MOLECULAR GENETIC ANALYSIS OF HUMAN HYPERTENSION

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THESIS SUBMITTED FOR THE DEGREE OF DOCTORATE OF PHILOSOPHY
TO THE UNIVERSITY OF GLASGOW, FACULTY OF MEDICINE

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May 1993

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To my father Taqleed Ullah Khan Nasir

DECLARATION

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ACKNOWLEDGEMENTS

I would like to extend my gratitude and appreciation to Professor Connor, Dr A Cooke, Dr R Davidson and Dr R Arngrimmson, for their supervision throughout the project and for useful comments in the preparation of the manuscipt. I would also like to thank the British Heart Foundation, for the funding of the project, Mrs M Crouch and Mrs N Tariq for their help in establishing lymphoblastoid cell lines, and all the patients whom took part in the study. My appreciation must also extend to all the staff and students at the Duncan Guthrie Institute for their friendship and support, in particular Mrs E O'Hare, Caspar Robinson and Marie Boyd, and to Kenny McCluskey, Janice and Anne Gillespie for the much needed light relief during the preparation of the manuscipt. Last but not least I would like to express my warmest thanks to my family, for their love and support, without whom none of this would have been possible or worthwhile.

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ABBREVIATIONS

A	adenine
ANP	atrial natruieretic peptide
APS	ammonium persulphate
ATP	2'-adenosine 5'-triphosphate
bp	base pair
BP	blood presure
C	cytosine
CCM	chemical cleavage of mismatch
Ci	Curie(3.7×10^{10} Becquerel)
cpm	counts per minute
cm	cemtimeter
cDNA	complementary deoxyribonucleic acid
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddNTP	dideoxyribonucleoside triphosphate
DEPC	diethyl pyrocarbonate
DGGE	denaturing gradient gel electrophoresis
dGTP	2'deoxyguanosine 5'-triphosphate
dH_2O	distilled water
DR	dahl resistant
DS	dahl sensitive
DBP	diastolic blood pressure
DMSO	dimethly sulfoxide
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease

DTT	dithiothreitol
dTTP	2'deoxythymidine 5'-triphosphate
DZ	dizygotic twin
FCS	Foetal calf serum
FH	family history
G	guanine
g	gram
GRA	Glucocorticoid remiable adolsteronism
HLA	human leucocyte antigen
kb	kilobase
M	molarity
min	minute
MOPS	3'(N-morpholino)propanesulfonic acid
mg	milligram
µg	microgram
ml	millilitre
µl	microlitre
mm Hg	millimeters mercury
mM	millimolar
MRC	medical research council
mRNA	messenger ribonucleic acid
MHS	milan hypertensive stain
MNS	milan normotensive strain
MZ	monozygotic twin
OD	optical density
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism

RNA	ribonucleic acid
Rnase	ribonuclease
rpm	revolutions per minute
RSP	restriction site polymorphism
RT-PCR	reverse transcription polymerase chain reaction
SBP	systolic blood pressure
SDS	sodium dodecyl sulphate
SHR	spontaneously hypertensive rats
SHRSP	sroke prone spontaneously hypertensive rat
SLC	sodium lithium countertransport
SSCP	single strand conformational polymorphism
T	thymidine
TBE	tris borate-ethylenediaminetetra acetic acid
TE	tris- ethylenediaminetetra acetic
TEMED	N,N,N,N'-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
u.v	ultra violet
WKY	wistar kyoto rat

SUMMARY

SUMMARY

Hypertension is a major public health problem affecting approximately 15-20% of adult western populations, and is a leading cause of cardiovascular disease and death due to cerebral haemorrage, cardiac failure and kidney disease. Evidence from twin studies, population-based epidemiological studies and adoption studies, demonstrate hypertension is inherited as a multifactorial polygenic trait, where several different genes may be involved in the regulation of blood pressure and the development of high blood pressure. The specific genes involved however are not known, and several potential candidate genes have been identified based on animal and human studies.

In the present study the candidate gene approach was employed to identify genetic determinants of high blood pressure in four groups of offspring with contrasting predispositions to high blood pressure; Group A (high offspring blood pressure, low parental blood pressure), Group B (high offspring blood pressure and high parental blood pressure), Group C (low offspring blood pressure and low parental blood pressure) and Group D (low offspring blood pressure high parental blood pressure), in parents with high and low blood pressures and in affected and unaffected sibling pairs.

The *HindIII* RFLP of the renin gene was examined in 192 offspring, including 45 high/high and 48 low/low offspring. The allele frequencies determined in the high/high and low/low offspring were 0.65 for the 9.0kb allele and 0.35 for the 6.2kb allele, in close agreement with the allele frequencies of 0.67 and 0.33 respectively, in all offspring examined. Chi-square analysis showed no statistically significant difference in the genotype distribution between the high/high

and low/low offspring (X^2_2=5.77 p=0.01–0.05). Analysis of this RFLP in 44 parents with high blood pressure and 47 with low blood pressure showed no statistically significant difference in the genotype distribution between the two groups (X^2_2=0.53 p=0.50–0.80).

The *BanI* RFLP of the beta-2-adrenergic receptor gene was analysed in 182 offspring, including 43 high/high and 47 low/low offspring. The allele frequencies in the high/high offspring were 0.27 for the 3.7kb allele and 0.73 for the 3.4kb allele, and in the low/low offspring were 0.16 for the 3.7kb allele and 0.84 for the 3.4kb allele, similar to the frequencies of 0.25 and 0.75 respectively in all offspring studied. Chi-square analysis showed no statistically significant difference in the genotype distribution between the high/high and low/low offspring (X^2_2=4.43 p=0.10–0.20). Analysis of this RFLP in 49 parents with high blood pressure and 48 with low blood pressure showed no statistically significant difference in the genotype distribution between the two groups of parents (X^2_2=2.88 p=0.20–0.50).

The *BglI* RFLP of the beta-1-adrenergic receptor gene was examined in 173 offspring, including 42 high/high and 45 low/low offspring. The allele frequencies in low/low offspring were 0.78 for the 6.2kb allele and 0.22 for the 4.7kb allele, and in the high/high offspring 0.75 for the 6.2kb allele and 0.25 for the 4.7kb allele, in close agreement with the frequencies of 0.78 and 0.22 respectively, in all offspring examined. Chi-square analysis showed no statistically significant difference in the genotype distribution between the high/high and low/low offspring (X^2_2=0.215 p=0.80–0.90). Analysis of this RFLP in 44 parents with high blood pressure and 47 with low blood pressure showed no statistically significant difference in the genotype distribution

between the two groups (X^2_2=0.46 p=0.50–0.80).

The *DraI* RFLP of the alpha-2-adrenergic receptor gene was examined in 151 offspring including 39 high/high and 40 low/low offspring. The allele frequencies of the high/high offspring were 0.90 for the 6.7kb allele and 0.10 for the 6.3kb allele, and for the low/low offspring 0.80 for the 6.7kb allele and 0.20 for the 6.3kb allele, in close agreement to the frequencies of 0.86 and 0.14 respectively, in all offspring examined. Chi-square analysis showed no statistically significant difference in the genotype distribution between the high/high and low/low offspring (X^2_2=4.36 p=0.10–0.20). Analysis of this RFLP in 45 parents with high blood pressure and 45 with low blood pressure showed no statistically significant difference in the genotype distribution between the two groups (X^2_2=0.216 p=0.90).

The *SacI* RFLP of the insulin gene was examined in 174 offspring including 35 high/high and 50 low/low offspring. The allele frequencies in the high/high offspring were 0.41 for the 7.5kb allele and 0.59 for the 6.0kb allele and in the low/low offspring were 0.25 for the 7.5kb allele and 0.75 for the 6.0kb allele, similar to the frequencies of 0.37 and 0.63 respectively in all offspring examined. Chi-square analysis showed no statistically significant difference in the genotype distribution between the high/high and low/low offspring (X^2_2=1.76 p=0.20–0.50). Analysis of this RFLP in 28 parents with high blood pressure and 26 with low blood pressure showed no statistically significant difference in the genotype distribution between the two groups (X^2_2=2.94 p=0.20–0.50).

The *TthIII* RFLP of the glucocorticoid receptor gene was examined in 117 offspring including 30 high/high and 25 low/low offspring. The

overall allele frequencies were 0.21 for the 3.8kb allele and 0.79 for the 3.4kb allele. The allele frequencies in the high/high offspring were 0.18 for the 3.8kb allele and 0.82 for the 3.4kb allele and in the low/low offspring were 0.32 for the 3.8kb allele and 0.68 for the 3.4kb allele, similar to the frequencies of 0.21 and 0.79 respectively, in all offspring studied. Chi-square analysis showed no statistically significant difference in the genotype distribution between the two groups (X_2^2=2.18 p=0.20–0.50). Analysis of this RFLP in 27 parents with high blood pressure and 28 with low blood pressure showed no statistically significant difference in the genotype distribution between the two groups (X_2^2=0.58 p=0.99).

42 parents with high blood pressure and 34 with low blood pressure were genotyped for the *BclI* RFLP of the glucocorticoid receptor gene, and no statistically significant difference in the genotype distribution between the two groups was demonstrated (X_2^2=0.56 p=0.50–0.80). Similarly, 40 parents with high blood pressure and 33 with low blood pressure were genotyped for the *BclI* RFLP of the beta-fibrinogen gene, and again no significant difference in the genotype distribution between the two groups was demonstrated (X_2^2=2.61 p=0.20–0.50).

Haplotype analysis of 119 offspring for the two RFLPs of the glucocorticoid receptor gene the *TthIII* and the *BclI* polymorphisms, showed no statistically significant difference between the observed and the expected distribution (X_2^2=2.04 p=0.10–0.20). Linkage equilibria was demonstrated between the two restriction sites; the *TthIII* and the *BclI* sites of the glucocorticoid receptor gene, with an estimated linkage disequilibrium parameter (D) of 0.012.

Sib-pair analysis in 19 affected sib-pairs and 14 unaffected sib-pairs

showed a statistically significant lack of allele sharing in affected sib-pairs for the alleles of a *HindIII*/renin RFLP (X_2^2=9.48 p<0.01). Similarly analysis of 16 affected sib pairs and 14 unaffected sib-pairs showed a statistically significant lack of allele sharing in affected sib-pairs for the alleles of a *BclI* RFLP of the beta-fibrinogen gene (X_2^2=6.46 p<0.05).

Sib-pair analysis also identified increased allele sharing in 18 affected sib-pairs for the alleles of the *BanI* RFLP of the beta-2-adrenergic receptor gene (X_2^2=4.65 p=0.1-0.05), in 10 affected sibs-pairs for the alleles of the *BglI* RFLP of the beta-1-adrenergic receptor (X_2^2=5.79 p=0.1-0.05) and in 16 affected sib-pairs for the alleles of the *BclI* RFLP of the glucocorticoid receptor gene (X_2^2=3.21 p=0.20). However, these differences were not statistically significant.

Mutational analysis of the angiotensinogen gene, by hydrolink gel electrophoresis in twelve offspring with polarised predispositions to high blood pressure identified two molecular variants in exon II of the gene. Analysis of these variants in 35 high/high and 40 low/low offspring showed an higher frequency in the low/low offspring, however this was not statistically significant (X_2^2=0.68 p=0.20-0.50). No molecular variants were identified in the remainder of the gene. Analysis of a T174M molecular variant of the angiotensinogen gene in 37 high/high offspring and 38 low/low offspring, showed no association with a predisposition to high blood pressure (X_2^2 =1.39 p=0.20-0.50). Similarly, analysis of a M235T molecular variant in 38 high/high and 36 low/low offspring showed no association (X_2^2 =1.33 p=0.20-0.50). Mutational analysis of the glucocorticoid receptor cDNA identified a molecular variant with an equal frequency in the high/high and

low/low offspring.

Therefore from the present study, sib-pair analysis and direct mutational analysis of candidate genes, seems the most promising way forward in the identification of genetic factors of etiological significance in hypertension.

CHAPTER 1: INTRODUCTION

Chapter 1 INTRODUCTION

1.1 Definition and prevalance

Hypertension is a sustained elevation of arterial blood pressure. Blood pressure is a quantitative trait that shows a normal distribution in the general population (Cammussi 1988), and the upper limit of normal in adults is usually taken as a diastolic pressure of 90mmHg and a systolic pressure of 160mmHg (Soubrier et al., 1988). The consequence of a blood pressure level in a given individual depends not only on the actual level, but also on other risk factors.

Hypertension is thought to represent the top 15-20% of the age/sex blood pressure distribution in industrialised countries (Rapp 1983). In the majority of hypertensive patients (95%) no detectable abnormality is present on clinical examination and this is defined as essential or primary hypertension (Sleigh 1978). Occasionally a definite cause, for example kidney disease can be found to explain the hypertension. Such cases are referred to as secondary hypertension (Sleigh 1978).

1.2 Evidence for a genetic contribution to high blood pressure

Evidence for a genetic contribution in the aetiology of human hypertension stems from several types of studies; animal models of hypertension, correlation of blood pressures in related and unrelated individuals, blood pressure tracking studies and studies of offspring with differing family histories of high blood pressure.

1.2.1 Animal studies

A variety of genetically hypertensive rodent strains have been developed where the level of blood pressure is inherited in a predictable manner (Table 1). The Spontaneously Hypertensive Rat (SHR) and its close relative, the Stroke Prone Spontaneously Hypertensive Rat (SHRSP), have been used extensively as animal models of human essential hypertension, and the Wistar-Kyoto (WKY) rats as the normotensive control strain.

Parental SHR and WKY strains represent genetically uniform populations, therefore blood pressure differences are due to environmental factors. Cross breeding hypertensive and normotensive strains, results in SHR-WKY F1 genetically uniform hybrids since they are homozygous at every locus at which parents are identical, and heterozygous at every locus that parents are different. The variance of blood pressure due to environmental factors can then be estimated, since all populations are genetically uniform. However, blood pressure variation in the SHR-WKY F2 progeny, as a result of F1 X F1 mating, is a result of the segregation of those loci at which F1 animals are heterozygous. This allows an estimation of the genetic contribution in the aetiology of hypertension (Harrap 1986). These forms of analyses have provided important clues to the genes involved in hypertension in animal models, as well as identifying a genetic basis for the disease.

The SHR strain has been developed from the normal Wistar-Kyoto (WKY) stock by selective inbreeding for higher blood pressure. A rapid response to selection has been demonstrated, with almost 100% hypertension by generation three, indicating that only a few loci are involved (Okomato & Aoki 1963). Studies of crosses of rodent strains

selectively bred for hypertension and normotensive strains and subsequent backcrosses further support the idea that few loci appear to be involved in the development of SHR hypertension.

Studies between SHR and normotensive rats have shown the genetics of transmission of high blood pressure to follow an additive mode of inheritance with a single major gene effect (Tanese et al., 1970). Other studies have however, demonstrated dominance effects of alleles (Harrap 1986). Louis et al., (1969) were the first to conclude that spontaneous hypertension had polygenic control. Tanese et al., (1970) then demonstrated the involvement of 2-3 genes in SHR hypertension and suggested the possibility of a major gene effect. More recently, cross-breeding experiments in the SHR suggested that about 4 independant loci are involved and that 64.5% of the variance of blood pressure within a segregating population is genetically determined (Harrap 1986). Pravnec et al., (1989) developed a set of recombinant inbred (RI) strains produced by brother-sister inbreeding between members of F2 generation from crosses of normotensive and hypertensive (SHR) strains, for the evaluation of markers of spontaneous hypertension. Statistical analysis demonstrated the involvement of multiple genetic loci estimated to number more than seven, with the effect of three major genes and multiple minor genes.

In general the genetic contribution to high blood pressure in the SHR has been reported to be between 46-96% and the number of loci involved between one and six (Rapp 1983). Such studies provide evidence for the involvement of genetic factors in animal models of hypertension.

Table 1: Examples of rodent strains selectively bred for hypertension

Strain	Lines*	Year 1st report
New Zealand (Dunedin) Genetically hypertensive rats (GH)	H, C	Smirk 1958
USA (Brookhaven) Salt sensitive (S) and Salt resistant (R)	H L	Dahl 1962
Japan (Kyoto) Spontaneously hypertensive rats	H	Okomata 1963
Israel (Jerusalem) DOCA salt sensitive (SHB) and salt resistant (SNB)	H L	Ben-Ishay 1972
France (Lyon) Hypertensive (LH) and normotensive (NL) and Low blood pressure (LL)	H,C,L	Dupont 1973
Italy (Milan) Hypertensive strain (MHS) normotensive (MNS)	H,C	Bianchi 1976

* the types of lines developed in each model were:
H=lines selected for high blood pressure
L=Lines selected for high blood pressure
C=Contol line, unselected, random bred

After Rapp JP. Genetics of experimental Human Hypertension. In Hypertension Pathophysiology and treatment. Edited by Genest Kucknel, Harlet and Larkin. 1983 2nd Eds. McGraw Hill book company

1.2.2 Family studies

Blood pressure as a normal physiological trait correlates well in families (Brandao et al., 1992). Correlation coefficients observed between parent-offspring and sibling relationships are greater than between spouse pairs and adopted offspring and parents (Johnson et al., 1965, Hayes et al., 1965, Biron et al.,1976, Feinlab et al., 1977, Feinlab et al., 1979). Table 2 shows blood pressure correlations in different family members. The greater correlation observed between related individuals can be explained by gene sharing and provides evidence for the involvement of genetic determinants in blood pressure variability. However, for correlations to be entirely due to genetic factors then the expected correlations between unrelated family members should be zero. As can been seen from Table 2 such individuals show some degree of correlation, suggesting that shared environmental factors complement genetic factors in determining familial aggregation of blood pressure.

Monozygotic twins (MZ) represent the only model where genetically identical individuals can be studied. A comparison with dizygotic twins (DZ) where 50% of the genes are in common allows an estimation of the genetic variance. Blood pressure correlations are higher in monozygotic twins compared to dizygotic twins consistent with a significant genetic effect (Table 2). The Medical College of Virginia Twin Study, analysed 251 eleven year old twins and demonstrated higher blood pressure correlations in monozygotic twins compared to dizygotic twins, and the authors provided heritability estimates of approximately 66% for both SBP and DBP (Schieken et al., 1989).

Analysis of data from adoption studies (Annest et al., 1979, Mongeau et al., 1986) have repeatedly demonstrated stronger correlations between biological parent-offspring than non-biological parents and their adopted children. This again provides strong evidence of a genetic role in blood pressure aggregation. Similarly, correlations between natural siblings are greater than between adopted children. The Montreal Adoption Study (Mongeau et al., 1986) analysed blood pressure in 756 adopted, 445 natural offspring and 1176 parents. Correlation coefficients reported are summarised in Table 3. The estimated genetic contribution for systolic blood pressure correlations between parent-offspring and sib-sib relationships were 61%, and the shared environmental effect, 39%. For diastolic blood pressure correlations between parent-offspring, the heritability estimate was 58% and the environmental contribution 42%.

Regardless of the different heritability estimates in different family members, data from the majority of population based studies provides evidence for a genetic basis for interindividual variability of blood pressure and the contribution from genes is proposed to be approximately 25-50%, and 50-75% is influenced by environmental factors (Corvol et al., 1989).

Table 2: Familial blood pressure correlations

Relationship	Correlation SBP	DBP
Spouse pairs	0.08	0.06
Adoptee-parent	0.03	0.09
Offspring-parent	0.18	0.18
Sib pairs	0.18	0.14
Dizygotic twins	0.25	0.27
Monozygotic twins	0.55	0.50

Data taken from Williams et al., (1990)

Table 3: Blood pressure correlations in adopted/natural offspring and their parents

Relationship	Correlation SBP	DBP
Natural child/mother	0.27	0.26
Natural child/father	0.24	0.21
Adopted child/mother	0.08	0.10
Adopted child/father	0.09	0.13
Natural child/natural child	0.38	0.53
Natural child/adopted child	0.19	0.27
Adopted child/adopted child	0.16	0.29

Data taken from the Montreal Adoption study (Mongeau et al.,1986)

1.2.3 Blood Pressure Tracking Studies.

Childhood blood pressure levels are a strong predictor of future blood pressure levels (Lauer et al., 1991). Evidence for this comes from longitudual epidemiological studies demonstrating blood pressure ranking or tracking in several different populations in cohorts of children, adolescents and adults (Lauer et al., 1986, Shear et al., 1987). Tracking refers to the maintenance in persons of specific levels of blood pressure distribution relative to their peer group with age, such that individuals that occupy the upper part of their age/sex blood pressure distribution maintain their position or rank into adult life (Szklo 1979). Correlation coefficients or tracking coefficients from such studies are in the range of 0.01-0.07 for SBP and 0.02-0.06 for DBP from early to late childhood (Szklo 1979). In adult populations this correlation is increased to 0.5-0.6 (Hames et al., 1971, Perrera et al., 1972, Hennekens et al., 1979, Zinner et al., 1979).

Some authors have reported a "horse racing effect" ie. the higher the initial blood pressure level the faster the blood pressure rises (Wu et al., 1980). In adults, initial blood pressure levels are the most powerful predictors of future blood pressure levels (Higgins et al., 1980, Szklo 1986), and blood pressure tracking coefficients in adults are stronger than in children. Similarly correlations in older children are stronger than in younger children (Szklo 1979).

This tracking phenomena suggests a universal environmental influence is acting on blood pressure levels causing them to rise, however the blood pressure distribution at birth may be genetically determined.

Therefore it seems likely that predictors of blood pressure levels operate from an early age (Anonymous 1981). A recent study by De Swiet et al., (1992) examined blood pressure tracking in a group of children in the South East of England from birth to the age of ten. Correlation coefficients gradually became stronger with age; up to the age of one, a weak correlation was demonstrated (<0.2), after the age of one, correlations gained strength such that the tracking coefficient from the age of nine to ten was 0.58. This suggested that at the age of ten blood pressure tracking accounts for 35% of the observed blood pressure variability. The Shimane Heart Study (Nishio et al., 1989) also demonstrated the tracking phenomena during childhood and adolescence in Japanese children, and its increase with age. Tracking coefficients in girls from the age of 6-9 years for SBP were 0.27 and this increased to 0.36 from the age of 9-12 years.

The factors controlling childhood blood pressures are still unclear, however familial factors have repeatedly been demonstrated with significant correlations between parent and offspring blood pressure levels.

1.2.4 Blood pressures in offspring with differing family histories of high blood pressure

Blood pressure levels of offspring with a positive family history of hypertension generally have higher blood pressure levels than offspring with a negative family history (Lauer et al., 1989). Hofman et al., (1983) demonstrated a difference of 3mmHg in SBP levels in newborn infants of parents with high blood pressure compared to newborns of parents with low blood pressure. A difference of 7mmHg for both SBP and DBP in 20 year-old offspring has also been reported (Van

Hooft et al., 1988), suggesting the magnitude of familial aggregation increases during adolescence and childhood probably reflecting environmental factors. The Minneapolis Blood Pressure study (Munger et al., 1988) also examined blood pressure levels of offspring with and without a family history of high blood pressure over a period of eight years. Offspring with a positive family history consistently showed a higher mean systolic blood pressure than offspring with a negative family history.

The Ladywell Blood Pressure Study is a large scale study based on a population initially recruited for the MRC Mild Hypertension Trial (Watt 1986). The present study is based within this project aimed at identifying genetic factors in offspring with contrasting predispositions to high blood pressure, and has demonstrated elevated blood pressure levels in offspring of parents with high blood pressure compared to offspring of parents with low blood pressure (Watt et al., 1992). The method employed for the selection of offspring for the Ladywell Study is based on the four corner approach (Watt 1986). The four corner approach, for the selection of offspring with negative and positive family histories of high blood pressure was first demonstrated in the mid-sixties by Deutscher et al., (1966), where blood pressure data from a large community of almost 9000 subjects was analysed. The data shows that the proportion of offspring in the upper 20% of their age/sex blood pressure distribution, is greatest when both parents lie in the upper 20% of their age/sex blood pressure distribution. Similarly, the proportion of offspring in the lower 20% is greatest when both parents lie in the lower 20% of their age/sex blood pressure distribution. A follow up study indicates this relationship

is maintained over time (Higgins et al., 1980).

Using this approach, four groups of offspring can be identified;

1) offspring with high personal and high parental blood pressure

2) offspring with low personal and low parental blood pressure

3) offspring with high personal and low parental blood pressure

4) offspring with low personal and high parental blood pressure.

Analysis of the blood presure data by Watt et al., (1992), has shown, that offspring whose parental blood pressure was high, generally had higher blood pressure levels compared to offspring whose parental blood pressure was low, with an average blood pressure difference of approximately 23mmHg for SBP and 19mmHg for DBP.

Thus it is evident that blood pressure tracking starts at an early age and is maintained into adult life. The observed familial aggregation of blood pressure levels, and the fact that offspring with a family history of high blood pressure generally have higher blood pressure levels compared to offspring with no family history, all provide evidence for a genetic contribution in the aetiology of hypertension. These observations have led to the hypothesis that a high-normal blood pressure level in childhood may be the antecedant of adult hypertension (Schieken et al., 1989).

1.3 The mode of inheritance

The number of genetic loci involved in the regulation of blood pressure is not yet fully understood (Corvol et al., 1989). At present the concept of the genetic control of high blood pressure is, that hypertension is likely a multifactorial disease that shows polygenic

inheritance and the interaction of several genetic loci (perhaps 4-6 loci) (Rapp 1983) with multiple environmental factors determine an individuals blood pressure level. However, it is not impossible that there are some genes with larger effects on the blood pressure distribution, thus the hypertensive population consists of a heterogeneous mixture of people with different genetic and environmental contributions (Williams et al., 1988, Williams et al., 1989). This hypothesis of a limited number of genes involved in the aetiology of hypertension lies mid-way between the two extremes of the oligogenic and polygenic models (Corvol et al., 1989).

1.4 Clues to the nature of the genetic contribution in the development of hypertension

Clues to the genes that might contribute to the determination of blood pressure levels have been deduced from analysis of biochemical phenotypes or, increasingly from molecular studies of candidate genes in animal models of hypertension, human pedigree studies or cross-sectional comparisons of hypertensives and normotensive controls.

1.4.1 Animal studies

Animal inbreeding results in homozygosity or fixation of previously heterozygous loci as a result of selection. Different animal strains will therefore fix contrasting alleles, such that genetic polymorphisms between strains of hypertensive and normotensive rats will exist. These strain specific genetic differences can be demonstrated to be responsible for the observed blood pressure differences between strains if they remain associated with blood pressure differences in segregating populations from crosses of hypertensive and normotensives

animal strains (Rapp et al., 1989). A series of criteria have been proposed by Rapp et al., (1989) to identify biochemical/physiological traits as primary genetic causes:

1) A blood pressure difference between the two parental strains must exist

2) The trait must follow Mendelian inheritance

3) Blood pressure in F2 hybrids must correlate with the trait

If cosegregation of phenotype and blood pressure is observed, then this establishes the expression of the gene in the aetiology of hypertension.

Several RFLP (Restiction Fragment Length Polymorphism) studies preformed in animal models of hypertension suggest that RFLPs may be useful markers for certain forms of hypertension. Genetic studies of hypertension have shown that the renin gene is associated with the development of hypertension in Dahl rats. Rapp et al., (1989) demonstrated that an RFLP in the first intron of the renin gene between Dahl salt sensitive (DS) and the Dahl salt resistant (DR) animal models cosegregates with a blood pressure increment, providing strong evidence that the renin gene or a gene closely linked to the renin gene is involved in blood pressure regulation.

Some evidence exists to suggest sex linked loci may be involved in the genetics of blood pressure. A study by Ely et al., (1990), compared blood pressure levels of F1 male and females from crosses of WKY mothers and SHR fathers compared to F1 progeny from WKY fathers and SHR mothers. Blood pressure levels F1 males from the first cross were significantly higher than blood pressure levels from the reciprocal cross. These results suggest a Y linked locus is involved in determin-

ing blood pressure levels in the SHR. A study by Hilbert et al., (1991) localised a gene on chromosome X, denoted BP/SP 2 that contributed significantly to blood pressure variation in the F2 population (SHRSP X WKY), providing evidence of the involvement of the X chromosome in the gender linkage of elevated blood pressure.

A number of studies in animal models of hypertension have provided strong evidence of the role of angiotensinogen converting enzyme (ACE) in the development of hypertension. Hilbert et al., (1991) developed a large collection of polymorphic DNA markers covering a large region of the rat genome. Linkage analysis in SHRSP, using a series of minisatellite and macrosatellite markers, identified two genes regulating blood pressure; BP/SP-1 maps to chromosome 10 and a BP/SP-2 gene mapping to the X chromosome in rats. Supporting these observations Jacob et al., (1991) have also identified markers linked to genes regulating blood pressure on chromosome 10 in rats (BP1). The BP/SP-1 loci is of particular interest since it maps close to the rat ACE gene which is involved in blood pressure regulation via the renin-angiotensin system. The results by Hilbert and Jacobs have been confirmed by Nara et al., (1991) and more recently by Deng et al. (1992) who demonstrated cosegregation of blood pressure in F2 rats from crosses between normotensive and Dahl Salt sensitive (DS) rats with alleles at the rat ACE locus. These studies are the first to carry out linkage analysis using a large fraction of the genome (approximately 70%), and thus have the potential to identify genes not suspected of being involved in the genetic control of blood pressure. In addition Jacobs et al., (1991) also provided preliminary evidence that a locus on chromosome 18 (BP2) influences blood pressure. Deng et al., (1992)

also demonstrated cosegregation of the atrial natriuretic peptide receptor with blood pressure in F2 rats derived WKY and DS rats. Abnormal thermoregulation occurs in hypertensive mice. Hamet et al., (1991) have demonstrated a polymorphism of the heat stress protein (hsp) gene, the hsp 70 gene, a member of the hsp family is associated with an 15mmHg increment in blood pressure in recombinant inbred rat strains.

The Sa gene has also been implicated in the development of hypertension. The exact function of the Sa gene protein remains to be determined, but it codes for a protein with a carboxyl terminal region closely resembling that of luciferase. The F2 offspring, obtained by crossbreeding SHR and WKY, showed a more frequent genotype of the SHR for the Sa gene in rats with high blood pressure compared to rats with low blood pressure (Iwai et al., 1991). This suggests a possible role of the Sa gene in hypertension in the SHR.

Additional RFLPs for some genes other than those described above have been detected between the genomes of hypertensive rats. These observations include RFLPs for the gene encoding adducin (Bianchi et al., 1990) and the phospholipase C gene (Yagisawa et al., 1991).

In addition to the observed genetic differences, several biochemical differences have also been reported in animal models of hypertension. Studies have shown Dahl salt sensitive (DS) rats to have lower plasma renin activity compared to Dahl salt resistant (DR) rats (Rapp 1980). Differences have also been reported for the P450 11-beta/18 adrenal steroid hydroxylating enzyme and abnormal sodium/potassium fluxes (De Mendonca et al., 1980).

Over recent years, transgenic animals have been used to study genes that are involved in blood pressure regulation. Creation of transgenic animals involves the introduction of foreign genes into the reproductive cell line of mammals primarily mice (Jaenish 1988). This may then allow for the phenotypic effects of potentially interesting genetic alterations to be studied in whole animals and provides a useful tool for studying molecular and cellular physiology in whole animals.

Mullins et al., (1990) introduced the mouse Ren-2 gene into fertilised rat embryos, such transgenic rats and their offspring were then shown to develop significant increases in arterial blood pressures, and raised levels of plasma aldosterone. A similar study by Steinhelper et al., (1990) demonstrated a reduction in blood pressure levels in transgenic mice carrying the atrial natriuretic factor gene and increased atrial natriuretic factor (ANF) production, suggesting that high levels of ANF peptide are capable of reducing blood pressure levels. Ohkubo et al., (1990) generated transgenic mice with elevated blood pressure levels by the introduction of the rat renin and angiotensinogen genes, suggesting the combined action of these two genes is responsible for an increase in blood pressure in transgenic mice. Such transgenic studies provide important clues to the genes responsible for animal hypertension.

Hence animal studies provide evidence that mutations at several loci may be responsible for high blood pressure, and allows the inference that such loci may be candidate genes that can be studied in human populations.

1.4.2 Human Studies

In human studies, several difficulties have been encountered in attempts to identify genes involved in the pathogenesis of high blood pressure. Such problems include phenotype definition, ascertainment of informative families and the heterogeneity of the disease (Hilbert et al., 1991). Thus human studies have generally provided contradictory evidence for the involvement of several loci in the genetic control of blood pressure.

1.4.2.1 Biochemical studies

In hypertensive subjects plasma renin levels have been shown to have an overall distribution that differs from normal (Morris & Griffiths, 1988). Offspring from hypertensive families have shown significantly lower plasma renin levels than offspring from normotensive parents (Van Hooft et al., 1989). Similarly, plasma angiotensinogen levels correlate well with blood pressure levels in humans, and higher plasma concentrations have been observed in hypertensives compared to normotensives (Walker et al., 1979). Biochemical findings of offspring of the Ladywell Study (Watt et al., personal communication 1990) show offspring of hypertensive parents to have raised levels of angiotensinogen compared to offspring of parents with low blood pressure. Raised levels of cortisol and 18-OH corticosterone have also been demonstrated to be associated with high blood pressure in the Ladywell offspring (Watt et al., personal communication 1990).

Levels of the atrial natriuretic factor (ANF) hormone have been reported to be significantly elevated in patients with essential hypertension (Sagnella et al., 1985). However Nillson et al., (1987) found

no significant difference in ANf levels between hypertensives and normotensives. The Dutch Hypertension study was also unable to demonstrate significant difference in offspring with differing probabilities of developing high blood pressure (Van Hooft et al., 1989).

Several biochemical traits thought to be involved in sodium metabolism have been studied in populations of hypertensives and normotensives. The rate of sodium-lithium countertransport (SLC) in human populations has been reported to be higher on average in hypertensive populations (Williams et al., 1983, Williams et al., 1987). Sodium potassium cotransport has also been reported to be higher in patients with essential hypertension than normotensive controls (Cusi et al., 1990). An altered distribution of sodium and calcium ions have been demonstrated in vascular smooth muscles in hypertensive patients (Nojma et al., 1989).

Kallikrein is a multigene family of highly homologous enzymes including a specific enzyme that hydrolyses kininogen to produce the vasoactive peptide kinin. Studies have shown that urinary kallikrein excretion is lower in hypertensives than normotensives (Zinner et al., 1976, Berry et al., 1989).

The sympathetic nervous system has also been widely studied in human populations and shows increased nervous activity in patients with genetic hypertension. Studies examining the role of the sympathetic nervous system in hypertension have studied levels of circulating catecholamines, raised levels of norepinephrine have been observed in hypertensive patients (Goldstein 1983), and total urinary catecholamine excretion is significantly higher in groups of offspring with a family history of hypertension (Ferrera et al., 1988). Higher densi-

ties of alpha-2-adrenergic receptors have been demonstrated in hypertensive offspring with a positive family history compared to hypertensives without a family history (Fritschka et al., 1987). The Dutch hypertension study showed approximately a 20% increase in alpha-2-adrenergic receptor density in offspring of hypertensive parents compared to offspring of normotensive parents. (Van Hooft et al., Personal communication). Increased density of beta-2-adrenergic receptors in lymphocytes have also been reported in patients with essential hypertension (Brodde et al., 1987). Michel et al., (1988) have demonstrated increased platelet alpha-2-adrenergic receptor densities in normotensive children with a genetic predisposition to high blood pressure, providing evidence that such changes are genetically determined.

A highly significant relationship has been demonstrated between blood pressure and insulin concentration (Rocchini 1991), such that prevalence of insulin resistance is greater with higher blood pressure levels (Julius et al., 1991), providing evidence for the insulin gene as a candidate gene for hypertnesion.

1.4.2.2 Genetic Studies

In light of the biochemical differences observed between hypertensives and normotensives a number of genetic studies have been performed in human populations. RFLP studies have been performed with the renin gene in human populations of normotensive and hypertensives to establish an association between hypertension and renin (Morris & Griffiths 1988, Naftilan et al., 1989). However no association between renin RFLPs and hypertension has been demonstrated.

Several intermediate phenotypes of hypertension have been identified which show major gene effects. Plasma ACE is one such intermediate phenotype and 50% of the interindividual variability of plasma ACE is determined by a major gene effect (Cambien et al., 1988).

Elevated sodium lithium conutertransport (SLC) is also a well studied hypertension intermediate phenotype demonstrating a major gene effect and 34% of the the total variation is due to the segregation of a recessive gene (Hasstedt et al., 1988). Normotensive individuals with recessive high SLC have been shown to develop hypertension four times more often than those with the dominant low SLC genotype (Hunt et al., 1991). It has been suggested that a mutation in the sodium-proton antiporter gene might be responsible for the elevated SLC and a contributor to hypertension.

A major gene effect has also been demonstrated for the kallikrein gene, and 51% of the phenotypic variation has been attributed to a dominant allele for total urinary kallikrein excretion and 27% due to polygenes and shared environment (Berry et al., 1989).

RFLP analysis of the ANF gene has been performed in two different ethnic populations (Barley et al., 1991). However no allelic association with blood pressure has been demonstrated.

Genetic studies performed in offspring with polarised predispositions to high blood pressure (Ladywell study), have shown an increased frequency of the larger allele of the *BclI* RFLP of the glucocorticoid receptor gene in offspring with the high blood pressure and high parental blood pressure compared to offspring with low personal and low parental blood pressure, suggesting that homozygosity of the larger allele is associated with hypertension.

Genetic studies have also been performed on the sodium-lithium countertransport gene and hypertension. However the two studies to date have reported no evidence of genetic linkage between the sodium-proton antiporter gene and hypertension (Dudley et al., 1990, Lifton et al., 1991).

Blood groups have been suggested to play a role in the development of hypertension. The human leucocyte antigens (HLA) and the alleles of the MNS blood group have both been the subject of investigation. HLA haplotype analysis has been performed in sib pairs and show significant distortion of the normal segregation patterns of inheritance of HLA haplotypes, suggesting a loci closely positioned or within the HLA complex is involved in the determination of blood pressure levels (Gerbase-DeLima et al., 1989). A difference in the distribution of MN alleles in hypertensives subjects compared to normotensive subjects has been observed (Heise et al., 1987) such that hypertensives have an excess of the N phenotype and a deficiency of the M phenotype. There is also evidence for linkage between the MN locus and the sodium lithium countertransport phenotype (Weder et al., 1991).

RFLP analysis of patients with hypertension and normotensive controls of the insulin gene have failed to demonstrate an association between the insulin gene and hypertension. However, a statistically significant association of a polymorphism of the insulin receptor gene with high blood pressure has been reported (Ying et al., 1991).

Other human genetic studies have suggested a connection between genetic markers and hypertension haptoglobin protein polymorphisms (Berettai-Picolli et al., 1986).

In addition, to the observed biochemical and genetic differences, a number of Mendelian hypertensive syndromes have been identified, that may provide clues to the genes involved in hypertension. Glucocorticoid Remediable Aldosteronism (GRA), is one such syndrome, where hypertension follows an autosomal mode of inheritance. A chimaeric gene duplication as a result of unequal crossing over between the 5' region of the 11B-hydroxylase gene and the distal sequences of the aldosterone gene is responsible for the disease (Lifton et al., 1992). These genes therefore represent potential candidate genes for hypertension.

Multiple candidate genes have therefore been identified from both human and animal studies, however the results from such studies generally provided conflicting evidence. This may be a result of the different genes involved in both animal and human hypertension.

1.5 Aims of the present study

a) To perform association analysis using restriction fragment length polymorphisms in candidate genes in groups of offspring with contrasting predispositions to high blood pressure on the basis of personal and parental blood pressures, and in their parents

b) To perform sib pair analysis using restriction fragment length polymorphisms of candidate genes in affected and unaffected sibling pairs with contrasting predispositions to high blood pressure

c) To seek new restriction fragment length polymorphisms in candidate genes of interest on the basis of a) and b)

d) To perform mutational analysis in candidate genes of interest on the basis of a) and b)

CHAPTER 2: PATIENTS AND METHODS

Chapter 2 PATIENTS AND METHODS

2.1 PATIENTS

2.1.1 Recruitment of subjects

In 1979, 6088 adults aged 35-64 years representing 76% of all individuals in this age group were selected from 24000 patients registered at the Ladywell Medical Centre, Edinburgh and screened to identify subjects for the Medical Research Council (MRC) Mild Hypertension Trial. All participants were identified via systematic selection from age/sex registers and patients on treatment for high blood pressure were included. 1809 husband/wife pairs were screened, with each person being given an age/sex adjusted blood pressure score. Parental blood pressure (Husband/wife) scores were combined to identify High/High and Low/Low families. Blood pressure was measured twice using a random zero sphygmomanometer and a standard adult cuff size. Diastolic blood pressure was recorded to the nearest 2mmHg after sitting for 10 minutes.

2.1.2 Phase 1

In 1986 the practice registers were reviewed. Of the original 1809 couples, 1473 were still resident in the Ladywell catchment area. Letters were sent to each couple asking for dates of births and current addresses of offspring. 2879 offspring were identified, of which 1169 were natural offspring to both parents and were aged between 16-24 years on 31-5-1986. Offspring were invited to the medical centre where their blood pressure, pulse, height and weight were recorded. They were asked to complete a questionnaire requesting information on age, sex, marital status, educational attainment, place of residence,

employment status, personal medical history, physical activity, cigarette smoking, health knowledge and alcohol consumption. 864 individuals took part. To enable a comparison to be made between blood pressures of parents and offspring a Z score was calculated by dividing an individuals blood pressure deviation from the mean of their age/sex blood pressure group by the standard deviation of blood pressure within the group. Individual scores and age/sex distribution were based on the arithmetic mean of all blood pressure data with a two year age groups for the offspring and a five year age groups for parents. The scores for women were based on roughly equal numbers of those taking or not taking the oral contraceptive pill. Parents on treatment for hypertension were given Z scores corresponding to the mean score of the top 5% of the distribution of scores in their age/sex group. Four groups of offspring were identified; Group A (122) Group B (118), Group C (122) and Group D (68) as shown in Figure 1.

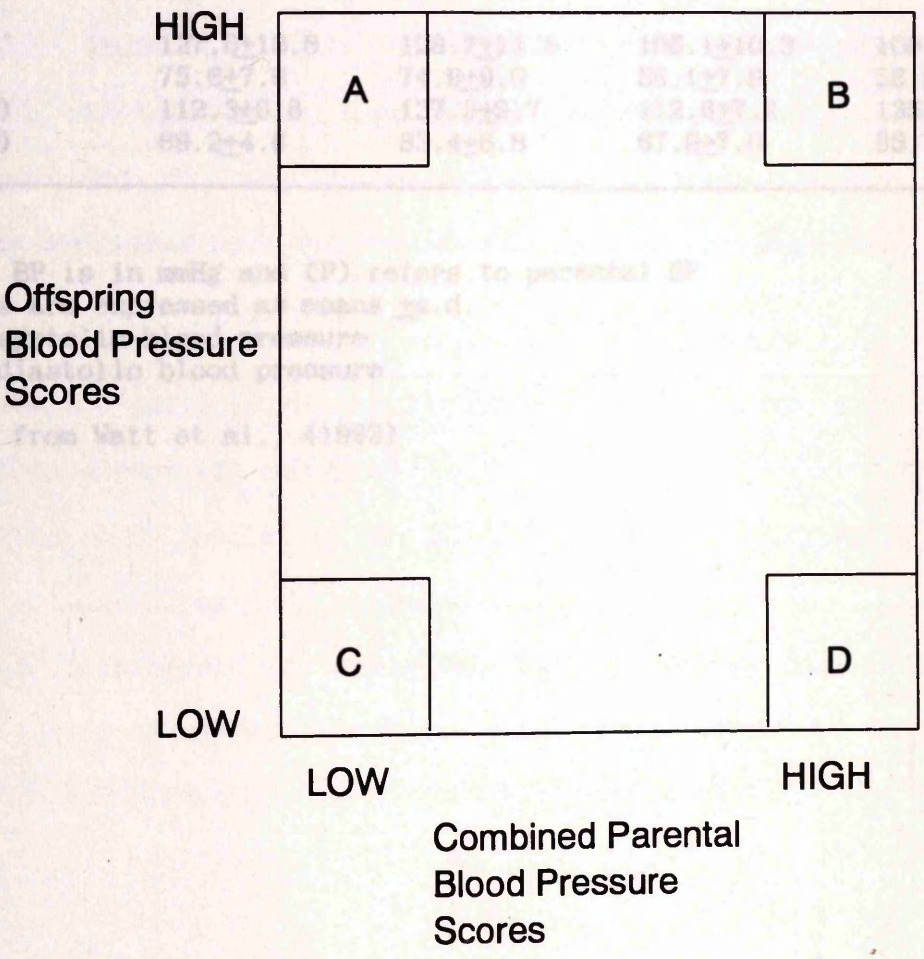
2.1.3 Phase II

Random samples were taken from the three largest groups (A, B and C) in order to identify four groups of approximately 70 individuals. Statistical analysis allowed 238 offspring to be approached at the final stage, of which 201 participated. Height, weight and blood pressure were recorded, a non fasting blood sample was taken and a 24 hour urine sample collected. The blood pressure characteristics of Phase II offspring are presented in Table 4.

2.1.4 Phase III

100 of the 200 offspring took part in detailed biochemical and physiological studies as inpatients at the MRC Blood Pressure unit, Glasgow..LS1

Figure 1: Diagrammatical representation of the four corner approach for the selection of offspring with contrasting predispostions to high blood pressure



Group	Personal BP	Parental BP
A	high	low
B	high	high
C	low	low
D	low	high

Table 4: Blood pressure characteristics of Phase II offspring

Group	A	B	C	D
n	50	51	48	52
SBP	127.6±10.8	128.7±11.8	105.1±10.3	109.7±9.4
DBP	75.6±7.8	74.9±9.0	56.1±7.9	56.9±8.2
SBP(P)	112.3±6.8	137.3±9.7	112.6±7.1	135.7±11.8
DBP(P)	69.2±4.6	83.4±6.8	67.9±7.0	83.1±6.9

where BP is in mmHg and (P) refers to parental BP
values are expressed as means ±s.d.
SBP, systolic blood pressure
DBP, diastolic blood pressure

Taken from Watt et al., (1992)

2.1.5 Phase IV

Parents of all phase II participants were invited to return to the Ladywell clinic for height, weight, blood pressure and pulse rate measurements and to donate a blood sample.

2.1.6 Phase V

Phase II siblings who had been characterised in phase I but whom were not included in Phase II were approached for a blood sample to increase the number of sibling pairs for stastistical analysis.

DNA extractions were carried out on all Phase II, Phase III and Phase V participants and lymphoblastoid cell lines (LCL) established as a constant source of DNA.

The final study population consists of 4 groups of individuals;

Group A (high offspring, low parent blood pressure)= 50

Group B (high offspring, high parent blood pressure)= 51

Group C (low offspring, low parent blood pressure)= 49

Group D (low offspring, high parent blood pressure)= 52

2.2 METHODS

2.2.1 Materials

A list of materials and solutions are presented in Appendix I. All chemicals used were Analar Grade and purchased from BDH limited or Sigma. Restriction enzymes were purchased from Gibco BRL, New England Biolabs, NBL Limited and Boehringer Mannheim. The source of the remaining materials can be found in the text.

2.2.2 Plasmid techniques

DNA Probes were supplied either as glycerol stocks or as plasmid DNA samples and were grown up within the Duncan Guthrie Institute and stored as glycerol stocks. The features of the probes used in this study are presented in Table 5.

2.2.2.1 Preparation of Competent Cells

5mls of prewarmed L broth (Luria broth) was innoculated with 10µl of glycerol stock and incubated overnight at 37°C in an orbital shaker. 100ml of fresh L broth was inoculated with 1ml of the overnight culture and incubated at 37°C until the absorbance of the culture was 0.6 ($6x10^8$ cells/ml). The culture was placed on ice and transferred to a chilled 30ml tube and spun at 4K for 5 minutes at 0°C The resulting pellet was resuspended in 12.5ml of ice cold 10mM magnesium sulphate and left on ice for 30 minutes. Cells were pelleted again and resuspended in 2ml of ice cold 50mM calcium chloride.

Table 5: Features of the DNA probes

Gene Probes	Vector/insert size/ release enzyme	Source
Alpha-2-adrenergic receptor	puC18/5.5kb/*BamHI*	ATTC
Beta-1-adrenergic Receptor	?/2.4kb/*EcorI*	ATTC
Beta-2-adrenergic Receptor	PMSG/2.6kb/*SmaI*	ATTC
Beta-fibrinogen	pFB5/1.4kb/*PstI*	Humphries
Glucocorticoid receptor	?/4.4kb/*BamHI*	Evans
	pMEG12/2.8kb/*NotI*	Detera-Wadleigh
Insulin	pBR327/0.88kb/*BamHI*	ATTC
Renin	PuC12/0.8kb/*EcorI* *HindIII*	Frossard

Humphries: Charing Cross Sunley Research Centre, Hammersmith, London, UK.
Evans: Howard Hughes Medical Institute, Gene Expression Laboratory Salt Lake Institute, La Jolla, CA, USA.
Detera-Wadleigh: National Institute of Health, Bethesda, Maryland USA.
Frossard:Calafornia Biotechnology Inc., Mountain View, Ca, USA.

2.2.2.2 Transformations

100µl of ice cold STET buffer was placed in a bijoux on ice. To this 10µg of plasmid DNA was added and thoroughly mixed. 200µl of competent cells were added and the bijoux placed on ice for 25 minutes. The cells were then subject to heat shock at 4°C for 2 minutes, followed by a 10 minute incubation at RT. 700µl of prewarmed L broth was added and the cells incubated in an orbital shaker for 1 hour. 200µl of the mixture was plated out onto X-Gal/ampicillin plates and left in a 37°C incubator overnight.

X-Gal/ampicillin plates were made as follows; 1.5% L-agar with ampicillin added to a final concentration of 100µl/ml. When the plates were set, 40µl of X-Gal was spread over the surface. All probes used were ampicillin resistant.

2.2.2.3 Preparation of glycerol stocks

The agar plates were inspected and a single colony was removed and grown overnight in 15ml of L broth containing ampicillin at 37 °C. After incubation the cultures were made 15% glycerol, and stored at -20°C.

2.2.2.4 Small scale plasmid preparation

15ml of L broth media containing 0.01mg/ml Ampicillin was innoculated with 5µl of glycerol stock and incubated overnight at 37°C with vigorous shaking. After incubation cultures were spun for 10 minutes at 1.5K at 4°C in an IEC DPR-6000 centifuge, and the supernatant discarded. The resulting pellet was resuspended in 400µl of ice cold STET buffer and transferred to a sarstedt tube. 25µl of fresh cold lysosyme

(10mg/ml) was added and the tubes left on ice for 5 minutes, followed by a 1 minute incubation at 100°C. The tubes were spun in a Scotlab Microcentaur centrifuge for 15 minutes and the supernatant transferred to a fresh tube. One tenth the volume of 3M sodium acetate was added together with an equal volume of phenol, and one sixth the volume of isopropanol. Tubes were placed at -20°C for 30 minutes, and spun again for 10 minutes. The supernatant was discarded and the pellet washed dry with 1ml 95% ethanol. The pellet was resuspended in 80µl of TE buffer.

2.2.2.5 CIRCLEPREP plasmid preparation

CIRCLEPREP (B10 101) kits were also used for the rapid isolation of plasmid DNA following the manufactures recommendations. 5ml cultures were grown overnight in Circlegrow medium provided in the kit and the cells pelleted in a centrifuge at 1.5K for 10 minutes at 4°C. The cells were then resuspended in 200µl of Pre-lysis buffer and lysed by the addition of 200µl of Alkaline lysis buffer. 200µl of neutralizing solution was added and the tubes mixed. After a 2 minute centrifugation the supernatent was transferred to a fresh tube by pipetting through a sieve, 400µl of isopropanol was added and the tubes centrifuged again. The resulting pellet was resuspended in 75µl of pre-lysis buffer. The tubes were then placed in a boiling water bath to denature linear cellular DNA and subsequently cooled on ice. 300µl of Lithium Chloride was added and left to incubate for 2 minutes to pellet rRNA and ssDNA precipitates. The supernatent was transferred to a fresh tube, 600µl of isopropanol added and centrifuged for 2 minutes, the pellet was dissolved in 0.5 ml of SDW. Plasmid DNA was then purified by the addition of 75µl of circleprep Glassmilk and a 5

minute incubation at RT. Tubes were spun for 10 seconds and the pellet was washed with binding buffer and then in wash solution, the pellet was then allowed to air dry. The plasmid DNA was resuspended in 10µl of TE buffer.

2.2.2.6 Releasing insert from plasmid

Plasmid concentrations were determined by measuring the optical density (OD) of each sample at 260nm in a dual beam spectrophotometer with a Deuterium lamp (LKB Biochem Ultrospec 4050). An OD reading of 1 corresponds to approximately 50µg/ml of DNA. 10ug of plasmid DNA was measured into a 0.5ml eppendorf tube, to which 8µl of enzyme buffer,4µl of 0.1M spermidine and 4µl of the appropriate enzyme was added (Table 5 lists release enzymes for various probes).

The plasmid digests were incubated overnight at the manufactures recommended temperature for each enzyme. The released insert was resolved on a 1.2% low melting point Seakem agarose gel (FCM bioproducts) along with 20µl of 1 kilobase marker (Boehringer Mannheim) at 120mA for 4 hours. The gel was visualised on a UV transilluminator (316nm), and the required band (released insert) was excised using a sterile scalpel blade and placed in a sarstedt tube. The weight of the insert was determined and a volume of SDW three times the weight of the insert was added. The probe was boiled for 10 minutes and stored at -20°C.

2.2.3 Preparation of cell cultures

Cell cultures were set up from previously established Lymphoblastoid cell lines (LCL) (Crouch M).

Since the culture media contains Epstein Barr Virus (EBV), and the

risk of HIV/Hepatitis infection, cultures were prepared in a specially designated Class II fume hood with only plastic ware. Great care was taken in the handling of these samples and all necessary safety precautions were adhered too.

2.2.3.1 Feeding of cell cultures

Vials were removed from liquid nitrogen and rapidly thawed at 37°C. The cells were washed by transferring the contents of the vials to a fresh Sterilin universal and adding 25ml RPMI (10%FCS), universals were spun at 1400 rpm for 10 minutes. The supernatant was discarded and the cells resuspended in 3ml of fresh media and transferred to a 50ml plastic culture bottle. The bottles were then left in a dry 37°C incubator overnight. The growth of the cells was assessed by the changing colour of the media. If the media changed colour, the cells were fed with 10ml RPMI (10%FCS) and left to grow for 3-4 days until the media colour changed again. When a volume of 30ml in the culture bottles was reached, the cells were resuspended and transferred to a 260ml culture bottle. Cells were fed every 3-4 days until 100ml of media was present.

2.2.3.2 Harvesting lymphoblastoid cells

When cell growth was sufficient, 30ml of media was gently removed from the bottles taking great care not to disturb the cells and discarded. The cells were suspended by gentle shaking and 40ml of the culture transferred to a sterile Falcon tube. Falcon tubes were spun at 1.5K for 10 minutes at 4°C in a IEC DPR-6000 centrifuge and the supernatent discarded. Cells were then prepared for DNA/RNA extraction. In order to maximise the quantity of DNA/RNA obtained the remaining cells in

the 260ml bottles were repeatedly fed ever 3-4 days with 20ml RPMI (10%FCS) until a volume of between 80-100ml of media was present, allowing further DNA/RNA to be obtained.

2.2.4 Genomic DNA techniques

2.2.4.1 Genomic DNA extraction

Genomic DNA was extracted from peripheral blood samples (fresh and frozen) using a variation of the method described by Kunkel et al. (1977).

10ml of whole blood was placed in a Falcon tube and 40ml of lysis mix added. Tubes were left on ice for 10 minutes and then centrifuged at 2.8K for 10 minutes at 4°C in a IEC DPR-6000 centrifuge. The resulting pellet was resuspended in 3ml of nuclei lysis mix, 200µl 10% SDS and 100µl proteinase K (10mg/ml). Tubes were incubated overnight at 37°C. After incubation 1ml of 6M sodium chloride was added with vigourous shaking,and tubes spun at 2.8K for a further 10 minutes at 4°C. The supernatent was then transferred to a fresh Falcon tube and 3ml of phenol/chloroform was added, and spun at 2.4K for 15 minutes. The upper aqueous phase was transferred to a fresh tube and DNA precipitated out by the addition of two volumes of ethanol. DNA was spooled out, washed in ethanol, allowed to air dry and resuspended in 0.5ml of TE buffer. DNA samples were stored at 4°C.

For a large number of subjects, DNA stocks obtained from the blood samples became exhausted, thus it was necessary to extract DNA from lymphoblastoid cell lines. The method used was the same as for extraction from blood samples, however the initial lysis step was omitted since the starting material was solely nucleated cells.

2.2.4.2 Optical densities of DNA

The concentration of each DNA sample was determined by measuring the optical density at 260nm in a dual beam spectrophotometer with a deuterium lamp. An OD reading of 1 corresponds to approximately 50µg/ml of DNA.

2.2.4.3 Digestion of genomic DNA

To a 10µg aliquot of DNA, 3µl of a restriction enzyme, 4µl of appropriate buffer and 2µl of 0.1M spermidine was added. Each digest was made up to a final volume of 40µl using SDW. Digests were incubated overnight at the suppliers recommended temperature for optimal enzyme activity.

2.2.4.4 Agarose Gel Electrophoresis

Digests were resolved on 0.8% EB Seakem agarose gels (FMC Bioproducts) containing 0.05% ethidium bromide (10mg/ml). Following addition of 4µl of loading mix, digests were loaded onto gels and run at 200mA for approximately 7 hours. The gels were visualised on a UV transilluminator (316nm) and photographed with a Polaroid type 667 film in a Polaroid instant print camera fitted with a Kodak 22A Wratten filter.

2.2.4.5 Southern Blotting

Southern blotting was carried out to transfer the digested DNA from the agarose gel to a Hybond N membrane (Amersham).

Gels were treated for 15 minutes with 0.25M hydrochloric acid with gentle agitation followed by a 30 minute wash in denaturation solution and a 30 minute wash in neutralisation solution. The gels were blotted

for a minimum of 16 hours according to the standard Southern Blotting procedure (Southern 1975). A tank was filled with 10xSSC. Gels were placed on a glass plate covered with 2 sheets of Whatman 3MM paper soaked in 10xSSC. Both ends of the 3MM paper were sitting in the tank full of buffer. Hybond N (Amersham, UK) membrane was placed on top of the gel and two sheets of 3MM paper soaked in 2xSSC placed on top of the membrane. The remaining area of the glass plate was covered in plastic and a stack of paper towels placed on top of the gel together with a small weight. When blotting was completed, the filter was removed, rinsed in 2xSSC and baked in a hot air oven at 80°C for 3 hours to fix the DNA to the filter.

2.2.4.6 Prehybridisation

This is necessary to prevent non specific binding of radiolabelled probe to the filter.

Filters were soaked in 2xSSC and placed on a mesh. The mesh was rolled up and placed in a hybridisation bottle (Hybaid), to which 5ml of 2xSSC was added and the bottle gently rolled so that the filter and mesh adhered to the walls of the bottle. Excess 2xSSC was poured off and replaced by 10ml of prewarmed prehybridisation solution containing 100µl of denatured salmon sperm DNA (10mg/ml). Bottles were placed in a Hybaid Hybridisation oven at 65°C overnight. Prior to purchasing a hybridisation oven, hybridisations were carried out in a water bath and filters were sealed in polythene bags.

2.2.4.7 Oligonucleotide Labelling Technique

Probes were labelled using random primed DNA labelling kits (Boehringer Mannheim) according to the method of Feinberg & Volgenstein (1984). 25µl of probe was boiled for 10 minutes and incubated at 37°C for 2 minutes. 2µl of the oligonucleotides dATP, dGTP, dTTP, 4µl of reaction mix, 1.5µl of Klenow enzyme and 50µCi (5µl) of ^{32}P dCTP isotope (Amersham International) were added. The mixture was incubated for a minimum of 1 hour, and the reaction stopped by the addition of 2µl 0.5M EDTA.

2.2.4.8 Separation of labelled probe from unlabelled probe

A column packed with Sephadex G50 equilibrated with 1xSSC/0.1%SDS was prepared. Once the column had settled excess 1xSSC was removed from the surface and the probe added. The effluent from the base of the column was monitored using a Seris 900 mini moniter and the first peak (>200cpm) corresponding to the labeled probe was collected.

2.2.4.9 Hybridisation of filters

The collected probe was denatured by boiling for 10 minutes and placed on ice for 2 minutes. The prehybridisation solution from the bottle was poured into a universal and the probe added. The contents of the universal were mixed gently and transferred back into the bottle. Hybridisations were carried out in the hybridisation oven at 65°C for a minimum of 16 hours.

2.2.4.10 Washing of Filters

Excess probe was removed by pouring off the solution in the bottles and washing in 2xSSC/0.1%SDS for 20 minutes. Filters were monitored using a Seris 900 minimonitor, if a signal greater than 10cpms was seen, filters were washed at increased stringency.

Washing solutions used were; 1xSSC/0.1% SDS, 0.5xSSC/0.1% SDS and 0.1xSSC/0.1%SDS. All washes were carried out at 65°C.

2.2.4.11 Autoradiography

Once filters were washed, they were placed between plastic and placed in light tight autoradiography cassettes with intensifying screens and exposed to Kodak Diagnostic AR imaging film. Cassettes were stored at -70°C for an average of 7 days. Films were developed in a Agfa Curix 60 X-Omat.

2.2.4.12 Stripping of filters

Filters were "stripped" by rinsing in 0.4M sodium hydroxide for 30 minutes followed by a 30 minute wash in neutralisation solution at RT. This removes hybridised probe and therefore allows for further probing.

2.2.5 RNA techniques

2.2.5.1 RNA extraction from lymphocytes

The Acid Guanidium Thiocynate Chloroform method.

This method is a variation of the procedure described by Chomczynski and Sacchi (1987).

All neccessary precautions were taken to avoid contamination by RNAses. Glassware and plasticware used for the preparation and storage

of RNA were soaked in 0.1% diethylpyrocarbonate (DEPC) in SDW for 14-16 hours and rinsed in SDW and autoclaved for 20 minutes at 15lb/sq in. All solutions used for RNA handling were also DEPC treated and autoclaved.

RNA was extracted from lymphoblastoid cell lines. Cells were initially washed in 10ml PBS and spun for 10 minutes at 1200 rpm. After this another wash was given and the supernatant discarded. The cell pellet was then dissolved in 1ml of Solution D (denaturing solution) and transferred into 2 sarstedt tubes. To each tube the following were added: 150µls of 2M sodium acetate, 500µls of phenol, 300µls of chloroform. Samples were mixed and left on ice for 15 minutes and then centrifuged at 10000g for 20 minutes at 4°C. The aqueous phase was transferred to a fresh tube and 1.5ml of isopropanol added. Tubes were mixed and placed at -20°C for at least 1 hour or at 4°C overnight. The tubes were then spun at 10000g for 20 minutes at 4°C and the supernatant discarded. The resulting pellet was dissolved in 0.6ml of Solution D and transferred to a fresh eppendorf to which 0.6ml isopropanol was added and the samples chilled at -20°C for 1 hour. The eppendorf tubes were spun in a microcentrifuge for 10 minutes at 4°C, the pellet was then washed in 75% ethanol twice, dried and dissolved in 100µl of SDW at 65°C for 10 minutes. RNA samples were stored at -20°C.

2.2.5.2 Optical densities of RNA

The concentration of each RNA sample was determined by measuring the optical density at 260nm in a dual beam spectrophotometer with a deuterium lamp. An OD reading of 1 corresponds to approximately 40µg/ml of RNA. Optical densities were measured at 260nm and 280nm to

determine the purity of the RNA sample. For a pure RNA sample the ratio of OD reading (260nm:280nm) is 2.

2.2.5.3 Visualisation of extracted RNA

A checker gel was run to determine if the RNA extraction was successful. A 30ml 1% agarose gel was prepared containing 5mls of formaldehyde and 3ml of 10xMOPS. The RNA sample was prepared as follows: 5uls formamide, 1.65µls formaldehyde, 1µl 10xMOPS, 1.3µl H20, 1µg RNA sample.

The preparation was heated to 55°C for 10 minutes and then placed on ice. 2µl of gel juice was added to the sample and loaded immediately. Gels were run in 1xMOPS at 75V. On completion of electrophoresis, gels were soaked in SDW for 1 hour to remove the formaldehyde, stained in ethidium bromide for 10 minutes destained in water overnight, visualised on a uv transilluminator (316nm) and photographed with a Polaroid type 667 film in a Polaroid Instant print camera fitted with a Kodak 22A Wratten filter.

An E. coli ribosomal RNA molecular weight marker (Boehringer Mannheim) was used to determine if RNA extraction was successful.

2.2.6 Reverse Transcription-Polmerase Chain Reaction amplification of the glucocorticoid receptor cDNA

2.2.6.1 Primer Design for amplification

Primers were designed with the aid of the OLIGO computer programme (Rychlich & Rhodes 1989) to determine the "best" primers for amplification of the human glucocorticoid receptor (HGR) cDNA. Three sets of primers were designed such that the cDNA including part of the 3'and 5' untranslated regions were amplified. The features of the oligonu-

cleotide primers are presented in Table 6.

Synthetic oligonucleotide primers were initially prepared by Oswell DNA sevices, Department of biochemistry, University of Edinburgh, and then within the Duncan Guthrie Institute using a Biosystems 380B DNA synthesiser using cyanoethyl phosphoramidites (E O'Hare). Figures 2 shows a diagrammatical representation of the regions amplified.

2.2.6.2 Deprotection of synthetic oligonucleotides

Primers prepared within the department required a deprotection stage, to remove the oligonucleotide from the columns.

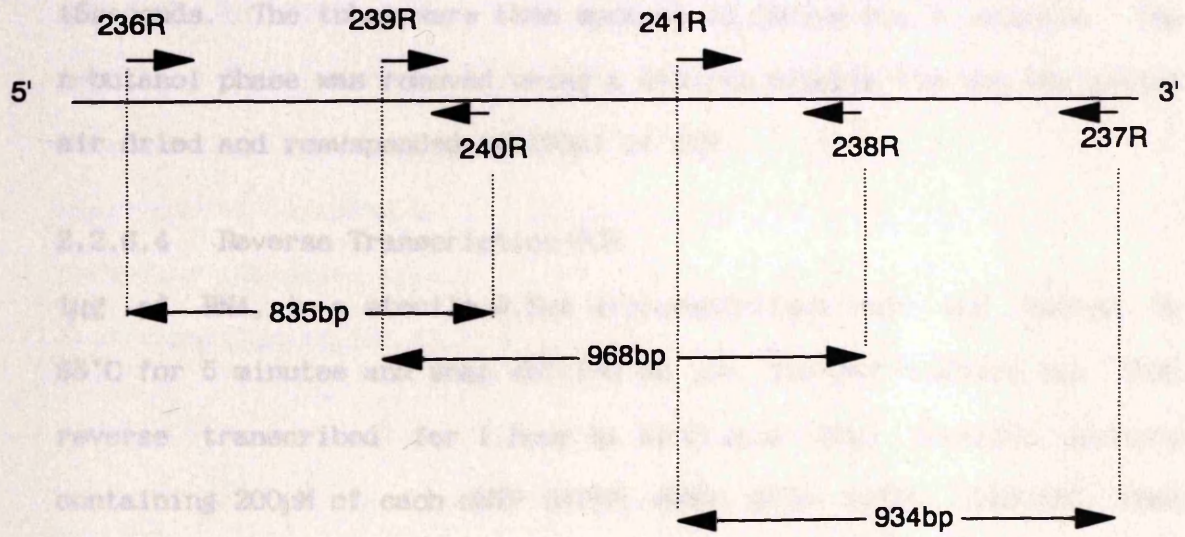
Using a 1ml syringe 0.3mls of ammonia solution was pushed into the column and left for 10 minutes. This was repeated several times until all the solution was in the column. A second syringe placed at the opposite end of the column was used to collect the solution containing the oligonucleotide. This was then placed in a Nunc tube and a further 1ml of ammonia solution was added. The tubes were incubated overnight at 55°C, and the ammonia allowed to evaporate off in a fume hood. The remaining oligonucleotide solution was made up to 1ml using SDW and the optical density determined (See section 2.2.4.2) and the concentration determined (Appendix II).

Table 6: Features of the primer sets used for the amplification of the glucocorticoid cDNA

Primer	Primer Sequence	Size of product
236R	F GCG TTC ACA AGC TAA GTT GT	
240R	R AGA GGC TTG CAG TCC TCA TT	835bp
239R	F GGA GAA GAC GAT TCA TTC CT	
238R	R ACA GAG CTA TCA TAT CCT GC	968bp
241R	F GTG TCA CTG TTG GAG GTT AT	
237R	R AGT GAT GAC GAC TCA ACT GC	934bp

Sequence of the primers are shown in the 5'——3' orientation
F: forward primer
R: reverse primer

Figure 2: Diagrammatic Representation of the relative positions of the amplified fragments of the glucocorticoid receptor cDNA



Primers 236R/240R amplify exon 1, 756bp of exon 2
Primers 239R/238R amplify 497bp of exon 2, exon 3 & 4, 188bp of exon 5
Primers 241R/237R amplify 151bp exon 5, exon 6, 7 & 8, 3349 bp exon 9

2.2.6.3 Purification of oligonucleotides

In some instances it was necessary to purify synthetic oligonucleotides after deprotection. This was carried out according to (Sawadogo 1991). 100µl of the deprotected oligonucleotide was placed in a 1ml microcentrifuge tube and vortexed together with 1ml of n-butanol for 15seconds. The tubes were then spun at 12,000rpm for 4 minutes. The n-butanol phase was removed using a sterile pipette tip and the pellet air dried and resuspended in 100µl of SDW.

2.2.6.4 Reverse Transcription-PCR

1µg of RNA, in a sterile 0.5µl microcentrifuge tube, was heated to 95°C for 5 minutes and snap chilled on ice. The RNA template was then reverse transcribed for 1 hour at 42°C in a 20µl reaction mixture containing 200µM of each dNTP (dTTP, dGTP, dCTP, dATP), 10mM DDT, 75mM KCl, 50mM Tris HCl (pH 8.4), 3mg MgCl, 100µg/µl Bovine serum albumen, 0.5µM of forward primer and 200 units of Murine Maloney Leukemia Virus (MMLV).

At the end of the transcription reaction, the reaction components were heated to 95°C for 5 minutes and placed on ice, and prepared for PCR. The reverse primer was added to a final concentration of 0.5µM and the volume adjusted to 50µls. After the addition of 2.5 units of Taq DNA polymerase, the reaction components were overlaid with 50µl of mineral oil and the cDNA PCR amplified for 30 cycles.

PCR reactions were carried out on a automated Cetus Perkin Elmer Thermo-cycler.

For oligonucleotide set 236R/240R the optimised thermo-cycler conditions were: 72°C for 10 minutes for 1 cycle, 94°C for 3 minutes, 60°C

for 1 minute, 72°C for 2 minutes for 1 cycle, 94°C for 1 minute, 60°C for 1 minute, 72°C for 2 minutes for 30 cycles followed by a final extension step of 72°C for 5 minutes. The optimised conditions for oligonucleotide set 238R/239R and 241R/237R were the same except for the annealing temperature, which were 58°C and 57°C respectively.

2.2.6.5 Identification of PCR products

Amplified PCR products were resolved on 1.4% Seakem agarose mini-gels containing 0.5µg/ml of ethidium bromide in 1xTBE buffer with the addition of loading mix. A 1kb molecular weight marker was used to determine the sizes of amplified products, and gels were visualised on a uv transilluminator (316nm) and photographed.

2.2.6.6 Precipitation of DNA from PCR product

The PCR product "band" was excised from the gel using a sterile blade and the DNA extracted by overnight elution in 100µl of TE buffer. The DNA was then precipitated by the addition of an equal volume of 4M ammonium acetate (pH 5.2) and 2 volumes of isopropanol and left at RT for 10 minutes, followed by a 10 minute spin at 12,000g. The resulting pellet was washed in 70% ethanol and allowed to air dry and resuspended in 100µls of SDW.

2.2.6.7 Generation of single stranded DNA templates

Asymmetric PCRs were performed for the generation of single stranded DNA for chain termination sequencing using precipitated DNA samples from standard PCR reactions by the method described by Mgone (personal communication) using an unequal ratio of 100:1 of the two oligonucleotide primers.

Asymmetric PCRs were set up as follows; to a 0.5ml eppendorf tube the following reagents were added: 10µls of Taq polymerase buffer, 10µls of 2.5mM dNTP mix, 5ng of forward primer and 500ng of reverse primer, or 500ngs of forward primer, 5ngs of reverse primer, 1µg of DNA, and a volume of SDW to give a total volume of 100µls. PCR reactions were then performed using the conditions described in section 2.2.6.4. The number of cycles was increased from 30 too 40, since the first 20-25 cycles results in double stranded DNA, but as the limiting primer is depleted, single stranded templates are produced.

2.2.6.8 Visulisation of Single stranded DNA

5µls of each PCR product was resolved on a 50ml 1% agarose TBE mini-gel containing 10% ethidium bromide with 5µls of loading mix, and the products viewed on a uv transilluminator. Single stranded DNA migrates slower through agarose gels than double stranded DNA and also shows less fluorescene. The single stranded DNA was then precipitated to remove primers and dNTPS prior to sequencing, using the same protocol described in section 2.2.6.6, but dissolving the DNA in 14µls of SDW.

2.2.7 PCR amplification of the Human Angiotensinogen gene

The angiotensinogen gene consists of five exons and four introns and spans a region of approximately 13kb (Gaillard 1989). Nine sets of synthetic oligonucleotides were manufactured within the department for the amplification of the five exons and the 5' region of the gene. Primer sequences were taken from Jeunemaitre et al., (1992), and are listed in Table 7.

Table 7: Features of the primers used for the amplification of the angiotensinogen gene

Primer	Primer sequence	size of product
5'a1	F ACC ATT TGC AAT TTG TAC AGC	255bp
5'a2	R GCC CGC TCA GGG ATG TG	
5'b1	F AAG ACT CTC CCC TGC CCC TCT	
5'b2	R GAA GTC TTA GTG ATC GAT GCA G	282bp
5'c1	F AGA GGT CCC AGC GTG AGT GT	
5'c2	R AGA CCA GAA GGA GCT GAG GG	238bp
2a1	F GTT AAT AAC CAC CTT TCA CCC TT	
2a2	R GCA GGT ATG AAG GTG GGG TC	252bp
2b1	F AAG CCA ATG CCG GGA AGC CC	
2b2	R ATC AGC CTG CCC TGG GCC A	381bp
2c1	F GAT GCG CAC AAG TCC TGT C	
2c2	R GCC AGC GAG AGG TTT GCCT	344bp
3a1	F TCC CTC CCT GTC TCC TGT CT	
3a2	R TCA GGA GAG TGT GGC TCC CA	335bp
4a1	F TGG AGC CTT CCT AAC TGT GC	
4a2	R AGA CAC AGG CTC ACA CAT AC	275bp
5a1	F GTC ACC CAT GCG CCC TCA GA	
5a2	R GTG TTC TGG GGC CCT GGC CT	260bp

Primer sequences are shown in the 5'——3' orientation
F: Forward primer
R: Reverse primer

PCR reactions were performed by adding 1µg of DNA to a 50µl reaction mix containing 50mM KCl, 10mM Tris HCl (Ph 8.4), 1.5mM MgCl, 100µg/ml gelatin and 200µM of each dNTP (dTTP, dCTP, dGTP, dATP) and 0.5µM of each primer. To this 2.5 units of Taq DNA polymerase was added the reaction mixture overlaid with 50µl of mineral oil. Prior to the addition of DNA and enzyme the reaction components were uv irradiated for 5 minutes, and the DNA denatured by heating to 95°C for 5 minutes. The PCR conditions used were, denaturation for 1 minute at 94°C, annealing at 62°C for 1 minute and extension for 2 minutes at 72°C for primer sets 5c1/5c2, 2a1/2a1, 2b1/2b2, 2c1/2c2, 4a1/4a2, and 5a1/5a2. For primer sets 5a1/5a2, 5b1/5b2, and 3a1/3a2 the annealing temperature was alter to 56°C. Once PCR was complete, samples were resolved on 1.4% agarose gels as described previously.

2.2.8 Chain termination sequencing

All sequencing reactions were carried out using Sequenase Version 2.0 (United States Biochemical Corporation). Sequencing reactions were carried out in sterile 0.5ml eppendorf tubes with sterile pipette tips.

2.2.8.1 Single strand sequencing-Annealing template and primer

To an eppendorf tube 1µg of ssDNA was added together with 1pmol of the limiting primer and 2µl of reaction buffer. The final volume was made up to 10µls using SDW. The tube containing the sample was heated to 65°C for a minimum of 2 minutes and then allowed to cool slowly to room temperature (<35°C) over a period of approximately 30 minutes. Once the temperature was below 35°C annealing reaction was complete and the tubes were snap chilled on ice.

2.2.8.2 Labelling reaction

Labelling mix was diluted five fold and the sequenase version 2.0 enzyme diluted 8 fold with enzyme dilution buffer. To the annealed template-primer the following reagents were added; 1.0µls of 0.1 M DDT, 1.0µls of diluted labelling mix, 0.5µl of ^{35}S dATP and 2.0µls of diluted sequenase enzyme. The contents of the tube were mixed thoroughly and incubated at RT for 2-3 minutes.

2.2.8.3 Termination reaction

Four eppendorf tubes were prewarmed to 37°C and labelled G,A,T and C, 2.5µl of ddGTP termination mix was added to tube G, 2.5µl of ddATP termination mix was added to tube A etc. Once the labelling incubation was complete 3.5µl was transferred to each tube and the incubation

reaction continued for a further 5 minutes at 37°C. Reactions were terminated by the addition of 4µl of stop solution. Prior to loading of the samples, tubes were heated to 75°C for 2 minutes and placed on ice and loaded immediately.

2.2.8.4 Sequencing gel electrophoresis

8% polyacrylamide gels were used and prepared as follows: An original stock solution of 60mls was prepared with SDW containing 12mls of 40% bis acrylamide, 6mls of 10 x TBE and 25g of urea. The solution was thoroughly mixed to dissolve the urea. A Sequi-Gen nucleic acid sequencing cell (Biorad) was used for electrophoresis of sequencing reactions. The glass plates were thoroughly cleaned with soapy water and rinsed in distilled water. The glass plates were then dried and cleaned with ethanol. The bound glass plate was siliconised in a fume hood and allowed to dry. Once dry spacers were placed between the two glass plates and held together by side clamps. The bottom of the sequencing cell was sealed by pouring 10ml of the gel solution containing 50µl of 25% ammonium persulphate and 50µl of TEMED onto a paper strip and the sequencing cell placed on top and held in place until the gel solution had polymerised. To the remaining gel solution, 100µl of TEMED and 150µl of fresh ammonium persulphate were added and the gel poured immediately taking care to prevent bubbles from forming. Once poured the gel comb was positioned and the gel allowed to polymerise. Once the gel had polymerised, the comb was removed and the wells washed with 1xTBE. Gels were pre-run at 2000V for approximately 1-2 hours to stabilise the temperature of the gel (55°C). Prior to loading of samples, the samples were heated to 70°C for 5 minutes and

placed on ice. 1.5µl of the sample was immediately loaded onto the gel. Polyacylamide sequencing gels were electrophoresed in 1 x TBE at of 50°C for 2-4 hours depending on the size of the DNA fragment to be sequenced. On completion of electrophoresis, the gel plates were gently prised apart and the gel stuck to the outer plate were treated in methanol/acetic acid (3:1) for 15 minutes, then gently blotted and placed on a sheet of 3MM paper. A layer of Saranwrap was placed over the gel and the gel dryed for 45 minutes at 80°C.

2.2.8.5 Autoradiography

Dryed gels were placed in light tight autoradiography cassettes and exposed to Kodak Diagnostic AR imaging film. Cassettes were stored at -70°C overnight. Films were developed in a Agfa Curix 60X-Omat, if the signal was not very strong the gels were exposed for longer.

2.2.9 Hydrolink Gel Electrophoresis

Hydrolink Gel Electrophoresis was carried out using a variation of the method described by Keen (1991).

2.2.9.1 Generation of Heteroduplexes

PCR was performed as described in Section 2.2.6.4 and 2.2.7. After the final extension PCR cycles, the PCR products were heated to 90°C for 5 minutes and incubated at 70°C for a minimum of 1 hour for the generation of heteroduplexes.

2.2.9.2 Hydolink Gel Electrophoresis

Gels were prepared as follows; 40mls of Hydrolink (AT Biochem), 5mls 10xTBE, 750µls of fresh 10% ammonium persulphate, 70µls of TEMED. The gel solution was poured in between freshly cleaned glass plates (20cm

x 20cm) separated by a 2mm spacer. A 50ml syringe was used to pour the gel solution into the mould. Once the gel was poured a gel comb was positioned in the gel, and clamped securely in place. Gels were allowed to set for a minimum of 30 minutes, and then place on a vertical gel apparatus. The comb was then removed, and the wells washed thourougly with 1 X TBE buffer. 4µls of loading mix was added to 16µl of the PCR product and added to the wells. Gels were electrophoresed in 1 X TBE buffer at approximatley 200v for 3-4 hours. Gels were stained in ethidium bromide for 10 minutes and destained in SDW for several hours and visualised on a uv transilluminator, and photographed.

2.3 Statistical methods

2.3.1 Chi-square analysis

Genotype frequencies were determined from the collected genotype data and the allele frequencies calculated. Chi-square analysis was performed on the observed genotype data to test the significance of the recorded data, using the following equation;

$$\text{chi-square } (X) = \frac{(\text{observed no.} - \text{expected no})^2}{\text{expected no.}}$$

In some instance Yates Correction Thereom was applied when the expected counts were less than one, using the following equation;

$$\text{Chi-square } (X) = \frac{(\text{observed no.} - \text{expected number} - 0.5)^2}{\text{expected no.}}$$

CHAPTER 3: RESULTS

Chapter 3 RESULTS

3.1 RFLP analysis in offspring with contrasting predispositions to the development of high blood pressure.

Six polymorphisms of six candidate genes were analysed in four groups of offspring with contrasting predispositions to high blood pressure; Group A (high offspring/low parental blood pressure), Group B (high offspring/high parental blood pressure), Group C (low offspring/low parental blood pressure) and Group D (low offspring/high parental blood pressure. The observed genotypes for all offspring studied are presented in Appendix III.

Genotype and allele frequencies for each RFLP were calculated and analysis of the data was carried out by chi-square analysis using Yates correction theorem where indicated.

3.1.1 Renin Gene Polymorphism

Using a 0.8kb cDNA probe of the renin gene 192 offspring of the Ladywell study were genotyped for the *Hind III* RFLP of the renin gene (Frossard et al., 1987). Homozygote individuals for the polymorphism have either a 9.0kb or a 6.2kb band, and heterozygotes have both the 9.0kb and 6.2kb bands, identified by Southern blotting.

The genotype and deduced allele frequencies for the whole population and the four individual groups are presented in Table 8. The genotypes have been abbreviated AA, Aa and aa, where the 9.0kb allele of the *HindIII* polymorphism has been designated (A) and the 6.2kb allele (a). The *HindIII* polymorphism allele frequencies for the whole population were 0.67 for the 9.0kb allele (A), and 0.33 for the 6.2kb allele (a), similar to those reported previously (Frossard et al., 1987). The

overall genotype frequencies were compared with those predicted from the Hardy-Weinberg equilibrium by chi-square analysis. No statistically significant difference was noted such that the genotype frequencies satisfied the Hardy-Weinberg proportions (X^2_2=4.38 p=0.1-0.2).

A comparison of the genotype distribution was carried out between the four different groups in the Ladywell study, and no statistically significant difference between the genotype distribution of the four groups of offspring was found (X^2_6=12.34 p=0.01-0.05).

The two groups of offspring with maximum genetic predisposition to high blood pressure ie. Groups B (high/high) and C (low/low), were then tested for a difference in genotype distribution as presented in Table 9. However, a statistically significance difference was not found (X^2_2=5.77 p=0.01-0.05).

A comparison of the genotype distribution was also performed between offspring with low blood pressure and offspring with high blood pressure irrespective of parental blood pressures ie. (Group A and B) versus (Group C and D). Again no significant difference was found (X^2_2=0.89 p=0.5-0.8).

Table 8:Genotype and allele frequencies for the A (9.0kb) and a (6.2kb) alleles of the *HindIII*/renin gene RFLP in the four groups of offspring;
Group A (high offspring BP/low parental BP)
Group B (high offspring BP/high parental BP)
Group C (low offspring BP/low parental BP)
Group D (low offspring BP/high parental BP)

Group		Frequencies				
		Genotype			Alleles	
	n	AA	Aa	aa	A	a
A	49	21	23	5	0.66	0.34
B	45	14	30	1	0.65	0.35
C	48	20	22	6	0.65	0.35
D	50	23	27	0	0.73	0.27
Total	192	78	102	12	0.67	0.33

Table 9:Comparison of the *HindIII*/renin gene RFLP distribution between the two groups of offspring with polarised predisposition to high blood pressure: Group B (high offspring/high parental BP) and Group C (low offspring/low parental BP).

Genotype	Number of offspring	
	Group B	Group C
Observed		
9.0	14	20
9.0/6.2	30	22
6.2	1	6
Expected		
9.0	16.45	17.55
9.0/6.2	25.16	26.84
6.2	3.39	3.61
X^2_2	5.77	
p	0.01-0.05(NS)	

3.1.2 Beta-2-adrenergic receptor gene polymorphism

Using a 2.6kb genomic DNA probe of the beta-2-adrenergic receptor gene 182 offspring were genotyped for the *BanI* RFLP of this gene (Lentes et al., 1988). Homozygote individuals for the *BanI* polymorphism have either a 3.7kb allele or a 3.4kb allele, and heterozygotes have both the 3.7kb and 3.4kb bands, detected by Southern blotting.

Genotypes and deduced allele frequencies for the whole population and for the four individual groups are presented in Table 10. The genotypes have been abbreviated to BB, Bb and bb, where the 3.7kb allele of the *BanI* polymorphism has been designated (B), and the 3.4kb allele (b). The allele frequencies for the whole offspring population were 0.25 for the the 3.7 kb allele (B) and 0.75 for the 3.4kb allele (b). These allele frequencies are similar to those reported previously (Lentes et al., 1988). The overall genotype frequencies were compared with those predicted from the Hardy-Weinberg equilibrium by chi-square analysis. No significant difference between observed and expected numbers was seen, such that the genotype frequencies satisfied the Hardy-Weinberg proportions (X^2_2=0.05 p=0.98).

A comparison of the *BanI* RFLP genotypes was performed between the four groups of offspring of the Ladywell Study and no significant difference was found (X^2_6=8.76 p=0.20-0.50).

The two groups of offspring with contrasting predispositions to high blood pressure ie Groups B (high/high) and C (low/low), were then tested for a difference in genotype distribution (Table 11). Chi-square analysis revealed no statistically significant difference between the two groups (X^2_2=4.43 p=0.10-0.20).

A comparison of the genotype distribution between offspring with high blood pressure and offspring with low blood pressure, irrespective of parental blood pressure levels was also performed ie. Group (A and B) versus Group (C and D). Again, no statistically significant difference was detected (X^2_2=1.84 p=0.20–0.50).

Table 10:Genotype and allele frequencies for the B (3.7kb) and b (3.4kb) alleles of the *BanI*/beta-2-adrenergic receptor gene RFLP in the four groups of offspring;
Group A (high offspring BP/low parental BP)
Group B (high offspring BP/high parental BP)
Group C (low offspring BP/low parental BP)
Group D (low offspring BP/high parental BP)

Group		Frequencies				
		Genotypes			Alleles	
	n	BB	Bb	bb	B	b
A	45	51	13	27	0.26	0.74
B	43	3	17	23	0.27	0.73
C	47	0	15	32	0.16	0.84
D	47	4	21	22	0.31	0.69
Total	182	12	66	104	0.25	0.75

Table 11:Comparison of the *BanI*/beta-2-adrenergic receptor gene RFLP distribution between groups with polarised predisposition to high blood pressure; Group B (high offspring/high parental BP), Group C (Low ofspring/low parental BP).

Genotype	Number of offspring	
	Group B	Group C
Observed		
3.7	3	0
3.7/3.4	17	15
3.4	23	32
Expected		
3.7	1.43	1.57
3.7/3.4	15.29	16.71
3.4	26.28	28.72
X_2^2	4.429	
p	0.10-0.20(NS)	

3.1.3 Beta-1-adrenergic receptor gene polymorphism

Using a 2.4kb genomic DNA probe of the beta-1-adrenergic receptor gene 173 offspring of the Ladywell study were genotyped for the *BglI* RFLP of this gene (Frielle et al., 1987). Homozygote individuals for the *BglI* polymorphism have either a 6.2kb band or a 4.7kb band, and heterozygotes have both the 6.2kb and the 4.7kb band, detected by Southern blotting.

Genotype and deduced allele frequencies for the whole population and the four individual groups are presented in Table 12. The genotypes have been abbreviated to CC, Cc and cc, where the 6.2kb allele of the *BglI* polymorphism has been designated (C) and the 4.7kb allele (c). The *BglI* polymorphism allele frequencies were found to be 0.78 for the 6.2kb allele (D) and 0.22 for the 4.7kb allele (d), similar to those reported previously (Frielle et al., 1987). Overall genotype frequencies were compared with those predicted from the Hardy-Weinberg equilibrium by chi-square analysis. No statistically significant difference between the observed and expected numbers was seen, such that the genotype frequencies satisify the Hardy-Weinberg proportions (X^2_2 =0.98 p=0.50-0.80).

A comparison of the *BglI* polymorphism was carried between the four groups of the Ladywell Study, however no statistically significant difference in the genotype distribution was observed (X^2_6=7.02 p=0.20-0.50).

Chi-square analysis was also performed between the two groups of offspring with maximum genetic predisposition ie. Groups B (high/high) and C (low/low) (Table 13). A significant difference between the two

groups was not detected (X_2^2=0.22 p=0.80–0.90).

A comparison of the genotype distribution between offspring with high blood pressure and offspring with low blood pressure irrespective of parental blood pressures was also performed ie. Group (A and B) versus Group (C and D). Again no statistically significant difference was found (X_2^2=0.29 p=0.80–0.90).

Table 12:Genotype and allele frequencies for the C (6.2kb) and c (4.7kb) alleles of the *BglI*/beta-1-adrenergic receptor gene RFLP in the four groups of offspring;
Group A (high offspring BP/low parental BP)
Group B (high offspring BP/high parental BP)
Group C (low offspring BP/low parental BP)
Group D (low offspring BP/high parental BP)

Groups		Frequencies				
		Genotype			Alleles	
	n	CC	Cc	cc	C	c
A	41	28	13	0	0.84	0.16
B	42	26	11	5	0.75	0.25
C	45	29	12	4	0.78	0.22
D	45	26	17	2	0.77	0.23
Total	173	109	53	11	0.78	0.22

Table 13:Comparison of the *BglI*/beta-1-adrenergic receptor gene RFLP distribution between groups of offspring with polarised predisposition to high blood pressure; Group B (high offspring/high parent BP), Group C (low offspring/low parent BP)

Genotype	Number of offspring	
	Group B	Group C
Observed		
6.2	26	29
6.2/4.7	11	12
4.7	5	4
Expected		
6.2	26.55	28.45
6.2/4.7	11.10	11.90
4.7	4.34	4.66
X^2_2	0.215	
p	0.80-0.90 (NS)	

3.1.4 Alpha-2-adrenergic receptor gene polymorphism

Using a 5.5kb genomic DNA probe of the alpha-2-adrenergic receptor gene 151 offspring of the Ladywell Study were genotyped for the *DraI* RFLP of this gene (Hoehe et al., 1988). Homozygote individuals for the *DraI* polymorphism have either a 6.7kb band or a 6.3kb band, and heterozygotes have both a 6.7kb and 6.3kb band, detected by Southern blotting.

The genotypes and deduced allele frequencies for the whole population and the four individual groups are presented in Table 14. The genotypes have been abbreviated to DD, Dd and dd, where the 6.7kb allele has been designated (D) and the 6.3kb allele (d). The allele frequencies were found to be 0.86 for the 6.7kb allele (D), and 0.14 for the 6.3kb allele (d), similar to those reported previously (Hoehe et al., 1988). The overall genotype frequencies were compared with those predicted from the Hardy-Weinberg equilibrium by chi-square analysis. No statistically significant difference between the observed and expected numbers was seen, such that the genotype frequencies satisified the Hardy-Weinberg proportions (X^2_2=0.34 p=0.80-0.90).

A comparison of the *DraI* polymorphism was carried between the four groups of the Ladywell Study. No statistically significant difference in the genotype distribution was observed between the four groups (X^2_6 =7.51 p=0.20-0.50 using Yates correction theorem).

Chi-square analysis was also performed between the two groups of offspring with maximum genetic predisposition ie. Groups B (high/high) and C (low/low) (Table 15). A statistically significant difference between the genotype distribution of the two groups was not

detected (X^2_2 =4.36 p=0.10–0.20).

A comparison of the genotype distribution between offspring with high blood pressure and offspring with low blood pressure, irrespective of parental blood pressures was also performed ie. Group (A and B) versus Group (C and D). Again no statistically significant difference was found (X^2_2 =3.33 p=0.10–0.20 using Yates correction theorem).

Table 14:Genotype and allele frequencies of the D (6.7kb) and d (6.3kb) alleles of the *DraI*/alpha-2-adrenergic receptor gene RFLP in the four groups of offspring;
Group A (high offspring BP/low parental BP)
Group B (high offspring BP/high parental BP)
Group C (low offspring BP/low parental BP)
Group D (low offspring BP/high parental BP)

Groups		Frequencies				
		Genotype			Alleles	
	n	DD	Dd	dd	D	d
A	38	30	7	1	0.88	0.12
B	39	31	8	0	0.90	0.10
C	40	25	14	1	0.80	0.20
D	34	24	10	0	0.85	0.25
Total	151	110	39	2	0.86	0.14

Table 15:Comparison of *DraI*/alpha-2-adrenergic receptor gene RFLP distribution between the two groups with polarised predisposition to high blood pressure: Group B (high ofspring/high parental BP), Group C (low offspring/low parental BP)

Genotype	Number of offspring	
	Group B	Group C
Observed		
6.7	31	25
6.7/6.3	8	14
6.3	0	1
Expected		
6.7	27.65	28.35
6.7/6.3	10.86	11.14
6.3	0.49	0.51
X^2_2	4.36 *	
p	0.10-0.20 (NS)	

* Yates Correction theorem applied since chi-square approximation probably invalid since 2 cells with expected counts less than 1.00

3.1.5 Insulin gene polymorphism

Using a 0.88kb DNA probe of the insulin gene 174 offspring of the Ladywell study were genotyped for the *SacI* RFLP of the insulin gene (Bell et al., 1982). Homozygote individuals for the *SacI* polymorphism have either a 7.5kb band or a 6.0kb band and heterozygotes have both the 7.5kb and 6.0 kb band, detected by Southern Blotting.

Genotypes and deduced allele frequencies for the whole population and the four individual groups are presented in Table 16. The genotypes have been abbreviated to E, Ee and ee, where the 7.5kb allele of the *SacI* polymorphism has been designated (E) and the 6.0kb allele (e). The *SacI* polymorphism allele frequencies for the whole population were 0.37 for the 7.5kb allele and 0.63 for the 6.0kb allele, similar to those reported previously (Bell et al., 1982). The overall genotype frequencies were compared with those predicted from the Hardy-Weinberg equilibrium by chi-square analysis. No statistically significant difference between observed and expected numbers was seen, such that the genotype frequencies satisfied the Hardy-Weinberg proportions (X^2_2 =1.523 p=0.20–0.50).

A comparison of the *SacI* polymorphism distribution was carried out for all four groups and a significant difference was not detected (X^2_6=4.95 p=0.50–0.80).

A comparison of the genotype distribution was also carried out between the two groups of offspring with maximum predisposition to the development of high blood pressure ie. Groups B (high/high) and C (low/low) (Table 17). However, no significant difference was detected (X^2_2 =1.76 p=0.20–0.50).

A comparison of the genotype distribution between offspring with high blood pressure and low blood pressure, irrespective of parental blood pressures was also performed ie. group (A and B) versus group (C and D). Again, no statistically significant difference was found (X_2^2 =0.066 p>0.95).

Table 16:Genotype and allele frequencies of the E (7.5kb) and e (6.0kb) alleles of the *SacI*/insulin gene RFLP in the four groups of offspring;
Group A (high offspring BP/low parental BP)
Group B (high offspring BP/high parental BP)
Group C (low offspring BP/low parental BP)
Group D (low offspring BP/high parental BP)

Group		Frequencies				
		Genotype			Alleles	
	n	EE	Ee	ee	E	e
A	45	6	18	21	0.33	0.67
B	35	7	15	13	0.41	0.59
C	50	10	15	25	0.35	0.65
D	44	6	22	16	0.39	0.61
Total	174	29	70	75	0.37	0.63

Table 17:Comparison of the *SacI*/insulin gene RFLP genotype distribution between the two groups with polarised predisposition to high blood pressure; Group B (high offspring/high parental BP), Group C (low offspring/low parental BP).

Genotype	Number of offspring	
	Group B	Group C
Observed		
7.0	7	10
7.0/6.5	15	15
6.5	13	25
Expected		
7.0	7.0	10.0
7.0/6.5	12.35	17.65
6.5	15.65	22.35
X^2_2	1.762	
p	0.20–0.50 (NS)	

3.1.6 Glucocorticoid receptor gene polymorphism

Using a 4.4kb genomic DNA probe of the glucocorticoid receptor gene 117 offspring of the Ladywell study were genotyped for the *TthIII* RFLP of this gene (Detera-Wadleigh et al., 1991). Homozygote individuals for the *TthIII* polymorphism have either a 3.8kb or a 3.4kb band, and heterozygotes have both the 3.8kb and 3.4kb bands, detected by Southern blotting.

The genotype and deduced allele frequencies for the whole population and the four individual groups are presented in Table 18. The genotypes have been abbreviated to FF, Ff and ff, where 3.8kb allele of the *TthIII* polymorphism has deen designated (F) and the 3.4 kb (f). The *TthIII* polymorphism allele frequencies were 0.21 for the 3.8kb allele (G), and 0.79 for the 3.4kb allele (g), in close agreement with those reported previously (Detera-Wadleigh et al., 1991). The overall genotype frequencies were compared with those predicted from from the Hardy-Weinberg equilibrium by chi-square analysis. A statistically significant difference between the observed and expected numbers was not seen, such that the genotype frequencies satisfied the Hardy-Weinberg proportions (X^2_2=3.71 p=0.02-0.05).

A comparison of the polymorphism was carried out between the four groups of offspring of the Ladywell study. No statistically significant difference in the genotype distribution was observed between the four groups (X^2_6=4.68 p=0.50-0.80).

Chi-square analysis was also performed between the two groups of offspring with maximum genetic prediposition ie. Group B (high/high) and Group C (low/low) (Table 19), however no significant difference was found (X^2_2=2.18 p=0.20-0.50).

A comparison of the genotype distribution was also performed between offspring with high blood pressure and offspring with low blood pressure, irrespective of parental blood pressures ie. Group (A and B) versus Group (C and D). Again, no statistically significant difference was found (X_2^2=0.40 p=0.50-0.80).

Table 18:Genotype and allele frequencies of the F (3.8kb) and f (3.4kb) alleles of the *TthIII*/glucocorticoid receptor gene RFLP in the four groups of offspring;
Group A (high offspring BP/low parental BP)
Group B (high offspring BP/high parental BP)
Group C (low offspring BP/low parental BP)
Group D (low offspring BP/high parental BP)

Group	n	Frequencies Genotype FF	Ff	ff	Alleles F	f
A	28	2	7	19	0.20	0.80
B	30	2	7	21	0.18	0.82
C	25	4	8	13	0.32	0.68
D	34	2	6	26	0.15	0.85
Total	117	10	28	79	0.21	0.79

Table 19:Comparison of the *TthIII*/glucocorticoid receptor gene RFLP genotype distribution between the two groups with polarised predispositions to high blood pressure; Group B (high offspring/high parental BP), Group C (low ofspring/low parental BP)

Genotype	Number of offspring	
	Group B	Group C
Observed		
3.8	2	4
3.8/3.4	7	8
3.4	21	13
Expected		
3.8	3.27	2.73
3.8/3.4	8.18	6.82
3.4	18.55	15.45
X^2_2	2.179	
p	0.20–0.50 (NS)	

3.2 RFLP analysis in parents with high blood pressure and low blood pressure.

The genotype data of the RFLPs examined in the parent popluation is presented in Appendix III.

3.2.1 Renin Gene polymorphism

91 parents of the high/high and low/low groups of offspring were genotyped for the renin *HindIII* polymorphism. 44 parents were classified as having high blood pressure and 47 parents as having low blood pressure. Genotype and allele frequencies of the two groups are presented in Table 20. The allele frequencies from all subjects examined are 0.67 for the 9.0kb allele (A) and 0.33 for the 6.2kb allele (a). These frequencies are similar to those reported previously (Frossard et al., 1987). Chi-square analysis was performed to determine if a difference between the genotype distribution in the two parental groups exists, however no statistically significant difference was observed (X^2_2=0.53 p=0.50–0.80) (Table 21).

Table 20:Genotype and allele freqencies for the A (9.0kb) and a (6.2kb) alleles of the *HindIII*/renin gene RFLP in parents with high (HBP) and low (LBP) blood pressure.

Group	n	Frequencies Genotype AA	Aa	aa	Alleles A	a
HBP	44	22	18	4	0.70	0.30
LBP	47	27	16	4	0.74	0.26
Total	91	49	24	8	0.67	0.33

Table 21:Comparison of the *HindIII*/renin gene RFLP distribution in parental groups with high blood pressure (HBP) and low blood pressure (LBP).

Genotype	Number of subjects HBP	LBP
Observed		
9.0	22	27
9.0/6.2	18	16
6.2	4	4
Expected		
9.0	23.69	25.31
9.0/6.2	16.44	17.56
6.2	3.87	4.13
X^2_2	0.53	
p	0.50–0.80 (NS)	

3.2.2 Beta-2-Adrenergic receptor Gene polymorphism

97 parents of the high/high and low/low groups of offspring were genotyped for the beta-2-adrenergic receptor/*BanI* polymorphism. 49 parents were classified as having high blood pressure and 48 parents as having low blood pressure. Genotype and allele frequencies of the two parental groups are presented in Table 22. The allele frequencies from all subjects examined are 0.22 for the 3.7kb allele (B) and 0.78 for the 3.4kb allele (b), similar to those reported previously (Lentes et al., 1988). Chi-square analysis was performed to determine if a difference between the genotype distribution in the two parental groups exists (Table 23). No statistically significant difference was observed (X^2_2=2.88 p=0.20-0.50).

Table 22:Genotype and allele frequencies for the B (3.7kb) and b (3.4kb) alleles of the *BanI*/beta-2-adrenergic receptor gene RFLP in parents with high (HBP) and low blood pressure (LBP).

Group	n	Frequencies Genotype BB	Bb	bb	Allele B	b
HBP	49	2	18	29	0.22	0.78
LBP	48	0	21	27	0.22	0.78
Total	97	2	39	56	0.22	0.78

Table 23:Comparison of the *BanI*/beta-2-adrenergic receptor gene RFLP distribution in parental groups with high blood pressure (HBP) and low blood pressure (LBP).

Genotype	Number of subjects HBP	LBP
Observed		
3.7	2	0
3.7/3.4	18	21
3.4	29	27
Expected		
3.7	1.10	0.99
3.7/3.4	19.70	19.30
3.4	28.29	27.17
X^2_2	2.88 *	
p	0.20-0.50 (NS)	

*Yates Correction theorem applied since chi-square approximation probably invalid since one cell with expected counts less than 1.00

3.2.3 Beta-1-Adrenergic receptor gene polymorphism

93 parents of the high/high and low/low groups of offspring were genotyped for the beta-1-adrenergic receptor/*BglI* polymorphism. 44 parents were classified as having high blood pressure and 47 parents as having low blood pressure. Genotype and allele frequencies of the two groups are presented in Table 24. The allele frequencies from all subjects examined are 0.81 for the 6.2kb allele (C) and 0.19 for the 4.7kb allele (c), similar to those reported previously (Frielle et al., 1987). Chi-square analysis was performed to detect a difference between the genotype distribution of the two groups (Table 25). No statistically significant difference was detected (X^2_2 =0.46 p=0.50-0.80).

Table 24:Genotype and allele frequencies for the C (6.2kb) and c (4.7kb) alleles of the BglI/beta-1-adrenergic receptor gene RFLP in parents with high (HBP) and low blood pressure (LBP).

Group	n	Frequencies Genotypes CC	Cc	cc	Alleles C	c
HBP	47	30	16	1	0.81	0.19
LBP	46	30	14	2	0.80	0.20
Total	93	60	30	3	0.81	0.19

Table 25:Comparison of the *BglI*/beta-1-adrenergic receptor gene RFLP distribution in parental groups with high blood pressure (HBP) and low blood pressure (LBP).

Genotype	Number of subjects HBP	LBP
Observed		
6.2	30	30
6.2/4.7	16	14
4.7	1	2
Expected		
6.2	30.32	29.68
6.2/4.7	15.16	14.84
4.7	1.52	1.48
X^2_2	0.456	
p	0.50–0.80 (NS)	

3.2.4 Alpha-2-Adrenergic receptor Gene polymorphism

90 parents of the high/high and low/low groups of offspring were genotyped for the alpha-2-adrenergic receptor/*DraI* polymorphism. 45 parents were classified as having high blood pressure and 45 parents as having low blood pressure. Genotype and allele frequencies of the two groups are presented in Table 26. The overall genotype frequencies from all subjects examined are 0.81 for the 6.7kb allele (D) and 0.19 for the 6.3kb allele (d), similar to those reported previously (Hoehe et al., 1988). Chi-square analysis was performed to detect a difference between the genotype distribution of the two groups (Table 27). However, no statistically significant difference was observed (X^2_2=0.22 p=0.90).

Table 26:Genotype and allele frequencies for the D (6.7kb) and d (6.3kb) alleles for the *DraI*/alpha-2-adrenergic receptor gene RFLP in parents with high (HBP) and low blood pressure (LBP).

Group	n	Frequencies Genotypes DD	Dd	dd	Alleles D	d
HBP	45	31	12	2	0.82	0.18
LBP	45	30	12	3	0.80	0.20
Total	90	61	24	5	0.81	0.19

Table 27:Comparison of the *DraI*/alpha-2-adrenergic receptor gene RFLP distribution in parental groups with high blood pressure (HBP) and low blood pressure (LBP).

Genotype	Number of subjects HBP	LBP
Observed		
7.6	31	30
7.6/7.4	12	12
7.4	2	3
Expected		
7.6	30.50	30.50
7.6/7.4	12.00	12.00
7.4	2.50	2.50
X^2_2	0.216	
p	0.90 (NS)	

3.2.5 Insulin Gene polymorphism

54 parents of the high/high and low/low groups of offspring were genotyped for the insulin/*SacI* polymorphism. 28 parents were classified as having high blood pressure and 26 parents as having low blood pressure. The genotype and allele frequencies of the two groups are presented in Table 28. The allele frequencies of all subjects examined are 0.36 for the 7.5kb allele and 0.64 for the 6.0kb allele., similar to those reported previously (Bell et al., 1982). Chi-square analysis was performed to determine if a difference between the genotype distribution in the two parental groups exists (Table 29). However, no statistically significant difference was observed (X_2^2 =2.94 p=0.20-0.50).

Table 28:Genotype and allele frequencies for the E (7.0kb) and e (6.5kb) alleles for the *SacI*/insulin gene RFLP in parents with high (HBP) and low blood pressure (LBP).

Group	n	Frequencies Genotypes EE	Ee	ee	Alleles E	e
HBP	28	5	10	13	0.36	0.64
LBP	26	2	15	9	0.37	0.63
Total	54	7	25	22	0.36	0.64

Table 29:Comparison of the *SacI*/insulin gene RFLP distribution in parental groups with high blood pressure (HBP) and low blood pressure (LBP).

Genotype	Number of subjects HBP	LBP
Observed		
7.0	5	2
7.0/6.5	10	15
6.5	13	9
Expected		
7.0	3.63	3.37
7.0/6.5	12.96	12.04
6.5	11.41	10.59
X^2_2	2.943	
p	0.20-0.50 (NS)	

3.2.6 Glucocorticoid receptor gene polymorphism

3.2.6.1 *TthIII* RFLP

55 parents of the high/high and low/low group of offspring were genotyped for the Tth III/glucocorticoid receptor gene polymorphism (Detera Wadleigh et al ., 1991). 27 parents were classified as having high blood pressure and 28 as having low blood pressure. Genotype and allele frequencies of the two parental groups are presented in Table 30. The allele frequencies of all subjects examined are 0.21 for the 3.8kb allele (F), and 0.79 for the 3.4kb allele (f). These allele frequencies are similar to those previously reported (Detera-Wadleigh et al., 1991). Chi-square analysis was performed to determine if a difference between the genotype distribution in the two parental groups exists (Table 31). However, no statistically significant difference was observed (X^2_2=0.58 p=0.98).

3.2.6.2 *BclI* RFLP

Using a 4.4kb cDNA probe 76 parents were genotyped for the *BclI* polymorphism of the glucocorticoid receptor (Murray et al., 1987). 42 parents were classified as having high blood pressure and 34 parents as having low blood pressure. Homozygotes for the *BclI* polymorphism have either a 5.3kb band or a 4.2kb band, and heterozygotes have the 5.3kb and 4.2kb band. Genotype and allele frequencies of the two groups are presented in Table 32. The genotypes have been abreviated to GG, Gg and gg, where the 5.3kb allele has been designated (G) and the 4.3kb allele (g). The allele frequencies of all subjects examined are 0.40 for the 4.5kb allele (G) and 0.60 for the 2.3kb allele (g), similar to those reported previously (Murray et al., 1987). Chi-square

analysis was performed to determine if a difference between the genotype distribution in the two parental groups exists (Table 34). No statistically significant difference was observed (X^2_2 =0.56 P=0.50=0.80).

Table 30: Genotype and allele frequencies for the G (3.8kb) and g (3.4kb) alleles for the *TthIII*/glucocorticoid receptor gene RFLP in parents with high (HBP) and low blood pressure (LBP).

Group		Frequencies				
		Genotypes			Alleles	
	n	FF	Ff	ff	F	f
HBP	27	1	9	17	0.20	0.80
LBP	28	1	10	17	0.21	0.79
Total	55	2	19	34	0.21	0.79

Table 31: Comparison of the *TthIII*/glucocorticoid receptor gene RFLP distribution in parental groups with high blood pressure (HBP) and low blood pressure (LBP).

Genotype	Number of subjects	
	HBP	LBP
Observed		
3.8	1	1
3.8/3.4	9	10
3.4	17	17
Expected		
3.8	0.98	1.02
3.8/3.4	9.33	9.67
3.4	16.69	17.31
X^2_2	0.58 *	
p	0.98-0.99	

* Yates Correction Theorem applied since chi-square approximation probably invalid since 1 cell with counts less than 1.0

Table 32: Genotype and allele frequencies for the G (5.3kb) and g (4.2kb) alleles for the *BclI*/glucocorticoid receptor gene RFLP in parents with high (HBP) and low blood pressure (LBP).

Group	n	Frequencies Genotypes GG	Gg	gg	Alleles G	g
HBP	42	6	19	17	0.25	0.75
LBP	34	6	17	11	0.43	0.57
Total	76	12	36	28	0.40	0.60

Table 33: Comparison of the *BclI*/glucocorticoid receptor gene RFLP distribution in parental groups with high blood pressure (HBP) and low blood pressure (LBP).

Genotype	Number of subjects HBP	LBP
Observed		
4.5	6	6
4.5/2.3	19	17
2.3	17	11
Expected		
4.5	6.63	5.37
4.5/2.3	19.89	16.11
2.3	15.47	12.53
X^2_2	0.561	
p	0.50–0.80 (NS)	

3.2.7 Beta-fibrinogen Gene polymorphism

Using a 1.4kb genomic probe of the beta-fibrinogen gene 73 parents were genotyped for the *BclI* polymorphism of this gene (Cook et al., 1988). 40 were classified as having high blood pressure and 33 parents as having low blood pressure. Homozygotes for the *BclI* polymorphism have either a 5.3kb or a 4.2kb band, and heterozygotes have both the 5.3kb and 4.2kb bands. Genotypes and allele frequencies of the two parental groups are present in Table 34. The genotypes have been abbreviated to HH, Hh and hh, where the 5.3kb allele has been designated (H) and the 4.2kb allele (h). The allele frequencies of all subjects examined are 0.82 for the 5.3kb allele (H) and 0.18 for the 4.2kb allele (h), similar to those reported previously (Cooke et al., 1988). Chi-square analysis was performed to determine if a difference between the genotype distribution in the two parental groups exists (Table 35). However, no statistically significant difference was observed (X_2^2=2.61 p=0.20-0.50).

Table 34: Genotype and allele frequencies for the H (5.3kb) and h (4.2kb) alleles for the *BclI*/beta-fibrinogen gene RFLP in parents with high (HBP) and low blood pressure (LBP).

Group	n	Frequencies Genotypes HH	Hh	hh	Alleles H	h
HBP	40	24	15	1	0.79	0.21
LBP	33	23	10	0	0.85	0.15
Total	73	47	25	1	0.82	0.18

Table 35: Comparison of the *BclI*/beta-fibrinogen gene RFLP distribution in parental groups with high blood pressure (HBP) and low blood pressure (LBP).

Genotype	Number of subjects HBP	LBP
Observed		
5.3	24	23
5.3/4.2	15	10
4.2	1	0
Expected		
5.3	25.75	21.25
5.3/4.2	13.70	11.30
4.2	0.55	0.45
X^2_2	2.61 *	
p	0.20-0.50 (NS)	

* Yates Correction Theorem applied since chi-square approximation probably invalid since 2 cells with expected counts less than 1.00

3.3 Sib-Pair RFLP Analysis

The 200 offspring identified in Phase II of the the Ladywell Study included 20 sib-pairs. Phase V of the study identified an additional 17 sib-pairs, thus increasing the number of sib-pairs to give a total of 37. DNA from these offspring was isolated and genotyped for seven RFLPs, for sib-pair analysis. The sib-pairs were divided into two groups ie. like sib-pairs, where both sibs have either high blood pressure or low blood pressure, and unlike sib-pairs where one sib has high blood pressure and the other has low blood pressure. 19 of the sib-pairs were classified as like-sibs and 18 as unlike-sibs. The sib-pairs were then classified as marker concordant if they share the same allele, marker discordant if the marker genotype share no common alleles, and marker half concordant if neither concordant or discon-cordant. The number of observed and expected sib-pairs falling into each of the catergories was then tested by chi-square analysis. The expected probabilites are a function of the allele frequencies, and are calculated from mating type probabilites.

3.3.1 Renin gene polymorphism

A total of 33 sib-pairs were genotyped for the *HindIII*/renin gene RFLP. 19 of the sib-pairs were classified as like sib-pairs and 14 as unlike sib-pairs. The number of like and unlike sibs-pairs sharing 2 alleles (marker concordant), 1 allele (marker half concordant) and 0 alleles (marker disconcordant) are presented in Table 36. The allele frequencies for the offspring population were 0.67 for the 9.0kb allele (A) and 0.33 for the 6.2kb allele (a) as calculated in section 3.1.1. These frequencies were used to calculate the expected number of

sib-pairs falling into the three categories. The expected proportions of sib-pairs sharing 2, 1 and 0 alleles are 0.60, 0.35 and 0.05 respectively.

The observed and expected number of sib-pairs (like and unlike) for the three catergories are presented in Table 37. Chi-square analysis was performed between the observed and expected numbers and showed a statistically significant lack of allele sharing in like sibs-pairs (X_2^2=9.48 p<0.01).

Table 36:Number of marker concordant, discordant and half concordant like and unlike sib-pairs for the alleles of the *HindIII*/renin gene RFLP.

Category	Sib-Pairs	
	Like	Unlike
Discordant	0	0
Half concordant	11	3
Concordant	8	11
Total	19	14

Table 37:Comparison of the observed and expected number of like and unlike sib-pairs sharing 2, 1 or 0 alleles of the *HindIII*/renin gene RFLP.

Shared Alleles	Sib-pairs	
	Like	Unlike
Observed		
0	0	0
1	11	3
2	8	11
Expected		
0	0.95	0.70
1	6.65	4.90
2	11.40	8.40
X^2_2	9.48 *	
p	<0.01 (S)	

* Yates correction theorem applied, since chi-square approximation probably invalid since two cells with expected counts less than 1.00

3.3.2 Beta-2-adrenergic receptor gene RFLP

A total of 33 sib-pairs were genotyped for the *BanI*/beta-2-adrenergic receptor gene polymorphism. 18 of the sib-pairs were classified as like and 15 as unlike sib-pairs. The number of like and unlike sib-pairs sharing 2 alleles (marker concordant), 1 allele (marker half concordant) and 0 alleles (marker disconcordant) are presented in Table 38. The allele frequencies for the offspring population were 0.25 for the 3.4kb allele (B) and 0.75 for the 3.7kb allele (b) as calculated in Section 3.1.2. These frequencies were used to calculate the expected number of sib-pairs falling into the three categories. The expected proportions of sib-pairs sharing 2, 1 and 0 alleles are 0.66, 0.30 and 0.04 respectively.

The observed and expected number of sib-pairs (like and unlike) for the three catergories are presented in Table 39. Chi-square analysis was performed between the observed and expected numbers, however no statistically significant difference was found (X^2_2=4.65 p=0.1-0.05).

Table 38: Number of marker concordant, discordant and half concordant like and unlike sib-pairs for the alleles of the *BanI*/beta-2-adrenergic receptor gene RFLP.

Category	Sib-Pairs	
	Like	Unlike
Discordant	0	1
Half concordant	4	4
Concordant	14	10
Total	18	15

Table 39: Comparison of the observed and expected number of like and unlike sib-pairs sharing 2,1 or 0 alleles for the *BanI*beta-2-adrenergic receptor gene RFLP.

Shared Alleles	Sib-pairs	
	Like	Unlike
Observed		
0	0	1
1	4	4
2	14	10
Expected		
0	0.72	0.60
1	5.40	4.50
2	11.88	9.90
X^2_2	4.65 *	
p	0.1-0.05 (NS)	

* Yates correction theorem applied since chi-square approximation probably invalid since two cells with expected counts less than 1.00

3.3.3 Beta-1-adrenergic receptor gene RFLP

A total of 24 sib-pairs were genotyped for the *BglI*/beta-1-adrenergic receptor gene polymorphism. 10 of the sib-pairs were classified as like and 14 as unlike sib-pairs. The number of like and unlike sib-pairs sharing 2 alleles (marker concordant), 1 allele (marker half concordant) and 0 alleles (marker disconcordant) are presented in Table 40. The allele frequencies for the offspring population were 0.78 for the 6.2kb allele (C) and 0.22 for the 4.7kb allele (c) as calculated in Section 3.1.3. These frequencies were used to calculate the expected number of sib-pairs falling into the three categories. The expected proportions of sib-pairs sharing 2, 1 and 0 alleles are 0.68, 0.29 and 0.03 respectively.

The observed and expected number of sib-pairs (like and unlike) for the three catergories are presented in Table 41. Chi-square analysis was performed between the observed and expected numbers, and no statistically significant difference was found (X^2_2=5.79 p=0.1-0.05).

Table 40: Number of marker concordant, discordant and half concordant like and unlike sib-pairs for the alleles of the *BglI*/beta-1-adrenergic receptor gene RFLP.

Category	Sib Pairs	
	Like	Unlike
Discordant	0	0
Half concordant	2	6
Concordant	8	8
Total	10	14

Table 41: Comparison of the observed and expected number of like and unlike sib-pairs sharing 2, 1 or 0 alleles for the *BglI*/beta-1-adrenergic receptor gene RFLP.

Shared Alleles	Sib-pairs	
	Like	Unlike
Observed		
0	0	0
1	2	6
2	8	8
Expected		
0	0.30	0.42
1	2.90	4.06
2	6.80	9.38
X^2_2	5.79 *	
p	0.1-0.05 (NS)	

* Yates correction theorem applied since chi-square approximation probably invalid since two cells with expected counts less than 1.00

3.3.4 Alpha-2-adrenergic receptor gene RFLP

A total of 16 sib-pairs were genotyped for the *DraI*/alpha-2-adrenergic receptor gene polymorphism. 8 of the sib-pairs were classified as like and 8 as unlike sib-pairs. The number of like and unlike sib-pairs sharing 2 alleles (marker concordant), 1 allele (marker half concordant) and 0 alleles (marker disconcordant) are presented in Table 42. The allele frequencies for the offspring population were 0.86 for the 6.7kb allele (D) and 0.14 for the 6.3kb allele (d) as calculated in Section 3.1.4. These frequencies were used to calculate the expected number of sib-pairs falling into the three categories. The expected proportions of sib-pairs sharing 2, 1 and 0 alleles are 0.86, 0.14 and 0.01 respectively.

The observed and expected number of sib-pairs (like and unlike) for the three catergories are presented in Table 43. Chi-square analysis was perfomed between the observed and expected numbers and no statistically significant difference was found (X^2_2=1.40 p=0.5-0.8).

Table 42: Number of marker concordant, discordant and half concordant like and unlike sib-pairs for the alleles of the *DraI*/alpha-2-adrenergic receptor gene RFLP.

Category	Sib-Pairs	
	Like	Unlike
Discordant	0	0
Half concordant	2	1
Concordant	6	7
Total	8	8

Table 43: Comparison of the observed and expected number of like and unlike sib-pairs sharing 2,1 or 0 alleles for the *DraI*/alpha-2-adrenergic receptor gene RFLP.

Shared Alleles	Sib-pairs	
	like	unlike
Observed		
0	0	0
1	2	1
2	6	7
Expected		
0	0.08	0.08
1	1.68	1.68
2	6.24	6.24
X_2^2	1.40 *	
p	0.5-0.8 (NS)	

* Yates correction theorem applied since chi-square approximation probably invalid since two cells with expected counts less than 1.00

3.3.5 Insulin gene RFLP

A total of 19 sib-pairs were genotyped for the *SacI*/insulin gene polymorphism. 11 of the sib-pairs were classified as like and 8 as unlike sib-pairs. The number of like and unlike sibs pairs sharing 2 alleles (marker concordant), 1 allele (marker half concordant) and 0 alleles (marker disconcordant) are presented in Table 44. The allele frequencies for the offspring population were 0.37 for the 7.5kb allele (E) and 0.63 for the 6.0kb allele (e) as calculated in Section 3.1.5. These frequencies were used to calculate the expected number of sib-pairs falling into the three categories. The expected proportions of sib-pairs sharing 2, 1 and 0 alleles are 0.60, 0.35 and 0.05 respectively.

The observed and expected number of sib-pairs (like and unlike) for the three catergories are presented in Table 45. Chi-square analysis was performed between the observed and expected numbers, and no statistically significant difference was found (X^2_2=1.97 p=0.20-0.50).

Table 44: Number of marker concordant, discordant and half concordant like and unlike sib-pairs for the alleles of the *SacI*/insulin gene RFLP.

Category	sib-pairs like	unlike
Disconcordant	0	1
Half concordant	5	2
Concordant	6	5
Total	11	8

Table 45: Comparison of the observed and expected number of like and unlike sib-pairs sharing 2,1 or 0 alleles for the *SacI*/insulin gene RFLP.

Shared Alleles	Sib-pairs Like	Unlike
Observed		
0	1	1
1	5	2
2	6	5
Expected		
0	0.60	0.40
1	4.20	2.80
2	7.2	4.80
X^2_2	1.97 *	
p	0.20-0.50 (NS)	

* Yates correction theorem applied since chi-square approximation probably invalid since two cells with expected counts less than 1.00

3.3.6 Glucocorticoid receptor gene RFLPs

3.3.6.1 *Tth III* RFLP

A total of 15 sib-pairs were genotyped for the *TthIII*/glucocorticoid receptor gene polymorphism. 10 of the sib-pairs were classified as like and 5 as unlike sib-pairs. The number of like and unlike sib-pairs sharing 2 alleles (marker concordant), 1 allele (half concondant) and 0 alleles (marker discordant) are presented in Table 46. The allele frequencies for the offspring population were 0.21 for the 3.8kb allele (F) and 0.79 for the 3.4kb allele (f), as calculated in section 3.1.6. These frequencies were used to calculate the expected number of sib-pairs falling into the three catergories. The expected proportions of sib-pairs sharing 2, 1 and 0 alleles are 0.68 0.29 0.03 respectively.

The observed and expected numbers of sib-pairs (like and unlike) for the three catergories are presented in Table 47. Chi-square analysis was performed between the observed and expected numbers, and no statistically significant difference was found

(X^2_2=2.94 p=0.20-0.50).

3.3.6.2 *BclI* RFLP

A total of 27 sib-pairs were genotyped for the *BclI*/glucocorticoid receptor gene polymorphism. 16 of the sib-pairs were classified as like and 11 as unlike sib-pairs. The number of like and unlike sibs pairs sharing 2 alleles (marker concordant), 1 allele (marker half concordant) and 0 alleles (marker disconcordant) are presented in Table 48. The allele frequencies for the offspring population were 0.40kb for the 5.4kb allele (G) and 0.60 for the 2.3kb allele (g)

(Watt 1992). These frequencies were used to calculate the expected number of sib-pairs falling into the three categories. The expected proportions of sib-pairs sharing 2, 1 and 0 alleles are 0.58, 0.37 and 0.05 respectively.

The observed and expected number of sib-pairs (like and unlike) for the three catergories are presented in Table 49. Chi-square analysis was performed between the observed and expected numbers, and a statistically significant difference was not found (X^2_2=3.21 p=0.20).

Table 46: Number of marker concordant, discordant and half concordant like and unlike sib-pairs for the alleles of the *TthIII*/glucocorticoid receptor gene RFLP.

Category	Sib-Pairs	
	Like	Unlike
Discordant	1	1
Half concordant	3	1
Concordant	6	3
Total	10	5

Table 47: Comparison of the observed and expected number of like and unlike sib-pairs sharing 2,1 or 0 alleles for the *TthIII*/glucocorticoid receptor gene RFLP.

Shared Alleles	Sib-pairs	
	Like	Unlike
Observed		
0	1	1
1	3	1
2	6	3
Expected		
0	0.30	0.15
1	2.90	1.45
2	6.80	3.40
X^2_2	2.94 *	
p	0.2-0.50 (NS)	

*Yates correction theorem applied since chi-square approximation probably invalid since two cells with expected counts less than 1.00

Table 48: Number of marker concordant, discordant and half concordant like and unlike sib-pairs for the alleles of the *BclI*/glucocorticoid receptor gene RFLP.

Catergory	Sib Pairs	
	Like	Unlike
Discordant	0	1
Half concordant	5	5
Concordant	11	5
Total	16	11

Table 49: Comparison of the observed and expected number of like and unlike sib-pairs sharing 2,1 or 0 alleles for the *BclI*/glucocorticoid receptor gene RFLP.

Shared Alleles	Sib-pairs	
	Like	Unlike
Observed		
0	0	1
1	5	5
2	11	5
Expected		
0	0.88	0.55
1	5.92	4.07
2	9.28	6.38
X_2^2	3.21 *	
p	0.20	

* Yates correction theorem applied since chi-square approximation probably invalid since two cells with expected counts less than 1.00

3.3.7 Beta-fibrinogen gene RFLP

A total of 30 sib-pairs were genotyped for the *BclI*/beta-fibrinogen gene polymorphism. 16 of the sib-pairs were classified as like and 14 as unlike sib-pairs. The number of like and unlike sib-pairs sharing 2 alleles (marker concordant), 1 allele (marker half concordant) and 0 alleles (marker disconcordant) are presented in Table 50. The allele frequencies for the offspring population were 0.84 for the 5.3kb allele (G) and 0.16 for the 4.2kb allele (g) (Wood 1990). These frequencies were used to calculate the expected number of sib-pairs falling into the three categories. The expected proportions of sib-pairs sharing 2, 1 and 0 alleles are 0.74, 0.24 and 0.02 respectively. The observed and expected number of sib-pairs (like and unlike) for the three catergories are presented in Table 51. Chi-square analysis was performed between the observed and expected numbers, and showed a statistically significant lack of allele sharing in like sibs (X^2_2=6.46 $p<0.05$).

Table 50: Number of marker concordant, discordant and half concordant like and unlike sib-pairs for the alleles of the *BclI*/beta-fibrinogen gene RFLP.

Category	Sib-Pairs	
	Like	Unlike
Discordant	1	1
Half concordant	8	3
Concordant	7	10
Total	16	14

Table 51: Comparison of the observed and expected number of like and unlike sib-pairs sharing 2,1 or 0 alleles for the *BclI*/beta-fibrinogen gene RFLP.

Shared Alleles	Sib-pairs	
	Like	Unlike
Observed		
0	1	1
1	8	3
2	7	10
Expected		
0	0.32	0.28
1	3.84	3.36
2	11.84	10.36
X^2_2	6.46 *	
p	<0.05 (S)	

* Yates correction theorem applied since chi-square approximation probably invalid since two cells with expected counts less than 1.00

3.4 Restriction Fragment Length polymorphism screening

3.4.1 Beta-2-adrenergic receptor gene

A 2.6kb genomic DNA probe of the beta-2-adrenergic receptor gene, was used to detect polymorphisms by hybridisation to a series of Southern blot filters containing genomic DNA from 10-20 unrelated individuals digested with a series of restriction enzymes.

The restriction enzymes tested were;

AluI, ApaI, AvaI, AvaII,Bam HI, BclI, BglI, BglII, Bst NI, CfoI, DdeI, DraI, EcoRI, HaeIII,HgiIA, HinDIII, HinfI, HpaI, KpnI, MspI, NciI, NotI, PstI, PvuII, RsaI, SacI, SalI, TaqI, XbaI

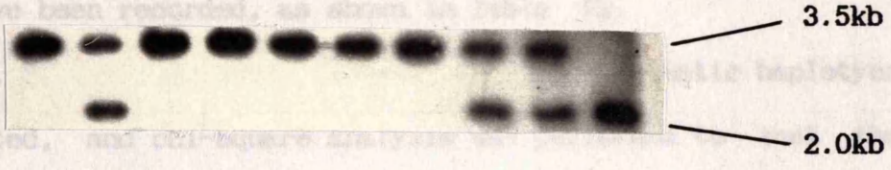
For all the above enzymes, no polymorphic band pattern was observed on autoradiography.

3.4.2 Glucocorticoid receptor gene

A 4.4kb cDNA probe of the glucocorticoid receptor gene was used to detect polymorphisms. The restriction enzymes used are listed in section 3.4.1. For the enzymes tested, with the exception of the enzyme *HgiIA*, no polymorphic band patterns were observed. However, *HgiIA* digested DNA on hybridisation to a radiolabelled glucocorticoid receptor probe identified a two allele polymorphism with band sizes of 3.5kb and 2.0kb. From the total of 20 individuals tested the frequency of the alleles were 0.75 for the 3.5kb allele and 0.25 for the 2.0kb allele.

Fig. 3 shows the polymorphic band pattern observed.

Figure 3: Polymorphic band pattern on hybridisation of radiolabelled glucocorticoid receptor gene probe to Southern blots of *HgiIA* digested DNA.



3.5 Haplotype analysis and linkage disequilibrium

The *BclI* RFLP of the glucocorticoid receptor gene has previously been examined in the offspring population (Watt et al., 1992). The allele frequencies were found to be 0.40 for the 5.3kb allele (G) and 0.60 for the 3.2kb allele (g). The *Tth III* polymorphism was analysed in the offspring population (See Section 3.1.6), with allele frequencies of 0.23 for the 3.8kb allele (F) and 0.77 for the 3.4kb allele (f). 119 offspring have thus been genotyped for both RFLPS and nine different haplotypes have been recorded, as shown in Table 52.

The number of, FG (++), Fg (+−), fG (−+) and fg (−−) gametic haplotyes were calculated, and chi-square analysis was performed to test the hypothesis that no difference between the observed and expected gametic haplotype distribution exists (Table 53). No statistically significant difference was found such that the observed gametic haplotype distribution is similar to the expected distribution for random association, and are therefore in linkage equilibrium (X^2 =2.04 p=0.10–0.20).

In order to confirm the linkage equlilibrium between the two markers of the glucocorticoid receptor, the gametic frequencies were calculated from observed numbers of gametic haplotypes, and the frequencies were FG=0.09, Fg=0.108, fG=0.302 and fg=0.50. These frequencies were then used to estimate the linkage disequilibrium parameter (D) as follows according to Hart (1988);

Linkage disequilibrium (D) = (a x b)- (c x d)

where a= frequency of FG haplotype
b= frequency of fg haplotype
c= frequency of Fg haplotype
d= frequency of fG haplotype

D=0 corresponds to linkage equilibrium

For the allele frequencies F, f, G, g the smallest (Dmin) and the largest (Dmax) possible values of D are as follows;

Dmin = -FG or -fg (whichever is largest)

Dmax = Fg or fG (whichever is smallest)

The estimated value of D is therefore 0.012. The Dmax value from the allele frequencies was calculated as 0.138, such that the amount of disequilibrium is 8.7% of its theoretical maximum. Therefore, linkage equlibrium exists between the *BclI* and the *TthIII* restriction site polymorphisms.

Table 52: The nine different halotypes of the two RFLPS of the glucocorticoid receptor and their distribution between the four groups of offspring, and in the whole popuation.

Group Haplotype	A	B	C	D	Total
1 (FFGG)	2	0	1	0	3
2 (FfGG)	2	2	0	2	6
3 (ffGG)	1	5	2	1	9
4 (FFGg)	0	2	5	0	7
5 (FfGg)	4	5	5	2	13
6 (ffGg)	8	11	9	12	40
7 (FFgg)	1	0	1	1	3
8 (Ffgg)	1	1	6	2	10
9 (ffgg)	9	4	2	13	28

Table 53: Comparison of observed and expected numbers of the four different gametic haplotypes (++/+−/−+/−−), for the two restriction sites.

Haplotype	No. chromosomes
Observed	
FG (++)	19
Fg (+−)	23
fG (−+)	64
fg (−−)	106
Expected	
FG (++)	19.50
Fg (+−)	29.26
fG (−+)	65.30
fg (−−)	97.94
X^2_1	2.042
p	0.10–0.20 (NS)

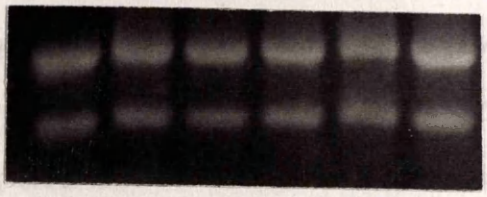
Where F and f are the alleles of the *TthIII* RFLP
where G and g are the alleles of the *BclI* RFLP

3.6 Mutational Screening of the Glucocorticoid receptor cDNA

3.6.1 RNA extraction and quantitation

Total cellular RNA was extracted from lymphoblastoid cell lines from 10 offspring of the Ladywell study. Five of the subjects were assigned to Group B (high personal and high parental blood pressure) and five to Group C (low personal and low parental blood pressure). The acid guanidium thiocynate chloroform method was used for the preparation of RNA samples (Chomczynski & Sacchi, 1989). The concentration of the RNA samples were determined by spectrophotometry at 260nm and the purity of the sample determined by the value of OD (260nm)/OD (280nm). The mean total cellular RNA concentration was 700ug/ml. RNA samples were electrophoresed on checker gels to assess the integrity of the RNA preparation. Results of fractionation of RNA samples are shown in fig. 4.

Figure 4:RNA electrophoresis



The upper band represents the 28S rRNA and the lower band the 18S rRNA, and the smear represents the presence of RNA

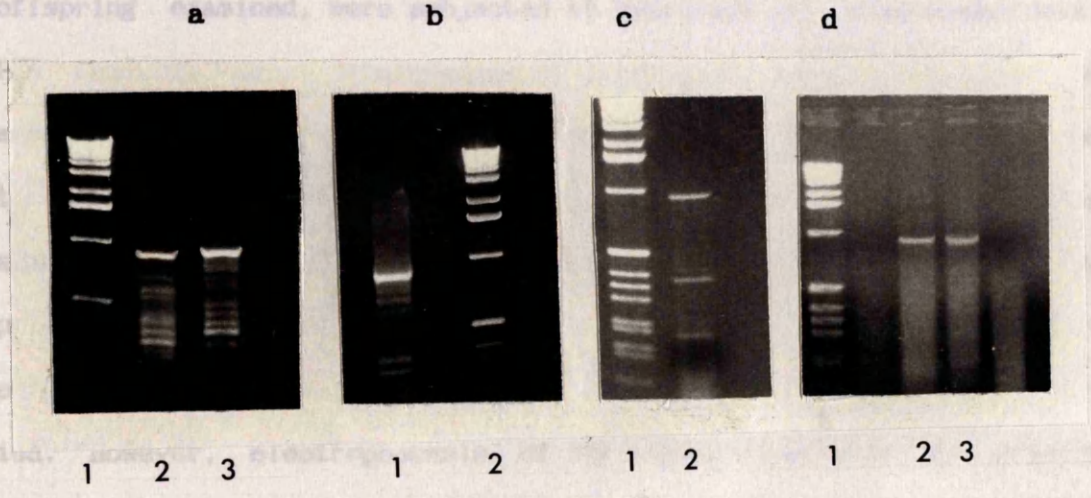
3.6.2 Reverse transcription-PCR Optimisation

Three sets of synthetic oligonucleotide primers were designed for amplification of the glucocorticoid receptor (HGR) cDNA with the aid of the Oligo computer program (Rychilk & Rhoads, 1989). For amplification it was necessary to transcribe the RNA template to produce cDNA. Reverse transcription (RT) was performed using the most upstream primer to initiate transcription.

Optimisation of RT-PCR (Polymerase Chain Reaction) was first performed on control RNA samples, and once optimised, RT-PCR was performed on total cellular RNA from 10 offspring of the Ladywell study, such that the cDNA of the HGR was amplified. A series of RT-PCR optimisation experiments were performed to determine the optimal annealing temperature for each PCR cycle, such that only the PCR product of interest was amplified and non-specific amplification was eliminated. The initial annealing temperature used was the average Tm (melting temperature) of each primer pair. Once the PCR product of interest was amplified, additional PCR products were minimised by increasing the annealing temperature. Fig. 5 shows a series of optimisation experiments performed for primers 238R/239R.

For all subjects examined the correct sized fragments were amplified for all three sets of primers, thus excluding any major gene deletions or truncated mRNA products.

Figure 5: PCR optimisation



5a: Lane 1, 1kb marker, lanes 2-3, RT-PCR amplification with primers 238R/239R, at an annealing temperature of 54°C

5b: lane 1, 1kb marker, lane 2, RT-PCR amplification with primers 238R/239R, at an annealing temperature of 56°C

5c: Lane 1, 1kb marker, lane 2 RT-PCR with primers 238R/239R, at an annealing temperature of 57°C

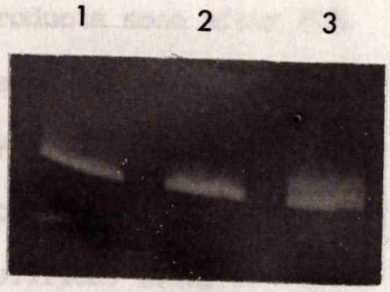
5d: Lane 1, 1kb ladder, lane 2-3, RT-PCR with primers 238R/239R at an annealing temperature of 58°C.

3.6.3 Hydrolink Gel Electrophoresis

The three PCR amplified regions of the HGR cDNA of the 10 Ladywell offspring examined, were subjected to Hydrolink gel electrophoresis. PCR products were denatured at 95°C for 5 minutes and allowed to anneal over a period of two hours at 70°C, and electrophoresed through Hydrolink gel matrixes. Ethidium bromide staining of the gels showed no evidence of heteroduplex formation for regions amplified by primers 236R/241R and 237R/240R. This suggests no DNA sequence differences exist in these regions of the HGR cDNA for the offspring studied. However, electrophoresis of the region amplified by primers 238R/239R, showed four of the ten offspring to have a second band migrating closely above the first band, representing the formation of a heteroduplex between a normal and mutant DNA strand. This additional band was not visible after agarose gel electrophoresis of the PCR products. Two of these offspring were assigned to Group B (high/high) (subject A and B) and two to group C (low/low) (Subject C and D). To ensure the validity of these results, each sample was run on several different gels, using different PCR products.

These results are presented in fig. 6.

Figure 6: Hydrolink gel electrophoresis



6: Lanes 1-2, ethidium bromide staining of a Hydrolink gel showing homoduplex formation in the region of the HGR cDNA amplified by primers 238R/239R, lane 3, ethidium bromide staining of a Hydrolink gel showing the presence of a second band above the homoduplex band ie. heteroduplex formation.

3.6.4 Asymmetric reamplification

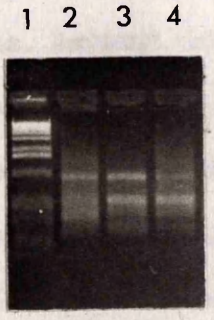
In attempts to characterise the additional band identified by Hydrolink gel electrophoresis in four of the offspring, asymmetric PCR were performed on dsDNA PCR products for the generation of ssDNA, for direct sequencing. In some instances RT-PCR resulted in non specific amplification products in addition to the expected PCR product, even after optimisation of the PCR parameters. Fig. 7 shows an example of the spurious PCR products seen after PCR. The PCR products were therefore fractionated on agarose gels and the cDNA eluted from agarose, for asymmetric PCR.

A series of asymmetric PCR optimisation experiments were performed for each region to be amplified, using differing ratios of the two synthetic oligonucleotides ie. 1:100, 1:75 and 1:50. Asymmetric PCR were performed using both primers limiting, such that both cDNA strands could be amplified. Asymmetric PCR using primer 239R as the limiting factor was unsuccessful at generating ssDNA, even when differing ratios of the two primers were used. However, asymmetric PCR was successful using primer 238R as the limiting primer. Fig.8 shows results from asymmetric PCR optimisation experiments.

Figure 7: Spurious PCR products

7: Lanes 1-3, RT-PCR using primers 238R/239R, the upper band represents the PCR product of interest, lanes 1 & 2 show spurious PCR products as a result of primer annealing to a non-target sequence. Lane 4, 1kb marker

Figure 8: Asymmetric PCR optimisation



8: Lane 1, 1kb marker, lane 2, asymmetric PCR using a ratio of 1:50 of primers 238R/239R, Lane 3, asymmetric PCR using a ration of 1:75 of primers 238R/239R, Lane 4, asymmetric PCR using a ratio of 1:50 of primers 238R/239R

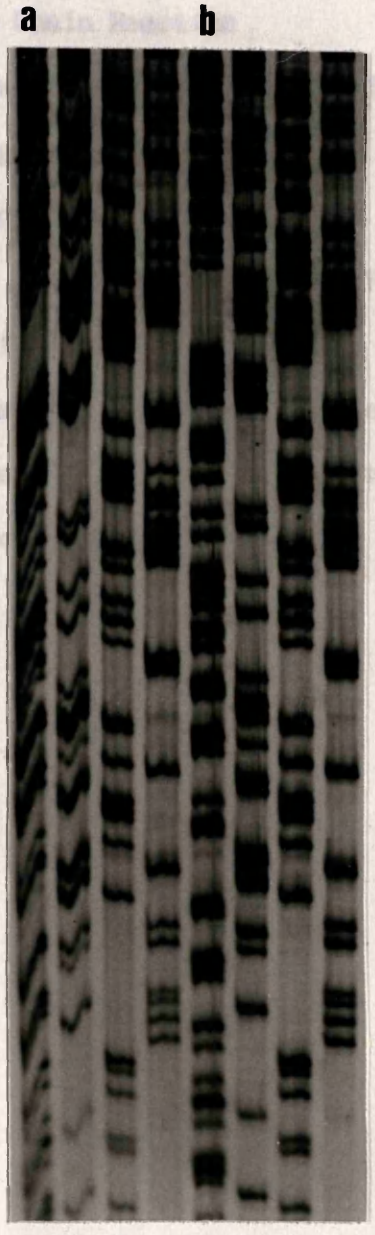
3.6.5 Direct sequencing of ssPCR amplified DNA templates

Single strand PCR products generated through asymmetric PCR, were sequenced. The PCR products were purified by selective precipitation in 2M ammonium acetate and isopropanol. Sequencing was performed with Sequenase enzyme using 100% of the purified PCR product and 1pmol of the limiting primer as a sequencing primer.

Several problems were encountered in attempts to perform direct sequencing of ssDNA products. Asymmetric PCR performed with primer 239R as the limiting primer was unsuccessful at generating ssDNA, however, when primer 238R was the limiting primer asymmetric PCR was successful, and a sequence was obtained. Fig.9 shows the sequence ladder produce from a Group B (subject A) and Group C (subject C) ladywell offspring using 238R as the sequencing primer. 400 bp of the 3' region of the PCR segment amplified by primers 238R/239R was sequenced in subjects A and C. No sequence difference was observed between these individuals, such that no molecular variants exist in the region sequenced in the subjects examined.

It was also found that although the asymmetric PCR was successful, sequencing was problematic due to the presence of ambiguous sequencing ladders caused by the presence of the same sequencing band in all four lanes.

Figure 9: Partial sequence of the glucocorticoid receptor cDNA



9a: Partial sequence of the HGR cDNA from RNA of subject A, using primer 238R as the limiting primer for sequencing

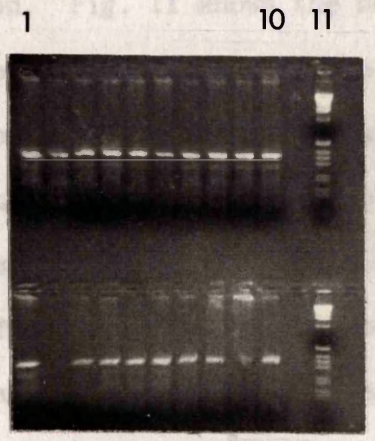
9b: Partial sequence of the HGRc DNA from RNA of subject B, using primer 238R as the limiting primer for sequencing

3.7 Mutational Screening of the angiotensinogen gene

3.7.1 Polymerase Chain Reaction

12 Ladywell subjects were identified with polarised predispositions to the development of high blood pressure ie. 6 from Group B (high/high) and 6 from Group C (low/low). All five exons and the 5' region of the angiotensinogen gene for all subjects were PCR amplified using nine sets of primers. Fig 10, shows an example of the PCR product generated using primers 2a1/2a2. All subjects examined for the nine sets of primers resulted in amplification of the correct sized fragments, thus excluding any major gene length mutations in these offspring.

Figure 10: PCR of a region of exon II of the angiotensinogen gene



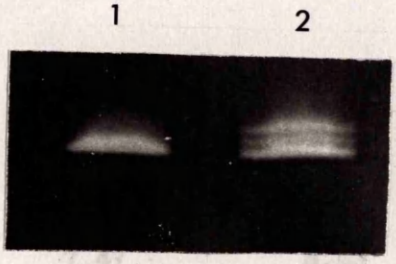
10: Lanes 1-10, PCR amplified DNA of Ladywell offspring using primers 2c1/2c2, Lane 11, 1kb marker

3.7.2 Hydrolink gel electrophoresis

The amplified products for each primer pair in all 12 offspring were subjected to hydrolink gel electrophoresis. No evidence for heteroduplex formation was seen for PCR products amplified by primer sets; 5a1/5a2, 5b1/5b2, 5c1/5c2, 2a1/2a2, 2b1/2b2, 3-1/3-2, 4-1/4-2 and 5-1/5-2. However, Hydrolink gel electrophoresis of the region amplified by primers 2c-1 and 2c-2 showed 4 out of the 12 offspring to show heteroduplex formation. Fig. 11 shows the heteroduplexes identified by Hydrolink gel electrophoresis. As can be seen from fig. 11, two additional bands are present above the homoduplex band, suggesting the presence of two molecular variants. This double heteroduplex band was seen in all four of the offspring.

Three of the four offspring exhibiting heteroduplex formation were assigned to Group C, and only 1 to Group B. Additional offspring from Group B, and Group C, were then analysed for heteroduplex formation. Thirty-five Group B subjects were studied, and six heteroduplexes detected, whereas in Group C, fourty subjects were studied and 10 heteroduplexes identified. Chi-square analysis was performed to determine if the distribution of offspring with heteroduplexes amongst the two groups was statistically significant. However, no statistically significant difference was observed (Table 54).

Figure 11: Hydrolink gel electrophoresis



11: Lanes 1, ethidium bromide staining of a hydrolink gel, showing the presence of a homoduplex band, in the region of the angiotensinogen gene amplified by primers 2a1/2a2, lane 2, shows the formation of two additional bands above the homoduplex band, representing heteroduplex formation.

Table 54: Comparison of the distribution of offspring showing heteroduplex formation between Group B (high offspring and high parental blood pressure) and Group C (low offspring and low parent blood pressure).

Heteroduplex Formation	Group B	Group C	Total
Observed			
Positive	6	10	16
Negative	29	30	59
Expected			
Positive	7.47	8.53	
Negative	27.53	31.47	
X_1^2	0.687		
p	0.20-0.50 (NS)		

3.8 Analysis of the T174M and the M235T molecular variants in exon II of the angiotensinogen gene

Two molecular variants in exon II of the angiotensinogen gene previously identifed by Juenemaitre et al., (1992b), by direct sequencing, were analysed in the high/high and low/low offspring by Soubrier et al., (Personal Communication 1992). The frequency of the variants in the two groups was compared by Chi-square analysis as presented in Tables 55 & 56, and no statistically significant difference was found.

Table 55: A comparison of the genotype distribution of the T174M molecular variant in group B (high/high) offspring and group C (low/low) ofspring.

Genotype	No of offspring Group B	Group C
Observed		
WW	30	32
WM	7	5
MM	0	1
Expected		
WW	30.59	31.49
WM	5.92	6.08
MM	0.49	0.51
X_2^2	1.385	
p	0.20–0.50 (NS)	

Table 56:A comparison of the genotype distribution of the M235T molecular variant in group B (high/high) offspring and group C (low/low) offspring.

Genotype	Group B	Group C
Observed		
WW	17	13
WM	17	16
MM	4	7
Expected		
WW	15.41	14.59
WM	16.95	16.05
MM	5.65	5.35
X_2^2	1.329	
p	0.20–0.50 (NS)	

CHAPTER 4:DISCUSSION

Chapter 4 DISCUSSION

The present study has utilised three different approaches to identify genetic determinants of high blood pressure; association analysis in subjects with polarised predispositions, sib-pair analysis and mutational analysis of candidate genes.

4.1 Genetic association studies

This form of genetic analysis is based on the determination of the prevalence of specific markers in groups of hypertensive and normotensive subjects and aims to identify an association between a DNA marker and hypertension. The present study has employed the candidate gene approach, where the frequency of RFLPs of candidate genes are compared, and may allow the demonstration of an association between the candidate gene or a closely linked locus and hypertension. For the majority of hypertension candidate genes, RFLPs have been reported and due to the ease of their identification have been used in many association studies.

There are several disadvantages encountered in association studies. The number of subjects studied must be large enough to demonstrate the smaller effects of some alleles. Association studies assume genetic homogeneity and hypertension itself is a highly heterogenous disorder, therefore absence of an association between a particular polymorphism and hypertension does not rule out the effect of some genes in subsets of hypertensive patients. Association studies assume linkage disequilibrium between the marker and a mutation at the disease locus, and if an association between a polymorphism and hypertension is found, then a second series of experiments are necessary using an additional

marker of the gene to determine if the association still holds. Association studies do not provide a definite causal link between a particular gene and hypertension, and further studies are necessary to confirm the precise genetic abnormality.

Another problem seen in association studies is in the definition of high blood pressure, and therefore in the interpretation of data. The majority of association studies in hypertension have examined patients with or without a family history, based on medical family history questionnaires. A review of the sampling methods by Watt (1986), has shown that only 3 of 31 studies verified reported family histories, therefore resulting in a large number of false positive and negative results. Negative family histories were usually defined as neither parent on treatment, and such individuals lie in the lower 75-80% of the blood pressure distribution, therefore individuals from the upper 15-20% of the distribution are compared to individuals from the lower 75-80% of the distribution. Based on such observations Watt (1986) has proposed the four corner approach for the identification of high/high offspring whose parents both have blood pressures in the upper part of the distribution and low/low offspring whose parents both have blood pressure in the lower part of the distribution.

The present study has employed the four corner approach for the selection of offspring with contrasting predispositions to high blood pressure. The unique design of the study has allowed the identification of individuals with high blood pressure and a strong genetic predisposition based on both parents having high blood pressure (high/high), and individuals with low blood pressure and a weak genetic predisposition based on both parents having low blood pressure

(low/low). The use of these groups allows for the most potentially informative comparisons to be made. In addition, parents with low blood pressure and parents with high blood pressure identified from opposite ends of the blood pressure distribution can be compared.

The number of studies to date on the RFLP analysis of candidate genes for hypertension has been limited. The renin gene RFLPs are the most widely studied. Several studies concerning renin gene polymorphisms in human hypertensive subjects have been reported. Morris & Griffiths (1988) were the first to compare the distribution of the renin *HindIII* RFLP genotypes in 29 hypertensive subjects and 202 adult normotensives. No association between the RFLP and hypertension was evident. Similarly, Naftilan et al., (1989) performed linkage analysis on 9 hypertensive relatives and 59 normotensives from a large Utah pedigree using three RFLPs of the renin gene (*HindIII, TaqI, BglI*), and failed to demonstrate an association. Haplotype analysis was also performed by Soubrier et al., (1990) on 102 hypertensives and 120 normotensives using three RFLPs of the renin gene (*TaqI, HinfI, HindIII*), and again no association was demonstrated. The study by Barley et al., (1991) examined four renin RFLPs (*BglI, BglII, TaqI, HindIII*) in hypertensives and normotensives from two different populations; Afro-Carribean and European. An association between blood pressure and the *BglI* RFLP was demonstrated in the Afro-Carribean population, but no association was observed for any of the other polymorphisms. The *BglI* RFLP of the renin gene was not examined in the present study, since it has previously been studied in the Ladywell offspring population and no association with hypertension was demonstrated (O'Hare Personal Communication 1988).

The *HindIII* RFLP of the renin gene was analysed in the present study in 192 offspring, including 45 high/high offspring and 48 low/low offspring, and in 44 parents with high blood pressure and 47 parents with low blood pressure. However, no statistically significant association between the *HindIII* polymorphism of the renin gene and hypertension was demonstrated.

The present study based on the analysis of offspring with contrasting predispositions to high blood pressure and parents with high and low blood pressures confirm previous findings of no association between *HindIII* alleles of the renin gene and hypertension.

A recent association study performed by Terhal (1992 personal communication) examined the *BglI* RFLP of the beta-1-adrenergic receptor gene, the *BanI* RFLP of the beta-2-adrenergic receptor gene and the *DraI* RFLP of the alpha-2-adrenergic receptor gene and hypertension in the Dutch Hypertension Study. Parents identified from the upper (P75) and the lower (P25) quintiles of the age/sex specific blood pressure distribution were genotyped for the three polymorphisms, and no significant difference in the genotype distribution was found. Similarly, the analysis of offspring with either high or low blood pressure, with either both parents as hypertensive, both parents as normotensive or one parent hypertensive and one parent normotensive showed no significant differences in the RFLP pattern. These three polymorphisms have been analysed in the present study, in four groups of offspring with contrasting predispositions to high blood pressure and in parents with high and low blood pressures. However, no association with hypertension was demonstrated. The design of the Dutch Hypertension Study, is similar to the approach used in the present study, where offspring

are identified from parents from opposite end of the blood pressure distribution, for maximal contrast in predisposition. However, in the present study a greater number of subjects were examined. Therefore the analysis of the three polymorphisms in the present study confirms the finding by Terhal (1992).

Several other RFLP association studies have been performed on hypertension candidate genes. Ying et al., (1991) analysed a *BglII* RFLP of the insulin gene in 68 hypertensives and 75 normotensives adults and failed to demonstrate an association between specific alleles and hypertension. An association between the alleles of a *RsaI* RFLP of the insulin receptor gene and hypertension was however determined (Ying et al., 1991). The *RsaI* RFLP of the insulin receptor gene was not examined in the present study because of problems associated with probe availability, however analysis of this polymorphism in the off spring population of the Ladywell study should be initiated. The present study has analysed the *SacI* RFLP of the insulin gene and demonstrated no association. This is the first study to perform RFLP analysis of the insulin gene in offspring with polarised predispositions to high blood pressure, and in parents with high and low blood pressures.

Association analysis by Watt et al., (1992) of the *BclI* RFLP of the glucocorticoid receptor gene in the offspring population of the Ladywell study, has previously shown a significant association between the larger allele of the RFLP and a predisposition to high blood pressure. However, the present study has demonstrated no such association between a second polymorphism of the gene ie. the *TthIII* RFLP and hypertension. The present study is the first to perform association analy-

sis of the *TthIII* polymorphism of the glucocorticoid receptor gene and hypertension. Haplotype analysis of the two RFLPs of the glucocorticoid receptor gene showed no significant difference between the observed and expected haplotype distribution, and linkage equilibria was demonstrated between the two restriction site polymorphisms (RSPs) with an estimated linkage disequilibrium parameter (D) of 0.012. Therefore the present finding of linkage equilibria between the two RSPs is in agreement with the lack of association observed between the *TthIII* RFLP of the glucocorticoid receptor gene and hypertension. If the two RSPs were in linkage disequilibrium then the analysis of the *TthIII* RFLP would be expected to show a similar association as observed with the *BclI* RFLP. This suggests that analysis of additional polymorphisms of the glucocorticoid receptor gene are necessary to confirm the original finding. To date no additional RFLPs of the glucocorticoid receptor gene have been identified. In the present study, RFLP screening of the glucocorticoid receptor gene using 30 different restriction enzymes was performed, and a polymorphism with the restriction enzyme HgiIA was found, with allele sizes of 3.5kb and 2.0kb. From a total 12 individuals tested the frequency of the alleles were 0.75 for the 3.5kb allele and 0.25 for the 2.0kb allele. However, due to the manufactures costly price of the enzyme, analysis of this polymorphism was not possible in the offspring population of the Ladywell study.

In the present study, analysis of parents with high and low blood pressure showed no association between the *BclI* RFLP of the glucocorticoid receptor and hypertension. These results are in contrast to the results obtained in the offspring population, by Watt et al.,

(1992) where the larger allele was associated with a predisposition to high blood pressure. This may reflect the two different approaches used ie. the four corner approach versus analysis of individuals with high blood pressure and low blood pressure. In the four corner approach 50 high/high offspring were compared to 50 low/low offspring, and in the latter study 44 parents with high blood pressure were compared to 33 with low blood pressure. Although slightly higher number of subject were studied in the four corner approach, if the association between the *BclI* RFLP and hypertension is true, then this provides evidence for the four corner approach as a more effective method of identifying genetic factors associated with hypertension compared to the more common approach of analysing parents with high and low blood pressure levels. However, before this can be established, the association between the *BclI* RFLP and predisposition to hypertension must be confirmed. The *BclI* RFLP of the glucocorticoid receptor gene has recently been examined in the Dutch Hypertension study (Terhal 1992), and no association was found. The lack of association demonstrated in the present study between the *TthIII* RFLP of this gene, suggests that the original findings of the Ladywell study may represents a chance finding and not a true association. If this is the case, then this highlights one of the pitfalls of association studies, were reported associations do not stand the test of time.
The *BclI* RFLP of the beta-fibrinogen gene has also been previously examined in the offspring population of the Ladywell study (Wood 1991 personal communication), and no association between the polymorphism and predisposition to hypertension was demonstrated. The present study has analysed this polymorphism in parents with high and low blood

pressures from opposite ends of the blood pressure distribution and again no association has been found. These two studies are the first to perform association analysis of the *BclI* RFLP of the beta-fibrinogen gene and hypertension.

The Atrial Natriuretic Factor (ANF) gene has also been the subject of investigation in association studies, and RFLP analysis of the *BglI* polymorphism in 32 normotensives and 51 hypertensives from two different ethnic populations, failed to demonstrate an association (Barley et al., 1991). The *BglI* RFLP of the ANF gene has previously been examined in the adywell offspring population, and no association was demonstrated (Personal Communication O'Hare).

A recent cross-sectional association study of 80 hypertensives and 93 normotensives has shown an association between hypertension and a 287bp insertion/deletion polymorphism of the Angiotensin Converting Enzyme (ACE) gene, suggesting the ACE locus or a gene closely linked to the ACE gene is implicated in hypertension (Zee et al., 1992). However, this polymorphism has also been previously examined in the Ladywell offspring population and no association was demonstrated (personal communication Soubrier et al,.)

Therefore the present analysis of offspring with contrasting predispositions to high blood pressure, suggests that allelic variations associated with the renin, the alpha-1, the beta-1, the beta-2-adrenergic receptors, glucocorticoid receptor and insulin genes identified by *HindIII, DraI, BglI, BanI, TthIII and SacI* RFLP respectively are not associated with human essential hypertension. The present study is the first to carry analysis of several RFLPs of candidate genes in

offspring with markedly contrasting predispositions to high blood pressure. In addition analysis of parents from opposite ends of the blood pressure distribution shows no association between the *HindIII* RFLP of the renin gene, *DraI* RFLP of the alpha-2-adrenergic receptor, *BgI* RFLP of the beta-1-adrenergic receptor, *BanI* RFLP of the beta-2-adrenergic receptor, *TthIII* and the *BclI* RFLP of the glucocorticoid receptor and the *BclI* RFLP of the beta-fibrinogen gene and hypertension. For all the polymorphisms studied, the allele frequencies observed in the offspring and parent populations were in close agreement with previously reported allele frequencies. The main limiting factor however, in the present study was the small number of offspring identified using the four corner approach, despite a total original population of 24,000.

The power of an association study may be increased by using more polymorphic markers. The majority of RFLPs are usually simple 2-allele site polymorphisms, and the strength of studies can be screening for additional polymorphisms at the marker locus. However, the majority of additional markers are usually in linkage equilibrium with the original and therefore no further additional information is gained. An alternative, is to identify new highly polymorphic markers at candidate genes. Simple tandem repeats have been shown to be very common in the human genome and are highly polymorphic with multiple alleles (Weber et al., 1989). The genetic heterogeneity of hypertension is another problem factor in association studies. One way to attempt to overcome this is to identify subsets of hypertensive patients where the same genes are more likely to operate.

In conclusion, the majority of association studies have been performed in case-control studies of hypertensive and normotensives. The present study has analysed offspring with polarised predispositions to high blood pressure in addition to parents with high and low blood pressures from opposite ends of the distribution. Several polymorphisms have been examined in the present study and no association between specific markers and hypertension has been demonstrated.

4.2 Sib pair analysis.

This form of analysis is based on Mendelian principles, where sib-pairs are typed for a number of genetic marker loci, and the number of marker alleles shared by affected sib-pairs is compared with the expected number assuming independent assortment of the marker and disease. At genetic loci contributing to the disease, siblings who both have the disease are expected to inherit the same alleles at the locus than would be expected from random segregation ie. 25% of sib-pairs would be expected to share identical alleles, 50% would be expected to share one haplotype and 25% would be expected to be non-identical. If a distortion of these ratios is seen, then this suggests the possible involvement of the locus in the aetiology of the disease. The method of sib-pair analysis is well established (Motro & Thomson 1985, Galton & Alcolado 1991), and has several advantages. It only requires the analysis of affected sibs, therefore if diagnosis is in doubt, the individual can be rejected from calculations. In addition, no assumptions are needed on the mode of transmission of the disease, which is essential for linkage analysis, and is therefore suited to diseases of polygenic inheritance. Sib-pair analysis requires no control group since the results are based on concordance of shared haplotypes. This is advantageous over association studies, where care fully matched control groups are necessary. It also overcomes to some extent the problem of genetic heterogeneity, since sib-pairs are more likely to be genetically homogeneous. The main disadvantage of this method is that a large number of affected sib-pairs are required, and the number has been suggested to be approximately 200, to detect

linkage (Lifton 1991).

In the present study sib-pair analysis was initiated in a limited number of affected sib-pairs ie. siblings who both have high blood pressure, for the identification of candidate loci involved in the development of hypertension.

Analysis of the *HindIII* RFLP of the renin gene in 19 affected sibs-pairs, showed a statistically significant lack of allele sharing. Similarly, a lack of allele sharing in 16 affected sib-pairs was also observed for the alleles of the beta-fibrinogen *BclI* RFLP. This suggests that the *HindIII* RFLP of the renin gene and the *BclI* RFLP of the beta-fibrinogen gene are not associated with hypertension. These results are in agreement with the analysis of the *HindIII* RFLP preformed in the present study in the offspring and parent population, demonstrating no association with high blood pressure. The *BclI* RFLP of the beta-fibrinogen gene has also previously been studied in the Ladywell offspring population (Wood 1992 Personal Communication), and demonstrated no association with hypertension, and in the present study in parents with high and low blood pressures again demonstrating no association.

A lack of allele sharing was evident for the alleles of the *TthIII* RFLP of the glucocorticoid receptor gene in 10 affected sib-pairs, however this was not statistically significant. Increased allele sharing was observed in 18 affected sib-pairs for the alleles of the *BanI* RFLP of the beta-2-adrenergic receptor gene polymorphism, in 10 affected sibs-pairs for the alleles of the *BglI* RFLP of the beta-1-adrenergic receptor gene , and in 16 affected sib-pairs for the alleles of the *BclI* RFLP of the glucocorticoid receptor gene. However,

the increased allele concordance for the three polymorphisms, was not statistically significant.

Although a significant lack of association between hypertension and the renin and beta-fibrinogen RFLPs has been observed, the number of sib-pairs studied is small, and therefore further studies are necessary on a larger number of affected sib-pairs to confirm or refute these finding. Similarly the lack of allele sharing in affected sib-pairs for the *TthIII* RFLP, requires to be confirmed in a larger number of affected sib-pairs. However, our preliminary analysis of the *TthIII* polymorphism in a limited number of sib-pairs is in agreement with no association, as demonstrated in the offspring and parent populations in the present study.

The increased allele sharing for the *BclI* RFLP of the glucocorticoid receptor gene, is also in agreement with the original findings of an association between the larger allele of the RFLP and hypertension as shown by Watt et al., (1992). Analysis of this polymorphism in a larger number of sib-pairs is again necessary to determine if a significant association can be demonstrated. The increased allele sharing observed in affected sib-pairs for the beta-1 and beta-2-adrenergic receptors gene RFLPs, however, is in contrast to the association analysis of these polymorphisms in offspring with contrasting predispositions to high blood pressure, and in parents with high and low blood pressures, where no significant association was demonstrated. Therefore, analysis of these two polymorphism in a greater number of subjects is necessary to confirm or refute our findings.

The sib-pair analysis performed has shown increased allele concordance in affected sib-pairs for three loci, suggesting the involvement of

these loci in the aetiology of hypertension. However, only one of these loci ie. the *BclI* RFLP of the glucocorticoid receptor gene, has been shown to be associated with hypertension, by studying offspring with differing risks of hypertension (Watt et al., 1992). If this is the case, then it points to sib-pair analysis as a more robust method of identifying genetic factors associated with hypertension compared to the four corner approach, however this needs to be confirmed by analysing a larger number of sib-pairs.

A number of recent studies have employed sib-pair linkage analysis in human hypertensives. Jeunemaitre et al.,(1992b), analysed 237 sib-pairs and provide evidence for absence of linkage between the Angiotensinogen Converting Enzyme and hypertension. However, a recent case-control association study performed by Zee et al.,(1992), has shown an association between a 287bp deletion/insertion polymorphism of the ACE gene, suggesting ACE or a locus closely linked to ACE may be implicated in the development of raised arterial pressure.

Another study has employed sib-pair analysis and has demonstrated linkage between the angiotensinogen gene and hypertension (Jeunemaitre 1992a). In this study, 215 sibships in two distinct geographical populations were analysed for the highly polymorphic VNTR of the AGT gene. Soubrier et al (1992 Personal Communication), have previously analysed this polymorphism in the four groups of offspring of the Ladywell Study, and no association between specific alleles and hypertension was demonstrated. Therefore linkage has been demonstrated using sib-pair analysis, yet no association has been identified in the association analysis using the four corner approach. This again suggests that sib-pair analysis provides a more robust method of identi-

fying genes of aetiological significance in hypertension compared to the Four Corner Approach.

The present sib-pair study is the first to analyse polymorphisms of the renin, beta-1, beta-2, alpha-2 adrenergic receptors, insulin, beta-fibrinogen and glucocorticoid receptor genes. The numbers studied are small and these results need to be confirmed in a larger number of subjects. The identification of more highly polymorphic markers for candidate genes, and their use in sibling pair analysis may improve the strength of the study. The strength of sib-pair studies can also be improved by identifying subsets of more severely hypertensives.

Thus, in conclusion, the present sib-pair analysis although only in a small number of sib-pairs seems to be suitable for identifying genetic factors associated with hypertension compared to the association studies performed in groups of offspring with contrasting predispositions to high blood pressure.

4.3 Mutational Screening

The ability to detect polymorphisms and mutations in individuals has become increasingly important for the characterisation of genes responsible for inherited disease and in DNA linkage studies. In the past, the detection of mutations was carried out by gene cloning and DNA sequencing. Although this provides 100% detection it is labour intensive and not rapid enough for routine diagnosis. Over recent years several techniques for mutation detection have been identified with the development of the Polymerase Chain Reaction (PCR) (Saiki et al., 1988), with the potential to provide simple, sensitive and rapid detection. The methods employed include Denaturing Gradient Gel Electrophoresis (DGGE) (Fischer et al., 1983), Single Strand Conformational Polymorphism Analysis (SSCP) (Orita et al., 1989), Chemical Cleavage of Mismatches (CCM) (Cotton et al., 1988) Hydrolink gel electrophoresis (Keen et al., 1991), and direct sequencing of asymmetric PCR products.

Denaturing Gradient gel electrophoresis (DGGE) involves the electrophoresis of heteroduplexes formed between mutant and normal DNA through denaturing gels. Heteroduplexes with regions of mismatches show altered migration pattern, therefore identifying mutations. The main disadvantage of this technique is the tendency of the heteroduplex ends to denature prematurely, such that mutations in these regions are not detected. The addition of GC clamps to the ends of heteroduplexes can overcome this (Myers et al., 1985), however this makes the procedure more time consuming.

Single strand conformation polymorphism analysis (SSCP), involves the

electrophoresis of single stranded DNA through non-denaturing gels. Mutations are identified as mobility shifts, due to conformational changes of single stranded DNA. However, the ability of SSCP to identify all mutations is unclear.

Chemical cleavage of mismatches (CCM) is a more recent developed technique. Regions of mismatches in heteroduplexes, showed enhanced sensitivity towards chemical cleavage at T by osmium tetraoxide and at C by hydroxylamine. Piperidine recognises these modified bases and cleaves these regions resulting in differing size fragment that can be resolved by electrophoresis. This method therefore allows for the detection of 100% of mutations, and also provide some information on the nature of the mutation. However, the chemicals used are highly toxic, making this procedure highly hazardous, and is also labour intensive.

Hydrolink Gel Electrophoresis is a very recent technique involving the electrophoresis of heteroduplexes through hydrolink gel matrices, and mutations are detected as mobility shifts, on ethidium bromide staining of gels. This is the simplest of all techniques to use, however, since it is a relatively new mutation detection method, its ability to detect all mutations is unclear, although some authors have suggested 100% of mutations can be identified (Boyd et al., 1992).

Whichever of the methods are used, confirmation and characterisation of mutations is necessary by sequencing. Sequencing has in recent years become simplified by the development of PCR. Asymmetric PCR, where the two oligonucleotide primers are used in differing ratios, results in single stranded DNA templates, which can then be directly sequenced, this method allows the detection of 100% of mutations.

The main advantages of these approaches is that mutations in candidate genes in hypertensive subjects can be directly identified. However, the inference that a mutation identified is causal to the development of hypertension, requires further analyses. In addition the detection of no mutations does not rule out the possibility that mutation involved in the development of hypertension is in an unscreened portion of the gene, such as introns or 5'-3' flanking regions.

Sequencing is the most powerful tool for the identification of molecular variants in hypertension candidate genes. The glucocorticoid receptor gene consists of 10 exons with a cDNA in the region of 3kb, and the angiotensinogen gene consists of five exons, therefore sequencing of such genes is a labour intensive process and time consuming. Hydrolink gel electrophoresis of PCR products has been successful at identifying several single base pair substitutions, both known and unknown (Boyd et al., 1992, Inglehearn et al., 1992, White et al., 1992).

In the present study, hydrolink gel electrophoresis and direct sequencing of the angiotensinogen gene and the glucocorticoid receptor cDNA, was employed to identify molecular variants in these genes, in offspring of the Ladywell study with contrasting predispositions to high blood pressure.

4.3.1 Mutational Screening of the Glucocorticoid receptor cDNA

Analysis of the *BclI* RFLP of the glucocorticoid receptor gene by Watt et al., (1992) demonstrated an association between the larger allele of the RFLP and hypertension. However, the present studied analysed a second polymorphism, the *TthIII* RFLP of this gene, and showed no

association. In order to identify additional DNA variation of the HGR gene, mutational screening of the HGR gene was initiated.

Using three sets of oligonucleotide primers the cDNA of the HGR gene was reverse transcription (RT)-PCR amplified in five high/high offspring and five low/low offspring. For all subjects examined, the correct size products were amplified, thus excluding any major gene deletions and truncated mRNA products.

Optimisation of the RT-PCR was necessary for successful amplification. Denaturation of the RNA sample followed by quenching on ice was found to be crucial for successful amplifcation since it allows for cDNA priming by reducing secondary structures in the RNA molecules (Kawasaki et al., 1990). Similarly, denaturation of the cDNA was also found to be necessary, since it inactivates the reverse transcriptase enzyme and denatures RNA-DNA hybrids (Kawasaki & Wang 1989). Once amplification was evident, the annealing temperature were optimised to reduce non-specific amplification, due to priming of a non-target sequence during the first few cycles of PCR. However, in some instances, non-specific amplification was seen, even at optimal annealing temperature.

Hydrolink gel electrophoresis of the three RT-PCR amplified regions of the HGR cDNA in 5 high/high and 5 low/low offspring showed no evidence of heteroduplex formation in the regions amplified by primers 237R/240R, and 236/241R, suggesting no molecular variants in these regions exist. However, two high/high offspring and 2 low/low offspring showed a mobility shift in the region amplified by primers 238R/239R, suggesting the presence of a mutation in this region in these individuals. Asymmetric PCR for the generation of single strand-

ed DNA, using primer 238R as the limiting primer allowed sequencing of 400bp of the 968bp PCR product in 1 high/high offspring and 1 low/low offspring showing the mobility shift, and no sequence variation was identified. Asymmetric PCRs were unsuccessful at generating single strand DNA when primer 239R was limiting, using ratios of 1:100, 1:75 and 1:50, and resulted in only the generation of dsDNA and no ssDNA. This was found to be the case in all subjects examined and not only one indiviual. The reason for this is not fully understood. One obvious way to overcome this would be to use alternative primers, however due to limited resources we were unable to attempt this.

For a mutation to be involved in the development of raised arterial pressure, then its frequency would be expected to be greater in high/high offspring compared to low/low offspring. The present variant identifed, however, showed an equal frequency in both groups of offspring, and is therefore unlikely to be associated with high blood pressure, and more likely a polymorphism.

Therefore we have screened the coding region of the cDNA of the HGR cDNA in 5 offspring with greatest genetic risk and 5 offspring with weakest genetic risk of hypertension by hydrolink gel electrophoresis. A molecular variant was identified in 4 offspring, with an equal frequency in both the high/high and low/low offspring. However characterisation of the variant was unsuccessful.

The present study is the first to inititate mutational screening of the glucocorticoid receptor cDNA in offspring with contrasting predispositions to high blood pressure. A study performed by Hurley et al., (1991), identifed a point mutation by direct sequencing of PCR products in the HGR cDNA in three family memebers with Familial gluco-

corticoid resistance (FGR). FGR is a hypertensive disorder, and the authors suggests that the point mutation which results in a single aminoacid substitution is the cause of FGR in this family. No previous mutations have been reported in the HGR cDNA in hypertensives subjects.

4.3.2 Mutational screening of the Angiotensinogen gene

Mutational analysis of angiotensinogen (AGT) gene has recently been performed by Jeunmaitre et al., (1992). Direct sequencing identified 15 molecular variants in the AGT gene, 5 of which were nucleotide substitutions in the 5' region, and the remaining 10, silent mutations. Two of these variants M235T and T174M, were significantly more frequent in hypertensives compared to controls, and in linkage disequilibrium. Haplotype analysis showed the haplotype carrying M235T, with or without T174M, was observed more frequently in hypertensives.

In the present study we mutational screening of the AGT gene in offspring with contrasting predispostions to high blood pressure was performed using Hydrolink gel electrophoresis (Keen et al., 1991).

PCR amplification of the five exons and the 5' region of the AGT in six high/high and six low/low offspring of the Ladywell Study, resulted in amplification of the correct sized PCR products, thus excluding any major gene mutations. Primer sequences were taken from Jeunemaitre et al., (1992b), and no optimisation was necessary.

Hydrolink gel electrophoresis of the PCR products showed no evidence for heteroduplex formation in the PCR products from primers 5a1/5a2, 5b1/5b2, 5c1/5c2, 2a1/2a2, 2b1/2b2, 3-1/3-2, 4-1/4-2 and 5-1.5-2. This suggests that no molecular variants are present in exon I, III, IV, V,

and 600bp of exon II and the 5' region of the gene, in the twelve subjects examined.

Hydrolink gel electrophoresis however, identified heteroduplex formation in the PCR products from primers 2c1/2c2, in one high/high offspring and three low/low offspring. Inspection of the gels, showed the presence of two additional bands above the homoduplex band in all four offspring, as shown in Fig. 11. The frequency of the molecular variants was determined in 40 low/low offspring and 35 high/high offspring, with frequencies of 25% (n=10) and 17% (n=6) respectively. However, the distribution of the molecular variant in the two groups of offspring was not statistically significant. The frequencies of the two molecular variants M235T and T174M identified by Jeuemaitre et al., (1992b), were previously analysed in the high/high and low/low offspring of the Ladywell study (Soubrier 1992 Personal Communication), and no statistically significant difference between the genotype distribution of the variants in the two groups was found, as shown in Tables 55 & 56. Therefore although the number of offspring examined in the Ladywell study were smaller, the results do not support the original findings of Jeunemaitre et al., (1992b), and further analyses are on a larger number of subjects are needed to confirm or refute the involvement of the AGT locus in the aetiology of hypertension.

Two of the molecular variants identified by Jeunemaitre et al., (1992b), M235T and T174M, were present in the same PCR region of the AGT gene as the double variant identifed in the present study. A comparison of the offspring possessing the double molecular variant with the genotype of the variants M235T and T174M deduced by Soubrier

et al., showed that all offspring who scored positive for heteroduplex formation (possess the double molecular variant) without exception were double heterozygotes for the M235T and the T174M variants ie. have the genotype wildtype/M235T wildtype/T174M. Those scored as negative were either wild type homozygotes for both variants or homozygote wild type for the T174M variant and heterozygotes for the M235T variant (wild type/M235T wild type/wild type) (Appendix IV). Therefore it is evident that the molecular variant we have identified is a double variant identifying both the M235T and the T174M molecular variants, and shows no association with hypertension.

The present study, and the previous studies performed in the Ladywell offspring population on the AGT gene suggest, that the AGT locus is not implicated in the aetiology of hypertension in the individuals studies. However, the number of subjects we have examined is small in comparison to other studies and therefore our results should be treated with caution and analysis of a larger number of subjects is required to confirm the original results of Jeunemaitre et al., (1992).

In conclusion, the present study has utilised three different approaches for the determination of genetic factors associated with hypertension; association study, sib-pair analysis and mutational screening. In the association analysis of several polymorphisms of several candidate genes in offspring with contrasting predispositions to high blood pressure and in parents with high and low blood pressures, no allelic association with hypertension was found. Where an association was previously demonstrated between hypertension and the *BclI* RFLP of the glucocorticoid receptor gene, analysis of an addi-

tional polymorphism of the same gene has failed to establish the same association. The sib-pair analysis in a limited number of sib-pairs has proved to be more advantageous, in this study, compared to the four corner approach, and has suggested an association between the *BgII* RFLP of the beta-1-adrenergic receptor gene, the *BanI* RFLP of the beta-2-adrenergic receptor gene and the *BclI* RFLP of the glucocorticoid receptor gene and hypertension. Mutational analysis of the glucocorticoid receptor cDNA identified a molecular variant, with an equal frequency in high/high and low/low offspring. Similarly mutational analysis of the angiotensinogen gene identified a double molecular variant in exon II of the gene. Analysis of this double variant in high/high and low/low offspring showed no statistically significant difference in the distribution.

The present study therefore suggests, sib-pair analysis in conjunction with direct mutational screening of candidate genes is the most promising way forward in identifying genetic factors of etiological significance.

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APPENDICES

APPENDIX I

Solutions

Plasmid solutions

Luria Broth(1000mls)	10g Bactotryptone 5g Bacto yeast extract 5g NaCl pH 7.5
Magnesium Sulphate	10mM-ice cold
Calcium Chloride	50mM-ice cold
STET Buffer	50mM Tris 50mM EDTA 8% Sucrose 5% Triton X-100
Glycerol	15%
Lysosyme	10mg/ml-ice cold-fresh
Sodium Acetate	3M
Phenol	water saturated protected from light stored at 4°C
Ethanol	95%
Ampicillin	0.1mg/ml

Genomic DNA solutions

Lysis Mix	0.32M Sucrose 10mM Tris 5mM Magnesium chloride 1% Triton X-100 pH 7.5
Nuclei Lysis Mix	10mM Tris 0.4M Sodium chloride 2mM EDTA pH 2.8
TE Buffer	10mM Tris 10mM EDTA pH 7.5

Loading Mix	30% Glycerol 0.25% Bromohpenol Blue
20 X SSC	3M Sodium chloride 0.3M Sodium citrate pH 7
Denaturation Solution	0.5M Sodium hydroxide 1.5M Sodium chloride
Neutralisation Solution	0.5M Tris Hydrochloric Acid 3M Sodium chloride pH 7.5
Depurination Solution	0.25M Hydrochloric Acid
Sephadex	30g Sephadex 500mls 2XSSC
Prehybridisation Solution	50% Formamide 5 X Denharts 5 X SSC 2% SDS
Salmon Sperm DNA	10mg/ml sonicated
SDS	10%
Proteinase K	10mg/ml
Sodium chloride solution	6M
Phenol/chloroform	50:50 pH 7.5 0.1% Hydroxyqinoline
Ethanol	95%
Spermidine	0.1M
Ethidium Bromide	10mg/ml
RNA extraction solutions	
PBS Buffer	0.14M Sodium chloride 3mM Potassium chloride 0.1mM sodium hydrogen phosphate 1mM potassium dihydrogen phosphate Stored at 4°C pH 7.2

Solution D	4M Guanidium thiocynate 25mM sodium citrate 0.1mM mercaptoethanol 0.5% sarkosyl
Sodium acetate	3M pH 4
Phenol	water saturated protected from light Stored at 4°C
Chloroform/isoamylalcohol	49:1
SDS	0.5%
ethanol	75%
isopropanol	
formaldehyde	37%
10xMOPS	0.2M MOPS (Sodium salt) 80mM Sodium acetate 10mM EDTA pH 7.0 protect from light
Formamide	deionised
Gel juice	2g Ficoll 1ml 2.5% Bromophenol blue 1ml 2.5% Xylene Cyanol 0.2ml 0.5M EDTA 8ml water
Ethidium bromide	10mg/ml

Reverse transcription solutions

Reverse transcription buffer (10x concentrate)	250mM Tris Hydrochloric acid (pH 8) 375mM Potassium chloride 15mM Magnesium chloride
DTT (Ditheriothiol)	0.1M
Reverse transcriptase	200units/ul
Rnase inhibitor	40units/ul

dNTPs (dATP,dTTP,dCTP,dGTP)	2.5mM
TBE	4.0M Tris Base 4.4M Boric Acid 1.3M EDTA
PCR solutions	
PCR Buffer (10 X concentrate)	100mM Tris Hydrochloric acid 15mM Magnesium chloride 500mM potassium chloride 1mg/ml gelatine pH 8.3
dNTPs	2.5mM
Taq polymerase	5 units/ul
Sequencing Reaction Solutions	
Sequenase Buffer (5x concentrate)	200mM Tris.HCl pH 7.5 100mM Magnesium chloride 250mM Sodium chloride
Dithiotheiol (DTT)	0.1M
Labelling Mix (dGTP) (5x concentrate)	7.5µM dGTP 7.5µM dCTP 7.5µM dTTP
Labelling mix (dITP) (5x concentrate)	15µM dITP 7.5µM dCTP 7.5µM dTTP
ddG termination mix	80µM dGTP 80µM dATP 80µM dCTP 80µM dTTP 8µM ddGTP 50mM NaCl
ddA termination mix	80µM dGTP 80µM dATP 80µM dCTP 80µM dTTP 8µM ddATP 50mM NaCl

ddT termination mix	80µM dGTP 80µM dATP 80µM dCTP 80µM dTTP 8µM ddTTP 50mM NaCl
ddC termination mix	80µM dGTP 80µM dATP 80µM dCTP 80µM dTTP 8µM ddCTP 50mM NaCl
Enzyme Dilution buffer	10mM Tris.HCl pH 7.5 5mM DTT 0.5mg/ml BSA
Stop solution	95% formamide 20mM EDTA 0.05% Bromophenol blue 0.05% Xylene Cyanol FF

APPENDIX II

Calculation of oligonucleotide primer 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.

$$MW = [(A \times 312.2) + (G \times 328.2) + (C \times 288.2) + (T \times 303.2)] - 61.0$$

Where A,G,T and C are the number of each base in the sequence

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

$$\text{Concentration} = (N \times 0.037/MW \times 106$$

Where N is the number of OD units

APPENDIX III

Ladywell offspring results

Ladywell Number	RFLP data			
	BanI β_2-adreno receptor	HindIII Renin	BglI β_1-adreno receptor	SacI Insulin
10082B	3.7/3.4	9.0/6.2	6.2/6.2	6.0/6.0
10083B	3.7/3.4	9.0/6.2	6.2.6.2	6.0/6.0
10121C	3.7/3.7	9.0/9.0	4.7/4.7	6.0/6.0
10173A	3.7/3.4	6.2/6.2	6.2/4.7	6.0/6.0
10252B	3.7/3.4	6.2/6.2	6.2/4.7	6.0/6.0
10371B	3.7/3.4	9.0/6.2	6.2/6.2	6.0/6.0
10502A	3.7/3.4	9.0/6.2	6.2/4.7	7.5/6.0
10531D	3.7/3.4	9.0/9.0	4.7/4.7	7.5/6.0
10592B	3.4/3.4	9.0/6.2	6.2/6.2	7.5/6.0
10621D	3.7/3.4	9.0/6.2	6.2/4.7	7.5/6.0
10622B	3.7/3.4	9.0/9.0	6.2/6.2	6.0/6.0
10892A	3.7/3.7	9.0/6.2	6.2/6.2	
10921D	3.7/3.4	9.0/6.2	6.2/6.2	6.0/6.0
10943B	3.7/3.4	9.0/6.2	6.2/4.7	6.0/6.0
10952D	3.4/3.4	9.0/9.0	6.2/6.2	
10953B	3.4/3.4	9.0/6.2	6.2/4.7	6.0/6.0
11001C	3.4/3.4	9.0/9.0	6.2/6.2	7.5.6/0
11002C	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
11062A	3.7/3.4	9.0/9.0		7.5/6.0
11232B	3.4/3.4	9.0/9.0	6.2/6.2	
11252C	3.7/3.4	9.0/9.0	4.7/4.7	7.5/6.0
11291D	3.4/3.4	9.0/9.0	6.2/6.2	7.5/7.5
11352B	3.7/3.4	9.0/6.2	4.7/4.7	7.5/7.5
11362D	3.4/3.4	9.0/9.0	6.2/6.2	
11453D	3.7/3.4	9.0/6.2		7.5/6.0
11543A	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
11611C	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
11722D	3.7/3.4	9.0/9.0	6.2/4.7	6.0/6.0
11842A	3.4/3.4	9.0/6.2		
11914D	3.7/3.7	9.0/9.0	6.2/4.7	7.5/6.0
11924A	3.7/3.7	9.0/9.0	6.2/6.2	
11951B	3.7/3.7	9.0/9.0	6.2/6.2	6.0/6.0
11952D	3.4/3.4	9.0/9.0	6.2/4.7	6.0/6.0
12062B	3.4/3.4	9.0/9.0	6.2/6.2	6.0/6.0
12063D	3.7/3.4	9.0/9.0	6.2/6.2	6.0/6.0
12111C	3.4/3.4	9.0/9.0	6.2/6.2	6.0/6.0
12112C	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
12132B	3.7/3.4	9.0/9.0	6.2/4.7	6.0/6.0
12203D	3.7/3.7	9.0/6.2	6.2/4.7	7.5/6.0
12251B	3.4/3.4	9.0/6.2	6.2/6.2	7.5/6.0
12252B	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
12281D	3.7/3.4	9.0/6.2		6.0/6.0

12513A	3.7/3.7	9.0/6.2	6.2/6.2	6.0/6.0
12594A	3.7/3.4	9.0/9.0	6.2/6.2	6.0/6.0
12792D		9.0/6.2		6.0/6.0
12902C	3.4/3.4	9.0/9.0	6.2/6.2	7.5/6.0
12922A	3.7/3.4	9.0/9.0	6.2/6.2	7.5/6.0
12942C	3.4/3.4	9.0/9.0	6.2/4.7	7.5/7.5
12982C	3.7/3.4	9.0/6.2	6.2/6.2	6.0/6.0
12983A	3.4/3.4	9.0/6.2	6.2/6.2	7.5/7.5
13003B	3.4/3.4	9.0/6.2	6.2/6.2	7.5/6.0
13101D	3.7/3.4	9.0/9.0	6.2/6.2	6.0/6.0
13102B	3.7/3.4	9.0/9.0	6.2/6.2	6.0/6.0
13123C	3.4/3.4	9.0/9.0	6.2/6.2	7.5/6.0
13222D				
13241C	3.7/3.4	9.0/9.0	6.2/4.7	7.5/6.0
13421A	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
13423A	t3.4/3.4	6.2/6.2	6.2/6.2	6.0/6.0
13642D		9.0/6.2	6.2/6.2	
13654D	3.7/3.4	9.0/9.0	6.2/4.7	6.0/6.0
13684A	3.4/3.4	9.0/6.2	6.2/6.2	7.5/6.0
13802D	3.4/3.4	9.0/9.0	6.2/6.2	6.0/6.0
13862C	3.4/3.4	6.2/6.2	6.2/6.2	6.0/6.0
13911A	3.7/3.4	9.0/9.0	6.2/6.2	7.5/6.0
14013B	3.7/3.4	9.0/6.2	6.2/4.7	7.5/6.0
14024C	3.4/3.4	9.0/9.0	6.2/4.7	7.5/6.0
14051A		9.0/6.2		7.5/7.5
14241A	3.4/3.4	6.2/6.2	6.2/4.7	6.0/6.0
14242A	3.7/3.4	9.0/6.2	6.2/6.2	6.0/6.0
14323C	3.4/3.4	9.0/9.0	6.2/6.2	6.0/6.0
14332D	3.4/3.4	9.0/6.2	6.2/4.7	6.0/6.0
14542A	3.4/3.4	9.0/9.0	6.2/4.7	7.5/6.0
14582B	3.7/3.7	9.0/6.2	6.2/4.7	7.5/6.0
14622B	3.7/3.4	9.0/6.2	6.2/6.2	6.0/6.0
14642A	3.4/3.4	9.0/6.2	6.2/6.2	7.5/6.0
14672B	3.4/3.4	9.0/9.0	6.2/6.2	7.5/7.5
14763C	3.7/3.4	6.2/6.2	6.2/6.2	7.5/6.0
14792C	3.4/3.4	9.0/6.2	6.2/6.2	7.5/7.5
14882D	3.7/3.7	9.0/6.2	6.2/6.2	6.0/6.0
14911D	3.4/3.4	9.0/9.0	6.2/6.2	7.5/7.5
14974D	3.4/3.4	9.0/9.0	6.2/6.2	7.5/6.0
15082B				6.0/6.0
15282D	3.4/3.4	9.0/6.2	6.2/6.2	7.5/6.0
15502B				7.5/6.0
15601B	3.4/3.4	9.0/6.2	6.2/6.2	6.0.6/0
15691C	3.7/3.4	9.0/9.0	6.2/4.7	
15723D	3.4/3.4	9.0/9.0	6.2/4.7	7.5/6.0
15771B	3.4/3.4	9.0/9.0	6.2/4.7	6.0/6.0
15833A	3.7/3.4			7.5/7.5
15932A	3.4/3.4	9.0/9.0		7.5/6.0
16111B	3.7/3.4	9.0/9.0	6.2/6.2	6.0/6.0
16182C	3.4/3.4	6.2/6.2	6.2/6.2	7.5/6.0
16301A	9.0/6.2	6.2/6.2		6.0/6.0
16332A	3.4/3.4	9.0/9.0	6.2/6.2	
16382C	3.7/3.4	9.0/6.2	6.2/6.2	6.0/6.0
16531B	3.4/3.4	9.0/6.2	6.2/6.2	7.5/7.5

16621A		9.0/9.0		7.5/7.5
16701D	3.4/3.4	9.0/9.0	6.2/4.7	6.0/6.0
16702D	3.4/3.4	9.0/6.2	6.2/4.7	7.5/6.0
16771D	3.7/3.4	9.0/6.2	6.2/4.7	7.5/6.0
16841A	3.4/3.4	9.0/9.0	6.2/6.2	7.5/6.0
17052D				
17161A	3.7/3.4	9.0/6.2	6.2/6.2	6.0/6.0
17321A	3.4/3.4	9.0/9.0	6.2/6.2	6.0/6.0
17371A	3.4/3.4	9.0/9.0	6.2/6.2	7.5/6.0
17551B	3.4/3.4	9.0/9.0	6.2/4.7	6.0/6.0
17671B	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
17672D	3.7/3.4	9.0/9.0	6.2/4.7	6.0/6.0
17892A	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
20072D	3.7/3.4	9.0/6.2	6.2/6.2	7.5/7.5
20091C	3.7/3.4	9.0/6.2	6.2/6.2	6.0/6.0
20131B	3.4/3.4	9.0/6.2	6.2/4.7	6.0/6.0
20142A	3.4/3.4	9.0/9.0	6.2/4.7	
20152C	3.7/3.4	9.0/9.0	6.2/4.7	7.5/6.0
20252C	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
20253C	3.7/3.4	9.0/9.0	6.2/6.2	7.5/6.0
20351C	3.7/3.4	9.0/6.2	6.2/4.7	7.5/6.0
20422D	3.4/3.4	9.0/9.0	6.2/6.2	7.5/7.5
20423D	3.4/3.4	9.0/6.2	6.2/6.2	7.5/6.0
20472A	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
20553A	3.4/3.4	9.0/6.2	6.2/6.2	
20561C	3.4/3.4	9.0/6.2	6.2/6.2	7.5/6.0
20682C	3.4/3.4	9.0/9.0	6.2/6.2	7.5/6.0
20741D	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
21052C	3.7/3.4	9.0/9.0	6.2/4.7	6.0/6.0
21192D	3.7/3.4	9.0/6.2	6.2/6.2	7.5/6.0
21222B	3.7/3.4	9.0/9.0	6.2/6.2	7.5/6.0
21272A	3.4/3.4	9.0/6.2	6.2/4.7	6.0/6.0
21332C	3.4/3.4	9.0/9.0	6.2/6.2	7.5/6.0
21451C	3.7/3.4	9.0/6.2		
21522B	3.7/3.7	9.0/6.2	6.2/6.2	6.0/6.0
21702A	3.7/3.7	9.0/6.2	6.2/6.2	6.0/6.0
21714A	3.4/3.4	9.0/9.0	6.2/4.7	7.5/6.0
21722A	3.7/3.	9.0/9.0	6.2/6.2	7.5/6.0
21843B	3.7/3.4	9.0/6.2	6.2/6.2	6.0/6.0
22023B		9.0/6.2		7.5/6.0
22024D	3.7/3.7	9.0/6.2	6.2/6.2	7.5/6.0
22112B				
22381C	3.4/3.4	9.0/9.0	6.2/4.7	7.5/6.0
22752A	3.4/3.4	6.2/6.2	6.2/4.7	7.5/6.0
22762A	3.7/3.4	9.0/9.0	6.2/4.7	7.5/6.0
22942D	3.7/3.4	9.0/9.0	6.2/6.2	7.5/6.0
23061A	3.7/3.4	9.0/6.2	6.2/6.2	7.5/6.0
23082D	3.7/3.4	9.0/6.2	6.2/6.2	7.5/6.0
23256D	3.7/3.4	9.0/6.2	6.2/4.7	7.5/7.5
23293A	3.7/3.4	9.0/6.2	6.2/4.7	7.5/6.0
23393A	3.7/3.4	9.0/9.0	6.2/6.2	7.5/6.0
23403D	3.7/3.4	9.0/6.2	6.2/6.2	7.5/6.0
23452D		9.0/6.2		
23523B	3.4/3.4		6.2/6.2	7.5/6.0

23583C		6.2/6.2		
23662C	3.4/3.4	9.0/9.0	6.2/6.2	6.0/6.0
23691D	3.7/3.4	9.0/6.2	6.2/4.7	6.0/6.0
23793 C	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
23892A	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
23952B	3.4/3.4	9.0/6.2	6.2/4.7	7.5/6.0
24011C	3.7/3.4	9.0/9.0	6.2/4.7	7.5/7.5
24113D	3.4/3.4	9.0/9.0	6.2/6.2	7.5/6.0
24261A		9.0/9.0		6.0/6.0
24262A	3.4/3.4	9.0/9.0	6.2/4.7	6.0/6.0
24301B	3.4/3.4	9.0/6.2	6.2/6.2	7.5/7.5
24302D	3.4/3.4	9.0/6.2	6.2/6.2	
24363A	3.4/3.4	9.0/6.2	6.2/4.7	7.5/7.5
24372C	3.4/3.4	9.0/6.2	6.2/6.2	
24492A	3.4/3.4	9.0/6.2		7.5/6.0
24516B	3.7/3.4	9.0/6.2	6.2/6.2	
24521C	3.4/3.4	9.0/6.2		
24531C	3.4/3.4	9.0/6.2	6.2/6.2	7.5/7.5
24682B				
24721B	3.4/3.4	9.0/6.2	6.2/6.2	7.5/7.5
24751C	3.4/3.4	9.0/6.2	4.7/4.7	
24752C	3.4/3.4	9.0/9.0	6.2/4.7	7.5/6.0
24762C	3.4/3.4	6.2/6.2	6.2/6.2	
24821B	3.7/3.4	9.0/6.2	6.2/6.2	6.0/6.0
24931D	3.4/3.4	9.0/6.2	6.2/4.7	7.5/6.0
25142D	3.4/3.4	9.0/6.2	6.2/6.2	7.5/6.0
25143B	3.4/3.4	9.0/6.2	6.2/6.2	7.5/7.5
25202D	3.7/3.4	9.0/6.2	6.2/4.7	6.0/6.0
25203C	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
25311C	3.4/3.4	9.0/6.2	6.2/4.7	7.5/6.0
25331A	3.4/3.4	9.0/9.0		6.0/6.0
25361C	3.4/3.4	9.0/6.2	6.2/6.2	7.5/6.0
25371B	3.7/3.4	9.0/6.2	6.2/6.2	6.0/6.0
25544C	3.4/3.4	9.0/9.0	6.2/6.2	6.0/6.0
25553B		9.0/9.0		6.0/6.0
25684D	3.4/3.4	9.0/9.0	6.2/6.2	7.5/6.0
25685D	3.4/3.4	9.0/9.0	6.2/6.2	7.5/6.0
25751A	3.4/3.4	9.0/9.0	6.2/4.7	7.5/7.5
25814A		6.2/6.2		6.0/6.0
25852C	3.4/3.4	9.0/6.2	6.2/4.7	7.5/7.5
25863B	3.4/3.4			
25864D	3.7/3.4	9.0/9.0	6.2/6.2	7.5/6.0
25942B	3.4/3.4	9.0/6.2	6.2/4.7	7.5/7.5
25962B		9.0/9.0		7.5/6.0
26093C	3.7/3.4	9.0/6.2	6.2/6.2	7.5/7.5
26132B	3.4/3.4	9.0/6.2		
26191D	3.4/3.4	9.0/9.0		7.5/7.5
26241B	3.4/3.4	9.0/9.0	6.2/6.2	6.0/6.0
26262D	3.7/3.4	9.0/6.2	6.2/4.7	7.5/6.0
26331C				
26372C	3.4/3.4	6.2/6.2	6.2/6.2	
26521B		9.0/6.2		

24011C	6.7/6.3	3.4/3.4
24113D	6.7/6.3	3.4/3.4
24261A		
24262A	6.7/6.3	3.8/3.4
24301B	6.7/6.7	
24302D		3.4/3.4
24363A	6.7/6.3	3.8/3.4
24372C		
24492A		
24516B		
24521 C		3.8/3.4
24531C	6.7/6.7	3.4/3.4
24682B		
24721B	6.7/6.3	3.4/3.4
24751C	6.7/6.7	3.8/3.8
24752C		3.8/3.4
24762C	6.7/6.7	3.8/3.8
24821B	6.7/6.7	3.4/3.4
24931D	6.7/6.3	3.4/3.4
25142D	6.7/6.7	3.4/3.4
25143B	6.7/6.7	3.8/3.4
25202D		
25203C	6.7/6.7	
25311C	6.7/6.7	3.8/3.4
25331A		
25361C	6.7/6.7	
25371B	6.7/6.3	
25544C	6.7/6.7	
25553B		
25684D		
25685D		
25751A	6.7/6.7	
25814A		
25852C	6.7/6.3	3.4/3.4
25863B		
25864D		3.4/3.4
25942B		3.8/3.8
25962B		
26093C		
26132B		3.8/3.8
26191D		3.8/3.8
26241B	6.7/6.7	
26262D		
26331C		
26372C	6.7/6.7	
26521B		

Genotypes are given in allele sizes (kb)
The letters after the 5 digit numbers refers to groups;
A;high offspring/low parent blood presure B;high offspring/high parent blood pressure C;low offspring/low parent blood pressure
D;low offspring/high parent blood pressure

Ladywell number	DraI α_1-adreno receptor	TthIII glucocorticoid receptor
10082B	6.7/6.7	3.4/3.4
10083B	6.7/6.7	3.4/3.4
10121C	6.7/6.7	
10173A	6.7/6.3	3.8/3.4
10252B	6.7/6.7	3.8/3.4
10371B	6.7/6.3	3.4/3.4
10502A	6.7/6.3	3.4/3.4
10531D	6.7/6.7	3.4/3.4
10592B	6.7/6.7	3.4/3.4
10621D	6.7/6.7	3.4/3.4
10622B	6.7/6.7	3.4/3.4
10892A	6.7/6.7	3.4/3.4
10921D	6.7/6.3	3.4/3.4
10943B	6.7/6.7	
10952D		
10953B	6.7/6.7	3.8/3.8
11001C		3.8/3.8
11002C	6.7/6.7	3.8/3.8
11062A		3.8/3.8
11232B	6.7/6.7	
11252C	6.7/6.7	3.8/3.4
11291D	6.7/6.3	3.8/3.4
11352B	6.7/6.7	3.8/3.4
11362D	6.7/6.7	3.4/3.4
11453D		3.4/3.4
11543A	6.7/6.7	3.4/3.4
11611C	6.7/6.7	3.4/3.4
11722D	6.7/6.7	3.4/3.4
11842A	6.7/6.7	
11914D	6.7/6.3	
11924A	6.7/6.7	
11951B	6.7/6.7	3.8/3.4
11952D	6.7/6.7	3.8/3.4
12062B	6.7/6.7	3.8/3.4
12063D	6.7/6.7	
12111C	6.7/6.7	
12112C		
12132B	6.7/6.7	3.4/3.4
12203D	6.7/6.3	3.4/3.4
12251B	6.7/6.7	3.4/3.4
12252B	6.7/6.7	3.8/3.4
12281D		
12513A	6.7/6.7	3.4/3.4
12594A	6.7/6.7	
12792D		
12902C	6.7/6.7	3.8/3.4
12922A	6.7/6.7	
12942C	6.7/6.3	3.8/3.8

12982C	6.7/6.7	3.8/3.8
12983A	6.7/6.3	3.4/3.4
13003B	6.7/6.7	
13101D	6.7/6.7	
13102B	6.7/6.3	
13123C	6.7/6.7	
13222D		
13241C	6.7/6.7	3.4/3.4
13421A		
13423A	6.7/6.7	3.4/3.4
13642D		3.4/3.4
13654D	6.7/6.7	3.4/3.4
13684A	6.7/6.7	3.4/3.4
13802D	6.7/6.3	3.4/3.4
13862C	6.7/6.3	
13911A	6.7/6.3	3.8/3.8
14013B	6.7/6.7	3.4/3.4
14024C		
14051A		3.8/3.4
14241A	6.7/6.3	3.4/3.4
14242A		3.4/3.4
14323C	6.7/6.7	3.4/3.4
14332D	6.7/6.7	3.4/3.4
14542A	6.7/6.7	3.8/3.4
14582B	6.7/6.3	
14622B	6.7/6.7	3.4/3.4
14642A	6.7/6.7	3.4/3.4
14672B	6.7/6.7	3.8/3.4
14763C	6.7/6.7	3.8/3.4
14792C		3.8/3.8
14882D	6.7/6.3	
14911D	6.7/6.7	
14974D	6.7/6.7	3.4/3.4
15082B	6.7/6.7	
15282D	6.7/6.7	3.4/3.4
15502B		
15601B	6.7/6.7	3.4/3.4
15691C	6.3/6.3	3.4/3.4
15723D	6.7/6.7	3.4/3.4
15771B	6.7/6.7	3.4/3.4
15833A		
15932A	6.7/6.7	
16111B	6.7/6.3	3.4/3.4
16182C	6.7/6.3	3.8/3.4
16301A		
16332A		
16382C	6.7/6.3	3.4/3.4
16531B	6.7/6.7	3.4/3.4
16621A		3.8/3.4
16701D		3.8/3.4
16702D	6.7/6.7	3.8/3.4
16771D	6.7/6.7	3.4/3.4
16841A	6.7/6.7	3.4/3.4
17052D		

17161A	6.7/6.7	3.4/3.4
17321A	6.7/6.7	
17371A	6.7/6.7	3.8/3.4
17551B	6.7/6.3	3.8/3.4
17671B	6.7/6.7	3.4/3.4
17672D	6.7/6.7	3.4/3.4
17892A	6.7/6.7	3.4/3.4
20072D		3.4/3.4
20091C	6.7/6.7	3.4/3.4
20131B	6.7/6.7	3.4/3.4
20142A	6.7/6.7	3.4/3.4
20152C	6.7/6.3	
20252C	6.7/6.3	3.4/3.4
20253C	6.7/6.7	3.4/3.4
20351C	6.7/6.7	3.4/3.4
20422D		3.4/3.4
20423D	6.7/6.3	
20472A	6.7/6.7	3.4/3.4
20553A	6.7/6.7	
20561C	6.7/6.7	3.4/3.4
20682C	6.7/6.7	3.4/3.4
20741D	6.7/6.7	3.4/3.4
21052C	6.7/6.3	3.4/3.4
21192D		3.8/3.8
21222B	6.7/6.3	3.4/3.4
21272A	6.7/6.7	3.4/3.4
21332C	6.7/6.7	
21451C		
21522B	6.7/6.7	3.4/3.4
21702A	6.7/6.7	
21714A	6.7/6.7	3.8/3.8
21722A	6.7/6.7	
21843B	6.7/6.7	3.4/3.4
22023B		
22024D	6.7/6.7	3.4/3.4
22112B		
22381C	6.7/6.7	3.8/3.4
22752A	6.7/6.7	
22762A	6.7/6.3	3.4/3.4
22942D	6.7/6.7	3.8/3.8
23061A	6.7/6.7	3.4/3.4
23082D	6.7/6.3	3.4/3.4
23256D	6.7/6.7	3.4/3.4
23293A	6.7/6.7	3.8/3.4
23393A	6.7/6.7	3.4/3.4
23403D	6.7/6.7	3.8/3.4
23452D		
23523B		3.4/3.4
23583C		
23662C	6.7/6.3	3.8/3.4
23691D	6.7/6.7	3.4/3.4
23793C	6.7/6.3	
23892A	6.7/6.7	3.4/3.4
23952B	6.7/6.7	3.4/3.4

Ladywell parents RFLP Data

High blood pressure group

Ladywell number	BanI β_2-adreno receptor	HindIII Renin	BglI β_1-adreno receptor	SacI Insulin
1008F	3.4/3.4	9.0/6.2	6.2/6.2	7.5/6.0
1059F	3.4/3.4	9.0/9.0	6.2/6.2	6.0/6.0
1059M	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
1225F	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
1462M	3.7/3.4		6.2/6.2	6.0/6.0
1467F	3.4/3.4	9.0/6.2	6.2/6.2	7.5/7.5
1467M	3.4/3.4		6.2/6.2	7.5/7.5
1507M	3.7/3.4	9.0/9.0	6.2/4.7	
1507F	3.4/3.4		6.2/4.7	
1705F	3.7/3.7	6.2/6.2	6.2/6.2	
1705M	3.4/3.4	9.0/9.0	6.2/4.7	
2067M	3.4/3.4	9.0/9.0	6.2/4.7	
2067F	3.4/3.4	9.0/9.0	6.2/6.2	
2122F	3.7/3.4	9.0/9.0	6.2/6.2	7.5/7.5
2122M	3.7/3.4	9.0/9.0	6.2/6.2	6.0/6.0
2537F	3.4/3.4	9.0/6.2	6.2/4.7	7.5/6.0
2537M	3.7/3.4	9.0/9.0	6.2/6.2	7.5/6.0
1062F	3.4/3.4	9.0/9.0		
1095F	3.4/3.4		6.2/4.7	
1095M	3.4/3.4	9.0/6.2	6.2/4.7	6.0/6.0
1129F	3.4/3.4	9.0/9.0	6.2/6.2	7.5/7.5
1129M	3.4/3.4	9.0/9.0	6.2/6.2	
1195F	3.7/3.4	9.0/9.0	6.2/4.7	7.5/6.0
1195M	3.7/3.4	9.0/6.2	6.2/6.2	
1206F	3.7/3.4	9.0/9.0	6.2/6.2	7.5/6.0
1206M	3.4/3.4	9.0/9.0	6.2/6.2	7.5/6.0
1220F	3.7/3.4	6.2/6.2	6.2/6.2	7.5/6.0
1220M	3.4/3.4	9.0/6.2	6.2/4.7	6.0/6.0
1310F	3.7/3.4	9.0/9.0	6.2/6.2	6.0/6.0
1310M	3.7/3.7	9.0/6.2	6.2/6.2	
2202F	3.7/3.4	6.2/6.2	6.2/6.2	6.0/6.0
2430F	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
2430M	3.4/3.4	9.0/6.2	6.2/6.2	7.5/6.0
2514F	3.4/3.4	9.0/9.0	6.2/6.2	7.5/7.5
2520F	3.7/3.4	9.0/9.0	6.2/4.7	6.0/6.0
2520M	3.4/3.4	6.2/6.2	6.2/6.2	6.0/6.0
2586F	3.4/3.4	9.0/6.2	6.2/4.7	6.0/6.0
2586M	3.7/3.4	9.0/9.0	6.2/4.7	7.5/6.0
1136F	3.7/3.4	9.0/6.2	6.2/4.7	7.5/6.0
1136M	3.7/3.4	9.0/6.2	6.2/6.2	
1677M	3.4/3.4	9.0/9.0	6.2/4.7	
2042F	3.4/3.4	9.0/6.2	6.2/4.7	
2042M	3.7/3.4	9.0/6.2	6.2/4.7	
2325F	3.4/3.4	9.0/9.0	6.2/6.2	6.0/6.0
2325M	3.7/3.4	9.0/6.2	6.2/4.7	
2568F	3.7/3.4	9.0/6.2	6.2/6.2	

Ladywell number	DraI α_2-adreno receptor	TthIII glucocort -icoid receptor	BclI glucocort -icoid receptor	BclI Fibrinogen
1008F	6.7/6.7		4.5/4.5	5.3/5.3
1059F	6.3/6.3		2.3/2.3	
1059M	6.7/6.3	3.4/3.4	4.5/2.3	5.3/4.2
1225F	6.7/6.7		4.5/2.3	
1462M	6.7/6.7		2.3/2.3	4.2/4.2
1467F	6.7/6.7	3.8/3.4		
1467M	3.4/3.4		2.3/2.3	5.3/5.3
1507M	6.7/6.7	3.8/3.4	2.3/2.3	
1507F	6.7/6.3	3.8/3.4		5.3/4.2
1705M		3.4/3.4		
2067M	6.7/6.7	3.4/3.4	2.3/2.3	
2067F	6.7/6.7			
2122F	6.7/6.7	3.4/3.4	4.5/2.3	5.3/4.2
2122M	6.7/6.3	3.8/3.4	2.3/2.3	5.3/4.2
2537F	6.7/6.7	3.8/3.4		5.3/4.2
2537M	6.7/6.7	3.8/3.4		5.3/4.2
1026F				
1026M				
1095F	6.7/6.7		2.3/2.3	
1095M	6.7/6.3	3.8/3.8	2.3/2.3	5.3/4.2
1129F	6.7/6.3	3.8/3.4	4.5/2.3	5.3/5.3
1129M	6.7/6.7	3.4/3.4	4.5/4.5	5.3/4.2
1195F	6.7/6.7	3.8/3.4	4.5/2.3	5.3/4.2
1195M	6.7/6.3			
1206F	6.7/6.7	3.4/3.4	4.5/2.3	5.3/5.3
1206M	6.7/6.7	3.8/3.4	2.3/2.3	5.3/5.3
1220F	6.7/6.7		4.5/2.3	5.3/5.3
1220M	6.7/6.3	3.4/3.4	2.3/2.3	5.3/5.3
1310F	6.7/6.7	3.4/3.4	4.5/4.5	5.3/5.3
1310M	6.7/6.3		4.5/2.3	5.3/5.3
2202F	6.7/6.7	3.4/3.4	4.5/2.3	5.3/5.3
2430F	6.7/6.3	3.4/3.4	2.3/2.3	5.3/5.3
2430M	6.7/6.3		4.5/4.5	5.3/5.3
2514F	6.7/6.7		4.5/2.3	5.3/5.3
2520F	6.7/6.3	3.4/3.4	2.3/2.3	5.3/5.3
2520M	6.7/6.7	3.4/3.4	4.5/2.3	5.3/4.2
2586F	6.7/6.3	3.8/3.4	2.3/2.3	5.3/5.3
2586M	6.7/6.7	3.4/3.4	4.5/2.3	5.3/5.3
1136F	6.7/6.7		4.5/2.3	5.3/5.3
1136M	6.7/6.7		4.5/2.3	5.3/5.3
1677M	6.7/6.7		2.3/2.3	5.3/5.3
2042F	6.3/6.3		2.3/2.3	5.3/5.3
2042M	6.7/6.7		2.3/2.3	5.3/5.3
2325F	3.8/3.4		4.5/2.3	5.3/5.3
2325M	6.7/6.7	3.4/3.4	4.5/2.3	5.3/5.3
2568F	6.2/6.2		4.5/4.5	5.3/4.2
2568M	6.7/6.7		4.5/2.3	5.3/5.3
1670F	6.7/6.7		2.3/2.3	5.3/4.2
1670M	6.7/6.7	3.4/3.4	4.5/2.3	5.3/4.2

2568M	3.4/3.4	9.0/9.0	6.2/6.2
1670F	3.4/3.4	9.0/6.2	4.7/4.7
1670M	3.4/3.4	9.0/9.0	6.2/6.2

Low blood pressure group

Ladywell number	BanI β_2-adreno receptor	HindIII Renin	BglI β_1-adreno receptor	SacI Insulin
1184F	3.7/3.4	9.0/6.2	6.2/6.2	
1184M	3.7/3.4	9.0/6.2		
1342F	3.7/3.4	9.0/6.2	6.2/4.7	
1368F	3.4/3.4	6.2/6.2	6.2/6.2	
1368M	3.7/3.4	9.0/9.0	6.2/4.7	
1424M	3.7/3.4	6.2/6.2	6.2/6.2	
1583F	3.7/3.4	9.0/9.0	6.2/6.2	
1583M	3.4/3.4	9.0/9.0	6.2/6.2	
1593F	3.7/3.4	9.0/6.2	6.2/4.7	
1593M	3.7/3.4	9.0/6.2	6.2/6.2	
2426M	3.7/3.4	9.0/6.2	6.2/6.2	
1100F	3.4/3.4	9.0/9.0	6.2/6.2	
1100M	3.4/3.4	9.0/9.0	6.2/6.2	
1211F	3.4/3.4		6.2/6.2	7.5/6.0
1211M	3.4/3.4	9.0/9.0	6.2/6.2	6.0/6.0
1290F	3.4/3.4	9.0/6.2	6.2/6.2	7.5/6.0
1290M	3.4/3.4	9.0/6.2	6.2/6.2	6.0/6.0
2025F	3.7/3.4	9.0/9.0	6.2/6.2	6.0/6.0
2025M	3.4/3.4	9.0/9.0	6.2/6.2	7.5/6.0
2068F	3.4/3.4	9.0/6.2	6.2/6.2	7.5/6.0
2068M	3.4/3.4	9.0/9.0	6.2/6.2	6.0/6.0
2105F			6.2/4.7	
2105M	3.7/3.4	9.0/9.0	6.2/6.2	6.0/6.0
2133F	3.4/3.4	9.0/9.0	6.2/4.7	
2133M	3.7/3.4	9.0/9.0	6.2/6.2	7.5/6.0
2238F	3.4/3.4	9.0/9.0	6.2/6.2	6.0/6.0
2238M	3.4/3.4	9.0/9.0	6.2/4.7	
2401F	3.7/3.4	9.0/6.2	4.7/4.7	
2401M	3.4/3.4	9.0/9.0	6.2/4.7	7.5/6.0
2475F	3.4/3.4	9.0/9.0	6.2/4.7	7.5/6.0
2475M	3.4/3.4	9.0/9.0	6.2/6.2	7.5/6.0
2530F	3.7/3.4	9.0/9.0	6.2/6.2	7.5/6.0
2530M	3.4/3.4	9.0/9.0	6.2/6.2	
1259F	3.7/3.4	9.0/9.0	6.2/6.2	6.0/6.0
1259M	3.7/3.4	9.0/9.0	6.2/6.2	6.0/6.0
1298M	3.4/3.4	9.0/6.2	6.2/6.2	
2389F	3.7/3.4	9.0/6.2	4.7/4.7	7.5/7.5
2389M	3.4/3.4	6.2/6.2	6.2/4.7	
2449F	3.7/3.4	9.0/6.2	6.2/4.7	7.5/6.0
2449M	3.7/3.4	9.0/9.0	6.2/4.7	7.5/6.0
2476F	3.4/3.4	9.0/6.2	6.2/6.2	7.5/6.0
2476M	3.4/3.4	6.2/6.2	6.2/6.2	7.5/6.0
2609F	3.7/3.4	9.0/9.0	6.2/6.2	7.5/7.5
2609M	3.7/3.4	9.0/6.2	6.2/6.2	7.5/6.0
2637F	3.4/3.4	9.0/6.2	6.2/4.7	7.5/6.0
2637M	3.4/3.4	9.0/6.2	6.2/4.7	6.0/6.0
1684F	3.4/3.4	9.0/9.0	6.2/4.7	
1684M	3.4/3.4	9.0/9.0		

Ladywell number	DraI α_2-adreno receptor	TthIII glucocort -icoid receptor	BcI glucocort icoid receptor	BclI Fibrinogen
1184F			4.5/2.3	5.3/4.2
1342F	6.7/6.7	3.4/3.4		
1368F	6.7/6.7	3.8/3.4	4.5/4.5	5.3/4.2
1368M	6.7/6.7	3.8/3.4	4.5/2.3	5.3/4.2
1424M	6.7/6.7		2.3/2.3	
1583F	6.7/6.7	3.4/3.4		5.3/5.3
1583M	6.7/6.7	3.4/3.4	4.5/4.5	5.3/5.3
1593F	6.7/6.7	3.4/3.4	4.5/2.3	
1593M	6.7/6.3	3.4/3.4	4.5/2.3	5.3/5.3
2426M	6.7/6.7		4.5/2.3	5.3/5.3
1100F	6.7/6.7			
1100M	6.7/6.7			
1211F	6.7/6.7		2.3/2.3	
1211M	6.7/6.3	3.4/3.4	4.5/2.3	5.3/5.3
1290F	6.7/6.7	3.4/3.4	2.3/2.3	5.3/5.3
1290M	6.7/6.7	3.8/3.4	4.5/4.5	5.3/4.2
2025F		3.4/3.4	2.3/2.3	5.3/5.3
2025M	6.3/6.3	3.4/3.4		
2068F	6.7/6.3	3.4/3.4	2.3/2.3	5.3/5.3
2068M	6.7/6.7			5.3/5.3
2105F	6.3/6.3			5.3/5.3
2105M	6.7/6.7		2.3/2.3	5.3/5.3
2133F	6.3/6.3			
2133M			4.5/2.3	5.3/4.2
2238F	6.7/6.7		4.5/2.3	
2238M	6.7/6.7		2.3/2.3	5.3/5.3
2401F	6.7/6.3		4.5/2.3	
2401M	6.7/6.3			5.3/5.3
2475F	6.7/6.7			
2475M	6.7/6.3		2.3/2.3	5.3/4.2
2530F	6.7/6.3	3.8/3.4		5.3/5.3
2530M	6.7/6.7	3.8/3.4		
1259F	6.7/6.7	3.8/3.4	4.5/2.3	5.3/5.3
1259M	6.7/6.3		2.3/2.3	5.3/5.3
1298M	6.7/6.3	3.4/3.4	2.3/2.3	5.3/5.3
2389F	6.7/6.3	3.8/3.8	4.5/2.3	5.3/5.3
2389M	6.7/6.7	3.4/3.4	4.5/2.3	
2449F	6.7/6.3	3.4/3.4	2.3/2.3	5.3/5.3
2449M	6.7/6.3	3.8/3.4	4.5/4.5	5.3/4.2
2476F	6.7/6.7	3.8/3.4	2.3/2.3	5.3/5.3
2476M	6.7/6.7	3.8/3.4	4.5/2.3	5.3/5.3
2609F	6.7/6.7	3.4/3.4	4.5/2.3	5.3/4.2
2609M	6.7/6.7	3.4/3.4	4.5/2.3	5.3/5.3
2637F	6.7/6.7		4.5/2.3	5.3/4.2
2637M	6.7/6.7		4.5/2.3	5.3/5.3
1684F	6.7/6.7	3.4/3.4		
1684M	6.7/6.7	3.4/3.4		
1062F	6.7/6.7	3.4/3.4	4.5/4.5	5.3/4.2
1062M		3.4/3.4	4.5/2.3	5.3/4.2

APPENDIX IV

Angiotensinogen PCR data

Group B offspring (high offspring/high parent blood pressure)

Ladywell no.	Heteroduplex formation	Genotype M235T	T174M
10082	-ve	WW	WW
10083	-ve	WW	WW
10252	-ve	WW	MM
10371	-ve	WW	WW
10592	-ve	WW	WW
10622	-ve	WW	WM
10943	-ve	WW	MM
10953	+ve	WM	WM
11232	-ve		
11352	-ve	WW	MM
11951	-ve	WW	WM
12062	-ve	WW	WM
12132	+ve	WM	WM
12251	-ve	WW	WM
13003	+ve	WM	WM
14013	-ve	WW	WW
14582	-ve	WW	WW
14672	+ve	WM	WM
15082	-ve	WW	WW
15771	-ve	WW	WM
16111	-ve	WW	WW
16531	-ve	WW	WW
17551	-ve	WW	WW
17671	-ve		
20131	-ve	WW	MM
21222	-ve	WW	WM
23523		WW	WW
23952	-ve	WW	WW
24301	-ve	WW	WM
24516	-ve	WW	WM
24721		WW	WM
24821	-ve		
25143	-ve	WM	WM
25371	+ve	WM	WM
25942	-ve	WW	WM
25962	-ve	WW	
26132	-ve	WW	WM

Group C offspring (low offspring/low parent blood pressure)

Ladywell no.	Heteroduplex formation	Genotype M235T	T174M
10121	-ve		
11001	-ve	WW	WM
11002	-ve		
11252	-ve	WW	WM
11611	-ve	WW	WW
12111	-ve		
12112	+ve	WM	WM
12902	+ve	WM	WM
12942	+ve	WM	WM
12982	+ve	WM	WM
13122	-ve	WW	WW
13862	+ve	WM	WM
14024	+ve	WM	WM
14323		WW	WW
14672	+ve		
14763	-ve	WW	WM
14792	+ve	WM	WM
15691	-ve	WW	WM
16182	-ve	WW	WW
16382	-ve		
20091	-ve	WW	WW
20152	-ve	WW	MM
20252	-ve	WW	WM
20253		WW	WW
20351	-ve	WW	WW
20682	+ve	WM	WM
21052	-ve	WW	WW
21332	-ve	WW	WM
21451	-ve	WW	WW
22381	+ve	WM	WM
23662	+ve	WM	WM
23793	-ve	WW	WM
24011	-ve		
24521	-ve	MM	MM
24531		WW	WM
24751	-ve	WW	WM
24752	-ve	WW	WM
24762	-ve	WW	WW
25361	-ve	WW	MM
25544	-ve		
25852	-ve	WW	WM
26093	-ve		
26372	-ve	WW	WW

W;wild type M;mutant
+ve; scored positive for heteroduplex formation
-ve; scored negative for heteroduplex formation