run for approximately 60 minutes at 55-70 Watts, ensuring that the surface temperature of the gel did not rise above 50°C. For reading of sequences over 300 bases from the sequencing primer, gels were run for approximately 90 minutes.

### 2.10.6 Preparation and autoradiography of sequencing gels

After completion of the electrophoresis, gels were soaked in 5% glacial acetic acid/ 5% absolute methanol (approximately 2 litres) in a flat tank for 30 minutes and then washed in wated for 10 minutes. Washed gels were then absorbed onto 3mm Whatman paper, covered in clingfilm and then dried for 60 minutes. The clingfilm was removed and the dried gels were placed in a photographic cassette in direct contact with the emulsion side of the photographic film and left at -70°C for 16 hours. Following overnight exposure, autoradiographs were developed in a Kodak X-Omat.

### 2.10.7 Determination of DNA sequence from polyacrylamide gels

Sequences were determined by direct reading of the autoradiograph and comparison of the deduced sequence with the consensus sequence of the CYP11B1 and CYP11B2 genes.

## 2.11 Data and DNA sequence analysis

Calculations of statistical significance were performed using Minitab Statistical Software, Release 8.21 for the Macintosh (Minitab, Philadelphia, U.S.A.) Analysis of DNA sequences was performed using the GCG (Genetics Computer Group) sequence analysis software package (8.1) (University of Wisconsin, U.S.A.) on the University of Glasgow mainframe computer.

## Chapter 3

# GENOTYPIC IDENTIFICATION OF SUBJECTS WITH GLUCOCORTICOID-SUPPRESSIBLE HYPERALDOSTERONISM

## 3.1 Introduction

As stated previously, individuals with GSH are at increased risk of developing premature cerebrovasular disease. In addition, given the possible difficulties in diagnosing GSH, in particular lack of clinical suspicion and variable phenotype of GSH, the use of a screening test to detect affected individuals is of considerable importance. Such an investigation would require the test to have 100% specificity and sensitivity, i.e. all individuals with GSH should be detected and only individuals with GSH should be detected, i.e. no false negatives or positives. Two possible means of performing such a screen are 1) the use of plasma measurements or 24 hour urine collections to assess urinary excretion of 18-hydroxycortisol, 18-oxocortisol or determination of the 18-oxotetrahydrocortisol: tetrahydroaldosterone ratio (Connell et al, 1986, Gomez-Sanchez et al, 1987, Ulick et al, 1990); 2) the use of a simple genetic test on a single blood sample to identify affected individuals on the basis of the inheritance of a chimeric CYP11B1/B2 gene. The former method has been used in a single North American kindred to screen relatives of affected probands for the presence of GSH (Rich et al, 1992). It has been shown to be effective but has several limitations; 1) the need for accurate 24 hour urine collections, 2) assay of urinary steroids which are not widely available, 3) interpretation of results at the extremes of the age range where reference ranges are not well established. Thus, the use of genetic method with 100% specificity for the diagnosis of GSH (Lifton et al, 1992) which can be performed on a single blood or tissue sample would be a considerable advance.
In order to assess the utility of the genetic method in detecting cases of GSH, a study of genotyping of the extended kindreds of five probands with GSH was performed.

## 3.2 Methods

### 3.2.1 Subjects

Subjects from five apparently unrelated kindreds were studied.

Probands from four kindreds, kindreds 1,2,3 and 5, were patients under the care of Professor JMC Connell, MRC Blood Pressure Unit, Glasgow.

The largest kindred, kindred 1, were natives of the Isle of Lewis, Outer Hebrides, Scotland and have been geographically immobile for at least six generations. The members of kindred 2 are inhabitants of South Ayrshire, Scotland, but can trace their ancestry through a maternal grandmother who originally came from the Isle of Lewis. However, no connexion between kindreds 1 and 2 could be detected on close questioning of the kindred members. The members of kindred 3 originate from the Glasgow area in central Scotland and can trace no relatives who have lived outwith this region of Scotland. The members of kindred 5 originate from the Thurso region of Northern Scotland, also have no known antecedents from the Hebridean Islands or from central Scotland, and have been geographically immobile for 7 generations.

The proband of the fifth kindred, kindred 4, was under the care of Dr PM Stewart, Queen Elizabeth Hospital, Birmingham. This kindred originated in Belfast, Northern Ireland and subsequently migrated to the West Midlands area of England in the 1950's.

The five probands had been diagnosed as having GSH on clinical and biochemical grounds (Chapter 6). The kindreds of the 5 probands were extended as far as possible and subjects tested for the presence of GSH by genotyping of DNA extracted from stored whole blood.

### 3.2.2 DNA Preparation and Southern Blotting

Genomic DNA was prepared from EDTA-preserved blood and stored as described in 2.7.1. An aliquot of DNA was then digested with the enzyme BamHI, subjected to agarose gel electrophoresis and blotted onto a nylon membrane as described in 2.8.2, 2.8.4 and 2.8.5.

Placental DNA obtained at birth was used for the diagnosis of GSH in one individual (1-3-8). Chorionic villi were cut into manageable pieces, homogenised manually and subjected to the same extraction process as whole blood (2.7.1.)

### 3.2.3 Probe Preparation and Membrane Hybridisation

A $^{32}$P-labelled probe was prepared as described in 2.8.8 by PCR amplification of genomic DNA from a normal control using oligonucleotides 1 and 2 (Appendix III) as described in 2.9.2. This primer pair amplify a 800 bp region spanning exons 2-5 of CYP11B1.

Membranes were pre-hybridised as described in 2.8.7 and the probe hybridised to the membrane as described in 2.8.10.

### 3.2.4 Autoradiography

Hybridised membranes were washed, wrapped in clingfilm, placed in an X-ray cassette with Hyperfilm MP and left at -70$^0$C for 48 hours. Autoradiographs were developed in a Kodak X-Omat automated developer as described in 2.8.11.

## 3.3 Results

Studies of the families of the five probands resulted in the identification of 60 subjects related to the 5 probands who were regarded as suitable for genotyping. There was no apparent connexion between any of the kindreds following detailed pedigree analysis.

Figures 3.3 a-e show the pedigrees of the five kindreds with the subjects genotyped highlighted.

Figures 3.3 f and 3.3 g show a typical result of Southern blots of subjects from the 5 kindreds.

Affected individuals possess three hybridising species on the Southern Blot: a 8.4 kb species corresponding to CYP11B1, a 4.2 kb species corresponding to CYP11B2 and a third 6.3 kb band corresponding to the chimeric CYP11B1/CYP11B2 gene. Unaffected subjects exhibit only two species corresponding to the normal CYP11B1 and CYP11B2 genes.

In total, genotyping of kindred members by this method identified 19 individuals with GSH in the five kindreds studied. Nine subjects were identified as having GSH with no clinical or routine biochemical evidence suggestive of hyperaldosteronism, i.e. hypertension or hypokalaemia.

Included in this group was a newborn infant from kindred 1. GSH was diagnosed on the basis of a positive result from a Southern Blot of chorionic villous DNA obtained from the placenta directly after delivery, stored in TE buffer and transferred by air to our unit. This is the first time such an observation has been made and would appear, at present, to be unique (Jamieson et al, 1994).

The relevance of the relationship between the clinical and biochemical indices of the 19 patients with GSH is discussed in full in Chapter 6.

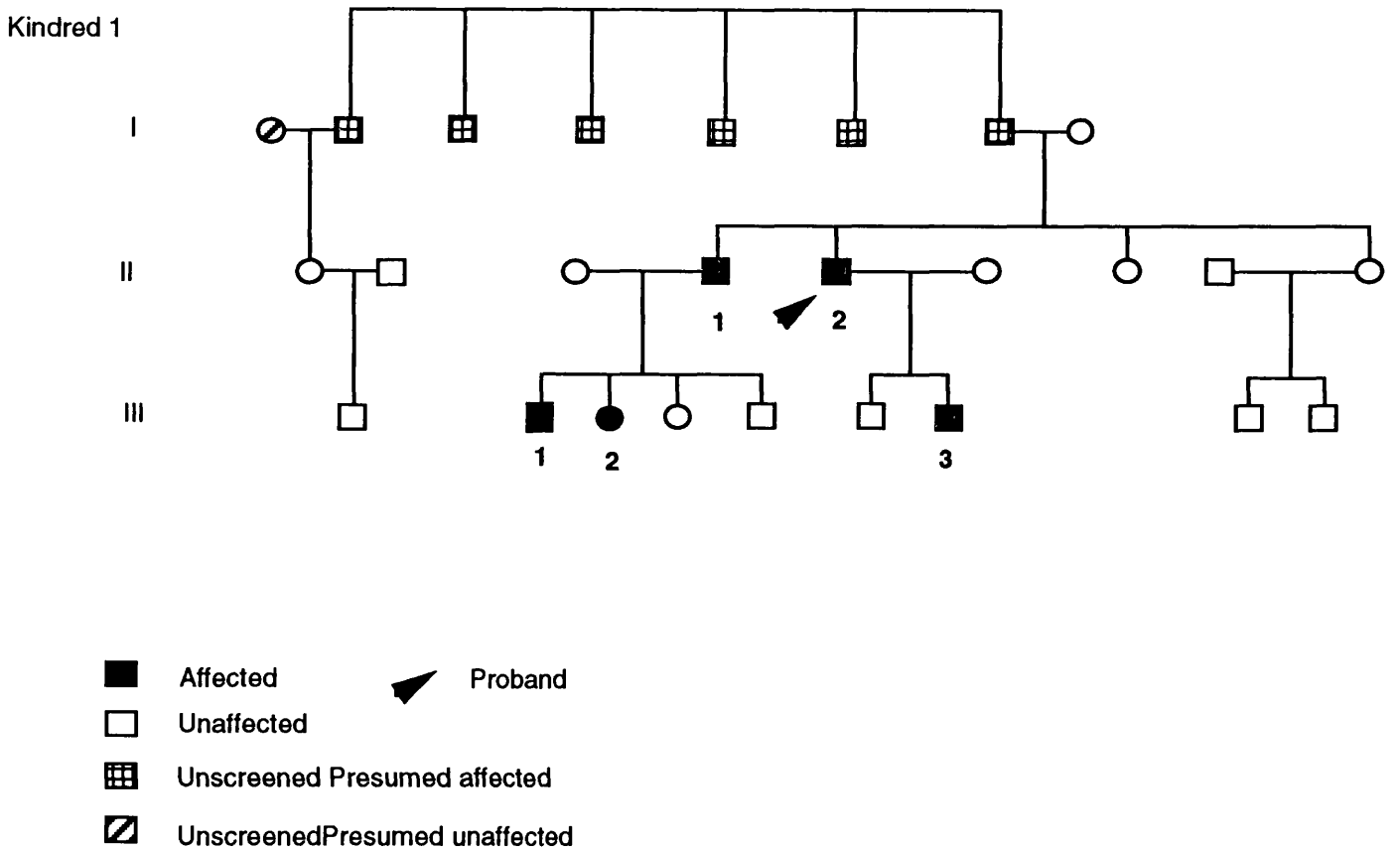
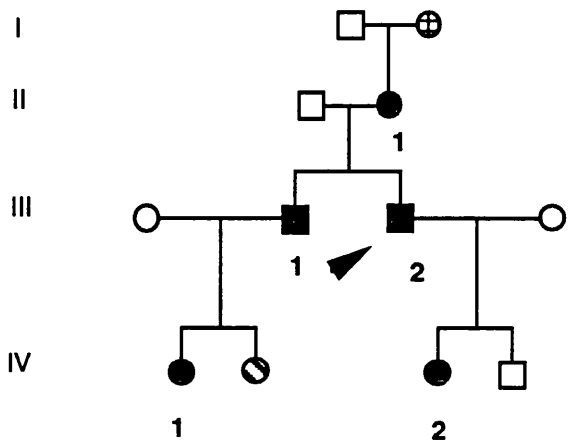



**Figure 3.3 a Kindred 1.**

**Affected individuals are identified by a three part identifier, i.e. Kindred Number-Generation-Member.**

**For example the proband of this kindred is 1-II-2.**

Kindred 2



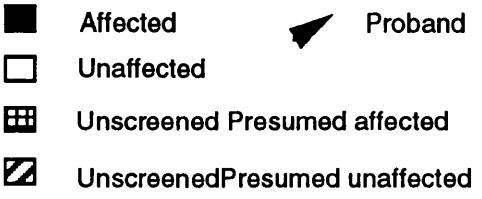




**Figure 3.3 b Kindred 2.**

**Affected individuals are identified by a three part identifier, i.e. Kindred Number-Generation-Member.**

**For example the proband of this kindred is 2-III-2.**

Kindred 3

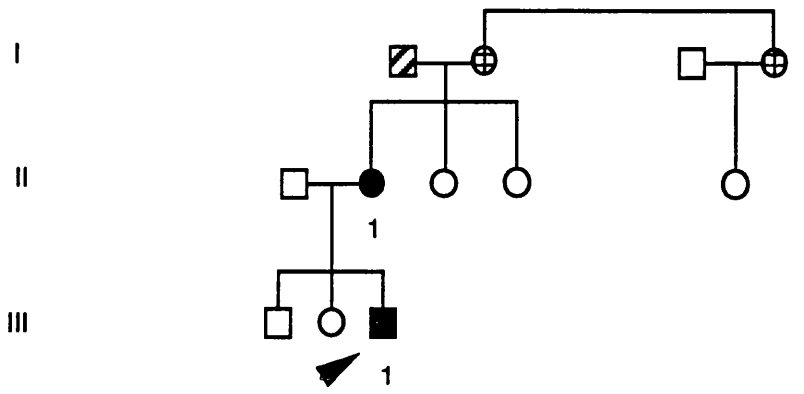
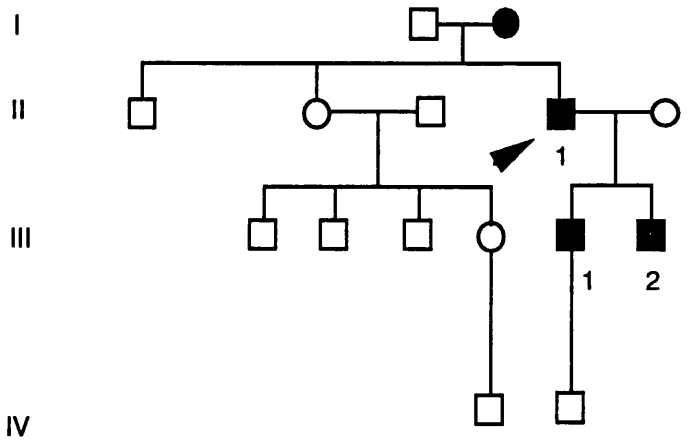



**Figure 3.3 c Kindred 3.**

**Affected individuals are identified by a three part identifier, i.e. Kindred Number-Generation-Member.**

**For example the proband of this kindred is 3-III-1.**

Kindred 4



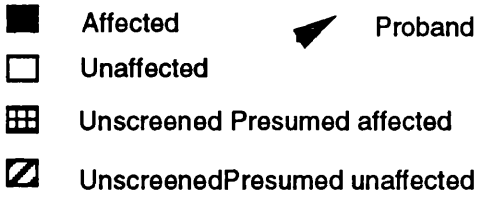
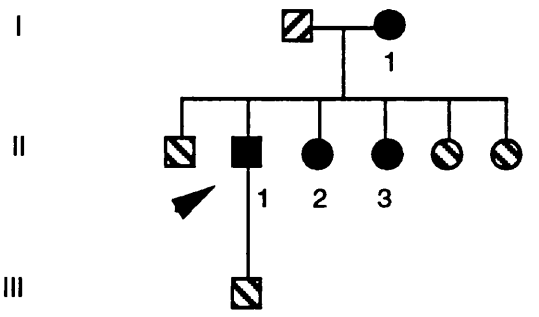




**Figure 3.3 d Kindred 4.**

**Affected individuals are identified by a three part identifier, i.e. Kindred Number-Generation-Member.**

**For example the proband of this kindred is 4-II-1.**

Kindred 5



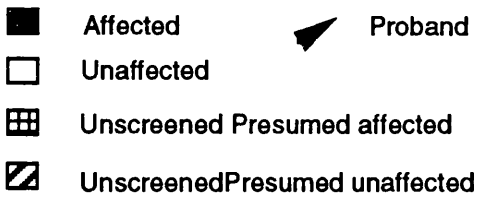
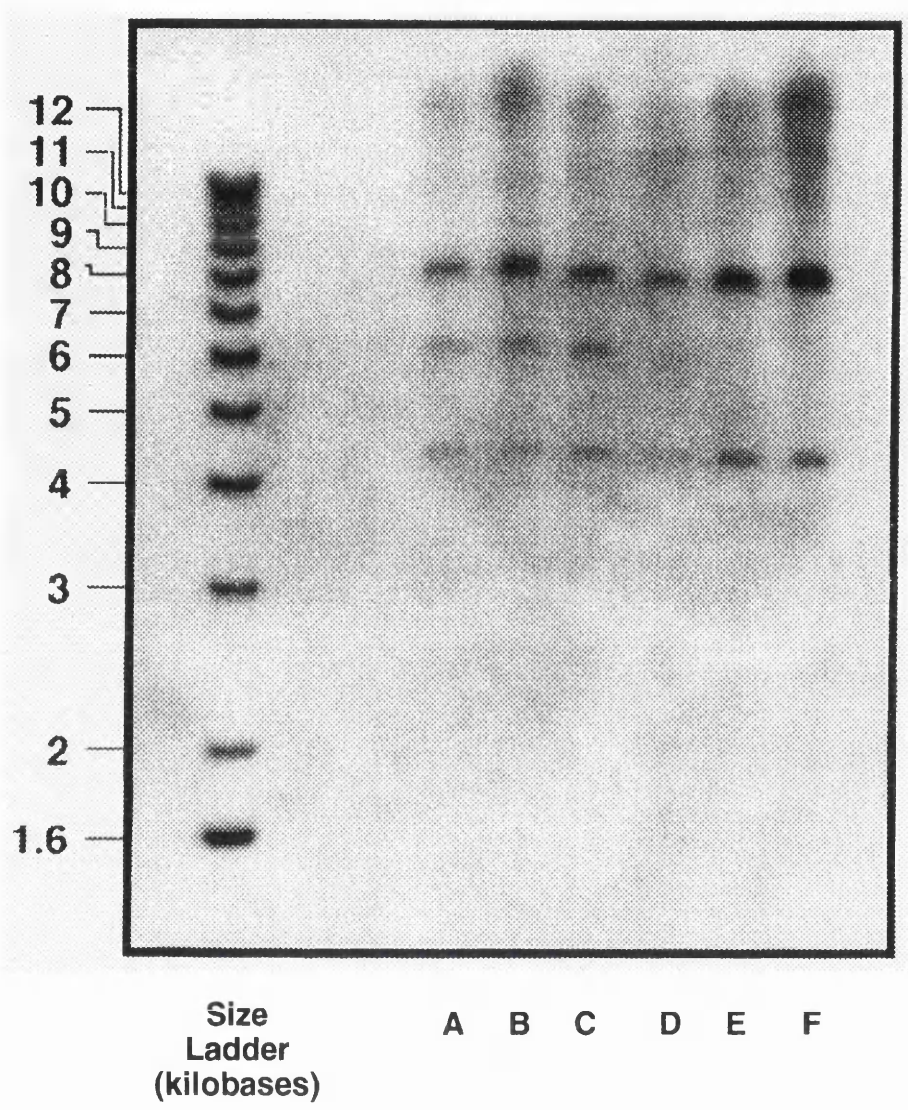




**Figure 3.3 e Kindred 5.**

**Affected individuals are identified by a three part identifier, i.e. Kindred Number-Generation-Member.**

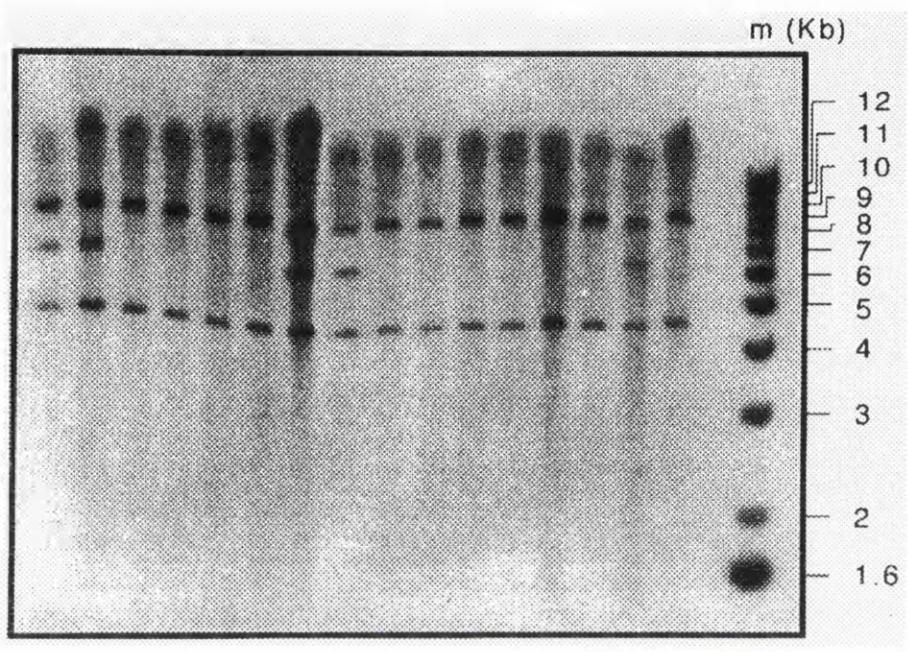
**For example the proband of this kindred is 5-II-1.**




A, B, and C possess three hybridising species,
i.e. 8.4 kb, 6.3 kb, 4.4 kb and therefore have GSH

D, E, and F possess two hybridising species,
i.e. 8.4 kb, 4.4 kb and therfore do not have GSH

Figure 3.3 f Southern blot of 6 individuals from kindred 4




A, B, G, H and P possess three hybridising species, i.e. 8.4 kb, 6.3 kb, 4.4 kb, and therefore have GSH

C, D, E, F, I, J, K, L, M, N, O and Q possess two hybridising species, i.e. 8.4 kb, 4.4 kb, and therfore do not have GSH

**Figure 3.3 g Southern blot of 16 individuals from kindreds 1, 2, 3 and 5**

## 3.4 Discussion

The method used in this study to detect the presence of a chimeric CYP11B1/B2 gene has been shown to have a 100% sensitivity and specificity for the diagnosis of GSH in all kindreds studied to date (Connell et al, 1986, Lifton et al, 1992, Lifton et al, 1992, Miyahara et al, 1992, Pascoe et al, 1992a). Lifton (Lifton et al, 1992) showed that the diagnosis of GSH was in strong linkage with the 6.3 kilobase band on the Southern blot in a single large kindred, lod score 5.23 for complete linkage, i.e. the odds of there being no linkage were greater than 100000 to 1. In addition, the method allows for the rapid diagnosis of individuals suspected of having GSH. The time taken from blood sampling to a result from autoradiography can be as little as 60 hours in our laboratory and gives results with 100% accuracy. I have also shown that a diagnosis of GSH in the perinatal period is possible (Jamieson et al, 1994), with the potential for appropriate counselling of the parents to be organised and delivered long before the affected child is at any risk from the effects of aldosterone excess. The potential for prenatal diagnosis of GSH from chorionic villus sampling material or amniocentesis-derived tissue should not be overlooked. However, the condition has a low early mortality and an effective, specific therapy and no *in utero* gene therapy for the condition is available. Therefore the need for such a diagnostic method is questionable as termination of pregnancy would be inappropriate given the high biological fitness of affected individuals.

The method is of particular benefit in assessing the relatives of an affected individual who do not have any obvious features of aldosterone-excess (e.g. hypertension or hypokalaemia) and can readily exclude or confirm the diagnosis in hypertensive relatives of affected patients. It also has the potential for identifying affected individuals prior to the onset of hypertension, allowing the clinician to monitor subsequently the blood pressure to detect any rise in blood pressure and consequently to institute appropriate therapy at an early stage with the hope of minimising the risks of stroke in these patients.

Finally, the identification of untreated, clinically silent patients with GSH allows for the detailed study of the effects of growth, sexual maturation, environmental and genetic factors on the severity of GSH phenotype. As will be shown in Chapter 6, there is a wide spectrum of clinical phenotypes in affected individuals with GSH. Environmental and epistatic genetic factors may be important in modulating the effect on blood pressure of the chimeric CYP11B1/B2 gene in a given individual.

Another intriguing observation regarding kindreds with GSH is the considerable excess of kindreds with a Celtic bias, i.e. originating from Ireland, Scotland or

possibly North-west France. Of those kindreds studied so far, an Irish origin is either established or strongly suspected in 9, Scottish ancestry in 6 and English in 2, approximately 75% of all kindreds described in the literature (Lifton et al, 1992, Woodland et al, 1985). Thus, it would appear that there is something peculiar to the inhabitants western aspects of northern Europe which predisposes to the formation of these chimeric genes. This predisposition may simply reflect the geographical distribution of the descendants of the index cases in relation to centres able to diagnose the condition, i.e. ascertainment bias, and the condition may be uniformly prevalent throughout all ethnic groups but simply underdianosed.

The majority of cases of GSH described in the literature are not the result of a single founder gene. Multiple chimeric genes have been found in the affected kindreds (Lifton et al, 1992, Miyahara et al, 1992, Pascoe et al, 1992a). However, a number of kindreds have chimeric genes indistinguishable by sequencing and analysis of linked markers has shown that in such kindreds there are haplotypic differences in the linked markers which confirm that they arise as the result of independent mutations (Lifton et al, 1992).

Similar work on a limited number of kindreds has shown that one SSCP marker, AldoX9, in exon 9 of CYP11B2 may be important in the development of a chimeric gene. In 9 pedigrees of Irish or English origin studied, all possessed allele 1 of the AldoX9 SSCP in the aldosterone synthase gene of the chromosome carrying the chimaeric gene, a significant deviation from that expected by chance ($p<0.04$); 8 out of 9 had allele 1 present in the chimeric gene. Among these 9 kindreds, 4 different crossover sites have been identified and three different haplotypes have been seen, indicating that within this subgroup independent duplications had occurred (Lifton et al, 1992). Thus, it would appear that the possession of certain marker haplotypes favours the formation of chimeric genes by, as yet, unknown mechanisms.

In the most common inherited disorder of adrenal steroid biosynthesis, steroid 21-hydroxylase deficiency, gene conversion events between the normal gene for steroid 21-hydroxylase (CYP21B) and a highly homologous pseudogene (CYP21A) lying in tandem, are a common cause of enzymatic deficiency (White et al, 1984, White et al, 1984). Gene conversion refers to a non-reciprocal recombination event in which a segment of one gene replaces the corresponding segment of a related genes, initially described in fungi . Such gene conversions have been described in cultured cells at a rate of $5x10^{-6}$ (Liskay and Stachelek, 1983), and suggested to occur in human ß-globin genes, human fetal $\gamma$-globin genes, and immunoglobulin

genes (Kourilsky, 1986). In 21-hydroxylase deficiency, recombination occurs between CYP21B and CYP21A to produce mutant alleles which introduce nucleotides into CYP21B which result in the production of truncated non-functional enzymes or alteration of key amino acids in the enzyme (White and New, 1992).

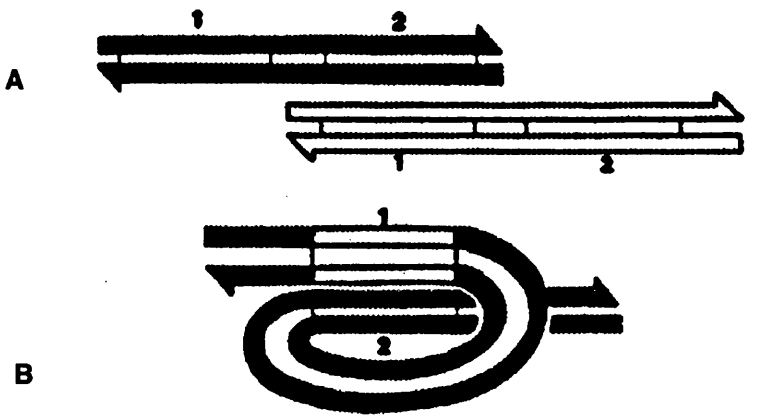
The mechanism whereby such transfer of genetic material occurs is not entirely clear, but two principal mechanisms have been suggested depending on whether the transfer of genetic material from the donor to the acceptor gene is via a single or a double stand and has been reviewed in detail previously (Kourilsky, 1986) (Figure 3.4 a, b).

First, a break in both strands of the acceptor gene double helix allows both strands to be repaired by the donor gene with the incorporation of donor genetic material into both strands of the acceptor helix. This is in effect a double recombination event and may result in the transfer of large pieces of DNA between genes. Such a break in the DNA helix is enlarged by nucleases; the 4 free ends of the DNA molecule are then free to invade the double stranded partner molecule and initiate the recombination event. This process favours the formation of extrachromosomal recombination in yeast and mouse models (Kourilsky, 1986).

Second, a break in a single strand of the donor gene may allow the advancing limb of the donor gene to invade the acceptor gene to form a heteroduplex. Subsequent repair of the donor strand leaves it intact and unaltered whilst the acceptor strand contains new material. Such a mechanism is thought to involve only short distances along the DNA strand and thus only a few nucleotides will be incorporated into the acceptor gene. However, many such conversion events may occur in areas of high homology leading to a 'patchwork' appearence of the acceptor gene such as that seen in certain forms of ß-thalassaemia (Kourilsky, 1986).

The chimeric gene in patients with GSH could have arisen from a non-homologous exchange of DNA between CYP11B1 and CYP11B2 on the same chromosome, or from recombination occuring between sister chromatids at meiosis. The mechanism is likely to be that of a double stranded break mechanism given the size of the conversion event although the precise mechanism remains to be determined.

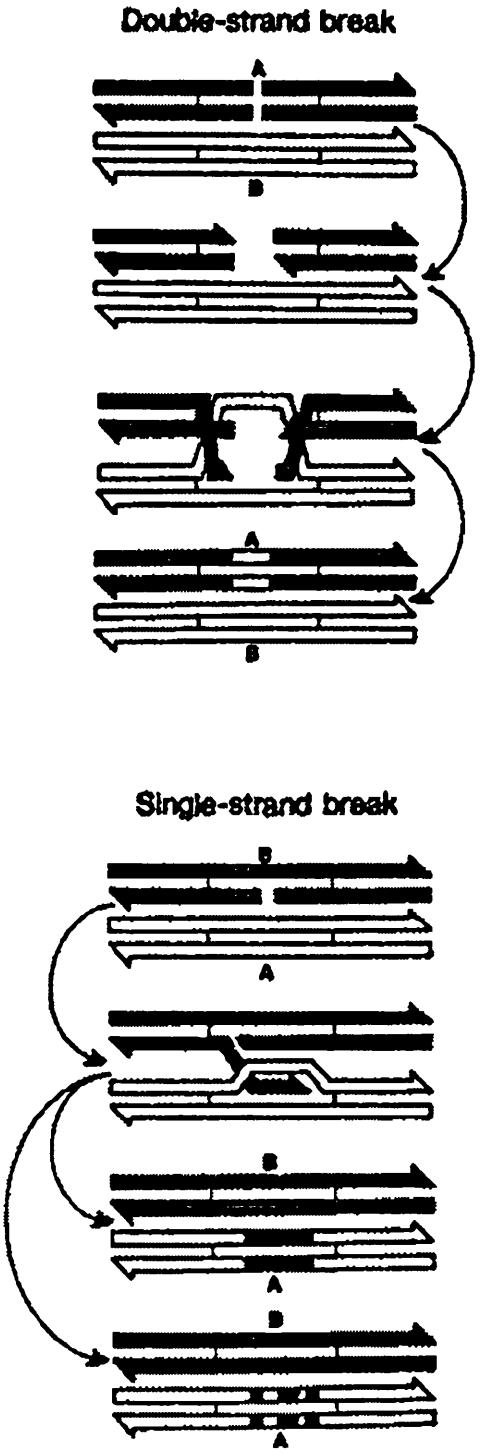
In summary, the use of a molecular genetic technique to identify individuals with GSH is straightforward and easily applicable to large numbers of 'at risk' individuals. It allows the identification of clinically 'silent' affected individuals which




A - gene conversion between genes on different chromosomes

B - gene conversion event between genes on the same chromosome

**Figure 3.4 a Schematic arrangements favouring gene conversion events**




A, B - non-homologous genes on different chromosomes involved in gene duplication event

**Figure 3.4 b Mechanisms of gene conversion events**

allows for the early institution of appropriate therapy when necessary and the identification of a cohort of patients who can be studied to allow assessment of the factors which may alter the observed phenotype of GSH.

## Chapter 4

# DETERMINATION OF CROSSOVER REGIONS IN CHIMERIC CYP11B1/B2 GENES IN KINDREDS WITH GLUCOCORTICOID-SUPPRESSIBLE HYPERALDOSTERONISM

### 4.1 Introduction

Identification of affected individuals with GSH by genotyping is of considerable benefit to the clinician dealing with the affected individuals and members of their kindred. However, while this method shows the presence of a chimeric gene it gives no information as to the exact nature of the crossover event giving rise to the gene. Without this information kindreds cannot be distinguished on the basis of the nature of their chimeric gene, i.e. the possibility of a founder effect giving rise to these 5 kindreds cannot be ruled out. Similarly the effect of a variable genotype (i.e. a variable site of crossover) on phenotype cannot be determined unless the exact composition of the chimeric gene is known.

To address these problems the nucleotide sequence of the chimeric CYP11B1/B2 gene in each affected kindred was determined by PCR amplification of the segment of the chimeric gene containing the crossover and sequenced using the dideoxy- chain termination method.

### 4.2 Materials and Methods

#### 4.2.1 Subjects

Subjects from each of the 5 kindreds were studied. In each case the proband of the kindred and one or more of the affected relatives identified by genotyping were studied.

#### 4.2.2 Methods

DNA was prepared from peripheral leukocytes as described in 2.7.1. Polymerase chain reactions were carried out in sterile Eppendorf tubes as described in 2.9.2.

A series of three reactions per chimeric gene were performed (Figure 4.2 a). Three sets of overlapping oligonucleotide primers were used to span the region of the chimeric gene (Appendix III). Each primer pair consisted a 5' sense primer specific to CYP11B1 and a 3' antisense primer specific to CYP11B2. A high ($65^0$C) annealing temperature was used to maximise the chance of annealing of each primer to the template DNA in regions of high homology between CYP11B1 and B2

and thus limit the possibility of non-specific amplification. Identical reactions were carried out on plasmid DNA containing pure clones of CYP11B1 and B2, DNA from unaffected relatives and reactions containing water only. Table 4.2 a summarises the findings of these reactions when carried out on an individual with GSH.

| | 1/2X | 3A/5N | 6/CC |
|---|---|---|---|
| Patient | negative | 1.4 kb product | negative |
| Unaffected Sib | negative | negative | negative |
| CYP11B1 clone | negative | negative | negative |
| CYP11B2 clone | negative | negative | negative |
| Water | negative | negative | negative |

**Table 4.2 a. Results of standard PCR reactions on DNA from a patient with GSH.**

Following each PCR reaction, 5µl of each reaction mix was loaded onto a 1% agarose gel and subjected to electrophoresis to check for the presence of a PCR product. In those reactions which showed the presence of a PCR product, the remainder of the PCR reaction product was loaded in to a 1% LMP gel and subjected to electrophoresis as described in 2.9.3. The PCR DNA was then purified from the agarose as described in 2.9.3 and reconstituted in 20µl of 1X TE buffer ready for sequencing. In general three PCR reactions, total volume 135µl (approimately 250 ng of amplified DNA), were used to ensure enough purified DNA was available for the sequencing protocol.

### 4.2.3 Sequencing of Chimeric Genes

Purified PCR product from affected patients was sequenced as described in 2.10.1-3. Three sequencing primers were used to sequence the amplified region (Figure 4.2 b). In all cases, the PCR amplification only occurred using primers spanning the intron 2 - intron 5 region and therefore sequencing primers that spanned this region were used.

Each primer hybridised to a non-unique exonic region of the chimeric gene, i.e. a region found in either CYP11B1 or B2. Primers 1178 and 4, both 5' sense primers, hybridised to the 5' end of exons 3 and 4 respectively, whilst primer 5N, a 3' anti-sense primer, hybridised to the 3' end of exon 5. These primers allowed the chimaeric PCR product to be sequenced in both the sense and anti-sense

directions to allow accurate determination of the region of change from CYP11B1-specific to CYP11B2-specific nucleotides.

Following completion of the sequencing reactions, the sequence of the PCR products was determined by polyacrylamide gel electrophoresis and autoradiography of dried blotted filters (2.10.4-6). The resulting sequence of the PCR product was compared with the consensus sequence of CYP11B1/B2 to determine the site of each crossover (2.10.7).

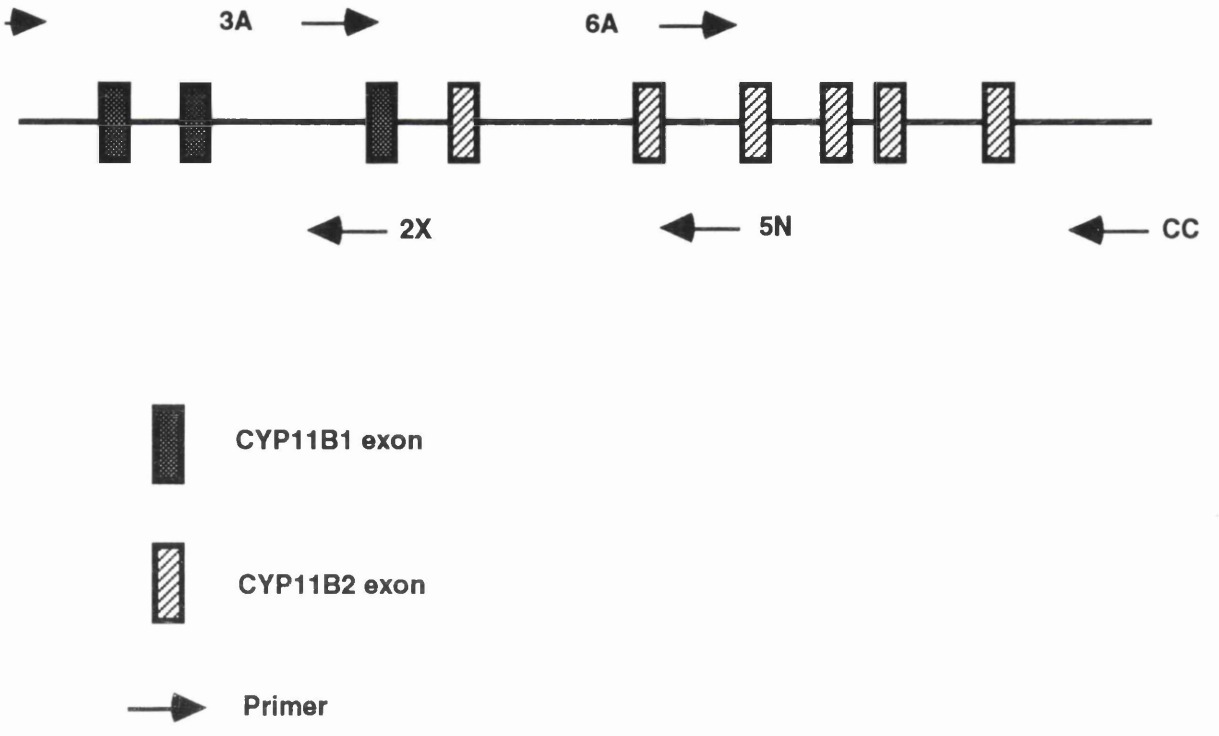



Figure 4.2 a PCR strategy to amplify crossover region

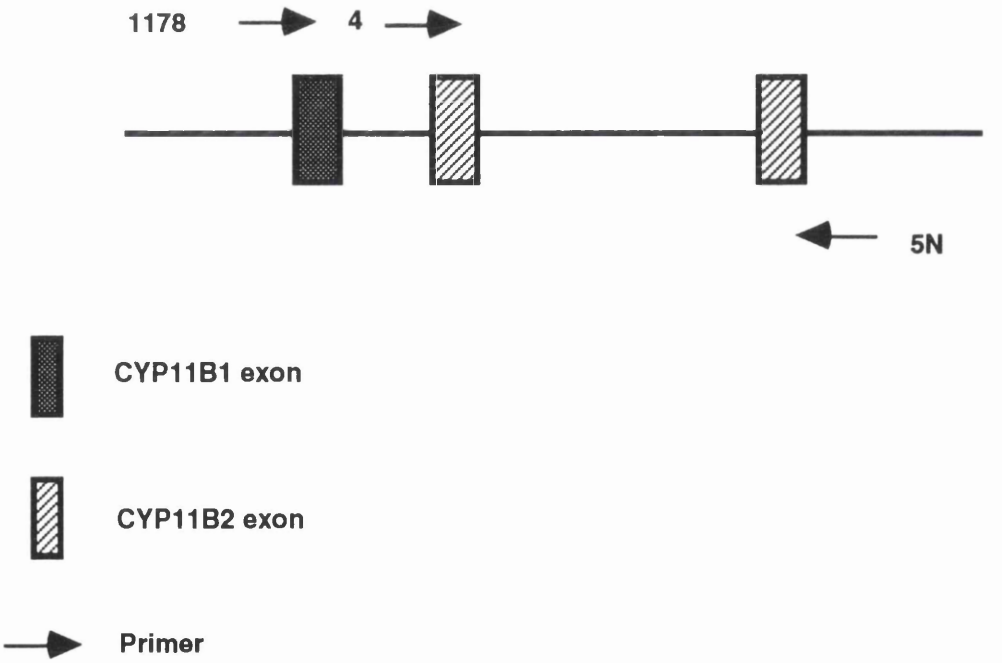



Figure 4.2 b Sequencing strategy to determine crossover site

## 4.3 Results

Sequencing reactions were carried out on PCR-amplified DNA from each of the 5 kindreds studied. Test reactions on unaffected individuals and test plasmids showed no evidence of non-specific amplification. Figure 4.3 a shows the result of a typical PCR amplification on 4 affected individuals from different kindreds.

In each affected individual a 1.4 kb PCR product was obtained using the primer pair 3A/5N, spanning the region containing intron 2 - intron 5 of the chimeric gene. No PCR products were detected when either 1/2X (spanning the 5' flanking region to intron 2) or 6A/CC (spanning intron 5 to the 3' flanking region) were used.

Direct sequencing of the PCR products using 1178, 4 and 5N allowed accurate delineation of the crossover region of the chimeric gene in all cases.

The crossover region in kindreds 1, 2 and 3 was localised to a region encompassing the exon 3 - intron 3 boundary of the chimeric gene (Figure 4.3 b,c,d). The region of the crossover in these kindreds spans some 89 nucleotides from an **A** residue in exon 3 of CYP11B1 at position 1335 (**G** in CYP11B2) to a **T** residue in intron 3 of CYP11B2 at position 1797 (**C** in CYP11B1).

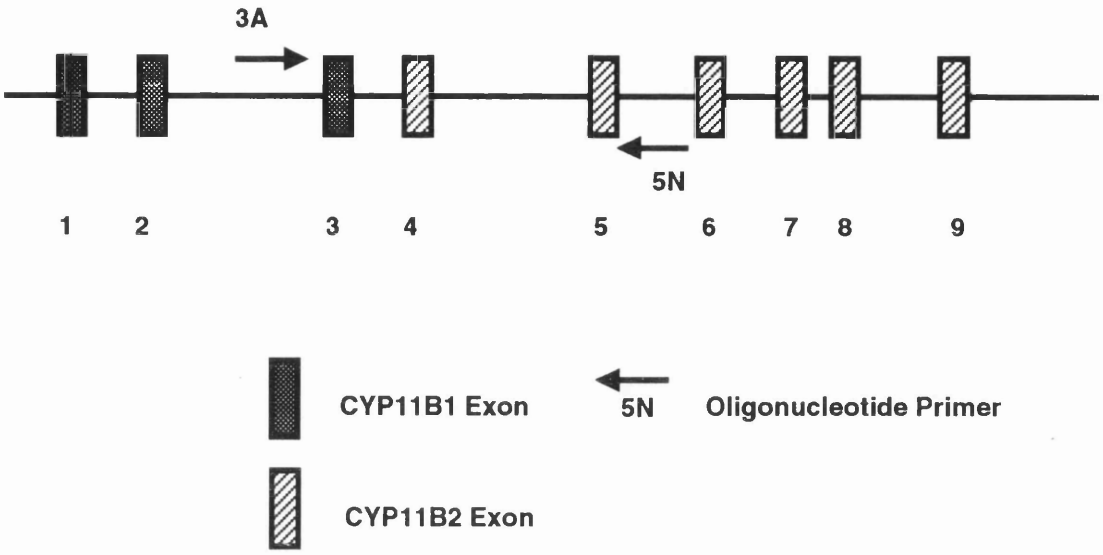
The crossover region in kindred 4 was localised to a region encompassing exon 4 of the chimeric gene (Figure 4.3 e). The region of the crossover in this kindreds spans some 138 nucleotides from a **G** residue in exon 4 of CYP11B1 at position 1558 (**A** in CYP11B2) to a **T** residue in exon 4 of CYP11B2 at position 2071 (**C** in CYP11B1).

The crossover region in kindred 5 was localised to a region encompassing the exon 4 - intron 4 boundary of the chimeric gene (Figure 4.3 e). The region of the crossover in this kindred spans some 94 nucleotides from a **C** residue in exon 4 of CYP11B1 at position 1646 (**T** in CYP11B2) to a **G** residue in intron 4 of CYP11B2 at position 2164 (**C** in CYP11B1).

The results of sequencing of the chimeric genes of the 5 kindreds are summarised in Figure 4.3 f. In all cases, the crossover points lie within the exon 3 - intron 4 region of the chimaeric gene. In no case is there evidence of a gene duplication or insertion of a DNA segment at the site of the crossover.

The degree of comparison of the predicted amino acid sequences of the translated chimeric genes is given in Table 4.3 a. This shows that the differences in predicted amino acid sequences between the chimeric proteins is minimal.

**Amplification of the chimaeric 11ß-hydroxylase/aldosterone synthase gene using the Polymerase Chain Reaction**



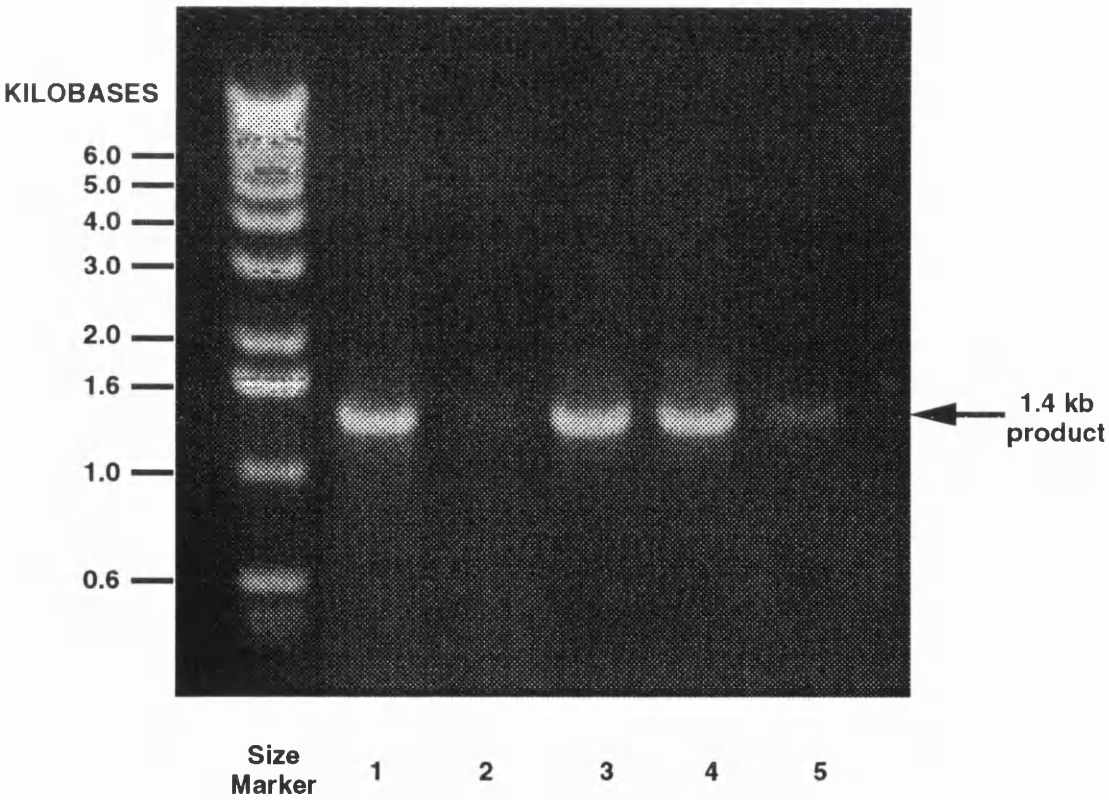




**Figure 4.3 a Specific amplification of CYP11B1/B2 crossover region by PCR.**

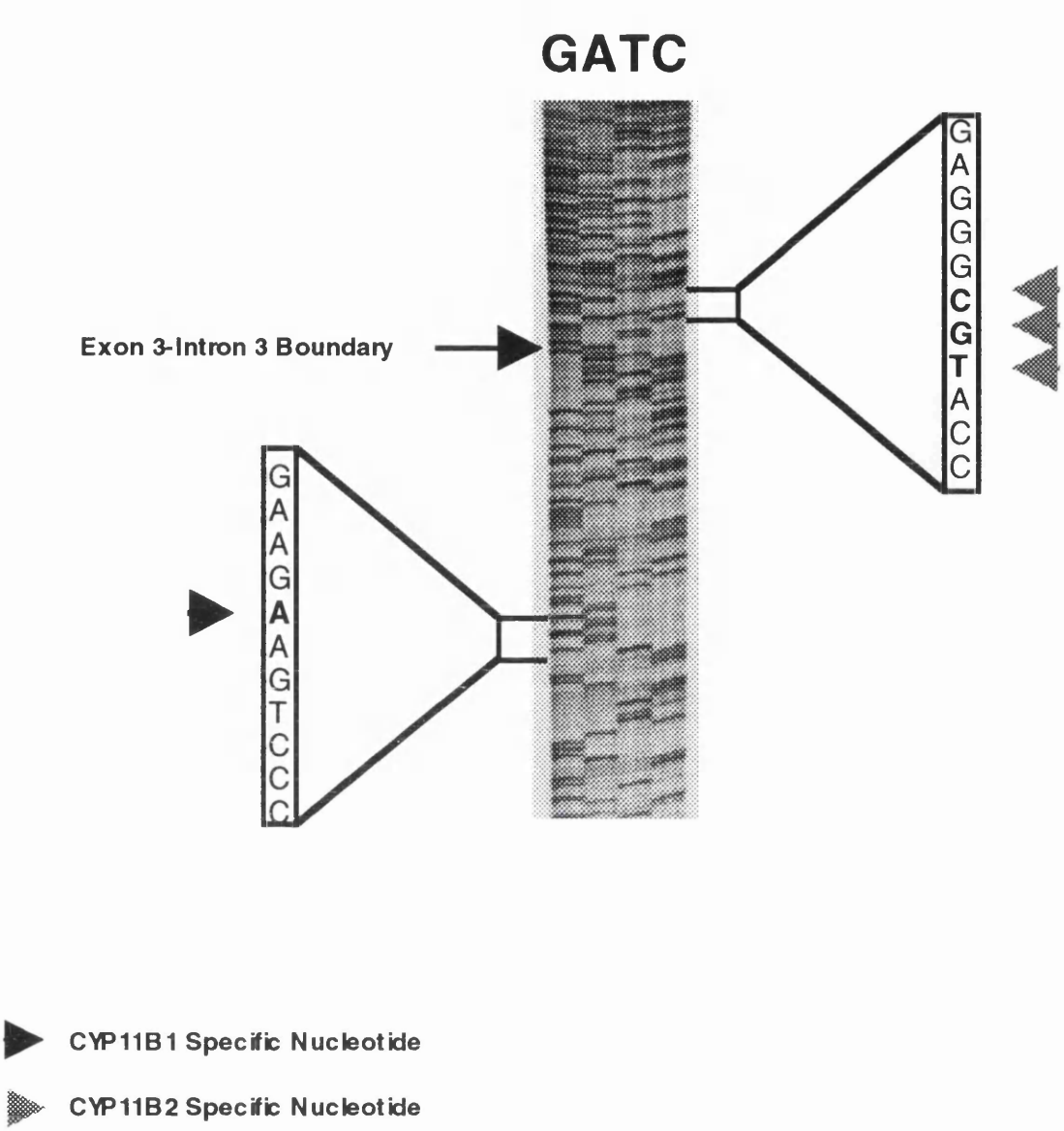



**Figure 4.3 b Sequence analysis delimiting the crossover region in the chimeric CYP11B1/B2 gene in Kindred 1**

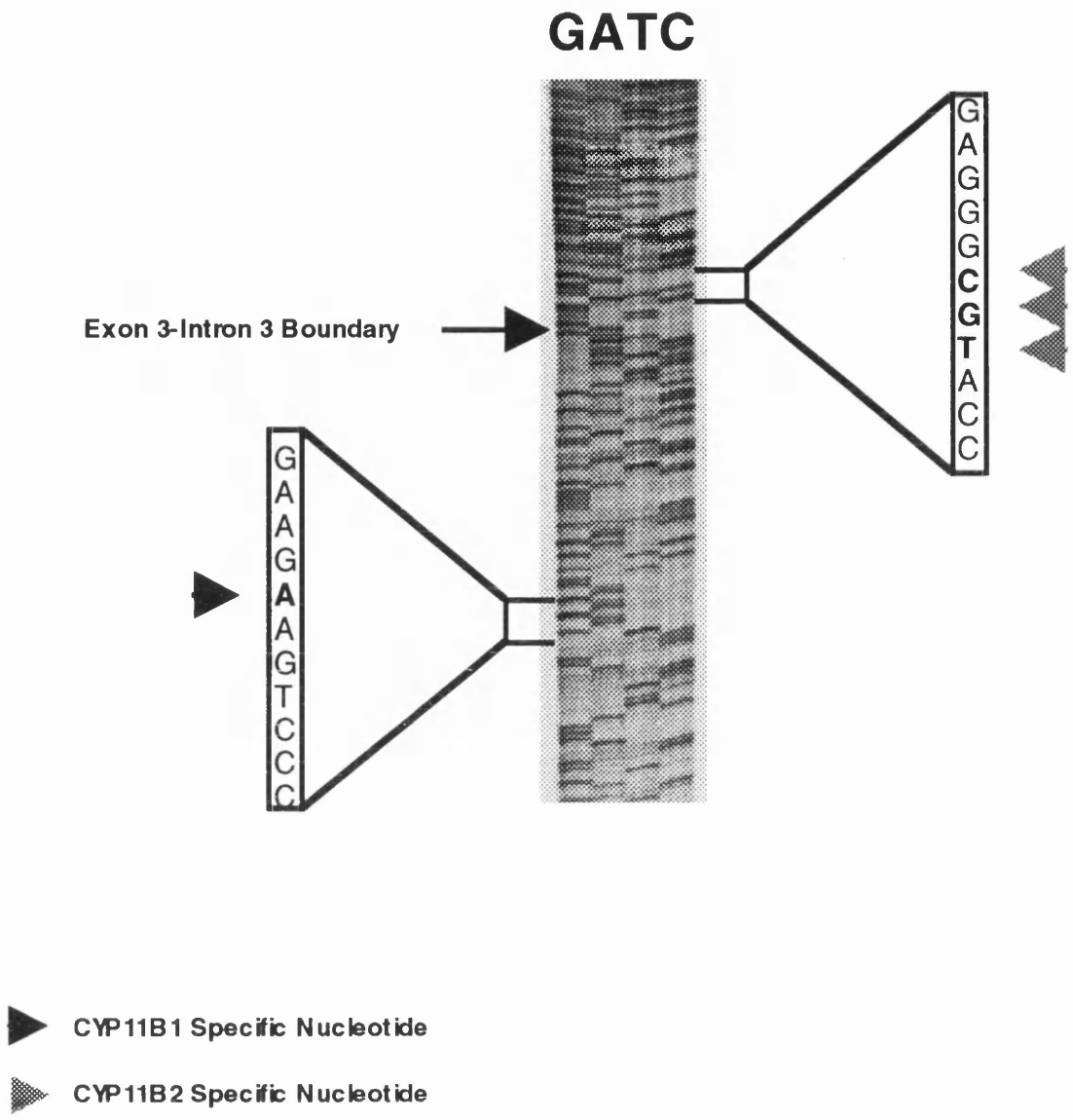



**Figure 4.3 c Sequence analysis delimiting the crossover region in the chimeric CYP11B1/B2 gene in Kindred 2**

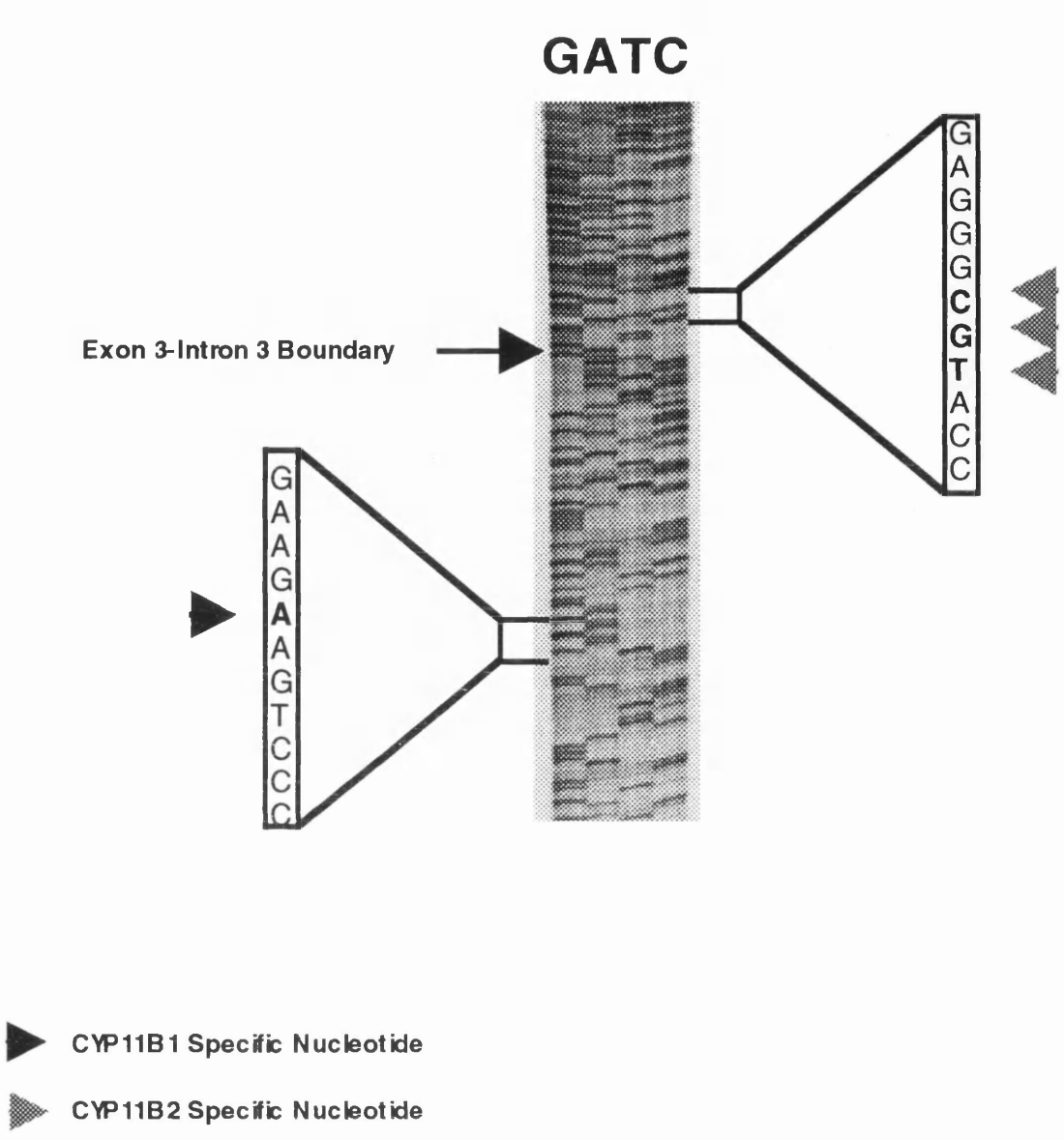



Figure 4.3 d Sequence analysis delimiting the crossover region in the chimeric CYP11B1/B2 gene in Kindred 3

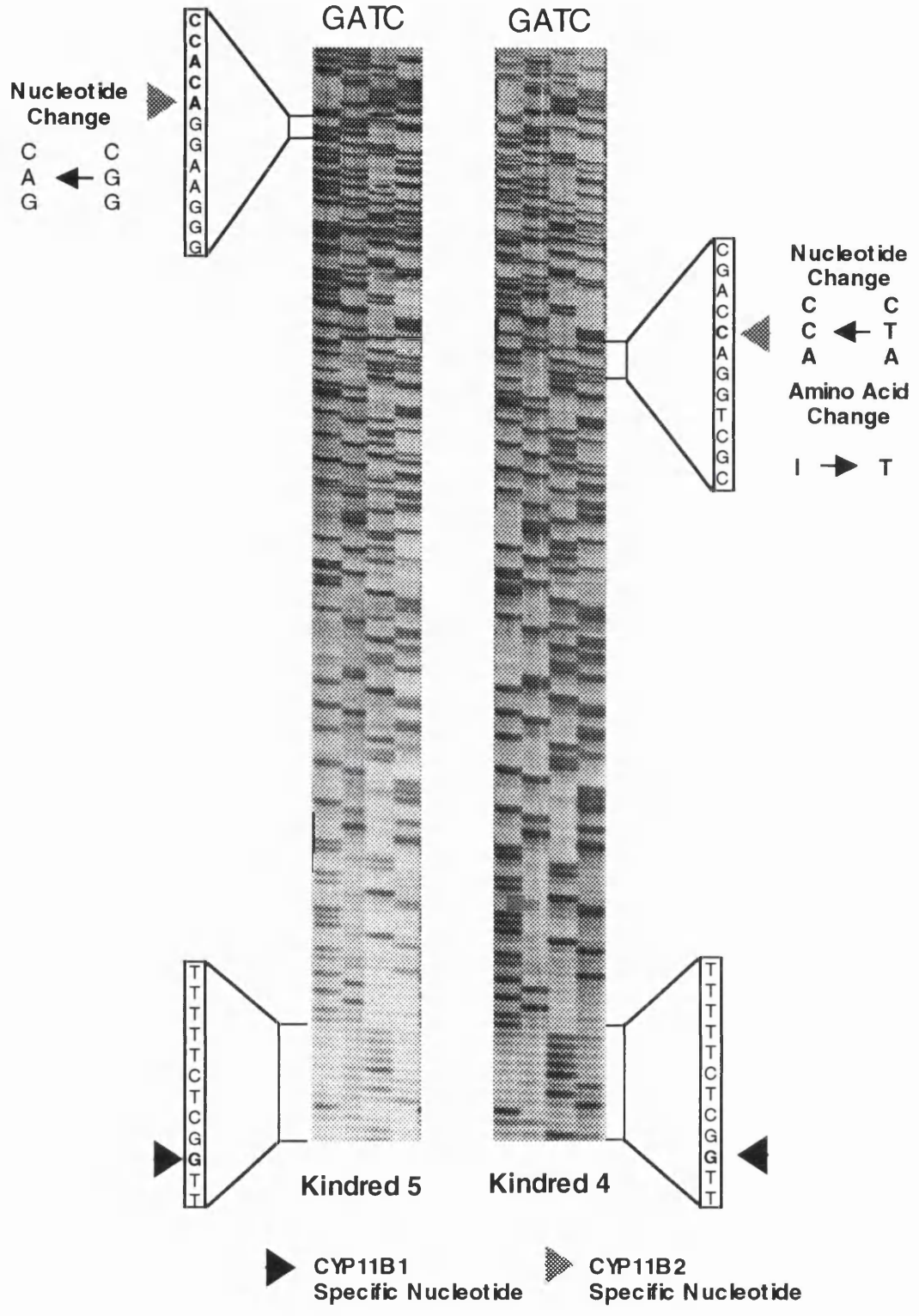
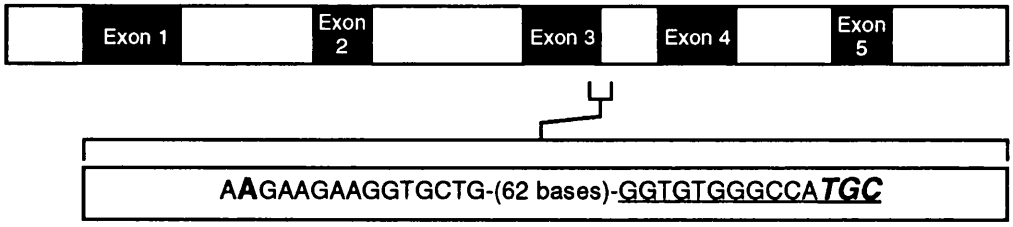
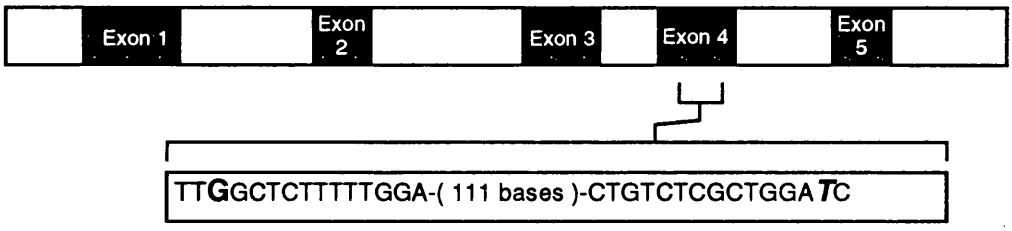



Figure 4.3 e Crossov er rgions in the chimericCYP11B1/B2 gene of Kindreds 4 and 5

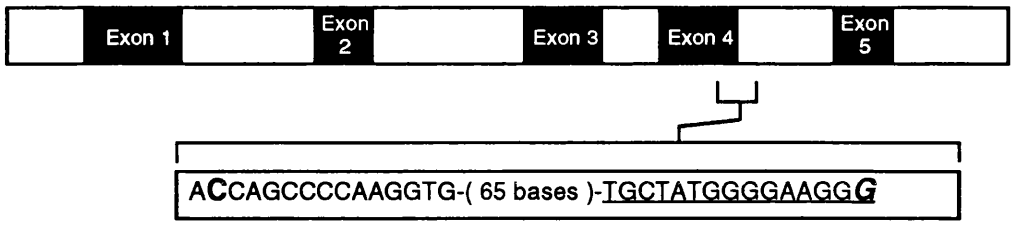
**Kindreds 1, 2, 3**




**Kindred 4**




**Kindred 5**




Bases in plain typeface are common to both CYP11B1 and CYP11B2.
Underlined bases (ATG) indicate intronic sequence.
Bases in larger bold typerface (**ATC**) are specific to CYP11B1, whilst bases in larger italic and bold typeface (***TGC***) are specific to CYP11B2.

The intervening region is the largest domain within which a crossover event could occur.

**Figure 4.3 f Schematic representation of sequence data for the crossover regions of kindreds 1 to 5.**

| | CYP11B1 | CYP11B2 | Kindreds -3 | Kindred 4 | Kindred 5 |
|---|---|---|---|---|---|
| Kindreds1-3 | 96.2 | 96.4 | 100 | 100 | 99.9 |
| Kindred 4 | 96.2 | 96.4 | 100 | 100 | 99.9 |
| Kindred 5 | 96.4 | 96.2 | 99.9 | 99.9 | 100 |

**Table 4.3 a. Comparison of predicted amino acid sequence homology (percent) between CYP11B1, CYP11B2 and the predicted amino acid sequence of the 5 chimeric genes detected in kindreds 1 - 5.**

## 4.4 Discussion

The results of these experiments yield two pieces of important information. Firstly, the distribution of the crossover regions in the chimeric genes is similar to that observed in previous studies (Figure 4.4 a) (Lifton et al, 1992, Miyahara et al, 1992, Pascoe et al, 1992a). The site of crossovers in all patients with GSH so far studied have been localised to the intron 2 - intron 5 region of the chimeric CYP11B1/B2 gene. This is not surprising if the evidence regarding the location of the active site of aldosterone synthase is taken into consideration. Observations of the *in vitro* steroidogenic capacity in COS cells of chimeric CYP11B1/B2 cDNA constructs comprising variable proportions of the CYP11B1 and CYP11B2 exonic DNA formed in a manner similar to those found in GSH (Pascoe et al, 1992a) have revealed that constructs comprising exons 1-3 of CYP11B1 coupled to the remainder from CYP11B2 possess aldosterone synthase activity (Figure 4.4 b). Constructs possessing exons 1-5 or more of CYP11B1 coupled to the remainder from CYP11B2 had no detectable aldosterone synthase activity, suggesting that exon 5 of CYP11B2 has residues important for conferring aldosterone synthase activity on the resultant gene product. Similar results have been obtained when chimeric rat CYP11B1/B2 cDNA hybrids were expressed in COS7 cells containing exons 1-5 of CYP11B1 (Zhou et al., 1994).

The steroidogenic ability of cDNA's of CYP11B1 in COS cells can also be altered by PCR-mediated site-directed mutagenesis of CYP11B1-specific residues to the corresponding CYP11B2-specific nucleotides. Alteration of a few nucleotides in the CYP11B1 cDNA to change the nucleotide sequence of exon 5 of CYP11B1 to that of CYP11B2 confers aldosterone synthase activity on the resultant cDNA when expressed *in vitro* (Pascoe and Curnow, 1994).

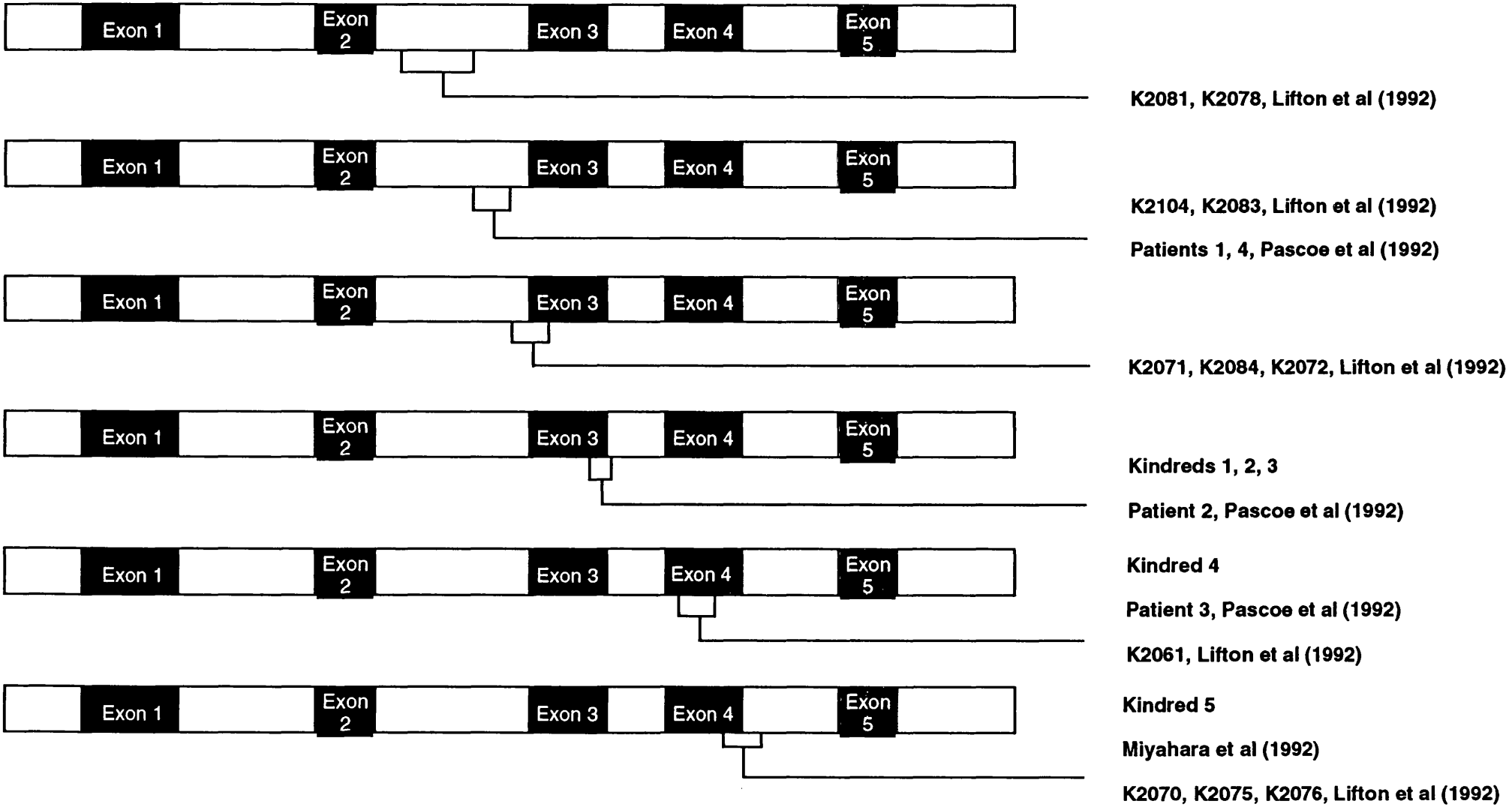
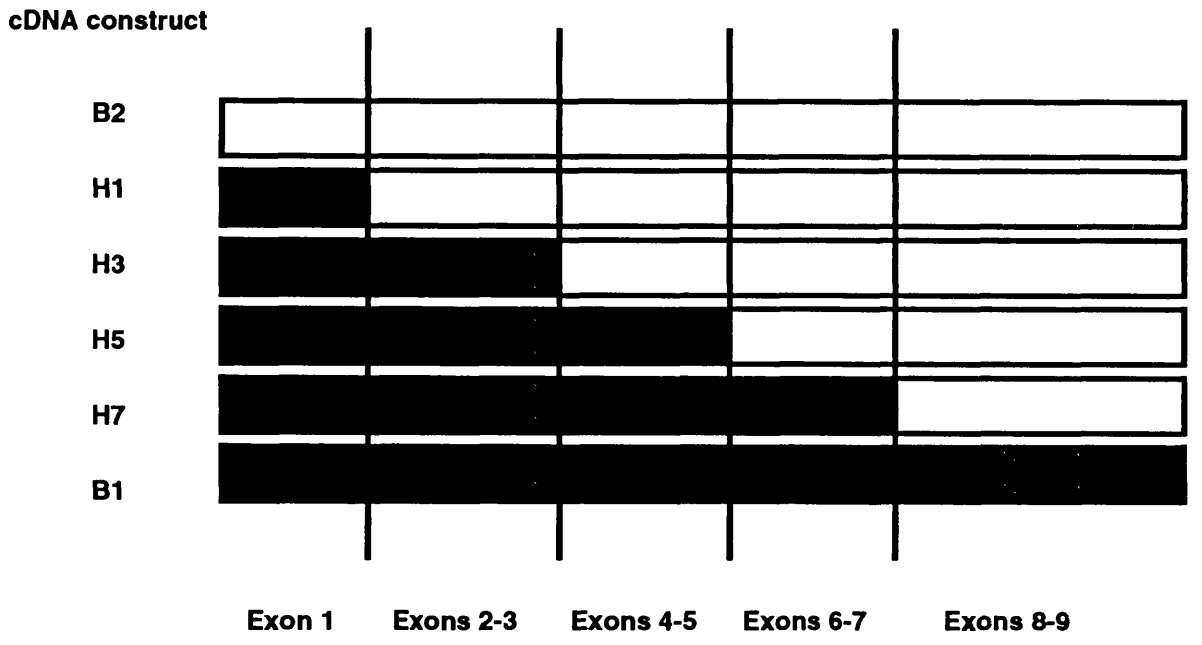



**Figure 4.4a Summary of published data on crossover sites in kindreds with GSH.**




| | % conversion from 11-DOC | | |
|---|---|---|---|
| | B | 18-OHB | Aldo |
| B2 | 30 | 22 | 11 |
| H1 | 21 | 7 | 5 |
| H3 | 28 | 9 | 6 |
| H5 | 8 | 0 | 0 |
| H7 | 13 | 0 | 0 |
| B1 | 74 | 0 | 0 |

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

**Figure 4.4 b Aldosterone synthase activity of chimeric CYP11B1/B2 cDNA's expressed in COS cells. (After Pascoe et al. 1992)**

In a single case of corticosterone methyloxidase type II deficiency, the condition has been shown to arise from a gene duplication altering exons 3 and 4 of the patients CYP11B2 gene (Fardella et al., 1994b). The resulting gene has exons 3 and 4 derived from CYP11B1 with resultant lack of aldosterone synthase activity. Three amino acids in the predicted protein product differ from that of the predicted aldosterone synthase and two of these, K152N and I248T, alter the nature of the amino acid side chains present and may also therefore have an important role in conferring aldosterone synthase activity on the protein.

Thus, as an alternative mechanism to unequal sister chromatid exchange and formation of a chimeric gene, it is possible that a GSH-like syndrome could result from the presence of a gene duplication event resulting in the incorporation of CYP11B2 sequences into exon 5 of CYP11B1 in a similar fashion to those seen in classical steroid 21-hydroxylase deficiency (White et al, 1984) although large duplications could give rise to deficient enzyme action rather than over activity. No such cases have been reported as yet but it may be that some cases of idiopathic hyperaldosteronism or low-renin essential hypertension could arise as a result of such mutations.

Similarly, classical GSH would appear not to result from the formation of chimeric genes containing exon 5 of CYP11B1. The possibility of 'formes fruste' of GSH resulting from crossovers which involve the first 5 or more exons of CYP11B1 should be considered. Such genes may be expressed in the adrenal glands and result in altered steroid 11ß-hydroxylation ability such as that previously demonstrated in patients with essential hypertension (De Simone et al, 1985) and in animal models of hypertension (Fraser et al., 1994), or low-renin forms of hypertension where subtle alterations in steroid secretion secondary to the expression of a CYP11B1-like gene throughout the adrenal cortex may lead to an alteration in the normal renin-aldosterone relationship. However, no evidence of such crossover events has so far been obtained.

Apparently unrelated kindreds may derive the condition from a common ancestor, i.e. a founder effect may exist even if not expected. Kindreds 1, 2, and 3 have indistinguishable crossover regions when sequenced. The chimeric gene in these kindreds may have a common ancestral origin, i.e. a single founder mutation has given rise to three kindreds. Kindreds 1 and 2, for example, share a common geographical ancestral origin in the Isle of Lewis and the possibility that these two kindreds arose from a common ancestor must be high. However, the possibility of

discrete independent mutational events, giving rise to similar chimeric genes cannot be ruled out by these sequencing studies.

To establish or refute the possibility of a single founder gene would require the study of linked markers in the region of the gene such as that carried out by Lifton previously (Lifton et al, 1992). This group used a series of linked markers detected by SSCP, present within exon 5 of CYP11B1 or in the 5' and 3' flanking regions of CYP11B2. Using this set of markers a haplotype for each kindred was established and the haplotype data for each kindred used to determine whether or not kindreds with indistinguishable crossover sequences were related.

By this method they were able to distinguish kindreds with identical chimeric gene nucleotide sequences on the basis of their linked markers and concluded that they must have arisen as the result of independent mutational events. A similar analysis of these kindreds will be undertaken in the future.

In summary, 5 kindreds with GSH have been studied. In all cases the nature of the chimeric gene has been determined. Three kindreds are indistinguishable on the basis of the nucleotide sequence of their chimeric gene whilst the other kindreds possess discrete mutations. The crossover regions of the 5 kindreds lie within the exon 3 to intron 4 region of the chimeric gene, in keeping with previous observations in this condition.

## Chapter 5

# DISCOVERY OF AN HAEIII POLYMORPHISM IN THE CYP11B2 PROMOTER REGION. RELEVANCE TO PATIENTS WITH AND WITHOUT GLUCOCORTICOID-SUPPRESSIBLE HYPERALDOSTERONISM.

## 5.1 Introduction

In patients with GSH, there is ectopic expression of a chimeric CYP11B1/B2 gene throughout the adrenal cortex (Lifton et al, 1992). In addition, patients with GSH show a wide variation in phenotype despite possessing similar or identical chimeric genes (Jamieson et al, 1994) (Chapter 6). This may arise through differences in environmental factors such as diet, including salt intake and alcohol ingestion, or through phenotypic expression of small genetic differences.

One possible mechanism altering the phenotype of individuals with GSH is through differential expression of the chimeric CYP11B1/B2 gene or the normal CYP11B1 and B2 genes in the adrenal cortex. Such differential expression might arise through differences in the 5' flanking regions of the chimeric gene which could alter the degree of response to transcriptional regulation factors such as cAMP or SF-1 (Lala et al, 1992). In addition, it has been suggested that allelic differences in the flanking regions of CYP11B1 and B2 may predispose to the formation of the chimeric gene, perhaps by altering the secondary structure of the DNA at this point and favouring unequal crossing over at meiosis (Lifton et al, 1992).

A putative SF-1 binding region in the 5' flanking region of CYP11B2 has been identified (Figure 5.1 a) (Kawamoto et al, 1992, Mornet et al, 1989, Parker and Schimmer, 1993). The role of SF-1 has been discussed earlier (1.7.3). It is an important transcriptional regulation factor regulating steroid hydroxylase gene expression. Studies using gene targeting to disrupt the SF-1 gene in mice have shown that SF-1 deficient mice do not survive the neonatal period and do not develop adrenal glands or gonads (Ikeda et al, 1994). The absence of SF-1 is also clearly detrimental to the post-embryonic development of mice and the adrenal in particular (Lala et al, 1992, Parker and Schimmer, 1993). Any alteration of the affinity of SF-1 for its binding site in CYP11B2, e.g. by a polymorphism in the binding region, should therefore alter the regulatory potency of SF-1 for this gene and thus alter the degree of expression of aldosterone synthase in the zona glomerulosa.

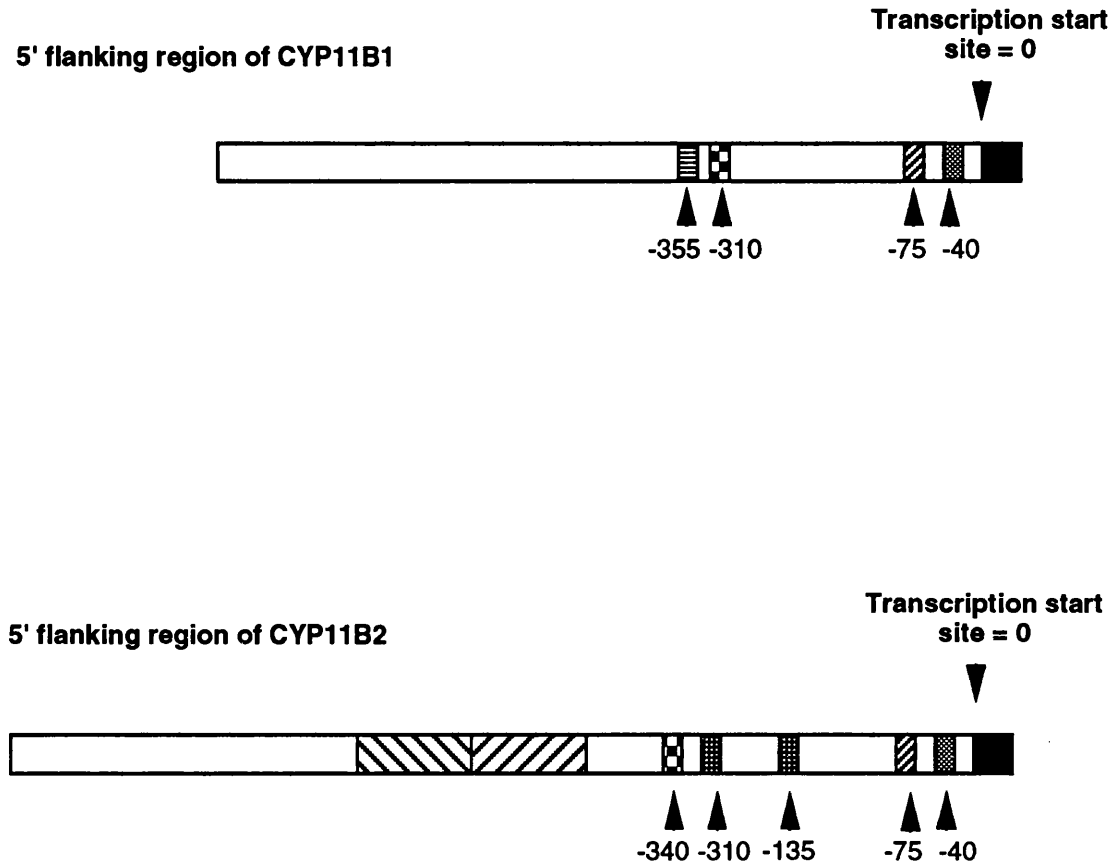



Figure 5.1 a Structure of promoter region of CYP11B1 and CYP11B2

Within the putative SF-1 binding region in the 5' flanking region of CYP11B2, a C/T single base pair change polymorphism has been detected at the -340 position by direct sequencing (-340C/T) (Figure 5.1 b) (PC White, personal communication). Gel mobility shift assays have shown that oligonucleotides carrying the -340C allele bind SF-1 approximately 4 times as strongly as the -340T allele (White et al., 1994). Thus it is possible that the C/T polymorphism may result in different rates of expression of CYP11B2 in the adrenal and alter the risk for the development of hypertension in an individual. It has been reported previously that patients with essential hypertension show enhanced aldosterone secretory responses to infusion of Ang II compared with normal subjects suggesting that aldosterone secretion is upregulated in essential hypertension (Kisch et al, 1976, Zoccali et al, 1983).

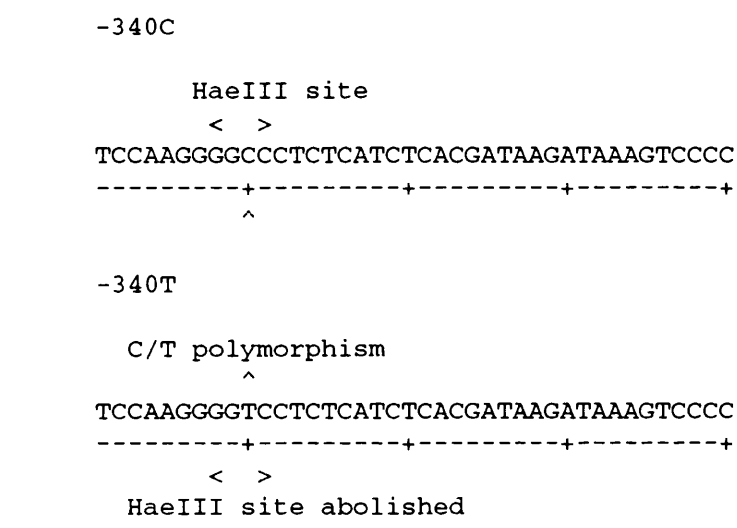



**Figure 5.1 b -340 C/T polymorphism in CYP11B2 promoter region**

Analysis of the nucleotide sequence in the region of this polymorphism shows that the C/T polymorphism confers or abolishes the presence of an HaeIII recognition site, i.e. the sequence found will either be -GGCC- (-340C, HaeIII +ve) or -GGTC- (-340T, HaeIII -ve). Therefore, if a means of identifying the two haplotypes could be found which avoids the need for direct sequencing, this might provide a convenient means of screening large populations to quantify the role, if any, of this polymorphism in the pathogenesis of hypertension.

Two methods were chosen to simplify the process:

1) Allele-specific oligonucleotide (ASO) hybridisation. This techniques is a variation on the basic method of Southern blotting (Curnow et al, 1993, Kogan et

al, 1987, Southern, 1975, White et al, 1991). A region of the desired gene containing the region of the polymorphism is amplified by PCR and blotted to a nylon membrane. It is then hybridised with oligonucleotides which are identical to the predicted nucleotide sequence flanking the polymorphism but differ by a single nucleotide at the polymorphism, i.e. either C or T. Two oligonucleotides are used to detect either allele of the polymorphism. This method has already been used successfully to identify disease alleles in congenital disorders of steroid hydroxylase function (Curnow et al, 1993, Pascoe et al, 1992b, White et al, 1991).

2) Restriction enzyme digest of a PCR-amplified portion of the CYP11B2 promoter encompassing the polymorphism. The digested PCR product is resolved on a high percentage agarose gel and alleles assigned on the basis of observed fragment length.

The results of both of these methods in identifying the C/T polymorphism are reported with the results of haplotype analysis of the five kindreds with GSH.

## 5.2 Materials and Methods

Subjects from all 5 GSH kindreds were typed for the presence of a dialleleic polymorphism in the promoter region of CYP11B2. DNA was prepared from peripheral leucocytes as described in 2.7.1. A portion of the CYP11B2 promoter region including nucleotides -576 to +6 was specifically amplified using oligonucleotide primers 2523 and 2524 by PCR as described in 2.9.2 (Figure 5.2 a).

A 5µl aliquot of the reaction was run on a 1% agarose gel to confirm the presence of the desired PCR product. In all cases a single band of desired length, 578 bp was observed after completion of the PCR.

### 5.2.1 Allele-specific oligonucleotide hybridisation

PCR products were subjected to electrophoresis on a 1% agarose gel and then blotted to a nylon membrane as described in 2.8.6. Filters were then hybridised with allele-specific oligonucleotides (ASO) recognising either the C (2360) or the T (2361) allele of the polymorphism (2.8.9). Following hybridisation, filters were washed and autoradiographs exposed as described in 2.8.10-11. Filters were stripped, checked to ensure the absence of residual hybridisation and re-hybridised with the alternative oligonucleotide.

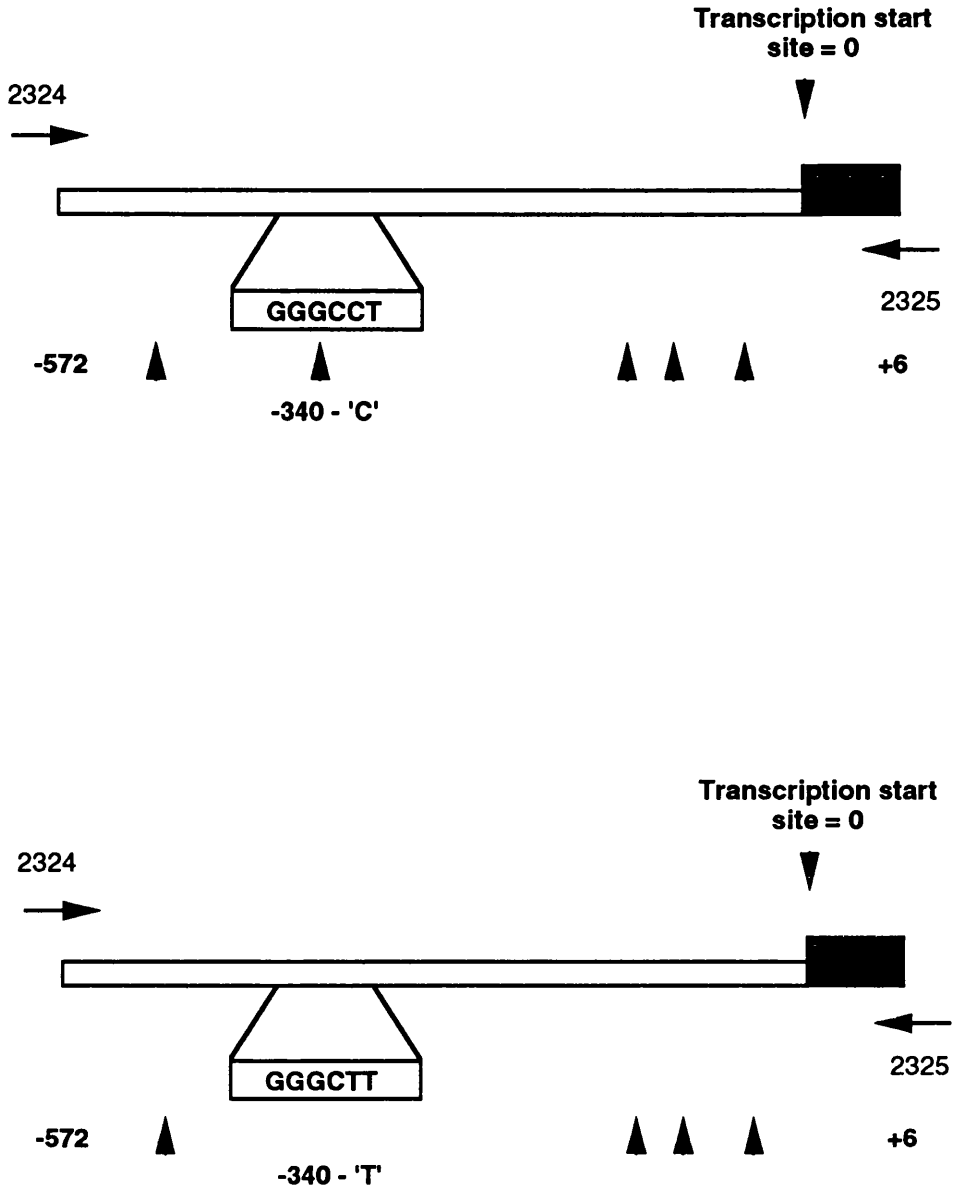



Figure 5.2 a -340C/T polymorphism in CYP11B2 promoter region

The ASO used to detect the -340C allele (2360) hybridised exclusively to the antisense strand of the PCR product containing the -340C allele, whilst the ASO used to detect the -340T allele (2361) hybridised exclusively to the sense strand of the PCR product containing the -340T allele. This strategy was chosen to eliminate the possibility of false positives occurring. That is, if an ASO intended to hybridise specifically to the *antisense* strand of the -340T promoter (-GGCT-) were used, it could also hybridise to the *antisense* strand of the -340C allele(-CCGG-) because G-T bonding forces are relatively stronger than those of G-A or C-A bonds. The possibility of a false positive is avoided if this strategy is followed.

### 5.2.2 Detection of HaeIII polymorphism

An aliquot of the completed PCR reaction was placed in a sterile Eppendorf tube with 10Units of HaeIII in the presence of an appropriate buffer and incubated at $37^0$C for 4 hours (2.8.3). On completion of the digest, 10µl of the reaction mix was run on an ethidium bromide-stained, 3% Metaphor agarose gel to determine the haplotype of the individual. A 3% agarose gel was chosen to ensure adequate resolution of the relatively low molecular weight digestion products so that naked eye assignment of haplotype was possible.

## 5.3 Results

All available members of the 5 kindreds were typed using both methods to determine the CYP11B2 promoter haplotypes.

The PCR protocol used yielded large quantities of a single product of the desired length, i.e. 578 base pairs (Figure 5.3 a).

### 5.3.1 ASO Hybridisation

The C and T allele of the polymorphism were demonstrated by ASO hybridisation as shown in Figure 5.3 b. The lower band corresponds to hybridisation of the probe to the C allele (2360) in the PCR product, the upper band the T allele (2361).

This technique allowed homozygotes and heterozygotes for the -340C/T polymorphism to be identified easily.

### 5.3.2 HaeIII digestion of CYP11B2 promoter region

The C allele of the polymorphism corresponds to the presence of an HaeIII recognition site at position -340 to -337 in the 5' flanking region of CYP11B2.

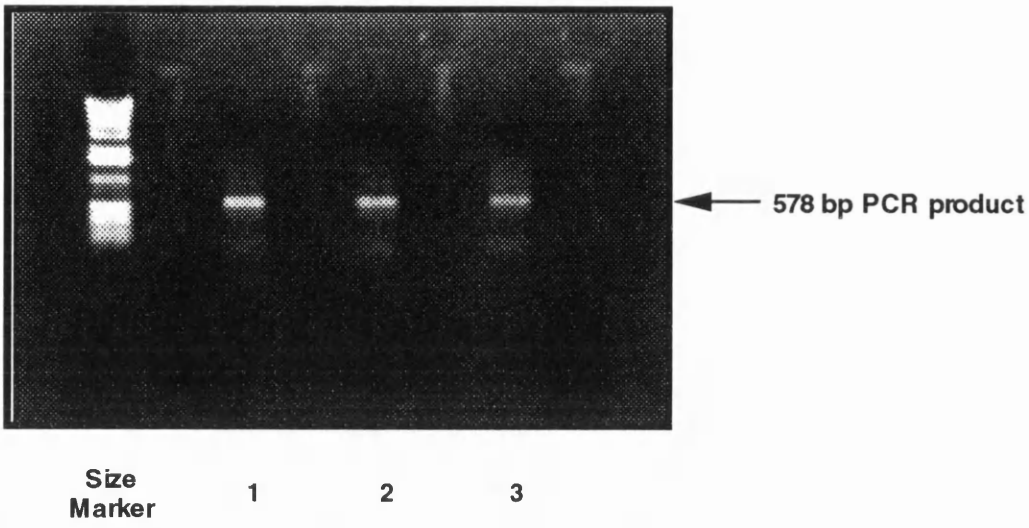
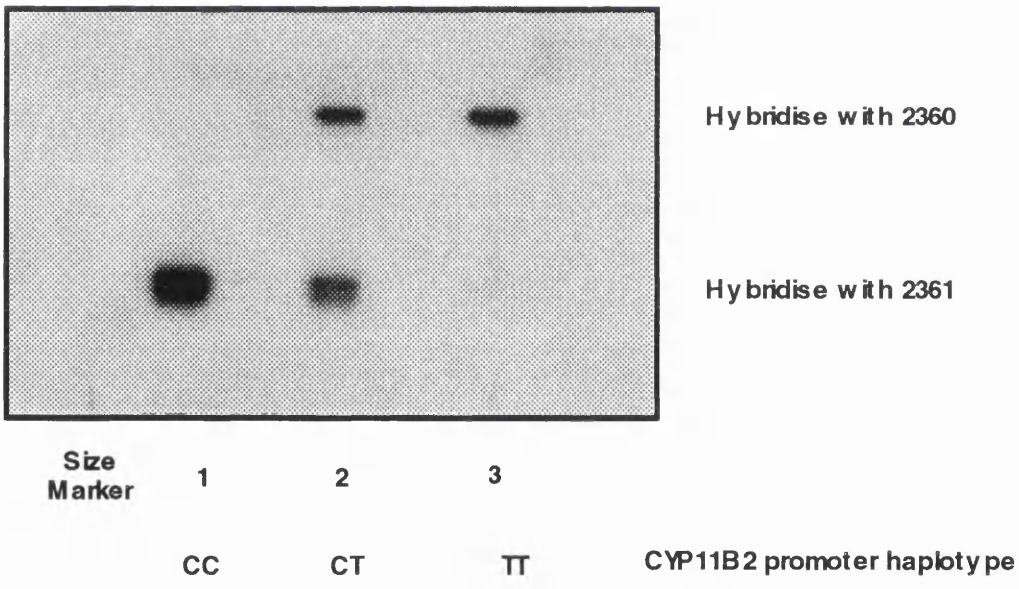



Figure 5.3 a PCR amplification of CYP11B2 promoter region




2360, 2361 - ASO's used to determine haplotype
C, T - Haplotypes assigned on presence of a C or T nucleotide at position -340

Figure 5.3 b ASO hybridisation to CYP11B2 promoter region

Digestion of the 578 bp PCR product in subjects with the C allele yields a 245 bp fragment in addition to lower molecular weight products, termed 'a'.

Possession of the T allele of the polymorphism abolishes the presence of an HaeIII recognition site at position -340 to -337 in the 5' flanking region of CYP11B2. HaeIII digestion of the PCR product in this case yields a 324 bp product along with low molecular weight products, termed 'A'.

The result of a typical digestion of PCR products from individuals homozygous for 'A', 'a', and a heterozygote are shown in Figure 5.3 c. It illustrates that the CYP11B2 promoter haplotype of individuals is readily established by HaeIII digestion of the PCR product without recourse to ASO hybridisation.

### 5.3.3 Haplotype analysis of GSH kindreds

Individuals in all 5 kindreds were typed for the presence of this polymorphism and the results shown in Figures 5.3 d-h. A total of 45 subjects were studied.

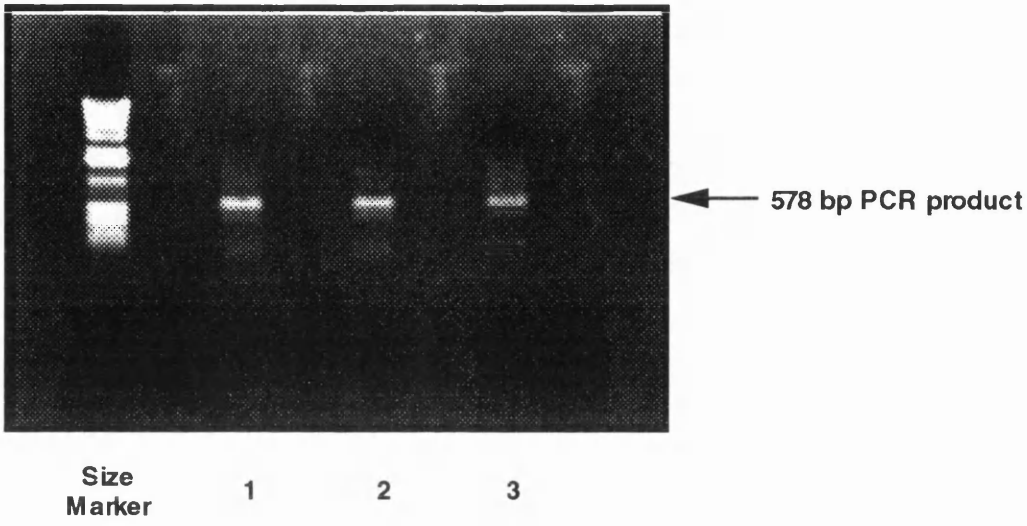
The frequencies of each allele and haplotype are given in Table 5.3 a.

| | Affected | Unaffected |
|---|---|---|
| **Allele** | | |
| C | 25 | 22 |
| T | 9 | 34* |
| **Haplotype** | | |
| CC | 8 | 8 |
| CT | 9 | 6 |
| TT | 0 | 14† |

* $\chi^2$ = 9.94 df=1, p=0.0016, † $\chi^2$ = 912.67 df=2, p=0.0018

**Table 5.3 a Frequency distribution of CYP11B2 promoter alleles and haplotypes in patients and unaffected relatives.**

The most striking feature of the distribution of the HaeIII haplotypes is the uniform inheritance of the 'a/-340C allele in association with the chimeric CYP11B1/B2 gene causing GSH. There is a significant excess of -340C alleles in the patients with GSH compared with unaffected relatives (or excess of -340T alleles in unaffected relatives) and a significant excess of CC homozygotes in the patients compared with the unaffected relatives (or an excess of non-CC homozygotes in the unaffected relatives) (Table 5.3 a).



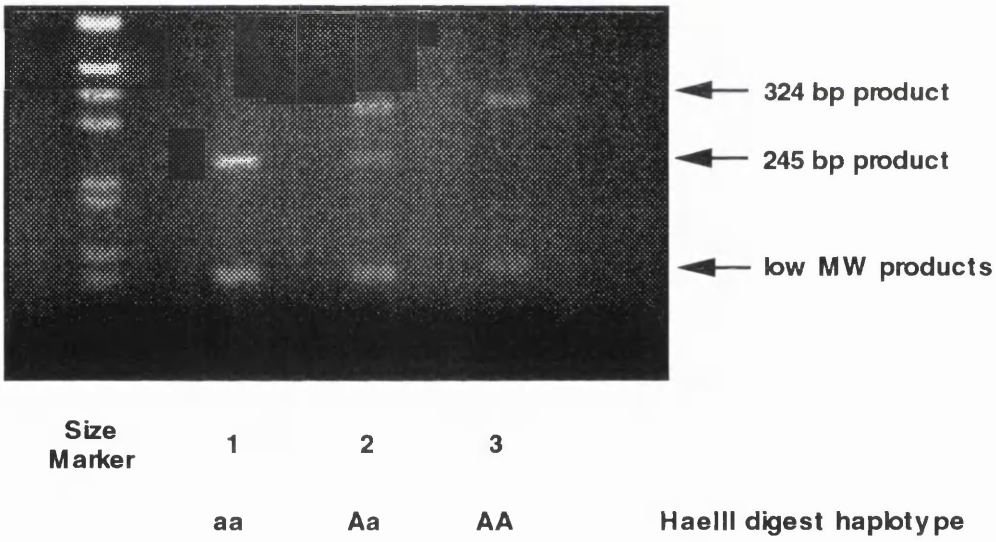




Figure 5.3 c HaeIII digest of CYP11B2 promoter region

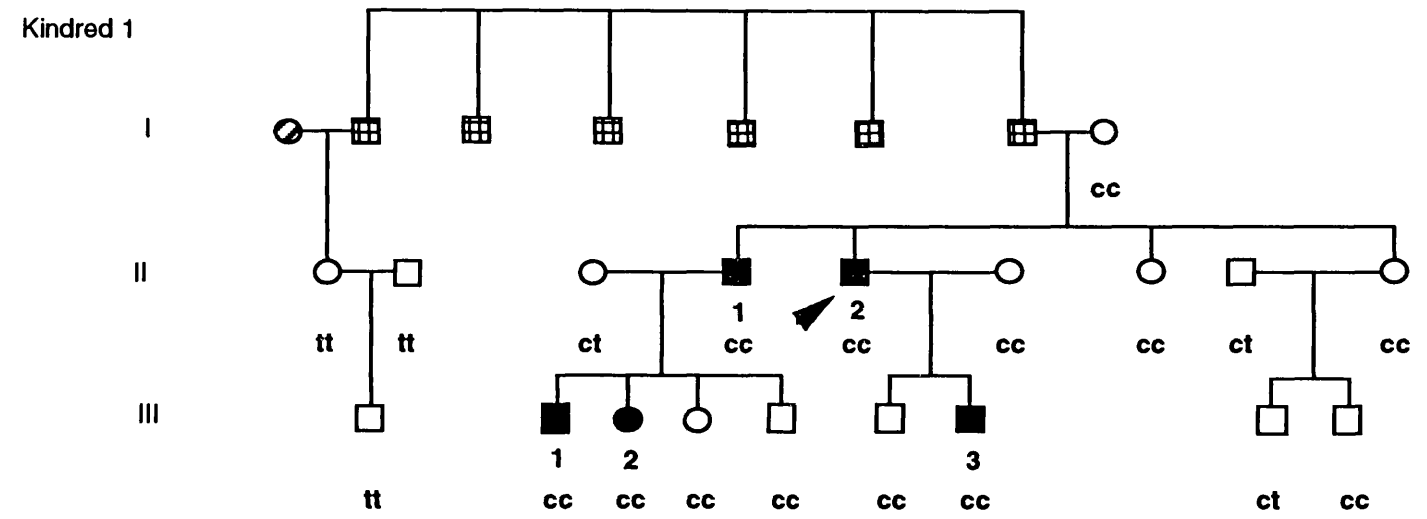



**Figure 5.3 d**

**Kindred 1. CYP11B2 promoter polymorphism haplotypes.**

Kindred 2

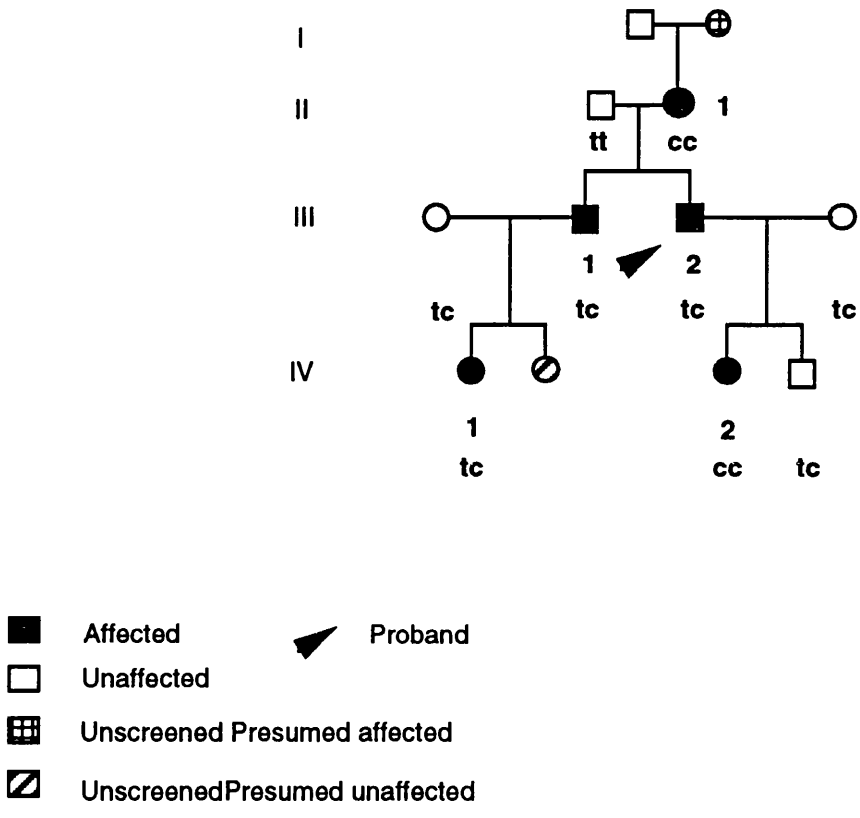



**Figure 5.3 e**

**Kindred 2. CYP11B2 promoter polymorphism haplotypes.**

Kindred 3

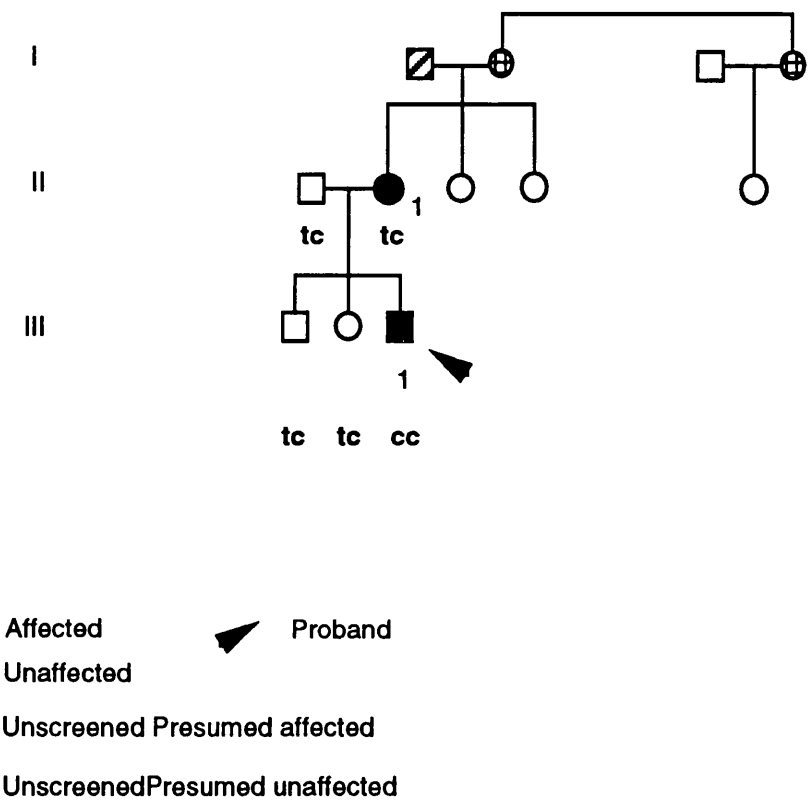
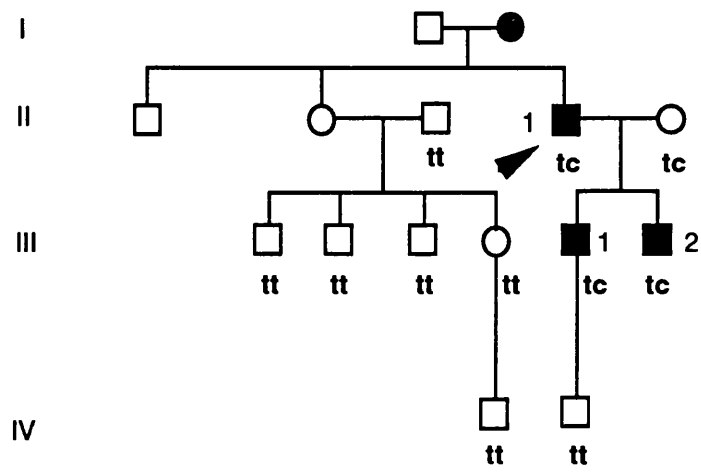



**Figure 5.3 f**

**Kindred 3. CYP11B2 promoter polymorphism haplotypes.**

Kindred 4



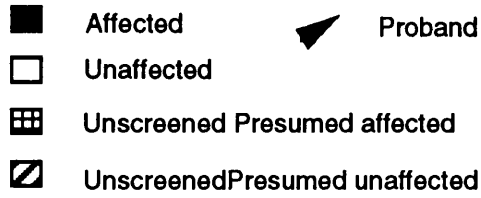




**Figure 5.3 g**

**Kindred 4. CYP11B2 promoter polymorphism haplotypes.**

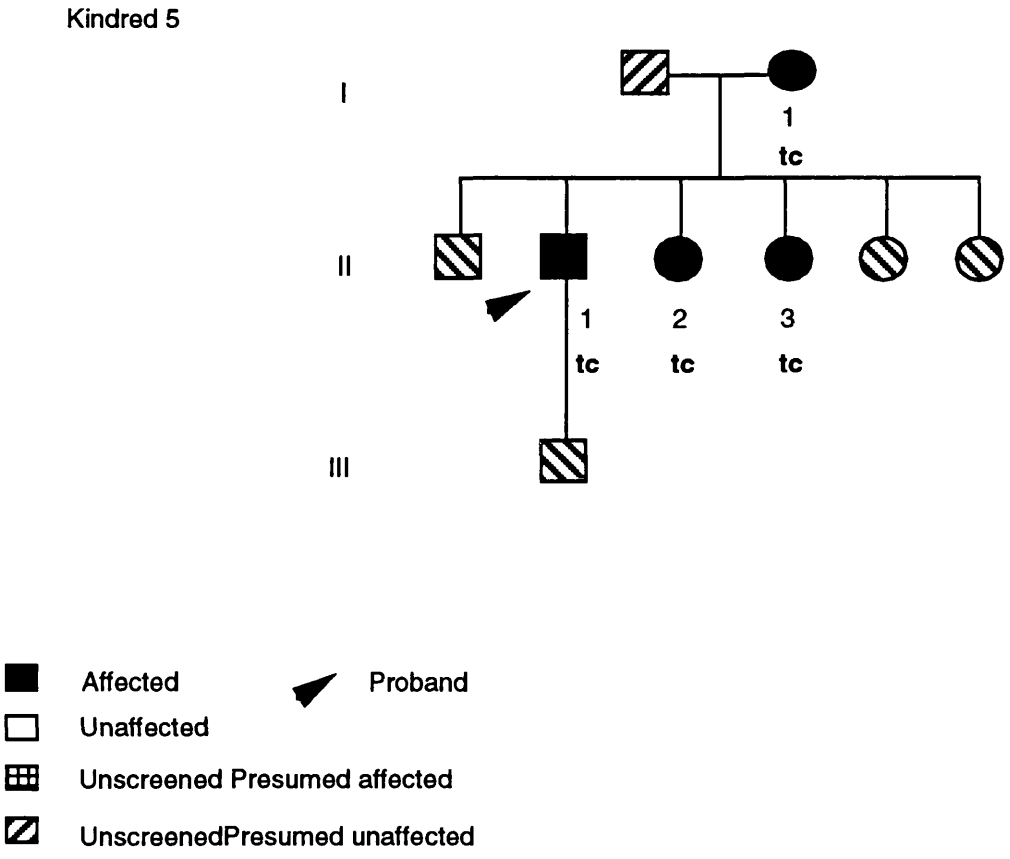



**Figure 5.3 h**

**Kindred 5. CYP11B2 promoter polymorphism haplotypes.**

The uniformity of the co-inheritance of the 'a' allele with GSH suggests that the -340C and GSH are in linkage disequilibrium.

## 5.4 Discussion

Both of the techniques employed to distinguish the two alleles in the CYP11B2 promoter were equally effective. The method based on restriction enzyme digestion of the promoter PCR product was quicker and avoided the need for end-labelling of ASO's eliminating the requirement for radioactivity. Both methods were used to haplotype the members of the 5 kindreds for the polymorphism and 100% concordance was seen. Thus I recommend that the HaeIII digestion of the promoter region PCR product be used as the initial screening tool and that the ASO hybridisation method be used in cases where this fails. Similar methods have been used previously to determine the presence or absence of disease causing mutant alleles in steroid 11ß-hydroxylase deficiency (Curnow et al, 1993, White et al, 1991), corticosterone methyloxidase type II deficiency (Pascoe et al, 1992b) and sickle cell anaemia (HbS) (Connor and Ferguson-Smith, 1987). In some of these cases, the method can also be used on material obtained at chorionic villus sampling or amniocentesis making prenatal diagnosis possible (Curnow et al, 1993).

The discovery of linkage disequilibrium between the 'a' / -340C allele of the polymorphism and inheritance of the chimeric CYP11B1/B2 gene is intriguing. A similar finding has been made by Lifton (Lifton et al, 1992) who described linkage disequilibrium between allele 1 of AldoX9, a SSCP marker in CYP11B2, and GSH. On chromosomes carrying the chimeric gene both the normal CYP11B2 gene and the portion of CYP11B2 contributing to the chimeric gene possessed allele 1 of AldoX9. This allele was present in the normal CYP11B2 on these chromosomes in 9 out of 9 kindreds and in the CYP11B2 portion of the chimeric gene in 8 out of 9 kindreds. In addition, 4 discrete crossover events and three different haplotypes for different CYP11B1/B2 SSCP markers were detected in these nine kindreds, suggesting that multiple independent crossovers had occurred within this group of kindreds. It would appear therefore that allele 1 of AldoX9 is a highly conserved feature in the chimeric genes of these kindreds.

However, a gene conversion event transferring most of intron 2 of CYP11B1 to intron 2 of CYP11B2 has been described (PC White, personal communication), i.e. in one allele CYP11B2 contains an intron virtually identical to that of CYP11B1. The existence of such an allele gives one possible explanation of how the chimeric gene might arise (Figure 5.4 a). In the normal situation where intron 2 of CYP11B2

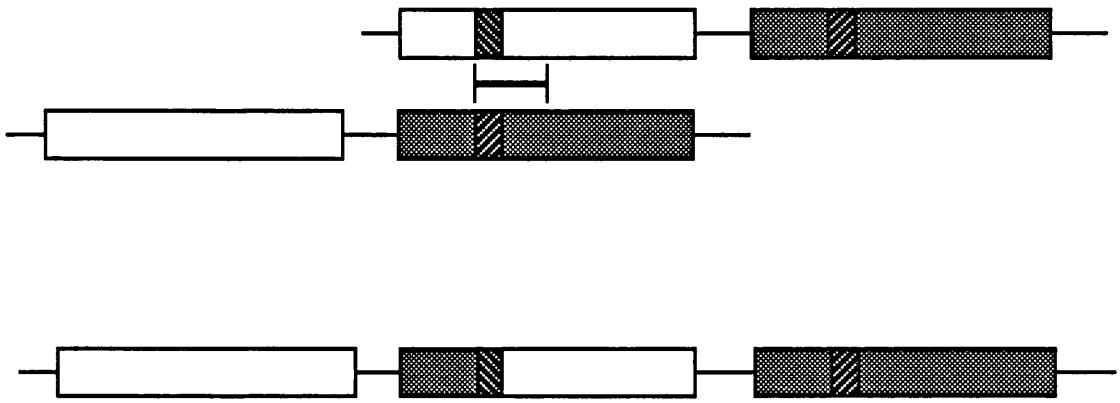


**Figure 5.4 a. Potential mechanism underlying formation of chimeric CYP11B1/B2 gene**

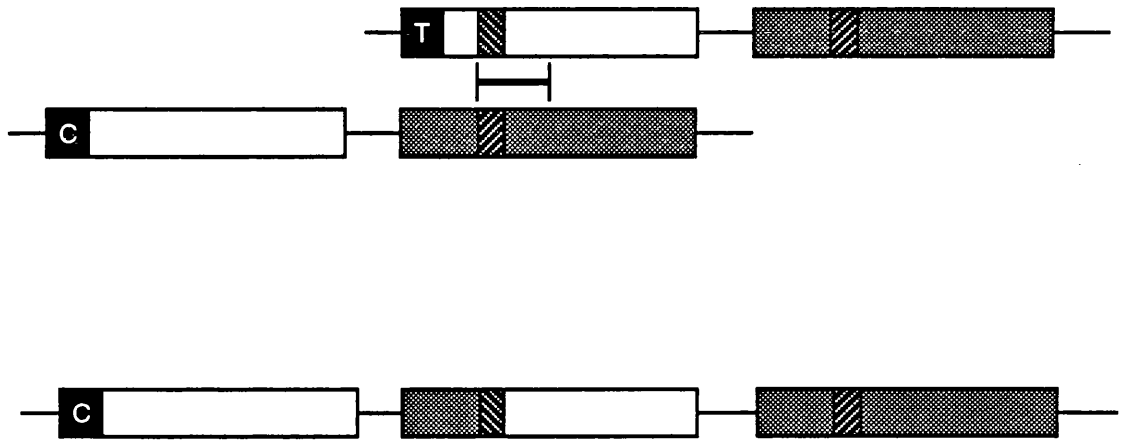
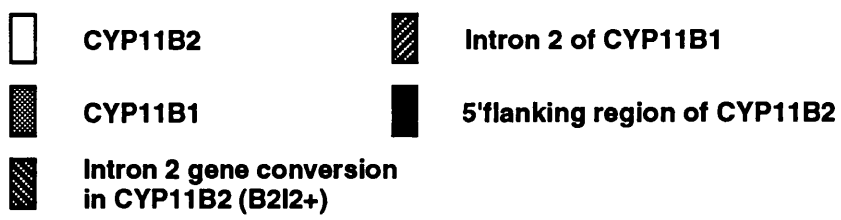



**Figure 5.4 b. Formation of chimeric CYP11B1/B2 gene and mechanism of linkage disequilibrium between -340C and GSH.**



does not contain the gene conversion (B2I2-), alignment of CYP11B1 and CYP11B2 is reduced because intron 2 of these genes is an area of sequence divergence between CYP11B1 and CYP11B2. However, in individuals with the CYP11B2 gene with the intron 2 conversion (B2I2+), CYP11B1 and CYP11B2 a region spanning approximately 2 kilobases from intron 2 - intron 4 can align with almost 100% identity and thus promote the likelihood of non-homologous recombination.

In all of the cases of GSH in this series, patients inherit the -340C allele of the CYP11B2 promoter polymorphism. This may be explained if the chromosome without the gene conversion in CYP11B2 also carries the -340C allele (Figure 5.4 b). Non-homologous recombination between a B2I2+ CYP11B2 gene and aCYP11B1 gene on a -340C chromosome results in a -340C-GSH haplotype. Observations in a limited number of patients suggests that -340T and B2I2+ are in linkage disequilibrium and that -340C and B2I2+ do not occur together on the same chromosome (White et al, 1994), thus strengthening the arguement in favour of the above mechanism.

Further work to investigate the prevalence of these alleles in other populations is planned.

Abnormalities in steroid secretory rates in response to intravenous ACTH infusion and Ang II infusion have been described in patients with essential hypertension (De Simone et al, 1985, Honda et al, 1977, Kisch et al, 1976, Zoccali et al, 1983). Such abnormalities could arise from differences in the rate of expression of genes encoding steroidogenic enzymes in the adrenal cortex of hypertensive patients, possibly determined by polymorphisms in the promoter regions of these genes. The -340 C/T polymorphism is in the region of the SF-1 binding site and alters binding of SF-1 to DNA (White et al, 1994). Such alteration in binding affinity might result in physiologically important differences in the expression of CYP11B2 in the zona glomerulosa and lead to detectable differences in aldosterone secretory responses to Ang II. At the present time no such polymorphisms have been detected in the promoter region of CYP11B1.

Abnormalities of 11ß-hydroxylase activity have been detected in rat models of hypertension (Fraser et al, 1994). In the Milan normotensive and hypertensive strains, the hypertensive strain showed evidence of enhanced 11ß-hydroxylase activity when adrenal steroid secretory rates were compared with those in the normotensive strain. Polymorphisms in the rat genes encoding enzymes with 11ß-hydroxylase activity have been described (Cicila et al., 1993, Matsukawa et al.,

1993, Mukai et al., 1991). In addition, in rats a third CYP11B like gene, CYP11B3, has been described which is highly homologous to CYP11B1 in both its coding and its 5' flanking region (Nomura et al., 1993). CYP11B2 and CYP11B3 in the rat lie in tandem approximately 24 kilobases apart. However, there was no detectable transcription of CYP11B3 in the adrenal glands of rats fed a normal or potassium-enriched diet (Nomura et al, 1993). This suggests that this gene may be a non-functional pseudogene. In bovine adrenal 5 different CYP11B genes have been described and three are pseudogenes (Hashimoto et al., 1993). The tandem arrangement of CYP11B genes has been demonstrated in the mouse (Domalik et al, 1991), and in man (Lifton et al, 1992, Pascoe et al, 1992a, Pascoe et al, 1992b) but as yet no human CYP11B pseudogenes have been identified.

In summary, in man and rat models of essential hypertension there are abnormalities of steroid secretion related to the enzymes $P450_{11\beta}$ and $P450_{aldo}$. Polymorphisms in the flanking regions of CYP11B2 and CYP11B1 have been described and may be important in the genesis of chimeric CYP11B1/B2 genes causing GSH and in influencing the secretory responses to infused ACTH and Ang II.

The mechanisms whereby these single base pair changes might influence the formation of a chimeric gene are not clear. However, the possibility of linkage disequilibrium with a gene conversion favouring non-homologous recombination may be an important feature favouring crossover formation.

## Chapter 6

# PHENOTYPIC OBSERVATIONS IN PATIENTS WITH GLUCOCORTICOID-SUPPRESSIBLE HYPERALDOSTERONISM

## 6.1 Introduction

The clinical and biochemical features of GSH are attributable to a primary aldosterone excess. However, detailed descriptions of affected individuals show that the clinical features of the condition, i.e. its phenotype, are variable even within the same kindred. The reasons for these differences are not entirely clear but could be genotype-environment interactions which alter the clinical expression of the genotype. Such influences include dietary sodium intake, alcohol ingestion and body weight, all of which may influence blood pressure in patients with essential hypertension and in mineralocorticoid-dependent hypertension (Brown et al, 1972b, Ferriss et al, 1983, Kremer et al, 1977, Oelkers et al, 1974, Whelton et al, 1994).

### 6.1.1 Phenotypic differences in GSH patients

The clinical phenotype of affected individuals may vary as a result of differences in the nature of the chimeric gene, i.e. different chimeric genes may confer variable degrees of aldosterone synthase activity and consequent variations in aldosterone concentration may determine the subsequent blood pressure and potassium status of individuals. Study of the *in vitro* expression of chimeric CYP11B1/B2 cDNA's in COS cells has shown that aldosterone synthase activity of the chimeric gene product is limited to those chimeric constructs containing exons 1 - 4 only of CYP11B1. Inclusion of exon 5 of CYP11B1 abolishes the aldosterone synthase activity of the constructs (Pascoe et al, 1992a). However, it is not clear if there is a dose-response relationship between aldosterone synthase activity and the number of exons donated from CYP11B2 is increased from 5 (exons 5 - 9) to 8 (exons 2 - 9).

### 6.1.2 The role of parental programming in the origin of hypertension

The role of parental inheritance of the chimeric gene may also be important in determining the phenotype of the disorder. Studies of pregnant rats given dexamethasone during pregnancy have shown that the birth weight of the glucocorticoid-treated rats is reduced by 14% and that the adult systolic blood pressure of the rats is elevated for many months after exposure to exogenous glucocorticoids (Benedicktsson et al, 1993). A similar effect on birth weight has

been observed in the offspring of dexamethasone treated mothers of fetuses with congenital adrenal hyperplasia (Seckl, 1994). In addition, epidemiological data suggests that hypertension develops most readily in those children born with a low birth weight and a large placenta, but not undernourished *in utero* (Law and Barker, 1994, Parker et al., 1990). Children likely to develop hypertension as adults can be detected at an early age by the phenomenon of 'tracking' of blood pressure (i.e. the blood pressure of a child likely to develop hypertension as an adult tends to be higher than average for their age and sex throughout childhood (Lever and Harrap, 1992)) and there is evidence that a child's blood pressure is more closely related to that of its mother than its father (Bengtsson et al., 1979, Gerson and Fodor, 1975, Law et al, 1991).

### 6.1.3 Impaired 11ß-hydroxylase activity in hypertension

There is evidence in patients with essential hypertension that conversion of DOC to corticosterone and 11-deoxycortisol to cortisol is impaired compared with normotensive controls, particularly following ACTH stimulation. This would appear to be secondary to impaired 11ß-hydroxylase activity in hypertensive patients (De Simone et al, 1985, Honda et al, 1977) and chapter 1.3.4. Similarly, in patients with GSH there is evidence of high basal plasma DOC concentrations in untreated patients (Connell et al, 1986, Connell et al, 1986, Oberfield et al, 1981, Ulick et al, 1990). ACTH infusion in patients with GSH leads to marked increases in plasma aldosterone and 18-hydroxycortisol concentrations (Connell et al, 1986) and might be expected to exaggerate defective 11ß-hydroxylase activity if it is present in patients with GSH.

Therefore, there are several potential mechanisms whereby the clinical phenotype of individuals with GSH may vary both between and within kindreds. In addition, increased DOC levels may indicate an additional defect in 11ß-hydroxylase activity in GSH which might influence the clinical phenotype of individual patients with GSH and may also contribute to its pathogenesis.

From these introductory sections, it is clear that the rare diseases, GSH and Conn's syndrome, and the much more common essential hypertension have in common a degree of disturbance of corticosteroid synthesis and control of secretion but that, within each group, the phenotypic result may be strongly influenced by a series of complex interactions with a large number of environmental factors. The following section compares the basal levels and responses to ACTH infusion of corticosteroids and other variables of groups of subjects with GSH, essential hypertension, Conn's syndrome and a matched group

of normotensive subjects. An examination of the effects of crossover site and parental inheritance on GSH phenotype is also presented.

## 6.2 Subjects and Methods

### 6.2.1 Methods

Blood pressure, serum electrolytes and plasma measurements of renin, aldosterone and other steroid hormones were obtained and assayed as described in 2.5.1-3 and 2.6.1-4.

Intravenous ACTH infusion studies were performed as described in 2.5.5 and blood taken for steroid measurements and assayed as described in 2.5.4, 2.6.1-4.

### 6.2.2 Subjects

A) GSH

All patients with genotypically proven GSH were studied in the basal state.

Twelve patients received oral dexamethasone 0.5mg q.d.s. for 4 weeks. Blood pressure measurement and determination of serum potassium and plasma concentrations of renin and aldosterone performed.

Nine patients received an intravenous ACTH infusion with blood sampling and blood pressure measurements as described in 2.5.5.

Ten first degree relatives of the patients in kindred 1 underwent basal state blood sampling for measurement of plasma steroids as described in 2.5.3.

B) Essential Hypertension

Eight patients with essential hypertension received an intravenous ACTH infusion with blood sampling and blood pressure measurements as described in 2.5.5. These patients had hypertension requiring hypotensive drug therapy, discontinued 1 month prior to study. Secondary causes of hypertension were excluded by relevant investigations.

C) Normotensive control subjects

Five normotensive volunteers, matched for age and sex range of the hypertensive subjects, with no family history of hypertension received an intravenous ACTH

infusion with blood sampling and blood pressure measurements as described in 2.5.5.

D) Conn's syndrome

Four patients with aldosterone-secreting adrenal adenoma demonstrated by CT scanning of the adrenal glands received an ACTH infusion with blood sampling and blood pressure measurements as described in 2.5.5.

### 6.2.3 Statistical Methods

Data are presented as median with the associated range. In cases where the data were normally distributed, tests of statistical significance were performed by means of a two-way paired t-test. If data were not normally distributed then a non-parametric test, either Mann-Whitney U-test or Wilcoxon rank test, was applied.

In section 6.3.1 C, the data for blood pressure and aldosterone were corrected for age by analysis of covariance with age as the covariate. Groups were then compared by a one-way t-test and $p<0.05$ regarded as significant.

## 6.3 Results

The data obtained from the studies are presented in Appendix V. Results are presented as median (range) unless otherwise stated.

### 6.3.1 Patients with GSH

In total 19 patients from 5 kindreds were identified by means of genotyping of DNA obtained from peripheral blood.

A) - Basal Data: patients untreated (Table 6.3 a)

Mean arterial blood pressure was considerably elevated as was plasma aldosterone concentration.

Plasma renin concentration was suppressed compared to normal.

The range of serum potassium concentrations included 5 below the lower end of the normal range (3.5 mmol/l) but the median serum potassium was 3.8 mmol/l (2.8-4.6) and the majority of the untreated values (14/19) were > 3.5 mmol/l.

In five patient under the age of 20 years, the mean blood pressure was not significantly elevated in spite of significantly elevated plasma aldosterone concentrations.

| | Mean Arterial Blood Pressure (mmHg) | Serum Potassium (mmol/l) | Plasma Aldosterone (pmol/l) | Plasma Active Renin (μU/ml) |
|---|---|---|---|---|
| **Normal Range** | | 3.5-5.1 | 140-500 | 5-50 |
| **All patients with GSH** | 120 (70-170) | 3.8 (2.8-4.6) | 999 (452-2466) | 1 (0-4) |
| **Patients with GSH and normal BP** | 76* (70-92) | 4.2¶ (3.6-4.6) | 763¶ (452-971) | 1 (1-4) |

* $p<0.0001$, ¶ $p<0.05$

**Table 6.3 a Baseline data for patients with GSH, n=19, and normotensive patients with GSH, n=5.**

B) - After treatment with oral dexamethasone (Table 6.3 b)

Treatment with oral dexamethasone led to a significant reduction in mean arterial blood pressure (Figure 6.3 a) and lowering of plasma aldosterone concentration into the normal range (Figure 6.3 b). This change following treatment was statistically significant, $p<0.001$.

| | Mean Arterial Blood Pressure (mmHg) | Serum Potassium (mmol/l) | Plasma Aldosterone (pmol/l) | Plasma Active Renin (μg/ml) |
|---|---|---|---|---|
| **Normal Range** | | 3.5-5.1 | 140-500 | 5-50 |
| **Pre-treatment** | 130 (113.5-170) | 3.8 (2.8-4.4) | 999 (452-2466) | 2 (1-3) |
| **Post-treatment** | 89 * (74-112) | 4.2 ¶ (3.7-5.0) | 203 † (28-252) | 23 * (16-60) |

* $p<0.0001$, †, $p<0.001$, ¶ $p<0.005$

**Table 6.3 b Effect of dexamethasone 0.5 mg q.d.s. for 4 week in patients with GSH. n=12.**

Plasma renin concentrations rose into the normal range (Figure 6.3 c). This change following treatment was statistically significant, $P<0.0001$.

Serum potassium concentration rose on treatment from 3.8 mmol/l (2.8-4.4) to 4.2 mmol/l (3.7-5.0). This change following treatment was statistically significant, $p<0.005$.

C) - Effect of parental origin of GSH on blood pressure (Table 6.3 c)

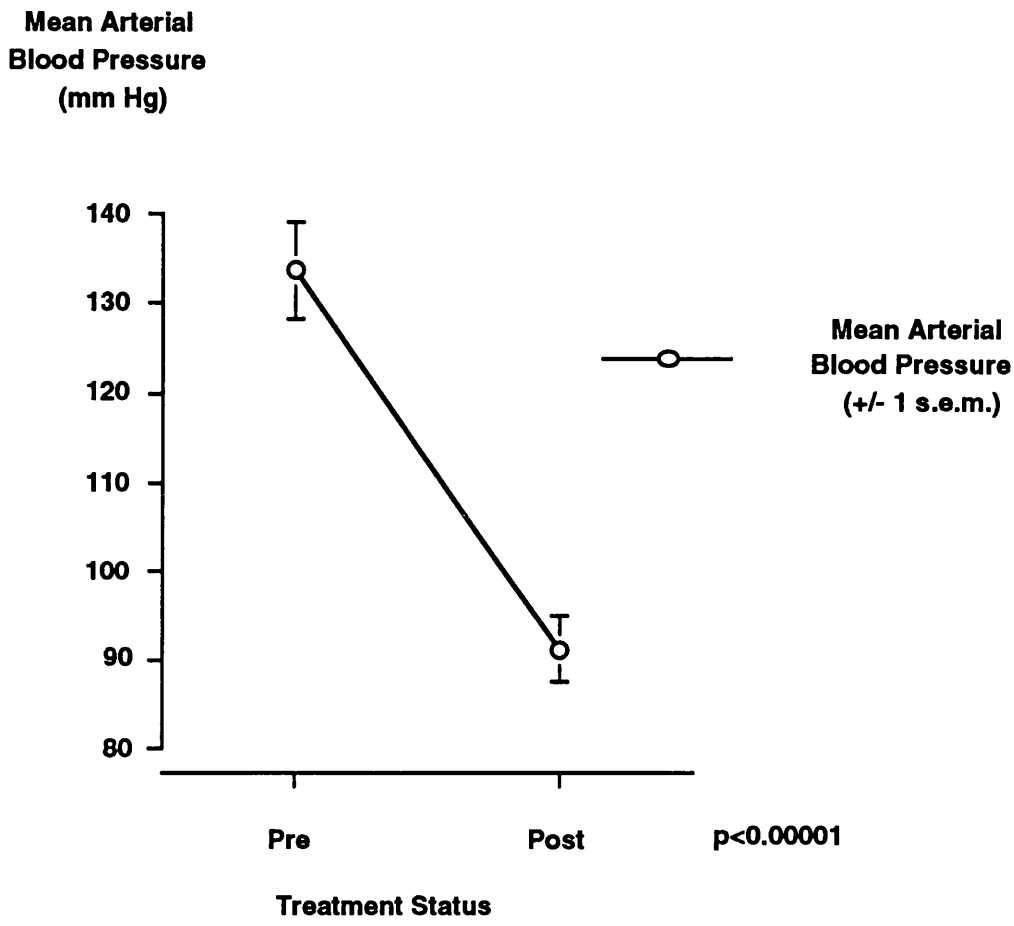



**Figure 6.3 a Effect of treatment with dexamethasone 0.5mg q.d.s on mean arterial blood pressure. (n=12)**

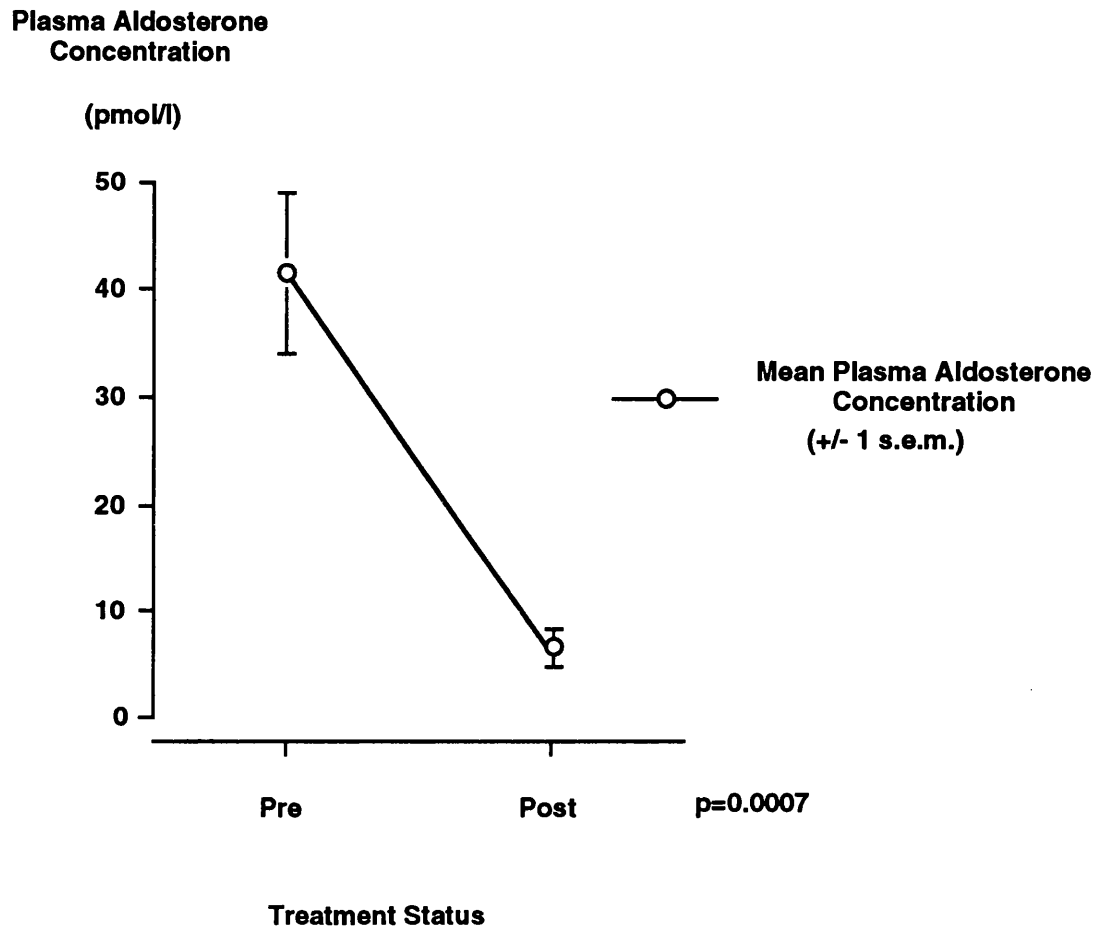



**Figure 6.3 b Effect of treatment with dexamethasone 0.5mg q.d.s on plasma aldosterone concentration. (n=12)**

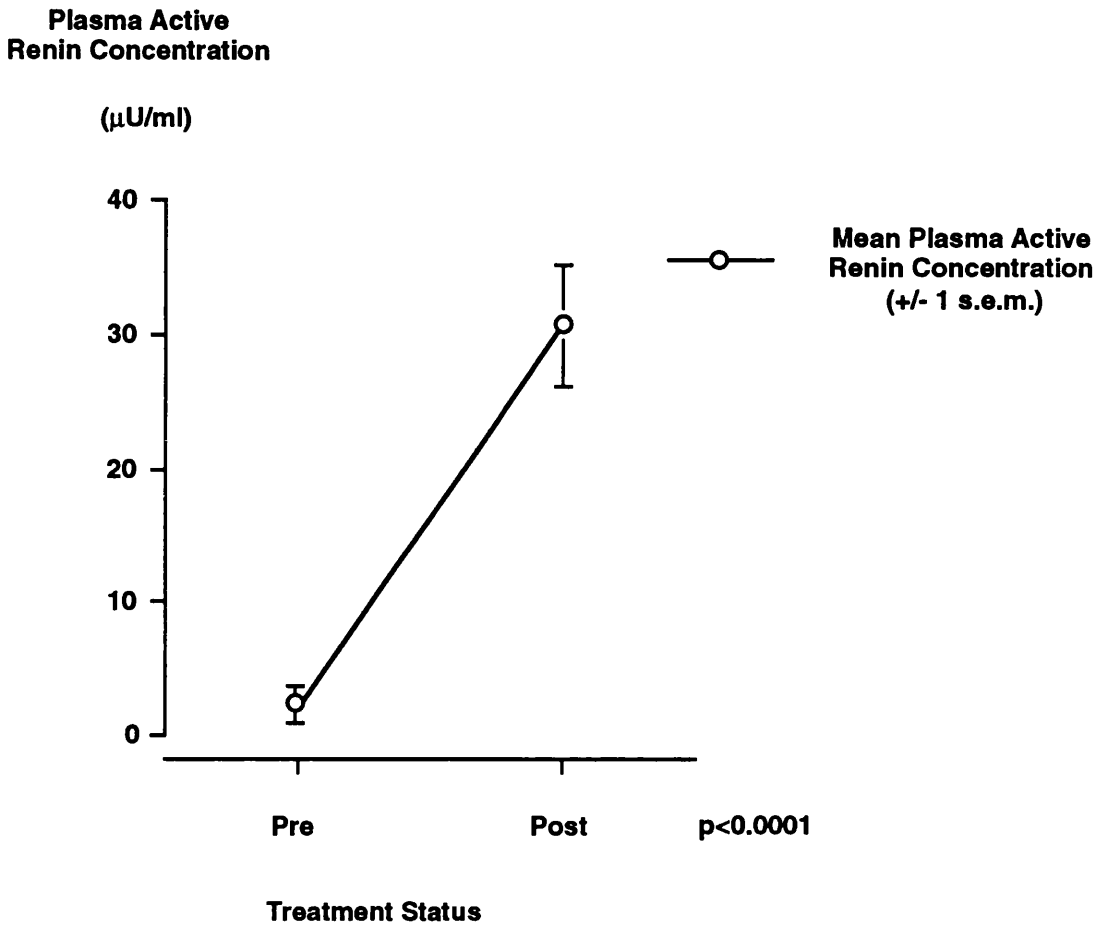



**Figure 6.3 c Effect of treatment with dexamethasone 0.5mg q.d.s on plasma active renin concentration. (n=12)**

The effect of parental origin of the chimeric CYP11B1/B2 on the mean blood pressure of affected individuals was determined by comparing age and weight corrected blood pressures of individuals who inherited the condition from their mother with those who inherited it from their father.

Those individuals who inherited GSH from their mother had significantly higher blood pressures than those inheriting the condition from their father, Maternal inheritance - 137 mmHg (115-170), Paternal inheritance - 103.5 mmHg (72-155), $p<0.05$.

| | Mean Arterial Blood Pressure (mmHg) | Plasma Aldosterone (pmol/l) |
|---|---|---|
| **Normal Range** | | **140-500** |
| **Maternal Inheritance n=9** | 137 (115-170) | 1185 (583-2466) |
| **Paternal Inheritance n=9** | 103.5 * (72-155) | 688 * (452-971) |

* $p<0.05$

**Table 6.3 c Effect of parental origin of chimeric CYP11B1/B2 gene on blood pressure and plasma aldosterone concentration in patients with GSH**

Plasma aldosterone concentrations were significantly higher in those patients inheriting GSH from their mother rather than their father, Maternal inheritance - 1185 pmol/l (583-2466), Paternal inheritance - 688 pmol/l (452-971), $p<0.05$.

D) Effect of crossover site on phenotype

Blood pressure and plasma aldosterone were analysed with respect to the crossover site in the chimeric gene as described in Chapter 4 (Table 6.3 d).

| | Mean Arterial Pressure (mmHg) | Plasma Aldosterone (pmol/l) |
|---|---|---|
| **A** | 122 (70-155) | 796 (452-1503) |
| **B** | 113 (113-153) | 971 (956-2466) |
| **C** | 138 (115-170) | 1040 (583-1806) |

A - kindreds 1,2,3, B - kindred 4, C - kindred 5

**Table 6.3 d Relationship of blood pressure and plasma aldosterone levels to crossover site**

There was no significant difference in blood pressure or plasma aldosterone concentration between any of the three groups of patients when assessed on the basis of crossover site.

E) Basal plasma steroid measurements in GSH patients and unaffected relatives

Patients with GSH in kindred 1 had significantly higher mean blood pressures and plasma aldosterone concentrations and lower plasma renin concentrations than their unaffected relatives (Table 6.3 e).

| | Mean Arterial Blood Pressure (mmHg) | Plasma Aldosterone (pmol/l) | Plasma Active Renin (μg/ml) |
|---|---|---|---|
| **Patients with GSH (n=5)** | 109 (76-136) | 666 (452-971) | 1 (0-4) |
| **Relatives** | 89† (66-118) | 166† (56-249) | 27* (19-51) |

* p<0.0001, †, p<0.001, ¶ p<0.005

**Table 6.3 e Blood pressure and biochemical data for patients and relatives in kindred 1**

The data for all patients were pooled and compared with those of the relatives in kindred 1. A similar difference observed (Table 6.3 f).

| | Mean Arterial Blood Pressure (mmHg) | Plasma Aldosterone (pmol/l) | Plasma Active Renin (μg/ml) |
|---|---|---|---|
| **All Patients with GSH** | 120 (70-170) | 999 (452-2466) | 1 (0-4) |
| **Relatives** | 89† (66-118) | 166† (56-249) | 27* (19-51) |

* p<0.0001, †, p<0.001, ¶ p<0.005

**Table 6.3 f Blood pressure and biochemical data for all patients with GSH and relatives in kindred 1**

Since previous observations have suggested that 11ß-hydroxylation is impaired in patients with GSH, calculation of the basal molar ratios of plasma 11-deoxycorticosterone:corticosterone (DOC:B) and 11-deoxycortisol:cortisol (S:F) was performed.

Patients with GSH had significantly higher basal molar plasma DOC:B and S:F ratios than their unaffected relatives and unrelated normotensive controls (Table 6.3 g).

| | Patients with GSH | Relatives | Normotensive |
|---|---|---|---|
| DOC:B | 0.22 (0.06) | 0.04 (0.01)* | 0.05 (0.02)*† |
| S:F | 0.004 (0.0007) | 0.0015‡ (0.0008) | 0.0016‡† (0.0007) |

Results are mean (sem)
* p<0.02 for comparison with GSH patients, ‡ p<0.05 for comparison with GSH patients,
† p= not significant for comparison between relatives and normotensive controls

**Table 6.3 g Comparison of molar ratios of DOC:B and S:F in patients with GSH, unaffected relatives and unaffected noromtensive controls**

There was no significant difference in blood pressure or plasma aldosterone when the data was analysed with respect to crossover site, i.e. interkindred differences in blood pressure were not found.

### 6.3.2 ACTH infusion studies

ACTH infusion resulted in significant increases in plasma concentrations of steroid hormones at 90 minutes in all groups studied (Table 6.3 h), (Figures 6.3 d-h).

| | DOC ng/dl | | B ng/dl | | Aldo ng/dl | | S ng/dl | | F nmol/l | |
|---|---|---|---|---|---|---|---|---|---|---|
| TIME mins | 0 | 90 | 0 | 90 | 0 | 90 | 0 | 90 | 0 | 90 |
| GSH | 11 | 84.9 | 97.4 | 1315 | 17.8 | 57.1 | 37.6 | 229.9 | 285 | 747 |
| n=9 | (2.9) | (16.8) | (51) | (148) | (4.4) | (9.1) | (7.9) | (13.8) | (25.9) | (34.7) |
| NT | 6.8 | 21.6 | 266.4 | 1344 | 9.0 | 28.2 | 22.8 | 74.8 | 368 | 793 |
| n=5 | (2.1) | (5.8) | (159) | (254) | (2.2) | (4.4) | (11.1) | (14.7) | (28.2) | (33.0) |
| HT | 7.6 | 23.9 | 227.3 | 1111 | 11.4 | 33.25 | 37.8 | 168.5 | 402 | 837 |
| n=8 | (2.1) | (6.7) | (80) | (179) | (1.9) | (4.8) | (11.1) | (31.6) | (30.7) | (41.5) |
| CONN | 16.7 | 63.3 | 292.7 | 1286 | 35.7 | 82 | 47.3 | 265 | 414 | 803 |
| n=3 | (0.3) | (20.8) | (130) | (259) | (4.9) | (18.5) | (47.3) | (66.7) | (5.2) | (49.9) |

GSH - patients with GSH, NT - normotensive controls, HT - patients with essential hypertension, Conn - patients with aldosterone-secreting adenomata
DOC - 11-deoxycorticosterone, B - corticosterone, Aldo - aldosterone, S - 11-deoxycortisol, F - Cortisol

**Table 6.3 h Effect of ACTH infusion on plasma steroid concentrations at +90 minutes**

### 6.3.3 11β-hydroxylation activity

Basal molar plasma DOC:B plasma ratios in patients with GSH were significantly higher than normotensive controls, patients with essential hypertension and patients with Conn's syndrome, p<0.02 (Table 6.3 g). In patients with GSH, essential hypertension and normotensive controls there was a significant fall in

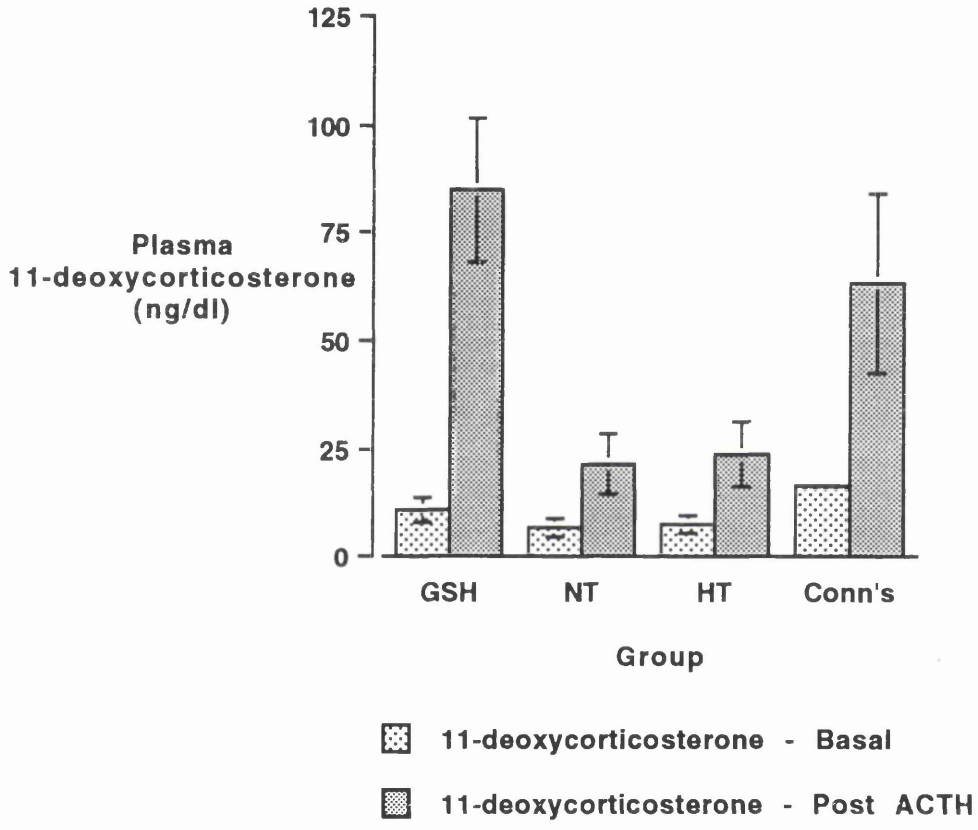



**Figure 6.3 d Plasma 11-deoxycorticosterone concentration pre- and post-ACTH infusion**

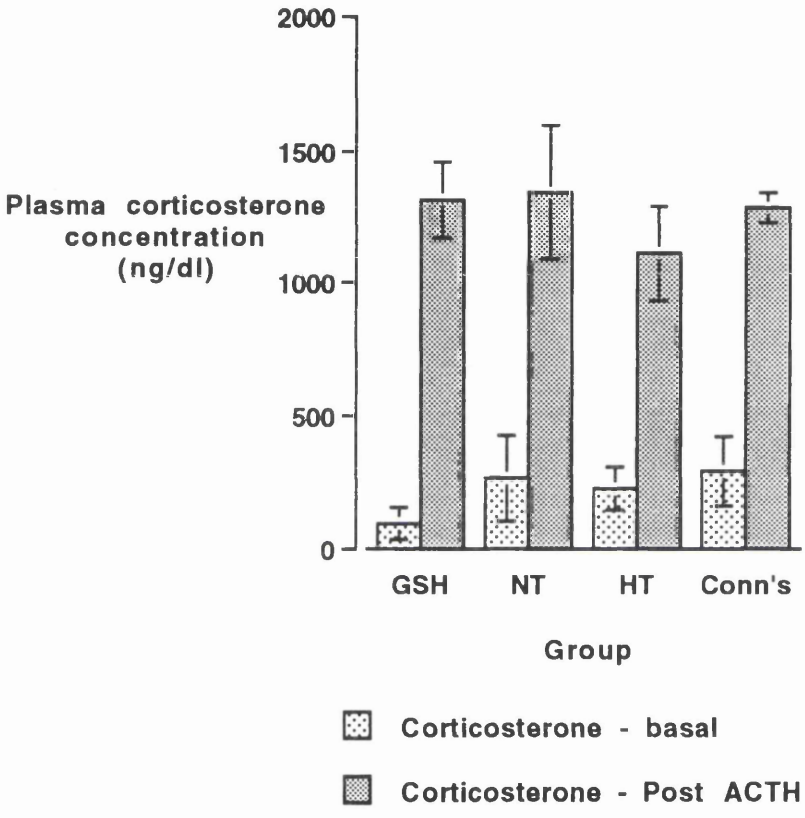



**Figure 6.3 e Plasma corticosterone concentration pre- and post ACTH infusion**

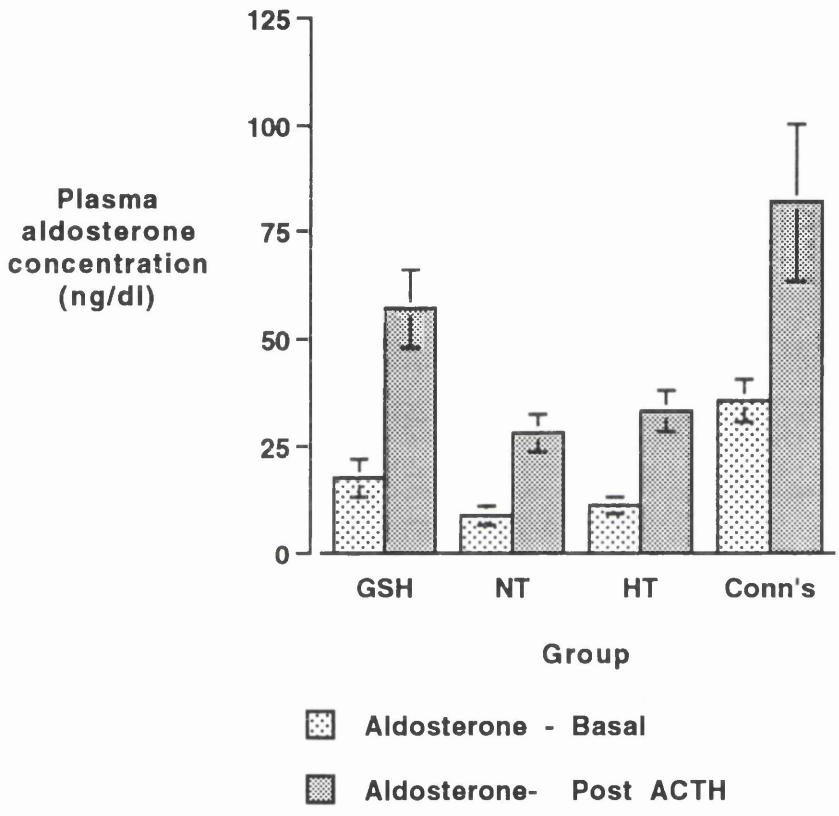



Figure 6.3 f Plasma aldosterone concentration pre- and post-ACTH infusion

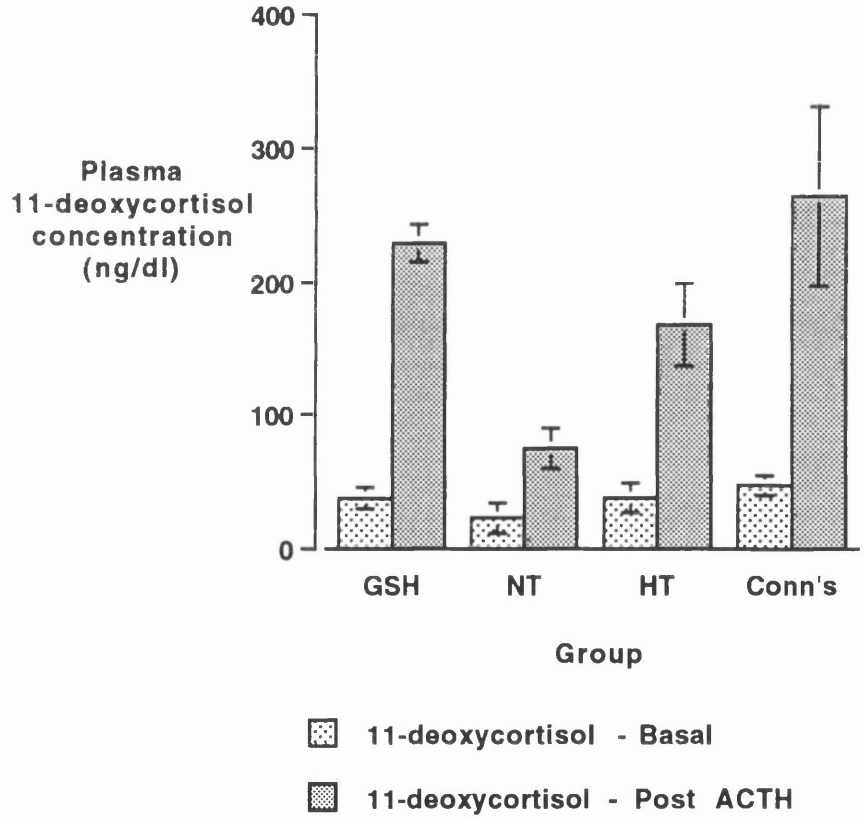



Figure 6.3 g Plasma 11-deoxycortisol concentration pre- and post- ACTH

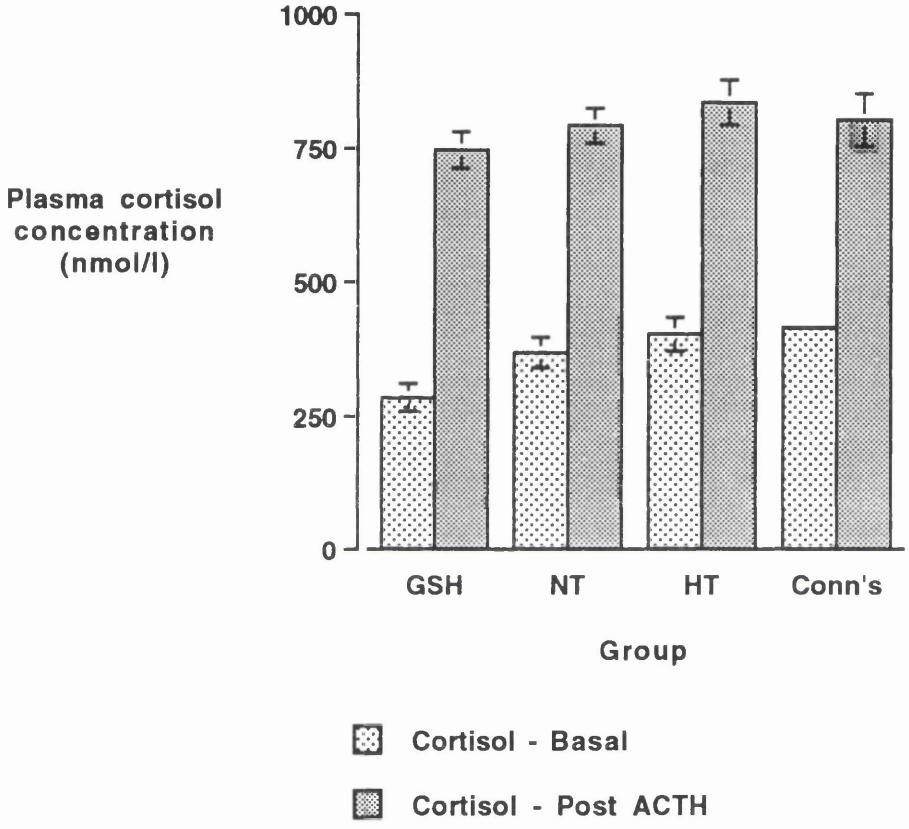



Figure 6.3 h Plasma cortisol concentration pre- and post ACTH

molar DOC:B ratio following ACTH infusion, whilst in patients with Conn's syndrome a significant rise in DOC:B ratio was observed (Figure 6.3 i). Following ACTH infusion, molar plasma DOC:B ratios were significantly higher in patients with GSH than in either normotensive or hypertensive subjects. The post-ACTH DOC:B ratio of patients with Conn's syndrome was higher than the other groups, but this did not reach statistical significance (Table 6.3 i).

Basal molar plasma S:F ratios were significantly higher in patients with GSH than normotensive controls. There was no significant difference in basal molar plasma S:F ratio between GSH patients and patients with essential hypertension or Conn's syndrome.

Following ACTH there was a rise in plasma S:F ratio in all 4 groups (Figure 6.3 j). Post-ACTH plasma S:F ratio was significantly higher in patients with GSH than normotensive and hypertensive subjects, and plasma S:F ratio was significantly greater in hypertensives than normotensive subjects. Patients with Conn's syndrome had higher plasma S:F ratios than both normotensive and hypertensive subjects post-ACTH, but this did not reach statistical significance (Table 6.3 i).

| | DOC:B | | S:F | |
|---|---|---|---|---|
| | 0 min | 90 min | 0 min | 90 min |
| GSH | 0.22 | 0.07* | 0.004 | 0.009† |
| | (0.06) | (0.01) | (0.0007) | (0.0009) |
| NT | 0.05 | 0.02‡ | 0.0016¶ | 0.003†‡ |
| | (0.02) | (0.01) | (0.0007) | (0.0005) |
| HT | 0.06 | 0.02‡ | 0.0023 | 0.006† |
| | (0.02) | (0.003) | (0.0007) | (0.001) |
| CONN | 0.08 | 0.22* | 0.003 | 0.009* |
| | (0.02) | (0.09) | (0.0006) | (0.005) |

GSH - patients with GSH, NT - normotensive controls, HT - patients with essential hypertension, Conn - patients with aldosterone-secreting adenomata
DOC - 11-deoxycorticosterone, B - corticosterone, S - 11-deoxycortisol, F - Cortisol

* $p<0.02$, † $p<0.01$ for comparison between basal and 90 mins
¶ $p<0.05$, ‡ $p<0.02$ for comparison with GSH

**Table 6.3 i Molar ratios of 11-deoxycorticosterone:corticosterone and 11-deoxycortisol:cortisol pre- and post-ACTH stimulation**

### 6.3.4 Aldosterone synthase activity

The conversion of DOC to B and S to F is performed in both the zona fasciculata and the zona glomerulosa. However, aldosterone synthesis in normal subjects only occurs in the glomerulosa catalysed by aldosterone synthase. Therefore, the molar plasma corticosterone:aldosterone ratio (B:ALDO), an index of aldosterone

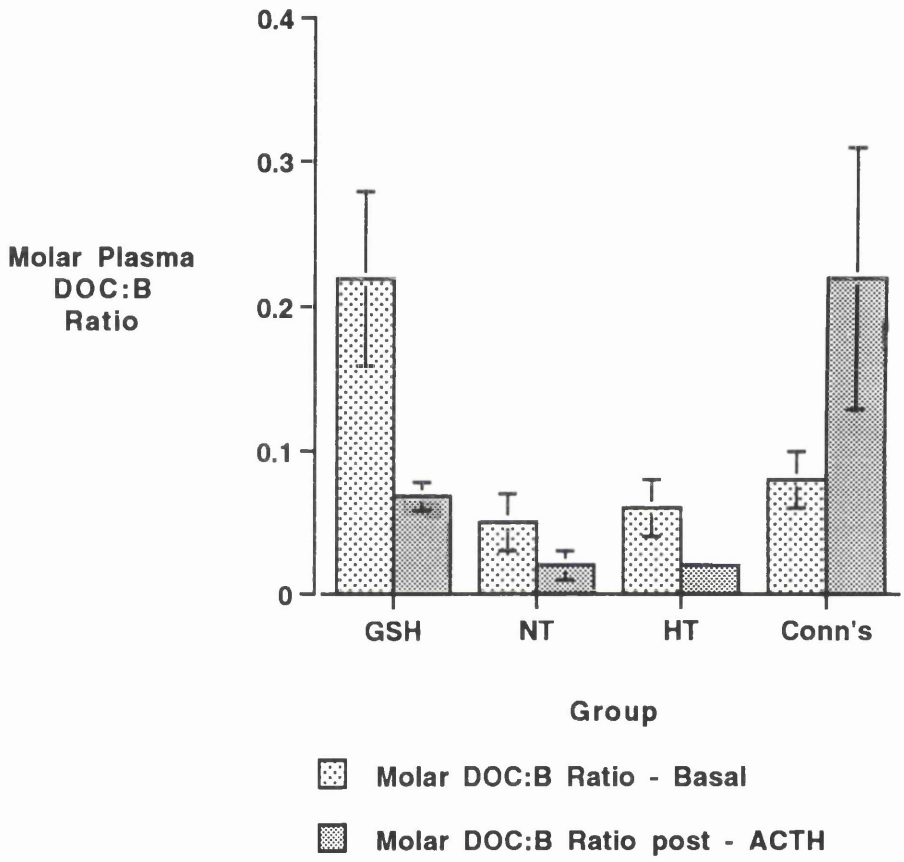



**Figure 6.3 i Molar plasma DOC:B ratio pre- and post ACTH infusion**

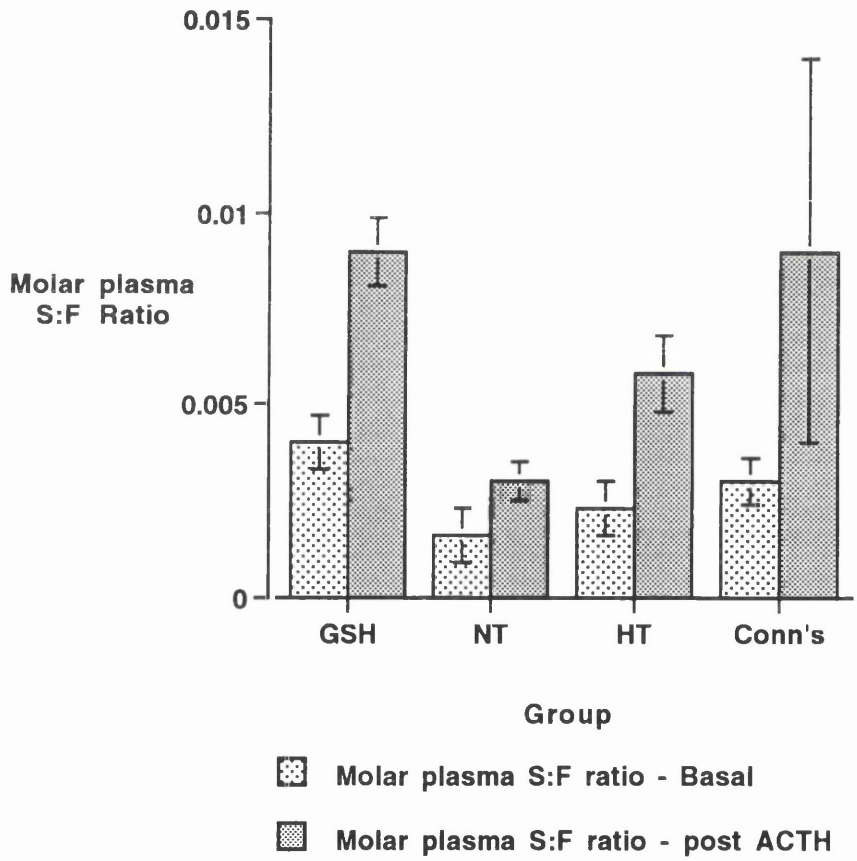



Figure 6.3 j Molar plasma S:F ratio pre- and post-ACTH

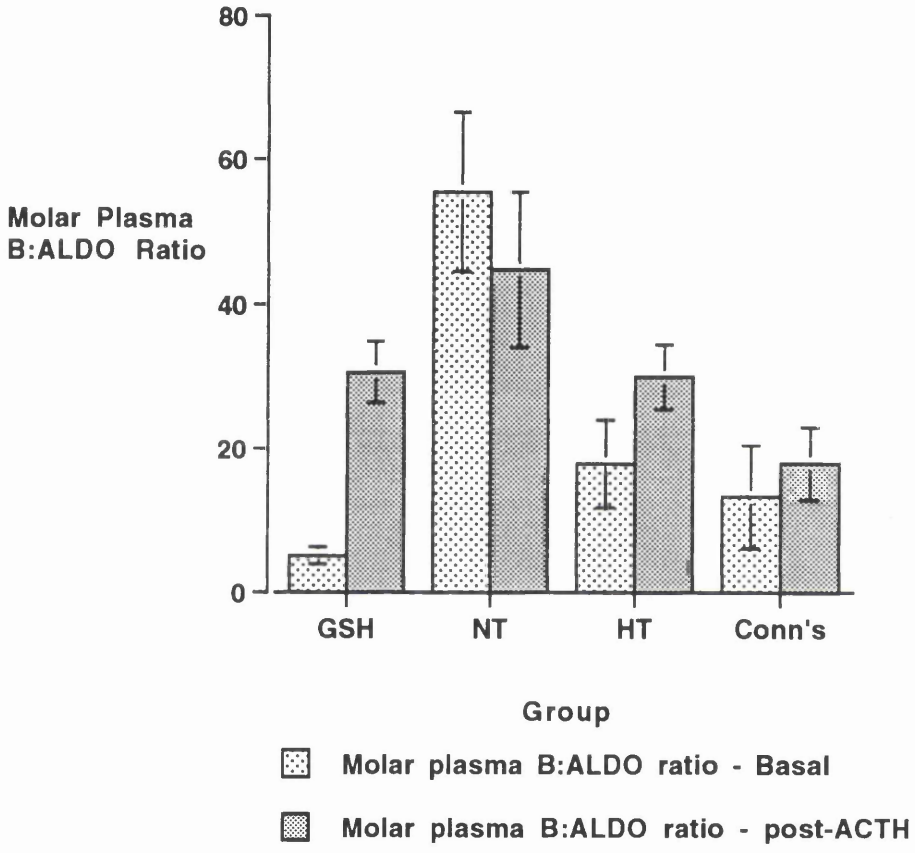



Figure 6.3 k Molar plasma B:ALDO ratio pre- and post- ACTH

synthase activity was calculated before and after ACTH in all 4 groups to assess the efficiency of aldosterone synthase (Table 6.3 j) (Figure 6.3 k).

Patients with GSH had the lowest basal B:ALDO ratios which rose significantly following ACTH administration. Normotensive individuals had the highest molar plasma B:ALDO ratios which fell following ACTH. Patients with essential hypertension and Conn's syndrome had intermediate B:ALDO ratios which rose following ACTH (Figure 6.3 k).

Basal B:ALDO ratios were significantly lower in GSH patients than normotensive subjects, whilst post-ACTH, B:ALDO ratios in patients with GSH were significantly lower than in normotensive subjects, but higher than patients with Conn's syndrome.

| | B:ALDO | |
|---|---|---|
| | **0 min** | **90 min** |
| **GSH** | **5.1** | **30.7*** |
| | (1.2) | (4.2) |
| **NT** | **55.5¶** | **44.8¶** |
| | (9.5) | (10.7) |
| **HT** | **17.9** | **30.1** |
| | (6.2) | (4.5) |
| **CONN** | **13.3** | **18.0** |
| | (7.2) | (5.1) |

GSH - patients with GSH, NT - normotensive controls, HT - patients with essential hypertension, Conn - patients with aldosterone-secreting adnenomata
B - corticosterone, ALDO - aldosterone

* $p<0.001$ for comparison between basal and 90 mins
¶ $p<0.05$ for comparison with GSH

**Table 6.3 j Molar ratio of plasma corticosterone to aldosterone before and after ACTH**

Blood pressure, electrolyte and steroid data for are contained in appendix V.

## 6.4 Discussion

Clinical descriptions of patients with GSH have previously highlighted the potential for affected individuals to develop severe hypertension, with the possibility of end-organ damage (Lee et al, 1982), and hypokalaemia (Connell et al, 1986, Fallo et al, 1985, Ganguly et al, 1981, Lee et al, 1982, New and Peterson, 1967, New et al, 1976, New et al., 1973, O'Mahony et al, 1989, Sutherland et al, 1966, Ulick et al, 1990, Ulick and Chu, 1982). However, in a number of cases the degree of

elevation of blood pressure and hypokalaemia may not be as profound as would be expected from the degree of plasma hyperaldosteronism (Rich et al, 1992).

### 6.4.1 Biochemical features of untreated patients with GSH

The current study examined the members of 5 kindreds with GSH. In 14 of the 19 affected individuals, plasma potassium concentrations were in the normal range. Five patients had blood pressure measurements which were normal for their age and sex in spite of plasma aldosterone concentrations elevated outwith the normal range. This supports data from the only previous study of a large number of patients with GSH in a single North American kindred, which suggested that hypokalaemia was an uncommon feature of GSH (Rich et al, 1992). However, the patients in that study were all found to be hypertensive in their teens whereas a number of the patients studied here were of similar age but normotensive. It is relevant that there is evidence to suggest that hypokalaemia may be less common than originally thought in patients with aldosterone-secreting adrenal adenomata (Gordon et al, 1994) and that plasma potassium measurements may be of little benefit in the identification of mineralocorticoid excess.

Treatment of GSH patients with dexamethasone resulted in a prompt and complete resolution of the clinical and biochemical features of the condition. Of interest is the small, statistically significant rise in plasma potassium level in dexamethasone treated patients, even where the untreated level was normal. This would suggest that plasma potassium levels in GSH are affected by the prevailing mineralocorticoid excess, although the effect is smaller than that seen in patients with similar plasma aldosterone concentrations due to Conn's syndrome (Kremer et al, 1977). This may reflect the absence of a significant diurnal variation of aldosterone secretion seen in Conn's syndrome; patients with GSH exhibit a nycthemeral rhythm of aldosterone secretion, similar to that of cortisol, and for many hours of the day aldosterone concentrations may be relatively normal (Connell et al, 1986, Connell et al, 1986). This and the tendency towards lower 24 hour plasma aldosterone levels may result in lower blood pressure levels and higher plasma potassium concentrations than those seen in Conn's syndrome (Brown et al, 1972b, Connell et al, 1987, Ferriss et al, 1983, Kremer et al, 1977, Oelkers et al, 1974).

### 6.4.2 Role of parental origin of chimeric gene on GSH phenotype

Perhaps the most intriguing phenotypic observation in patients with GSH in this series is the clear effect of parental inheritance of the condition on plasma

aldosterone concentration and blood pressure. The observation that a child's blood pressure more closely resembles its mother than its father has been reported previously in studies of hypertensive families (Bengtsson et al, 1979, Gerson and Fodor, 1975, Law et al, 1991). Although the reasons which underly this phenomenon are not fully understood, there is now good evidence to suggest that adult blood pressure may be partly determined *in utero*. Thus, epidemiological studies examining the relationship among birthweight, placental weight and adult blood pressure all suggest that hypertension most commonly develops in those adults that were undernourished *in utero* (Barker et al., 1990, Bengtsson et al, 1979, Law and Barker, 1994, Law et al, 1991). Indeed, it has been suggested that intrauterine retardation of growth may result in the syndrome of insulin resistance, a feature of essential hypertension and a risk factor for the development of coronary heart disease (Reaven, 1991): a reduction in total pancreatic ß-cell numbers resulting from impaired pancreatic growth, may predispose to the development of hyperinsulinaemia and insulin resistance occurs (Barker et al., 1993). In addition, fetal undernutrition leads to a reduction in trunk growth to sustain brain growth *in utero* and it has been suggested that a degree of growth hormone deficiency and/or resistance results and persists throughout adult life to amplify the hypertensive tendency (Barker et al, 1993).

Of greater possible relevance to the current studies is the apparent link between intrauterine exposure to glucocorticoids and later cardiovascular function. Offspring of rats given dexamethasone during pregnancy develop higher adult blood pressures than those of untreated rats (Benedicktsson et al, 1993, Edwards et al., 1993), and it was suggested that placental activity of 11ßOHSD may be important in regulating fetal growth. Placental 11ßOHSD inactivates circulating maternal cortisol to cortisone, but the glucocorticoid dexamethasone is unaffected by 11ßOHSD and can cross the placenta to induce changes in birth weight an adult blood pressure in the offspring. Furthermore, treatment of pregnant rats with carbenoxolone, an inhibitor of 11ßOHSD, leads to a reduction in birthweight in rats. This was most marked in those rats with the lowest levels of placental 11ßOHSD activity (Lindsay et al., 1995).

These studies emphasise the crucial effect of altered intrauterine environment on adult cardiovascular function, and provide a possible explanation for the more severe hypertension in offspring inheriting the condition maternally: such subjects will be exposed to excessive aldosterone (and 18-hydroxylated cortisol derivatives) concentrations during the whole of their gestation. However, the mechanism whereby excess intrauterine mineralocorticoid exposure results in higher plasma

aldosterone concentrations and blood pressure levels in patients inheriting the chimeric gene from their mothers remains unclear. Vascular endothelial and smooth muscle cells have recently been shown to possess CYP11B2 mRNA and mineralocorticoid receptor mRNA implying local transcription of aldosterone synthase and MR protein (Hatakeyama et al, 1994). In addition, aldosterone administration to cultured cells resulted in an increase in protein synthesis which was blocked by a specific MR antagonist. Therefore, local vascular aldosterone synthesis and increased aldosterone-induced vascular smooth muscle cell proliferation *in utero* may underly the tendency to higher blood pressure levels in the offspring of females affected with GSH. Other key organs such as the hippocampus of the brain will also be exposed to increased aldosterone concentrations, and this may be important in the the long term "setting" of blood pressure. Why plasma aldosterone levels are higher in the offspring of affected females is not known. Such an effect may arise from an altered level of expression of the chimeric CYP11B1/B2 gene product determined by unknown intrauterine factors.

Another possible explanation for the observed effect is the principal of genomic imprinting, i.e. the phenotype of a genetic mutation differs depending on the parental origin of the mutated gene or chromosome. The best known example of imprinting is the Angelman's - Prader-Willi syndrome. These syndromes arise from the inheritance of a deleted portion of chromosome 15 from the mother or father respectively (Nicholls, 1993). Such a hypothesis is difficult to test but cannot be excluded.

Thus, from the study of these five kindreds with GSH there is novel evidence to support a role for intrauterine corticosteroid exposure in the development of hypertension. However, further studies including animal studies of mineralocorticoid exposure similar to that seen in GSH are necessary in order to explore this phenomenon further.

### 6.4.3 Effect of crossover location on phenotype

The lack of a significant relationship between blood pressure and plasma aldosterone concentrations and chimeric gene crossover site is not unexpected. The degree of alteration in nucleotide sequence and subsequent amino acid sequence of the chimeric enzyme is minimal (See Chapter 4.4). Similarly the studies of *in vitro* expression of chimeric CYP11B1/B2 cDNA constructs revealed an 'all or nothing' response in aldosterone synthase activity of the constructs rather than a graduated response relative to the composition of the construct (Pascoe et

al, 1992a). Thus, it its unlikely that clinically significant variations in plasma aldosterone concentrations or blood pressure would arise from the relatively minor changes in chimeric gene sequence detected. The absence of *in vitro* aldosterone synthase activity of chimeric cDNA plasmids containing exons 1-5 or more of CYP11B1 suggests that classical GSH cannot arise from a chimeric gene with more than exons 1-3 of CYP11B1. However, the possibility of the existence of chimeric genes comprising more than the first 4 exons of CYP11B1 cannot be excluded, and such genes may have minor effects on aldosterone secretion which contribute to hypertension, particularly of the 'low-renin' form.

### 6.4.4 ACTH infusion studies

The ACTH infusion studies confirm the previous observations that 11ß-hydroxylase activity as determined by molar plasma S:F and DOC:B ratios is impaired in GSH and essential hypertension. In the basal state, both S:F and DOC:B ratios are elevated in patients with GSH compared to normotensive controls. Stimulation by ACTH results in further significant elevation in S:F ratios in patients with GSH and essential hypertension compared with normotensive controls, confirming the earlier observations in essential hypertension (De Simone et al, , Honda et al, 1977), and GSH (Connell et al, 1986). However, following ACTH stimulation the high basal DOC:B ratios in patients with GSH fall to levels similar to that seen in normotensive controls and patients with essential hypertension .

In normal subjects the formation of cortisol and corticosterone is performed by $CytP450_{11ß}$ in the zona fasciculata (the major source of corticosterone), whilst synthesis of corticosterone in the zona glomerulosa is performed by $CytP450_{aldo}$ only. In GSH, three enzymes with 11ß-hydroxylation capacity are present in the adrenal cortex: $CytP450_{11ß}$ in the zona fasciculata, $CytP450_{aldo}$ in the zona glomerulosa and the chimeric ACTH-responsive CYP11B1/B2 gene product in the zona fasciculata. Cortisol synthesis can be performed by only one enzyme in the fasciculata, $CytP450_{11ß}$, whilst conversion of DOC to B can be performed by three enzymes in two different zones of the adrenal cortex. In the basal, untreated state this key biosynthetic step is impaired compared with normal subjects.

In patients with GSH $CytP450_{11ß}$ synthesises F, but in the basal state F synthesis is impaired, as is the conversion of DOC toB. One possible explanation for the high basal S:F and DOC:B ratios is an excess of a pseudosubstrate which can compete with the usual precursor for the active site of the enzyme. For example 18-hydroxyDOC, 18-oxocortisol or 18-hydroxycortisol are all present in high concentrations and might inhibit the efficiency of 11ß-hydroxylation. Precedents for

pseudosubstrate inhibition are found in cases of classical 21-hydroxylase deficiency (Hornsby, 1989). Following ACTH, the marked increase in the local release and synthesis of 11-deoxycortisol and DOC, the normal enzyme substrates may overcome the pseudosubstrate inhibition in the case of corticosterone but not cortisol synthesis. No plasma measurements of 18-hydroxyDOC, 18-oxocortisol or 18-hydroxycortisol are available in this study but will be performed in the future to assess this possibility.

An alternative explanation may be that the relative amount of S to F and DOC to B conversion performed by each enzyme may be altered in GSH. Clearly the activity of the normal CYP11B2 gene product ($CytP450_{aldo}$) will be suppressed by the mineralocorticoid excess state produced by aldosterone excess consequent upon the expression of the chimeric CYP11B1/B2 gene product in GSH. Therefore F and B synthesis will only be performed by $CytP450_{11\beta}$ (F and B) and the chimeric gene product (B only) in the zona fasciculata.

ACTH levels are not elevated in GSH (Connell et al, 1986, New et al, 1973). In GSH, basal untreated S:F and DOC:B ratios are high. It has been suggested that the zona fasciculata is the major site of *in vivo* corticosterone synthesis (Tan and Mulrow, 1979), but *in vitro* both $CytP450_{11\beta}$ and $CytP450_{aldo}$ have equal corticosterone synthetic capacity (Kawamoto et al, 1992). Therefore, in the basal state there may be a relative deficiency of F and B synthetic capacity due to impaired basal activity of $CytP450_{11\beta}$ due to unknown factors, whilst activity of the chimeric gene is relatively low. When ACTH is administered there is an increase in the synthesis and release of DOC and B by the chimeric gene product sufficient to compensate for the basal deficiency. However, ACTH stimulation of $CytP450_{11\beta}$ leads to an incresae in S:F ratio because F synthesis is still impaired.

The impaired activity of $CytP450_{11\beta}$ may result from altered expression of CYP11B1 and gene transcription in the zona fasciculata or impaired responsiveness to ACTH. All patients with GSH in this study possessed the -340C allele of the CYP11B2 promoter region polymorphism which alters DNA binding to the SF-1 region *in vitro* (White et al, 1994). CYP11B1 also has a SF-1 binding region and sequences in the promoter region specific for other regulatory species such as cAMP (Parker and Schimmer, 1993). It is possible therefore, that polymorphisms exist which affect the expression of the CYP11B1 gene product in GSH and essential hypertension and result in impaired $CytP450_{11\beta}$ function. Similarly, other gene conversion events related to the primary unequal recombination may have altered important regions of the promoter which impair the

expression of CYP11B1 in GSH. Alternatively, as both CytP450$_{11\beta}$ and the chimeric gene product are expressed within the mitochondria there may be competition for available adrenodoxin and electron transport systems which results in an impaired basal activity of both enzymes. Following ACTH, selective activation of the chimeric gene may occur with exacerbation of the 11ß-hydroxylase activity of CytP450$_{11\beta}$ and enhaced B synthesis.

Molar plasma B:ALDO ratio was low in the untreated GSH patients compared with normal controls, and rose signifcantly following ACTH whereas the B:ALDO ratio in normotensive controls fell following ACTH (not statistically significant). Aldosterone synthesis in GSH presumably occurs in the zona fasciculata and is performed by the chimeric CYP11B1/B2 gene product. This is confirmed by observations in patients with GSH of tandem increases in aldosterone and 18-hydroxycortisol following ACTH (Connell et al, 1986). ACTH will increase aldosterone synthase activity in this case and should increase aldosterone biosynthesis. This is confirmed when the basal and post-ACTH aldosterone concentrations are examined. However, the incremental rise in aldosterone concentration following ACTH is approximately three-fold whereas the rise in corticosterone concentration is almost 13 times. The equivalent changes in normotensive controls were 3- and 6-fold increases respectively. Therefore it would appear that in GSH aldosterone synthase activity, i.e. 18-hydroxylation of corticosterone and other 17-hydroxy- and 17-deoxycorticosteroids, is impaired compared with normotensive controls when assessed by B:ALDO ratio. The explanation for this observation is not clear but *in vitro* expression studies of chimeric cDNA plasmids have shown that basal B:ALDO ratios are of a similar order to that seen in these patient studies (approximately 2-4) (Pascoe et al, 1992a) and that aldosterone synthase activity of the chimeric cDNA is relatively less than that of CYP11B2 (approximately 1/3 of the activity of CYP11B2 cDNA *in vitro*) (Miyahara et al, 1992). Thus, ACTH may increase the supply of precursor corticosterone but conversion to aldosterone may be limited by saturation of the aldosterone synthase activity of the chimeric gene product resulting in apparent inhibition of the synthetic capacity of the enzyme. It may be possible to assess this by using 18-hydroxycortisol measurements across the ACTH infusion as an index of the activity of the chimeric gene product compared to that of CytP450$_{11\beta}$. Therefore, variation in enzymatic activity may offer a plausible explanation for the disordered aldosterone synthase activity in GSH.

Patients with essential hypertension and Conn's syndrome exhibited evidence of impaired CytP450$_{11\beta}$ function as shown by the S:F ratios, both basally and

following ACTH, and the high DOC:B ratio after ACTH in the Conn's syndrome group. Similar findings have been reported previously and suggest that in both conditions 11ß-hydroxylation is impaired. Aldosterone synthase activity did not change following ACTH in these patients and the B:ALDO ratio was low suggesting relatively unimpaired aldosterone synthase activity.

In summary, patients with GSH exhibit signs of primay aldosterone excess which are abolished following glucocorticoid administration. Patients who inherit the condition from their mothers develop higher plasma aldosterone concentrations and blood pressure levels than those inheriting the condition from their father. The composition of the chimeric gene appears to have no effect on blood pressure levels or plasma aldosterone concentrations in affected individuals. There is evidence of impaired 11ß-hydroxylation of 17-hydroxy- and 17-deoxycorticosteroids in patients with GSH. Basal S:F and DOC:B ratios were elevated in patients with GSH. ACTH produced a rise in S:F ratio but a fall in DOC:B ratio. The impaired 11ß-hydroxylation activity may reflect pseudosubstrate inhibition of the 11ß-hydroxylase enzymes or altered gene expression in the adrenal cortex of patients with GSH. Similarly, aldosterone synthase activity may be impaired in GSH compared with normal subjects. The low basal B:ALDO ratios rise following ACTH, principally due to an excessive rise in corticosterone concentrations, reflecting impaired aldosterone synthetic capacity of the chimeric gene product.

## Chapter 7

# LEFT VENTRICULAR MASS IN GLUCOCORTICOID-SUPPRESSIBLE HYPERALDOSTERONISM

## 7.1 Introduction

Left ventricular hypertrophy is an important consequence of arterial hypertension and its presence is an important predictor of subsequent mortality from stroke, ischaemic heart disease and sudden cardiac death (Stokes et al, 1989). The basic mechanisms underlying the development of left ventricular hypertrophy are unclear but studies of animal models of mineralocorticoid-dependent hypertension suggest an important role for aldosterone in the development of myocardial fibrosis and left ventricular remodelling (Weber et al, 1992). For example, Brilla et al. (Brilla et al, 1993b) have shown that reactive accumulation of collagen within the intramyocardial arteries and neighbouring interstitial space occurs in both the pressure overloaded hypertrophied left ventricle as well as the normotensive, non hypertrophied right ventricle of rats with arterial hypertension due to either primary or secondary hyperaldosteronism. In addition, it has been shown that spironolactone, an aldosterone antagonist, can prevent the occurrence of myocardial fibrosis in doses which are insufficient to lower blood pressure in the same models (Brilla et al., 1993a). However, the role of aldosterone in the development of left ventricular fibrosis and hypertrophy in man remains unclear. A post-mortem study of five patients with adrenal adenomata demonstrated the presence of perivascular fibrosis in the coronary and systemic arteries in a similar distribution to that seen in animal models of mineralocorticoid-dependent hypertension (Campbell et al, 1992).

Patients with GSH are exposed to abnormally high plasma aldosterone levels from birth, and possibly also *in utero*, and most eventually develop hypertension. Therefore, these patients should be at high risk of developing left ventricular hypertrophy and myocardial fibrosis if the observations in animal models are applicable to man.

To determine whether prolonged hyperaldosteronism in GSH has effects on left ventricular mass, 11 patients with GSH and ten control subjects were studied by echocardiography.

## 7.2 Subjects and Methods

### 7.2.1 Subjects

Eleven subjects with GSH (6 males) and ten unaffected normotensive controls (6 males) with no family history of hypertension were studied.

Three hypertensive subjects with GSH had received glucocorticoid therapy prior to echocardiography (Dexamethasone 0.25mg daily) for a median of six months (range 3 months, 6 months and 2 years).

### 7.2.2 Genotyping

Patients with GSH were identified by genotyping based on a Southern blot of BamHI digested DNA prepared from peripheral leucocytes as described in Chapter 3.

### 7.2.3 Biochemical measurements

Measurements of plasma potassium, aldosterone and renin concentrations were performed on patients and controls at 9am following overnight recumbency (patients) or 90 minutes of supine rest (controls). No patients or controls were receiving any glucocorticoid, hypotensive or diuretic drugs at the time of the examination.

Plasma assays were performed in a single batch for each group as described in 2.6.1-3.

### 7.2.4 Echocardiographic measurements

Echocardiograms were performed by the same person in all 11 patients and 10 controls. Measurements were obtained from echocardiograms in a blinded fashion by two observers as described in 2.5.6. Height and weight were recorded at the time of echocardiography and left ventricular mass determined as described in 2.5.6.

### 7.2.5 Statistics

Differences in blood pressure, plasma aldosterone, renin and potassium concentration and left ventricular mass measurements between the two groups were assessed by the Mann-Whitney U-test.

## 7.3 Results

The two groups were matched for age range and sex distribution: median age of subjects was 27 years (Range 6-59 years, males=6), and the median age of the control subjects was 29 years (Range 20-54 years, males=6).

### 7.3.1 Blood pressure and biochemical analyses

Patients with GSH had elevated blood pressures, plasma aldosterone concentrations and suppressed plasma active renin concentrations compared to the normotensive subjects (Table 7.3 a).

| | Mean Blood Pressure (mmHg) | Renin (mU/ml) | Aldosterone (pmol/l) | Potassium (mmol/l) |
|---|---|---|---|---|
| **GSH** | 119 (9) | 1.0 (0.4) | 766 (111) | 4.07 (0.12) |
| **GSH-HT** | 133 (7) | 1.7 (0.3) | 796 (164) | 3.93 (0.16) |
| **GSH-NT** | 78 (6)* | 1.0 (0.1) | 712 (175) | 4.28 (0.15)† |
| **Controls** | 86 (4)* | 23 (2.5)‡ | 305 (34)‡ | 4.16 (0.11) |

GSH - GSH patients, n=11; GSH-HT - GSH patients with elevated blood pressure
GSH-NT - GSH patients with normal blood pressures
Results are expressed as mean ± s.e.m.
* $p<0.0001$ for comparison between GSH-HT patients and GSH-NT and controls
‡ $p<0.001$ for comparison between Controls and all GSH patients groups
† $p=0.057$ for comparison between GSH-HT and GSH-NT

**Table 7.3 a Blood pressure and biochemical data on GSH patients and controls.**

Subjects with GSH were subdivided into patients with GSH and hypertension, those patients whose resting pressure was <160 mmHg systolic or ≤90 mmHg diastolic, (GSH-HT) (n=7) and patients with GSH without hypertension (GSH-NT), (n=4). Plasma active renin and aldosterone concentrations in GSH-HT and GSH-NT were not significantly different from each other but plasma potassium in the GSH-NT group tended to be higher in the GSH-HT group ($p=0.057$).

### 7.3.2 Left ventricular mass index

The left ventricular mass index determined by echocardiography of subjects and its relation to mean arterial blood pressure is shown in Table 7.3 b.

The left ventricular mass index of subjects with GSH was not significantly different from each other or from the control group (GSH - 99.7 +/- 7.7 gram/$m^2$ vs. 100.3 +/- 5.4 gram/$m^2$, $p=0.76$).

| | Mean Blood Pressure (mmHg) | Left ventricular mass index (gram/m$^2$) |
|---|---|---|
| GSH | 119 (9)† | 99.7 (7.7)‡ |
| GSH-HT | 133 (7) | 100 (10.1)‡ |
| GSH-NT | 78 (6)* | 99.2 (13.6)‡ |
| Controls | 86 (4)* | 100.3 (5.4)‡ |

GSH - GSH patients, n=11; GSH-HT - GSH patients with elevated blood pressure
GSH-NT - GSH patients with normal blood pressures
Results are expressed as mean ± s.e.m.
* p<0.0001 for comparison between GSH(all patients) GSH-HT and other groups,
† p<0.001 for comparison between GSH(all patients) and GSH-NT and control groups
‡ p= not significant for comparison between all groups.

**Table 7.3 b Comparison of left ventricular mass index in patients with GSH and controls**

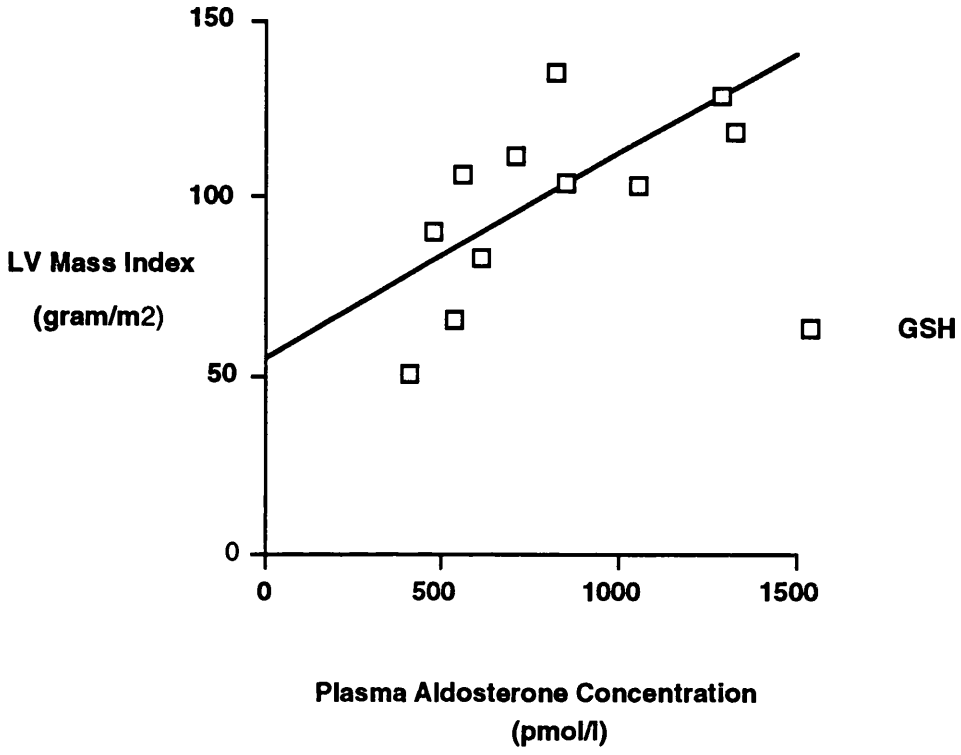
Left ventricular mass index was non-significantly different when patients were sub-divided on the basis of blood pressure; GSH-HT - 100.0 +/- 10.1 gram/m$^2$, GSH-NT - 99.2 +/- 13.6 gram/m$^2$, Control 100.3 +/- 5.4 gram/m$^2$, p=0.63. In addition, neither age at diagnosis nor duration of hyperaldosteronism before diagnosis of GSH had any demonstrable effect on left ventricular mass index.

However, a statistically significant positive correlation between basal plasma aldosterone concentration and LVMI in patients with GSH (Spearman rank correlation coefficient, r=0.66, p<0.03) (Figure 7.3 a).

## 7.4 Discussion

Echocardiography allows the accurate identification of left ventricular hypertrophy in patients with hypertension, a group at high risk of developing serious complications such as stroke, cardiac failure and sudden death (Stokes et al, 1989). Reversal of left ventricular hypertrophy can be achieved using a number of anti-hypertensive agents (MacMahon, 1994).

There is now a large body of animal data, predominantly in rat models of mineralocorticoid dependent hypertension in favour of a pathogenetic role for aldosterone in the development of myocardial fibrosis and left ventricular remodelling (Brilla et al, 1993a, Brilla et al, 1993b, Weber et al, 1992). Observations in rats with mineralocorticoid hypertension suggest that myocardial fibrosis can occur in both left and right ventricles in the presence of arterial hypertension (Brilla et al, 1993a, Brilla et al, 1993b). In these models the right ventricle is exposed to normal pressures and it is suggested that the fibrosis occurs




Spearman Rank Coefficient r = 0.66 ($p<0.03$)

**Figure 7.3 a Correlation between plasma aldosterone concentration and Left ventricular mass index in patients with GSH.**

as a direct result of mineralocorticoid action on the cardiac fibroblasts rather than an effect of pressure-overload mechanisms. Therefore, the aldosterone receptor antagonist, spironolactone may have an important and specific role in the prevention and treatment of aldosterone-induced myocardial fibrosis (Brilla et al, 1993a, Brilla et al, 1993b). Administration of spironolactone in doses that do not lower blood pressure prevents the development of myocardial fibrosis such that left ventricular hypertrophy occurs in the absence of significant collagen accumulation. If spironolactone is given in high doses which prevent mineralocorticoid-induced hypertension, both myocardial fibrosis and left ventricular hypertrophy are prevented. Left ventricular hypertrophy reflects an increase in cardiac myocyte mass whereas myocardial fibrosis may have no effects on LVMI but may affect cardiac function. For example, cardiac fibrosis is an important determinant of ventricular diastolic function in animal models of ventricular hypertrophy, particularly in association with hypertension of renal origin (Swales, 1994). Fibrosis may also contribute to sudden death in patients with normal gross cardiac anatomy by acting as a substrate for the development of re-entrant ventricular arrhythmias or areas of focal arryhthmogenic myocardial ischaemia. Biopsy of ventricular muscle from patients with grossly normal hearts and life-threatening arrhythmias showed areas of intramyocardial fibrosis (Strain et al., 1983).

Clinical information in human subjects on the effects of prolonged primary hyperaldosteronism is limited. In a post-mortem study of five patients with adrenal adenomata, the presence of perivascular fibrosis in the coronary and systemic arteries in a similar distribution to that seen in animal models of mineralocorticoid-dependent hypertension was reported (Campbell et al, 1992). Such patients usually have marked hyperaldosteronism and hypertension and usually also have associated left ventricular hypertrophy. Pre-existing left ventricular hypertrophy in these patients can be reversed upon treatment of the hyperaldosteronism, either by removal of the adenoma or by the administration of aldosterone antagonists (Pringle et al., 1988).

Furthermore, studies of patients with essential hypertension in association with left ventricular hypertrophy suggest a positive correlation between plasma aldosterone concentration and left ventricular mass index (Duprez et al., 1993). In the study of hypertensive subjects, the mean arterial pressure of hypertensive subjects was similar to that observed in the patients with GSH but plasma aldosterone levels were within the normal range. The mean left ventricular mass index of the patients with essential hypertension was significantly greater than in the subjects with GSH,

but both groups showed a similar correlation between plasma aldosterone concentration and left ventricular mass index.

It is clear therefore that primary aldosterone excess in man can cause hypertension, left ventricular hypertrophy and myocardial fibrosis. The relationship among these features is variable and may be influenceded by factors other than aldosterone. Thus, the plasma aldosterone concentrations seen in patients with GSH are only modestly elevated compared to those seen in the majority of cases of Conn's syndrome (Ferriss, 1992), and may in themselves be insufficient to provoke significant pressure- mediated LVH. Previous observations suggest that blood pressure in many patients with GSH is only modestly elevated and may require 2-3 decades to develop despite the presence of hyperaldosteronism (Jamieson et al, 1994). Therefore, it may require the interaction of the genetically-determined hyperaldosteronism and other factors, either genetic or environmental, such as salt intake, to provoke significant LVH in patients with GSH. However, GSH patients may have myocardial fibrosis present, even at this level of aldosteronism, which could be detected by myocardial biopsy. Detection of myocardial fibrosis would confirm the observations in animal studies of aldosterone-dependent, hypertrophy-independent myocardial fibrosis.

The measured LVMI in our patients with GSH was not significantly raised compared with the normotensive controls. This may reflect the relatively small size of our sample. Alternatively, as aldosterone may have predominant effects in producing cardiac fibrosis rather than pressure-mediated hypertrophy, which cannot be detected by conventional transthoracic echocardiography, this may be the principal consequence in GSH. Thus, significant LVH may not occur because pressure-dependent effects are not prominent in these patients in distinction to those patients with adrenal adenomata (Ferriss, 1992).

The literature on GSH consists of case reports; systematic evaluations of cardiovascular risk are not available. It is thought that there is an increased risk of premature stroke in these patients, although the reasons for this are unclear (Connell et al, 1986, New and Peterson, 1967). In addition, severe renal impairment as a consequence of GSH has been reported (Lee et al, 1982).

Because of the relative rarity of GSH and the geographical isolation of many of our affected subjects, the numbers studied were limited. However, the results of these observations suggest that, genetically determined, prolonged elevation of plasma aldosterone concentrations does not necessarily lead to left ventricular hypertrophy. However, plasma aldosterone concentrations do have a definite effect

on left ventricular mass index as shown by the positive correlation between plasma aldosterone concentration and left ventricular mass. Other factors, however, may be important in determining the absolute level of blood pressure attained and the risk of subsequent end-organ damage.

## Chapter 8

## CONCLUSIONS

Until recently, diagnosis of GSH relied on the accurate documentation of primary aldosterone excess and demonstration of its complete reversal following glucocorticoid administration. This process requires frequent visits for blood sampling and the need to take dexamethsone for 1 month. Elevated urinary excretion of 18-hydroxylated cortisol derivatives has been shown to identify reliably the affected relatives of index cases of GSH (Rich et al, 1992). This method requires an accurate 24 hour urine collection and a readily available assay, features which may limit its applicability to patients unable to provide accurate urine collections, for example young children, or patients remote from a major endocrine centre. In addition to the relatively cumbersome technical aspects of these approaches, they rely to a greater or lesser extent on the presence of a proband whose relatives can be screened for GSH rather than being applicable to wider clinical practice for detection of the disorder in the general hypertensive population.

The development of the molecular genetic method which can be used to diagnose GSH from a single blood sample or from placental tissue is a significant advance in the detection of GSH. This method is 100% specific and sensitive for GSH (Lifton et al, 1992). In addition, these studies have demonstrated that hypertension is not a *sine qua non* of the condition in childhood or the second decade. Similarly plasma potassium measurements are a poor indicator of hyperaldosteronism in GSH. However, given the strong family history of hypertension and related problems in all the kindreds, patients under 25 years of age or those with a strong family history of hypertension or premature cerebrovascular disease GSH should always be considered as a possible cause of hypertension and action taken to exclude or confirm its presence.

The discovery of a potential influence of the parental origin of the chimeric gene on plasma aldosterone concentration and blood pressure is novel and intriguing. As discussed there are a number of lines of evidence to suggest that hypertension may have its origin, or at least its potential course, modified *in utero*. The origin of this programming effect is unclear but may arise from fetal vascular effects of intrauterine mineralocorticoid excess or a genomic effect, i.e. imprinting. Further studies in an animal model to assess the effect of maternal mineralocorticoid excess on offsprings blood pressure are planned.

The nucleotide sequences of the chimeric genes are in keeping with the previously published data. Similarly the lack of apparent variation in clinical phenotype in individuals with differing chimeric genes is not surprising when the effects of nucleotide changes on the predicted amino acid structure of the chimeric gene product are examined.

The discovery of a polymorphism in the promoter region of CYP11B2 and its linkage to GSH is interesting. This polymorphism and the gene conversion involving intron 2 of CYP11B1 and B2 indicate a possible mechanism whereby the formation of a chimeric gene might occur. Preliminary unpublished data suggest that the intron 2 duplication is in linkage disequlibrium with -340C only in whites, not black subjects, thus reinforcing the suggestion of a Celtic bias in the origin of GSH (PC White, personal communication). The presence of the intron 2 gene conversion would explain the observed data on the structure of the chimeric genes. As yet no chimeric CYP11B1/B2 gene in patients with GSH containd exon 2 from CYP11B2 (Jamieson et al., 1995, Lifton et al, 1992, Miyahara et al, 1992, Pascoe et al, 1992a). This would suggest that the presence of the gene duplication, whilst facilitating the formation of chimeric genes also limits the composition of the gene to a minimum of exons 1-2 from CYP11B1 and the remainder from CYP11B2. Other data on the origin of aldosterone synthase activity in the CYP11B2 gene product may explain why there is a limit to GSH activity of chimeric genes (Pascoe and Curnow, 1994). When exon 5 of a chimeric gene originates from CYP11B1 aldosterone synthase activity of the chimeric gene product is abolished (Pascoe et al, 1992a). Thus it would appear that the genes causing GSH are likely always to contain exons 1-2 from CYP11B1, exon 5 from CYP11B2 and a variable portion of intron 2-4 from CYP11B1 and B2.

Impaired 11ß-hydroxylase activity is a feature of essential hypertension. These studies have demonstrated the presence of impaired 11ß-hydroxylation in GSH also. The basis for this apparent deficiency is unclear but may arise from pseudosubstrate inhibition of 11ß-hydroxylase enzymes in the adrenal cortex, or altered expression of the genes encoding enzymes with 11ß-hydroxylase capacity, i.e. $CytP450_{11ß}$, $CytP450_{aldo}$, and the CYP11B1/B2 chimeric gene product.

There are clear similarities between essential hypertension and GSH in this regard and the abnormalities underlying impaired 11ß-hydroxylation in both conditions may have a similar etiology.

End-organ damage is the cause of mortality and morbidity in hypertension. Left ventricular hypertrophy is a significant independent risk factor for sudden death.

Animal studies and *in vitro* observations on cardiac fibroblasts suggest that aldosterone may be an important mediator of myocardial fibrosis. Thus aldosterone excess may have adverse effects on cardiac remodelling, particularly if present from childhood. Studies in this thesis were unable to show an effect of aldosterone excess on absolute left ventricular mass index (LVMI) in patients with GSH, although LVMI was correlated with plasma aldosterone concentration. However, these studies did not address the role of such aldosterone excess on the fibrous tissue content of the ventricles or the coronary arteries of patients with GSH, sites of prominent aldosterone-induced myocardial fibrosis.

A international registry of cases of GSH has been established in Harvard Medical School, and British cases of GSH are logged by the Blood Pressure Group, Department of Medicine and Therapeutics, Western Infirmary, Glasgow. The establishment of such registers will allow data from centres world wide to be pooled and important questions about GSH to be answered. For example, how great is the effect of parental inheritance of GSH on phenotype and what other factors interact to alter the phenotype; are there prominent effects of aldosterone on LVMI or myocardial fibrosis in patients with GSH; do individuals inheriting chimeric genes which differ significantly in composition, e.g. exon 1-2 or exon 1-4 from CYP11B1, exhibit any phenotypic differences?

Furthermore, a gradual increase in awareness of GSH will undoubtedly lead to an increase in diagnoses of GSH aided by the simplicity and reliability of the molecular genetic method. This makes possible accurate assessment of population prevalence of the condition.

Since commencing the writing of this thesis a further kindred has been discovered and is in the process of being characterised. The work carried out in this thesis has characterised the basic clinical and genetic features of our local kindreds with GSH and will act as a catalyst for further work on this and related topics such as:

1) The need to establish the population prevalence of GSH in target populations, e.g. hypertension clinic, Isle of Lewis population

2) A further examination of the role of intrauterine influences on blood pressure in GSH by the use of animal models and the detailed analysis of birthweight data and adult blood pressure in patients and offspring with and without GSH

3) Study of the effects of mineralocorticoid exposure *in utero* on myocardial fibrosis in the offspring of aldosterone-treated rats.

## APPENDICES

**APPENDIX I**

**Genomic DNA solutions**

| | |
|---|---|
| Cell Lysis Buffer | 0.32M Sucrose<br>10mM TRIS<br>5mM Magnesium Chloride<br>1% Triton X-100<br>pH 7.5 |
| Nuclei Lysis Buffer | 10mM TRIS<br>0.4M Sodium Chloride<br>2mM EDTA<br>pH 2.8 |
| RNAse enzyme | 100µg/ml |
| Sodium acetate | 3M |
| Phenol/Chloroform | 50:50, pH 7.5, 0.1% hydroxyquinolone |
| TE buffer | 10mM TRIS<br>10mM EDTA<br>pH 7.5 |
| Loading Buffer | 30% Glycerol<br>0.25% Bromophenol Blue |
| 20xSSC | 3M Sodium Chloride<br>0.3M Sodium Citrate<br>pH 7 |
| Denaturation Buffer | 0.5M Sodium Hydroxide<br>1.5M Sodium Chloride |
| Neutralisation Buffer | 0.5M Tris- HCl<br>3M Sodium Chloride<br>pH 7.5 |
| Depurination buffer | 0.25M Hydrochloric Acid |
| Sephadex | 30g Sephadex<br>500 ml 2X SSC |
| Prehybridisation Buffer (100ml) | 30ml 20X SSC<br>10ml 50X Denhardts<br>5ml 10% SDS<br>1.2ml Denatured<br>Sonicated Salmon Sperm (50µg/100ml) |
| Salmon sperm DNA | 10mg/ml sonicated |
| SDS | 10% Solution |

| | |
|---|---|
| Proteinase K | 10μg/ml |
| Saturated Sodium Chloride | Approximately 6M |
| Ethanol | 95% |
| Ethidium Bromide | 10mg/ml |

## APPENDIX II

### Molecular Biology Reagents

### Restriction enzymes

| Enzyme | Concentration | Recognition Site |
|---|---|---|
| **BamHI:** | 50U/µl | G^GATCC<br>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**

60mM Tris-HCl, 60mM $MgCl_2$, 1M NaCl, 10mM DTT, pH7.4

| Enzyme | Concentration | Recognition Site |
|---|---|---|
| **HaeIII:** | 10U/µl | GG^CC<br>CC^GG |

**Storage Buffer**

10mM Tris-HCl, 300mM NaCl, 0.1mM EDTA, 1mM DTT, 0.5mg/ml BSA, 50% glycerol, pH 7.4

**10X Reaction Buffer**

100mM Tris-HCl, 100mM $MgCl_2$, 0.5 M NaCl, 10mM DTT, pH7.9

### DNA Polymerase Enzyme

**Taq polymerase:** 2-5U/µl

**Storage Buffer**

50mM Tris-HCl, 100mM NaCl, 0.1mM EDTA, 5mM DTT, 50% glycerol, Triton X-100, pH 8.0

**10X Reaction Buffer**

100mM Tris-HCl, 2.5mM $MgCl_2$, 500 mM KCl, Triton X-100, pH 9.0

**Nucleotides:**

dATP, dCTP, dGTP, dTTP: 0.2 mM

## Oligonucleotide labelling

**T4 Polynucleotide kinase**: 5-10μU/ml

**10X Reaction Buffer**

700 mM Tris-HCl, 100mM $MgCl_2$, 50mM DTT, pH 7.6

## DNA labelling for Southern Blotting

**Random Primer Labelling Kit (Gibco-BRL Life Technologies)**

**Klenow Enzyme (Large fragment of DNA Polymerase I):** 3U/μl

**Storage Buffer:** 100mM $K_3PO_4$, 10mM 2-mercaptoethanol, 50% glycerol

**Random Primers Buffer Mixture:** 0.67M HEPES, 0.17M Tris-HCL,

17mM $MgCl_2$, 33mM 2-mercaptoethanol, 1.33mg/ml BSA,

18 $OD_{260}$ units/ml oligodeoxyribonucleotide primers (hexamers)

pH 6.8

**Nucleotides:**

dATP, dGTP, dTTP: 0.5 mM in 3mM Tris-HC, 0.2 mM $Na_2EDTA$

## Purification of PCR products

**GELase**: 1U/μl

**Reaction Buffer:**

40mM bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane,
1mM EDTA, 40mM NaCl, pH 6.0

**Storage Buffer:** 50mM Tris-HCl, 0.1mM EDTA, 0.1M NaCl, 0.1%

Triton X-100, 50% (w/v) glycerol, pH 7.5

## DNA Sequencing

**DNA Sequencing Kit (Sequenase Version 2.0)**

**Storage Buffer**: 13 U/µl in 20mM $KPO_4$, pH 7.4, 1mM DTT, 0.1mM EDTA, 50% glycerol

**Enzyme Dilution Buffer**: 10 mM Tris-HCl, pH 7.5, 5 mM DTT, 0.5 mg/ml BSA

**Sequenase Buffer (5X)**: 200mM Tris-HCl, pH 7.5, 100mM $MgCl_2$, 250 mM NaCL

**Labelling Mix (5X)**: 7.5µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP

**Termination Mixes (5X):**

**ddG**: 80 µM dGTP, 80 µM dATP, 80 µM dCTP, 80 µM dTTP, 40 µM ddGTP, 50 mM NaCl

**ddA**: 80 µM dGTP, 80 µM dATP, 80 µM dCTP, 80 µM dTTP, 40 µM ddATP, 50 mM NaCl

**ddT**: 80 µM dGTP, 80 µM dATP, 80 µM dCTP, 80 µM dTTP, 40 µM ddTTP, 50 mM NaCl

**ddC**: 80 µM dGTP, 80 µM dATP, 80 µM dCTP, 80 µM dTTP, 40 µM ddCTP, 50 mM NaCl

**Stop Solution**: 95% formamide, 20 mM EDTA, 0.05% Bromophenol blue 0.05% Xylene Cyanol FF

## APPENDIX III

## Oligonucleotides used in this thesis

| Oligonucleotide | Sequence 5' › | Site | Use |
|---|---|---|---|
| 1 | TTTGAATTCTCGAAG GCAAGGCACCAG | CYP11B1 5' UTR (+) | PCR of chimeric CYP11B1/B2 gene |
| 2X | CTCGGATCCCAGCTC TCAGCTCCCAA | CYP11B2 Intron 2 (-) | PCR of chimeric CYP11B1/B2 gene |
| 3A | GCAGAAAATCCCTCC CCCCTA | CYP11B1 Intron 2 (+) | PCR of chimeric CYP11B1/B2 gene |
| 5 | GACACGTGGGCGCC GTGTGA | CYP11B1 Inton 5 (-) | PCR of chimeric CYP11B1/B2 gene |
| 5N | TGGAGCATGGATCC CCACACA | CYP11B2 Intron 5 (-) | PCR/ Sequencing chimeric CYP11B1/B2 gene |
| 6A | GTCTAACCCTGCAGC TGTGT | CYP11B1 Intron 5 (+) | PCR of chimeric CYP11B1/B2 gene |
| CC | AATCTAGATTTGTGC AGGAGCTGGCTGG | CYP11B2 3'UTR (-) | PCR of chimeric CYP11B1/B2 gene |
| 3 | ACAAGGAGGATGCC ATA | CYP11B1/2 Intron 2 (+) | Sequencing chimeric CYP11B1/B2 gene |
| 4 | GGACTGAAGGGAGT GTG | CYP11B1/2 Intron 3 (+) | Sequencing chimeric CYP11B1/B2 gene |
| 2523 | TTCGGATCCTCCCTC CACCCTGTTC | CYP11B2 5' UTR (-) | PCR of CYP11B2 promoter region |
| 2524 | TTTGTCGACATGTGT TCAAAACCCACA | CYP11B2 5' UTR (+) | PCR of CYP11B2 promoter region |
| 2360 | ATCCAAGGCCCCCTC TCAT | CYP11B2 5' UTR (+) | ASO Probe |
| 2361 | ATGAGAGGGAGCCT TGGAT | CYP11B2 5' UTR (-) | ASO Probe |

**Key:**

**PCR - polymerase chain reaction amplification, ASO Probe - allele specific oligonuceotide probe, UTR - untranslated region of gene, either 5' or 3'.**

**(+) or (-) - sense of oligonucleotide, i.e. (+) elongates the sense strand 5' to 3' and (-) elongates the antisense strand 5' to 3'**

## Calculation of oligonucleotide concentrations

The concentration of an oligonucleotide of known sequence can be calculated when the molecular weight (MW) of the oligonucleotide and the quantity synthesised is determined.

MW = [(A x 312.2) + (G x 328.2) + (C x 288.2) + (T x 303.2)] - 61.0
Where A, G, T, and C are the number of each base in the sequence

The quantity synthesised is determined from the optical density (OD) reading. OD is the amount synthesised in 1ml volume. An OD reading of 1.00 corresponds to approximately 37mg/ml of oligonucleotide.

Concentration = ( OD x 0.037/MW) x 106

## APPENDIX IV

## 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: | 5' flanking region and exon 1 | D10169, D90428, X55765 |
| | Exon 1 and 2 | M32863, J01540 |
| | Exon 3 -8 | M32878, J01540 |
| | Exon 9 | M32879 J01540 |
| CYP11B2: | 5' flanking region and exon 1 | D10170, D90429 |
| | Exon 1 and 2 | M32864, J05140 |
| | Exon 3 -8 | M32880, J05140 |
| | Exon 9 | 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 bounded by double <>>; the double >> indicates the sense of the oligonucleotide, e.g. < **oligo 1** >> indicates that oligo 1 is a sense oligo.

### CYP11B1

```
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  1 ---------+---------+---------+---------+---------+---------+ 60

    TTGTGGGCATTTAGTGCTATAAATTTCCCTCTACACACTGCTTTGAATGTGTTCCAGAGA
 61 ---------+---------+---------+---------+---------+---------+ 120

    TTCTGGTATGCTGTGTCTTTGTTCTCGTTGGTTTCAAGAACATCTTTATTTCTGCCTTCA
121 ---------+---------+---------+---------+---------+---------+ 180

    TTTTGTTACGTACCCAGTAGTCATTCAGGAGCAGGTTGCTCAGTTTCCATGTAATTGAGC
181 ---------+---------+---------+---------+---------+---------+ 240

    GGTTTTGAGTGAGTTTCTTAATCCTGAGTTCTAGTTTGATTGCACTAAAATTTTTAAAAA
241 ---------+---------+---------+---------+---------+---------+ 300

    GTAAAAAAAATACATGTGGTTTAATACAATTCATGCCAACTCATTCCCTCGTTTTTTGCT
301 ---------+---------+---------+---------+---------+---------+ 360

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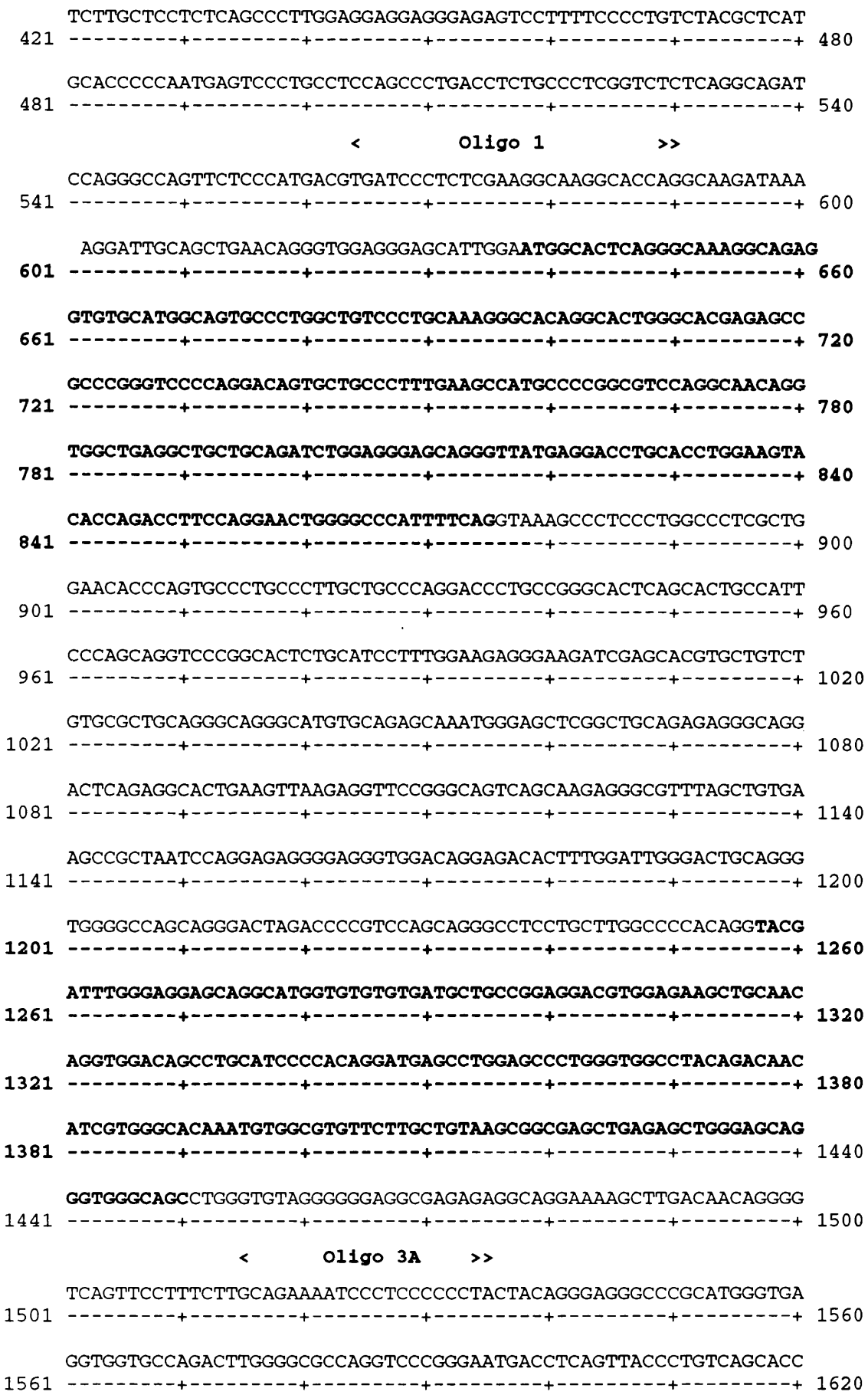
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601 ---------+---------+---------+---------+---------+---------+ 660
GTGTGCATGGCAGTGCCCTGGCTGTCCCTGCAAAGGGCACAGGCACTGGGCACGAGAGCC
661 ---------+---------+---------+---------+---------+---------+ 720
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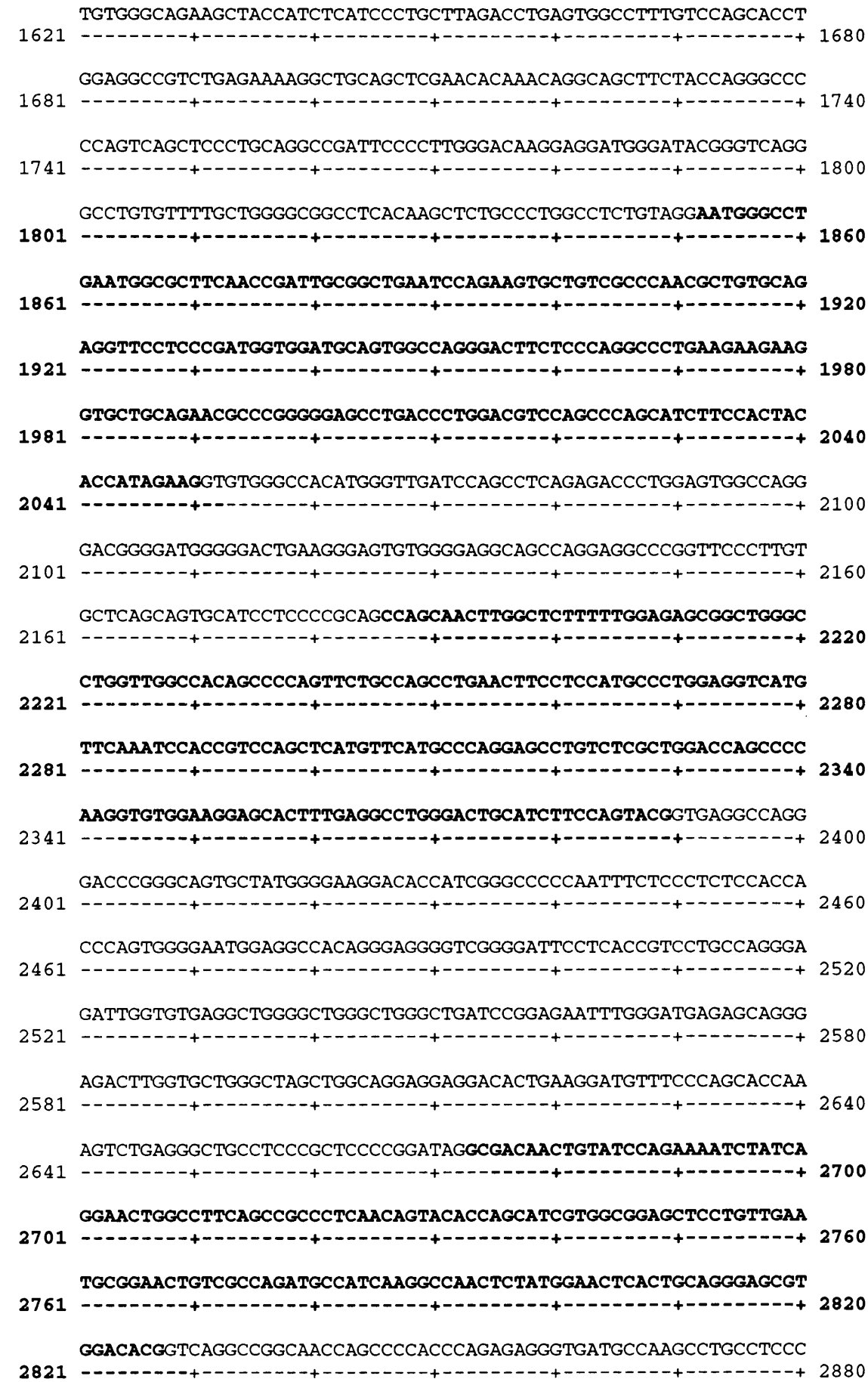
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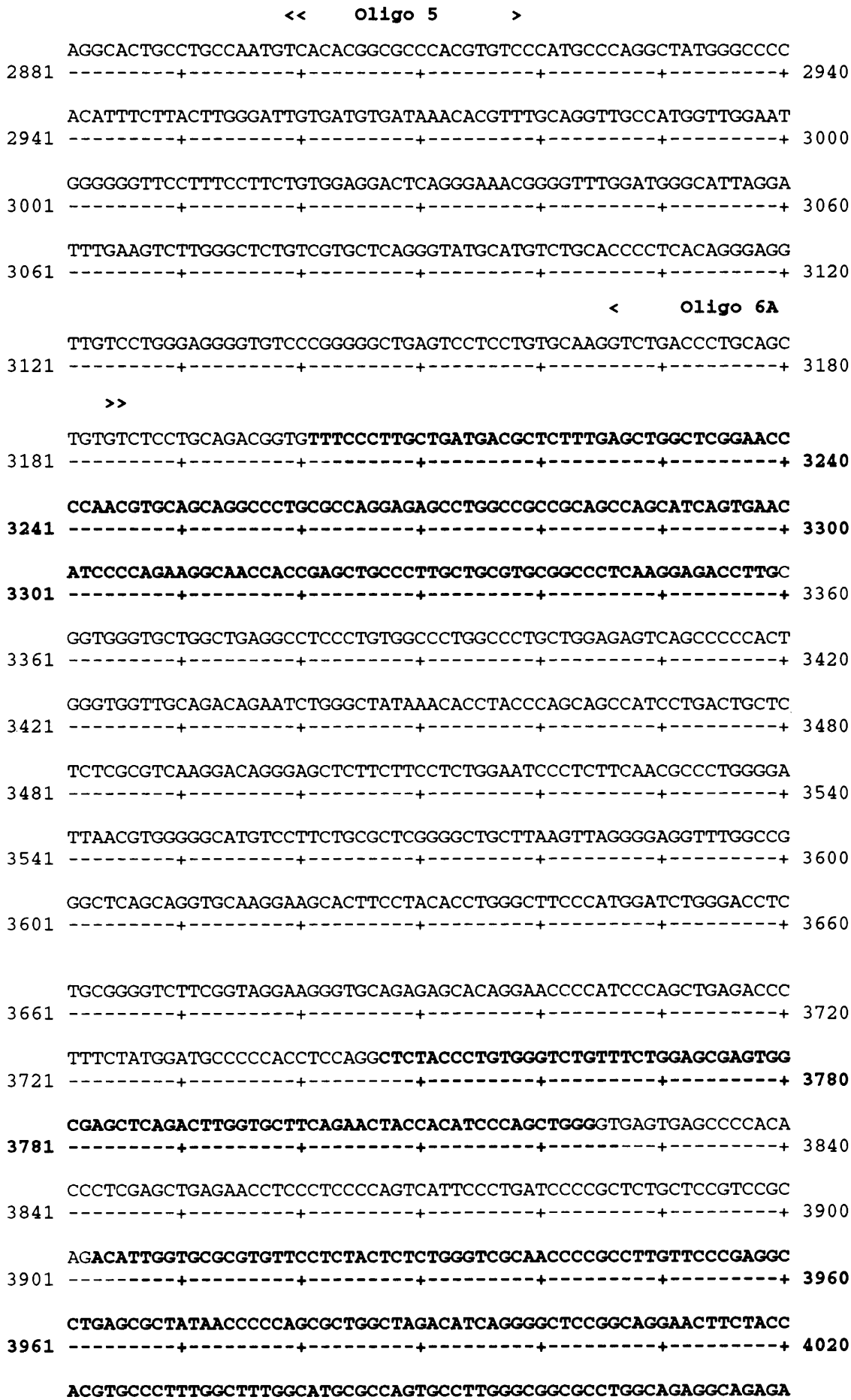
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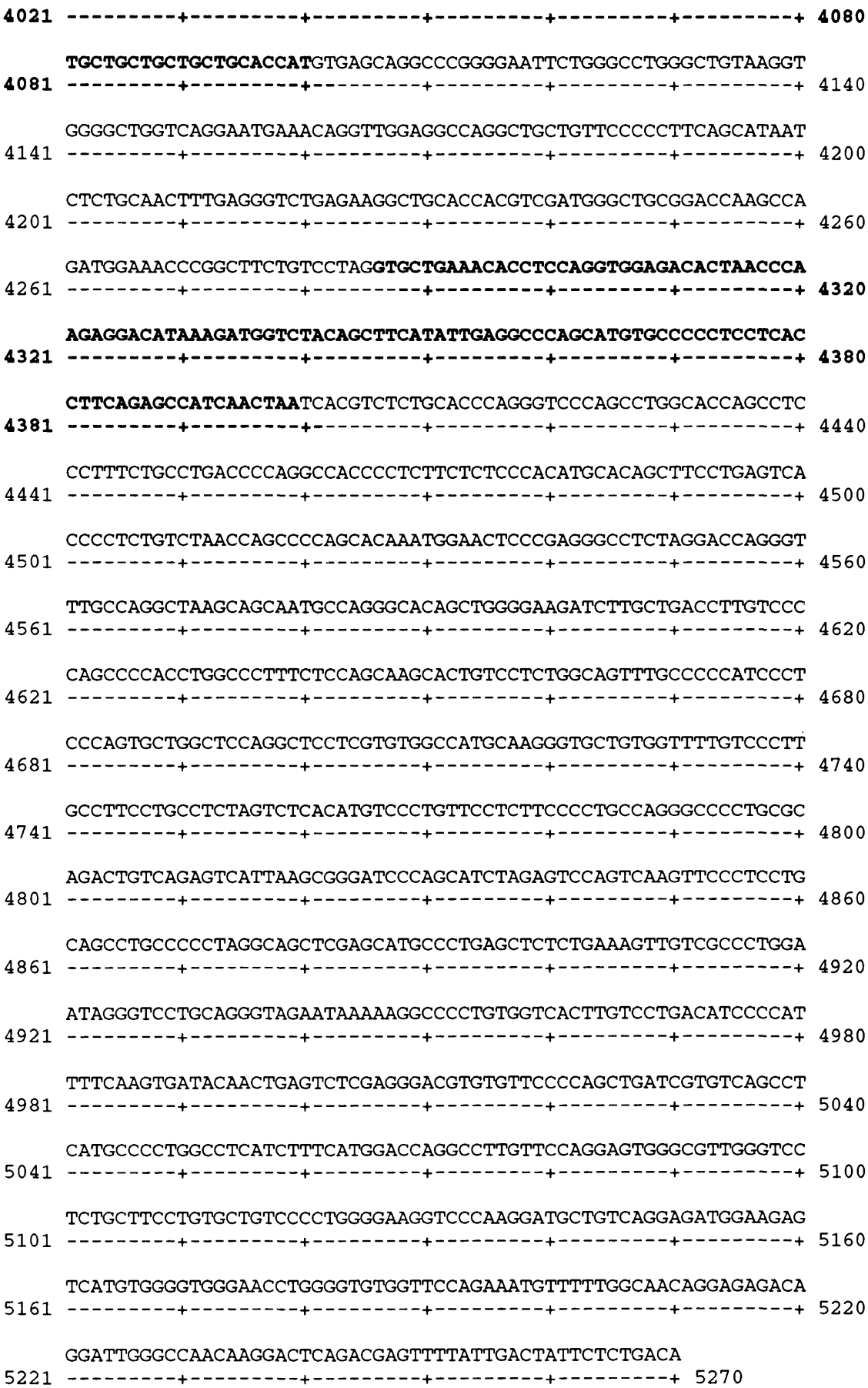

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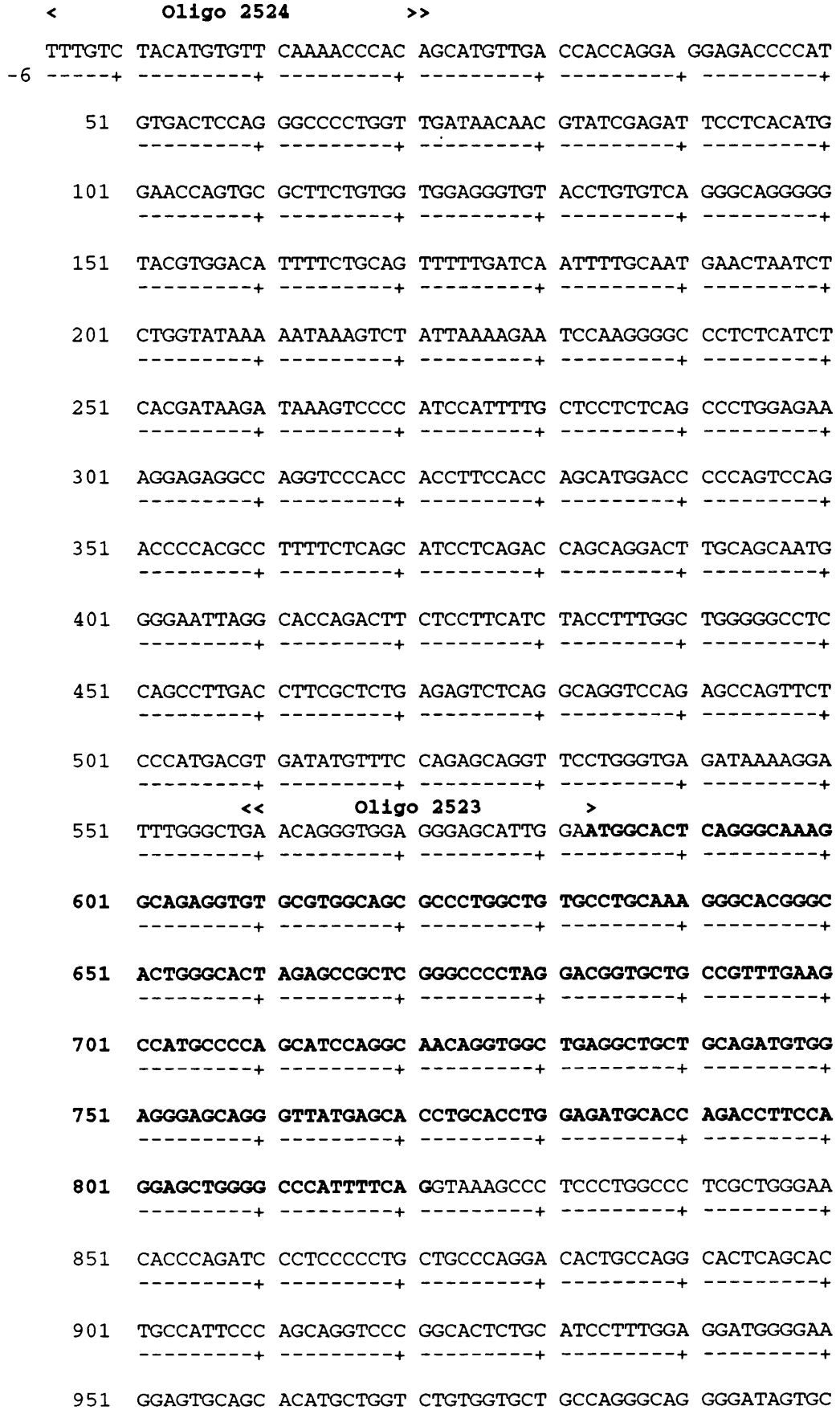
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801 GGAGCTGGGG CCCATTTTCA GGTAAAGCCC TCCCTGGCCC TCGCTGGGAA
851 CACCCAGATC CCTCCCCCTG CTGCCCAGGA CACTGCCAGG CACTCAGCAC
901 TGCCATTCCC AGCAGGTCCC GGCACTCTGC ATCCTTTGGA GGATGGGGAA
951 GGAGTGCAGC ACATGCTGGT CTGTGGTGCT GCCAGGGCAG GGGATAGTGC

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         ---------+ ---------+ ---------+ ---------+ ---------+

1001     AGAGAAAACC CCAGCTCACT GCAGAGAGGG CAGGACTCAG AAGCACTAAA
         ---------+ ---------+ ---------+ ---------+ ---------+

1051     GTTGAAAGGT TCCAGGGAGC CAGCAGGAGG GCTTTAGCTG TGAAGCCGCT
         ---------+ ---------+ ---------+ ---------+ ---------+

1101     AATCCAGGAG CAGGGAGGGT GGACAGGAGA CACTTTGGAT TGGGACTGCA
         ---------+ ---------+ ---------+ ---------+ ---------+

1151     GGGTGGGGCC ACGAGGGACA TGACCCCGTC CAGCAGGGCC TCCTGCTTGG
         ---------+ ---------+ ---------+ ---------+ ---------+

1201     CCCCACAGGT ACAACTTGGG AGGACCACGC ATGGTGTGTG TGATGCTGCC
         ---------+ ---------+ ---------+ ---------+ ---------+

1251     GGAGGATGTG GAGAAGCTGC AACAGGTGGA CAGCCTGCAT CCCTGCAGGA
         ---------+ ---------+ ---------+ ---------+ ---------+

1301     TGATCCTGGA GCCCTGGGTG GCCATCAGAC AACATCGTGG GCACAAATGT
         ---------+ ---------+ ---------+ ---------+ ---------+

                                        <<     Oligo 2X       >

1351     GGCGTGTTCT TGTTGTAAGC GGCGAGTTGG GAGCTGAGAG CTGGGAGCAG
         ---------+ ---------+ ---------+ ---------+ ---------+

1401     GGTGGGCAGC CTGGGTGTAG GGGGGAGGCG AGAGAGGTAG GACCCAAAAG
         ---------+ ---------+ ---------+ ---------+ ---------+

1451     CACATCTGCC CTGGGCCCCT GTGGTGGGCA GTGAGGGTGA GCACCCGGCC
         ---------+ ---------+ ---------+ ---------+ ---------+

1501     CAGAGGACGG CCATCCTGTG GGGTCGCGTC TGCACTGTGG GTTGGGGAAG
         ---------+ ---------+ ---------+ ---------+ ---------+

1551     CAGGGCGGTG GTGGAGAAAT GGGCAGGGGC ACCTCTGCAG AGAAGACGCA
         ---------+ ---------+ ---------+ ---------+ ---------+

1601     GAGCAATGAG CCCTTCTGTG TAGTGAGAAC CCGCTCTGCA CCAACCTCGG
         ---------+ ---------+ ---------+ ---------+ ---------+

1651     CGGCTGCTTT CTCTTGCGGT CTGGGGACTC TCCTTCCCAT AGGTCAGAAA
         ---------+ ---------+ ---------+ ---------+ ---------+

1701     ACTGAGGCCC TGAGAAGGGG ACTTCCACTG GCCCAGGTCA CAGGCTGAGT
         ---------+ ---------+ ---------+ ---------+ ---------+

1751     ACTGAGCCTG GTGTTCGCCG GGGCCACAGC CTCCCTCAGG GCGCTCAGGG
         ---------+ ---------+ ---------+ ---------+ ---------+

1801     TCCCTGCAGA ACAGGGGTCA CCTCCTTTCT TGGAGAAAAG CCCTACCCTG
         ---------+ ---------+ ---------+ ---------+ ---------+

1851     TTACTACAGG GAGGGCCTGC ATGGGTGAGG TGGTGCCAGA CTTGGGTCGC
         ---------+ ---------+ ---------+ ---------+ ---------+

1901     CAGGTCCCAG GAATGACCTC AGTTACCCTG TCAGCACCTG TGGGCAGAAG
         ---------+ ---------+ ---------+ ---------+ ---------+

1951     CTACAGTCTC ATCCCTGCTT AGACCTGAGC GGCCTTTGCC CAGCACCTGG
         ---------+ ---------+ ---------+ ---------+ ---------+
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2001 AGGTCGCTCT GAGAAAAGGT CTGCAGCTCG AACACAAACA GGCAGCTTCT

2051 ACCAGGGCCC CAGTCACTCC TGCAGGCCGA TTCCCCTTGG GTACAAGGAG

2101 GATGGGATAC GGGGTCAGGG CCTGTGTCTT GCTGGGGCGG CCTCACAAGC

2151 TCTGCCCTGG CCTCTGTAGG **AATGGGCCTG AATGGCGCTT CAACCGATTG**

**2201 CGGCTGAACC CAGATGTGCT GTCGCCCAAG GCCGTGCAGA GGTTCCTCCC**

**2251 GATGGTGGAT GCAGTGGCCA GGGACTTTTC CCAGGCCCTG AGGAAGAAGG**

**2301 TGCTGCAGAA CGCCCGGGGG AGCCTGACCC TGGACGTCCA GCCCAGCATC**

**2351 TTCCACTACA CCATAGAA**GG TGTGGGCCAT GCGGGAAGGT CCAGCCCCAG

2401 AGACCCTGGA GTGGCCAGGG ATGGGGATGG AGGACTGAAG GGAGTGTGGG

2451 GAGGCAGCCA GGAGGTCCGG GGCTGCCTTG TGCTCAGCAG TGCATCCTCC

2501 CCGCAG**CCAG CAACTTAGCT CTTTTTGGAG AGCGGCTGGG CCTGGTTGGC**

**2551 CACAGCCCCA GTTCTGCCAG CCTGAACTTC CTCCATGCCC TGGAGGTCAT**

**2601 GTTCAAATCC ACCGTCCAGC TCATGTTCAT GCCCAGGAGC CTGTCTCGCT**

**2651 GGATCAGCCC CAAGGTGTGG AAGGAGCACT TTGAGGCCTG GGACTGCATC**

**2701 TTCCAGTACG** GTGAGGCCAG GGACCCGGGC AGTGCTATGG GGAAGGGACA

2751 CCATGGGGGC CCAATTTCTC CCTCTCCACC ACCCAGTGGG GAATGGAGGC

2801 CACAGGGAGG GGTCGGGGAT TCCTCACCTT CCTGCCGGGG AGATTGGTGC

2851 GAGGCTGGGG CTGGGCTGGG CTGATCCGGA GAATTTGGGA TGAGAGCAGG

2901 GAGATTTGGG TGTCGGGGCA GTCTCGGCAG GAGGAGGACA CTGAAGGATG

2951 CTTCCCAGCA CCAAGATCTA GGGCTGTCCC CTGCTCCCTG TACAG**GTGAC**

**3001 AACTGTATCC AGAAAATCTA CCAGGAACTG GCCTTCAACC GCCCTCAACA**

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3051  CTACACAGGC ATCGTGGCAG AGCTCCTGTT GAAGGCGGAA CTGTCACTAG
      ---------+ ---------+ ---------+ ---------+ ---------+

3101  AAGCCATCAA GGCCAACTCT ATGGAACTCA CTGCAGGGAG CGTGGACACG
      ---------+ ---------+ ---------+ ---------+ ---------+

3151  GTCAGGCCAG CAACCAGCCC CACCCAGAGA GGGTGATGCC AAGCCCTGCC
      ---------+ ---------+ ---------+ ---------+ ---------+

3201  TCCCAGCACT GCCTGCCAAT GCCACACGGC ACCCACGTTC CCCATCCCCA
      ---------+ ---------+ ---------+ ---------+ ---------+

3251  GGCTACAGGC CCCACATTTC TGTTGCCCTC AGCCTTCCCC CTCCTTTGTT
      ---------+ ---------+ ---------+ ---------+ ---------+

3301  AAGGGATGAG ATTTGCAGGG GAGGGGAAAT GTGAGCTCCC CCTCACATGA
      ---------+ ---------+ ---------+ ---------+ ---------+
                          <<      Oligo 5N      >
3351  GACTGAGTTT GCAGTTACCT GTGTGGGGAT CCATGCTCCA GGCTGGAAGA
      ---------+ ---------+ ---------+ ---------+ ---------+

3401  AAGTTGGATG AGGCCCTGGA CACACAGCAG CTCTGTCCCC ACTGGAAAGC
      ---------+ ---------+ ---------+ ---------+ ---------+

3451  TCTGGGTGTA CAAGGAGAAG GAGGGTTGAG AGGCAGCTGG AGGACTCCAC
      ---------+ ---------+ ---------+ ---------+ ---------+

3501  TGGGCACCCT TCCCAGTGTG CCCGGTCACC TTGGGCCAGA AATGTACATG
      ---------+ ---------+ ---------+ ---------+ ---------+

3551  CATGGGAGGG CAGGGTTGTG GGGAAGGCAG CAGCACGGGC TCCAGCCAGT
      ---------+ ---------+ ---------+ ---------+ ---------+

3601  GCAGAGGGGC CTGTGGGTGC ACAGTGGGGA GAACTCAATG GAAGCAGAGG
      ---------+ ---------+ ---------+ ---------+ ---------+

3651  GAGCTGGGGC TCCAGAACTC CCAGGATGAT GCTGAGGTCT GGCCCCCTTT
      ---------+ ---------+ ---------+ ---------+ ---------+

3701  TCTAAGGTGG CTGTGAGAAC CCGCCTGAAG AGGCTGCAGG GGACCTGGGC
      ---------+ ---------+ ---------+ ---------+ ---------+

3751  CTTGGTGGAG ATGGGGGTCA GCTTTGCGTG AAGAAGTCAG GGAATCTGGC
      ---------+ ---------+ ---------+ ---------+ ---------+

3801  CCAAGTGGTC ATCAAGGTTT CAGATCCGGC GTCCCAGGGC TCTGTCGTGC
      ---------+ ---------+ ---------+ ---------+ ---------+

3851  TCAGGGCATG GATGTCTCCA CCCCTCAGAG GGAGGTTGTC CTGGCTGGGG
      ---------+ ---------+ ---------+ ---------+ ---------+

3901  TGTCCCGGGG GCTGAGTCCT CCTGTGCAAG GTCAGACCCT GCAGACATGG
      ---------+ ---------+ ---------+ ---------+ ---------+

3951  CTTCTGTAGA CAGCGTTTCC GTTGCTGATG ACGCTCTTTG AGCTGGCTCG
      ---------+ ---------+ ---------+ ---------+ ---------+

4001  GAACCCCGAC GTGCAGCAGA TCCTGCGCAA GGAGAGCCTG GCCGCCGCAG
      ---------+ ---------+ ---------+ ---------+ ---------+

4051  CCAGCATCAG TGAACATCCC CAGAAGGCAA CCACCGAGCT GCCCTTGCTG
      ---------+ ---------+ ---------+ ---------+ ---------+
```

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5151  TTCCCTCTGC CTGACCCCAG GCCACCTGTC TTCTCTCCCA CGTGCACAGC
      ---------+ ---------+ ---------+ ---------+ ---------+

                           <<          Oligo CC         >

5201  TTCCTGAGTC ACCCCTCTGT CCAGCCAGCT CCTGCACAAA TGGAACTCCC
      ---------+ ---------+ ---------+ ---------+ ---------+

5251  CAGGGCCTCC AGGACTGGGG CTTGCCAGGC TTGTCAAATA GCAAGGCCAG
      ---------+ ---------+ ---------+ ---------+ ---------+

5301  CGCACAGCTG GAGCGATCTT GCTGCAGGCC TGCCTTGTCC CCAGCCCCAC
      ---------+ ---------+ ---------+ ---------+ ---------+

5351  CTGGCCCCTT CTCCAGCAAG CAGTGCCCTC TGGACACTTG ACTCTACTCC
      ---------+ ---------+ ---------+ ---------+ ---------+

5401  TCCCAGCGCT GGCTCCAGGC TCCTCATGAG GCCATGCAAG GGTGCTGTGA
      ---------+ ---------+ ---------+ ---------+ ---------+

5451  TTTTGTCCCZ DTTGCCTTCC TGCFINDCTA GTCTCACATG TCCCTGTCCC
      ---------+ ---------+ ---------+ ---------+ ---------+

5501  TCTCGCCCTG GCCAGGGCCT CTGTGCAGAC AGTGTCAGAG TCATTAAGCG
      ---------+ ---------+ ---------+ ---------+ ---------+

5551  GGATCCCAGC ATCTCAGAGT CCAGTCAAGT TCCCTCCTGC AGCCTGACCC
      ---------+ ---------+ ---------+ ---------+ ---------+

5601  CAGGCAGCTC GAGCATGCCC TGAGCTCTCT GAAAGTTGTC ACCCAGAAAT
      ---------+ ---------+ ---------+ ---------+ ---------+

5651  ACGATCCTGC AGGGTAGACT AAAAAGGCCC CTGTGGTCAC TTATACTGAC
      ---------+ ---------+ ---------+ ---------+ ---------+

5701  ACATTTTAAG TGATACAACT GAGTCTCGAG GGGCGTGTGT TCCCCAGCTG
      ---------+ ---------+ ---------+ ---------+ ---------+

5751  ATCATGTCAG CCTCATGCCC CAGGCCTCGT CTTTCATGGA CCAGGTCTTG
      ---------+ ---------+ ---------+ ---------+ ---------+

5801  TTCAAGCAGC GAGTGTTGGG TCCTCTGCTT CCTGAGCTGT CCCCTGGAAA
      ---------+ ---------+ ---------+ ---------+ ---------+

5851  AGGTCCCGAG GATGCTGTCA GGAGATGGAA GAGTCATGTG GGGTGGGAAC
      ---------+ ---------+ ---------+ ---------+ ---------+

5901  CTGGGGTGTG GTTCCAGAAA TGTTTTTGGC AACAGGAGAG ACAGGATTGG
      ---------+ ---------+ ---------+ ---------+ ---------+

5951  GCCAACAAGG ACTCAGATGA GTTTATTGAC TCATTCCTCT GGAAGATACG
      ---------+ ---------+ ---------+ ---------+ ---------+

6001  CAGC
      ---------+
```

## CYP11B2 promoter region

The 5'-untranslated flanking region of CYP11B2 containing the -340C/T polymorphism is listed below. The sequence strands -340C and -340T denote the sequence between -452 to -383 containing either a C or T at -340. Restriction sites for the enzyme HaeIII are marked in bold at the site i.e. **GGCC**.

```
        <        Oligo 2524         >>

        TTTGTC TACATGTGTT CAAAACCCAC AGCATGTTGA CCACCAGGA GGAGACCCCAT GTGACTCCAG

-588    -----+ ---------+ ---------+ ---------+ ---------+ ---------+ ---------+

    GGACCCTGGT TGATAACAAC GTATCGAGAT TCCTCACATG GAACCAGTGC GCTTCTGTGG TGGAGGGTGT
-522 ---------+ ---------+ ---------+ ---------+ ---------+ ---------+ ---------+

    ACCTGTGTCA GGGCAGGGGG TACGTGGACA TTTTCTGCAG TTTTTGATCA ATTTTGCAAT GAACTAATCT
-452 ---------+ ---------+ ---------+ ---------+ ---------+ ---------+ ---------+

-340C
                                          HaeIII site
                                            <   >
    CTGGTATAAA AATAAAGTCT ATTAAAAGAA TCCAAGGGGC CCTCTCATCT CACGATAAGA TAAAGTCCCC
-382 ---------+ ---------+ ---------+ ---------+ ---------+ ---------+ ---------+
                                            ^
-340T                                C/T polymorphism
                                            ^
    CTGGTATAAA AATAAAGTCT ATTAAAAGAA TCCAAGGGGT CCTCTCATCT CACGATAAGA TAAAGTCCCC
-382 ---------+ ---------+ ---------+ ---------+ ---------+ ---------+ ---------+

                                       HaeIII site
                                         <  >
    ATCCATTTTG CTCCTCTCAG CCCTGGAGAA AGGAGAGGCC AGGTCCCACC ACCTTCCACC AGCATGGACC
-312 ---------+ ---------+ ---------+ ---------+ ---------+ ---------+ ---------+

    CCCAGTCCAG ACCCCACGCC TTTTCTCAGC ATCCTCAGAC CAGCAGGACT TGCAGCAATG GGGAATTAGG
-242 ---------+ ---------+ ---------+ ---------+ ---------+ ---------+ ---------+

                                    HaeIII site
                                      <  >
    CACCAGACTT CTCCTTCATC TACCTTTGGC TGGGGGCCTC CAGCCTTGAC CTTCGCTCTG AGAGTCTCAG
-172 ---------+ ---------+ ---------+ ---------+ ---------+ ---------+ ---------+

    GCAGGTCCAG AGCCAGTTCT CCCATGACGT GATATGTTTC CAGAGCAGGT TCCTGGGTGA GATAAAAGGA
-102 ---------+ ---------+ ---------+ ---------+ ---------+ ---------+ ---------+
                              <<       Oligo 2523        >

    TTTGGGCTGA ACAGGGTGGA GGGAGCATTG GAATG
-32  ---------+ ---------+ ---------+ -----
                           Start >
```

HaeIII digest of the PCR product results in products of the following sizes:

| -340T | -340C |
|---|---|
| 324 | 245 |
| 138 | 138 |
| 88 | 88 |
| | 69 |

## APPENDIX V

## Data for patients studied in this thesis

A) Patients with GSH

| | Mean arterial blood pressure (mm Hg) | Serum potassium (mmol/l) | Plasma active renin concentration (μU/ml) | Plasma aldosterone concentration (pmol/ll) |
|---|---|---|---|---|
| **Normal range** | | **3.6-5.1** | **5-50** | **140-500** |
| **Individual** | | | | |
| 1-II-1 | 155 | 3.4 | 1 | 904 |
| 1-II-2 | 124 | 3.6 | 1 | 971 |
| 1-III-1 | 94 | 3.9 | 1 | 666 |
| 1-III-2 | 76 | 4.6 | 1 | 662 |
| 1-III-3 | 70 | 4.4 | 4 | 452 |
| 2-II-1 | 120.5 | 4.2 | | 1503 |
| 2-III-1 | 124 | 3.7 | 3 | 1288 |
| 2-III-2 | 139 | 4.4 | 1 | 1266 |
| 2-IV-1 | 82 | 4.2 | 0 | 688 |
| 2-IV-2 | 80 | 4.6 | 1 | 648 |
| 3-I-1 | 136 | 4.2 | 1 | 596 |
| 3-II-1 | 144 | 3.7 | 1 | 1100 |
| 4-II-1 | 153 | 2.8 | 1 | 2466 |
| 4-III-1 | 113 | 3.0 | 1 | 971 |
| 4-III-2 | 113 | 3.4 | 2 | 956 |
| 5-1-1 | 120.5 | 3.6 | 4 | 583 |
| 5-II-1 | 170 | 3.3 | 1 | 1248 |
| 5-II-2 | 155 | 2.8 | 1 | 832 |
| 5-II-3 | 115 | 3.9 | 1 | 1806 |

Data at presentation, n=19.
Individuals are identified by their kindred identifier as described in figure 3.3 a

B) ACTH infusion data

Below are listed the steroid biochemical data obtained from the ACTH infusion studies carried out in Chapter 6.

1) Plasma aldosterone response to ACTH

| Time (mins) | 0 | 15 | 30 | 45 | 60 | 75 | 90 |
|---|---|---|---|---|---|---|---|
| GSH | 17.8 | 33.3 | 41 | 61 | 70.3 | 76.3 | 57.1 |
| | 4.4 | 7.2 | 6.9 | 11.8 | 10.9 | 10.3 | 9.1 |
| NT | 9 | 6.8 | 12 | 21.2 | 23.4 | 32.4 | 28.2 |
| | 2.2 | 2.1 | 2.5 | 4.0 | 2.6 | 5.2 | 4.4 |
| HT | 11.4 | 12.8 | 16.1 | 20.9 | 27.6 | 33.9 | 33.3 |
| | 1.9 | 2.0 | 2.6 | 3.8 | 4.0 | 5.4 | 4.8 |
| Conn's | 35.7 | 28 | 32 | 50.7 | 70 | 88.7 | 82 |
| | 4.9 | 8.3 | 7.4 | 10.3 | 18.6 | 15.9 | 18.4 |

Data are mean ± s.e.m. Units are ng/dl. To convert to pmol/l - divide by 0.0361
GSH - patients with GSH, NT - normotensive controls, HT - patients with essential hypertension, Conn - patients with aldosterone-secreting adenomata

2) Plasma corticosterone response to ACTH

| Time (mins) | 0 | 15 | 30 | 45 | 60 | 75 | 90 |
|---|---|---|---|---|---|---|---|
| GSH | 97.4 | 218.7 | 367.3 | 612 | 783.3 | 999.3 | 1315 |
| | 51 | 67 | 95 | 136 | 110 | 178 | 145 |
| NT | 266 | 218 | 478 | 790 | 1076 | 1483 | 1344 |
| | 159 | 73 | 76 | 157 | 99 | 282 | 254 |
| HT | 227 | 242 | 380 | 646 | 847 | 942 | 1111 |
| | 80 | 62 | 100 | 152 | 200 | 200 | 179 |
| Conn's | 293 | 274 | 351 | 722 | 972 | 1061 | 1286 |
| | 130 | 175 | 134 | 113 | 65 | 50 | 39 |

Data are mean ± s.e.m. Units are ng/dl. To convert to pmol/l - divide by 0.0346

GSH - patients with GSH, NT - normotensive controls, HT - patients with essential hypertension, Conn - patients with aldosterone-secreting adenomata

3) Plasma 11-deoxycorticosterone response to ACTH

| Time (mins) | 0 | 15 | 30 | 45 | 60 | 75 | 90 |
|---|---|---|---|---|---|---|---|
| GSH | 11 | 17.7 | 26.7 | 33.3 | 50.7 | 54.7 | 84.9 |
| | 2.9 | 5.0 | 7.6 | 11.2 | 16 | 14.8 | 16.8 |
| NT | 6.8 | 8.2 | 9.4 | 16.4 | 17.2 | 16.6 | 21.6 |
| | 2.1 | 1.3 | 2.3 | 4.1 | 4.1 | 4.3 | 7.0 |
| HT | 7.6 | 8.4 | 9.5 | 14.3 | 20.3 | 21.3 | 23.9 |
| | 2.0 | 1.8 | 1.8 | 2.8. | 5.1 | 4.7 | 4.6 |
| Conn's | 16.7 | 19 | 16 | 37 | 56.7 | 53.3 | 63 |
| | 0.3 | 3.6 | 2.6 | 13 | 17 | 19.1 | 20.8 |

Data are mean ± s.e.m. Units are ng/dl. To convert to pmol/l - divide by 0.033

GSH - patients with GSH, NT - normotensive controls, HT - patients with essential hypertension, Conn - patients with aldosterone-secreting adenomata

4) Plasma 11-deoxycortisol response to ACTH

| Time (mins) | 0 | 15 | 30 | 45 | 60 | 75 | 90 |
|---|---|---|---|---|---|---|---|
| GSH | 37.6 | 51.3 | 120.3 | 145.7 | 181.3 | 204 | 230 |
| | 7.9 | 11.8 | 47 | 25 | 11 | 6 | 14 |
| NT | 22.8 | 28 | 37.6 | 61.6 | 55.2 | 73.2 | 74.8 |
| | 11 | 12.5 | 8.0 | 16.4 | 17.6 | 16.7 | 14.7 |
| HT | 32.8 | 41.9 | 53 | 110.8 | 109 | 118.5 | 168.5 |
| | 11.1 | 12 | 17.4 | 26.8 | 24 | 25 | 31.5 |
| Conn's | 47.3 | 38.7 | 78.3 | 128.3 | 277 | 202.3 | 265 |
| | 7.6 | 7.5 | 26.7 | 25.3 | 79 | 59 | 66.7 |

Data are mean ± s.e.m. Units are ng/dl. To convert to pmol/l - divide by 34.6

GSH - patients with GSH, NT - normotensive controls, HT - patients with essential hypertension, Conn - patients with aldosterone-secreting adenomata

5) Plasma cortisol response to ACTH

| Time (mins) | 0 | 15 | 30 | 45 | 60 | 75 | 90 |
|---|---|---|---|---|---|---|---|
| GSH | 285 | 426 | 491 | 570 | 639 | 721 | 747 |
| | 26 | 49 | 54 | 59 | 59 | 70 | 35 |
| NT | 368 | 387 | 561 | 656 | 753 | 788 | 793 |
| | 28 | 30 | 37 | 33 | 46 | 40 | 33 |
| HT | 402 | 454 | 548 | 654 | 763 | 783 | 838 |
| | 31 | 35 | 48 | 48 | 45 | 43 | 42 |
| Conn's | 414 | 452 | 498 | 602 | 680 | 719 | 803 |
| | 5 | 51 | 23 | 19 | 48 | 55 | 50 |

Data are mean ± s.e.m. Units are nmol/l.

GSH - patients with GSH, NT - normotensive controls, HT - patients with essential hypertension, Conn - patients with aldosterone-secreting adenomata

C) Left ventricular mass data

| Patient | LVIDd (cm) | PWTd (cm) | IVSTd (cm) | LVM (gram) | Height (cm) | Weight (kg) | BSA ($m^2$) | LVMI ($g/m^2$) |
|---|---|---|---|---|---|---|---|---|
| 2-IV-1 | 4.14 | 0.80 | 0.72 | 104.02 | 141 | 33 | 1.15 | 90.61 |
| 2-IV-2 | 4.05 | 0.82 | 0.70 | 99.69 | 110 | 19 | 0.76 | 131.46 |
| 2-II-1 | 4.10 | 0.80 | 0.71 | 101.10 | 158 | 54 | 1.54 | 65.78 |
| 2-III-1 | 5.21 | 1.02 | 1.09 | 252.10 | 182 | 75 | 1.96 | 128.75 |
| 2-III-2 | 4.83 | 0.88 | 1.07 | 197.85 | 167 | 60 | 1.67 | 118.25 |
| 1-II-1 | 4.30 | 1.10 | 1.20 | 205.89 | 178 | 80 | 1.98 | 103.97 |
| 1-II-2 | 5.19 | 1.08 | 1.11 | 264.33 | 179 | 77 | 1.96 | 135.11 |
| 1-III-1 | 5.08 | 0.98 | 0.90 | 206.19 | 180 | 82 | 2.83 | 72.83 |
| 3-I-1 | 3.25 | 0.86 | 0.99 | 89.59 | 166 | 68 | 1.76 | 50.99 |
| 3-III-2 | 4.56 | 0.95 | 1.13 | 195.84 | 179 | 72 | 1.90 | 103.00 |
| 3-II-1 | 4.80 | 1.00 | 1.10 | 217.46 | 177 | 88 | 2.05 | 105.88 |

LVIDd - diastolic left ventricular internal diameter, PWTd - diastolic posterior left ventricular wall thickness, IVSTd - diastolic ventricular septal thickness, LVM - leftventricular mass, BSA - body surface area, LVMI - left ventricular mass index.

Echocardiographic measurements for calculation of left ventricular mass in 11 patients with GSH (Chapter 7).

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