DEVELOPMENT OF CONGENIC LINES AND APPLICATION OF PHYSICAL MAPPING STRATEGIES FOR THE DISSECTION OF BLOOD PRESSURE QUANTITATIVE TRAIT LOCI IN THE STROKE-PRONE SPONTANEOUSLY HYPERTENSIVE RAT

by

Cervantes Domingo Negrín Deus, M.D.

This being a thesis submitted for the degree of Doctor of Philosophy in the Faculty of Medicine, University of Glasgow

Department of Medicine and Therapeutics

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

DECLARATION

I declare that this thesis has been written entirely by myself and is a record of work performed by myself. It has not been submitted previously for a higher degree. The research was carried out in the Department of Medicine and Therapeutics, University of Glasgow, under the supervision of Professor Anna Dominiczak.

Cervantes D. Negrín D.

October 2000

ACKNOWLEDGEMENTS

I would like to make extensive my gratitude and sincere appreciation to each of the following persons who helped me in the preparation of this thesis:

Professor Anna Dominiczak for her infinite intellectual support, her teaching, and enthusiasm while supervising throughout the course of my PhD.

Drs. James Clark and Baxter Jeffs for their expert guidance in all the molecular techniques that were completely unknown for me, and for their good humor to confront the everyday scientific challenge. Special thanks to Dr. Delyth Graham who performed and taught me all the surgical procedures involved in the radio-telemetry and guided me through the intricate breeding protocols used to develop congenic animals.

Dr. Niall Anderson for his help in all the complex analysis performed in the cardiovascular phenotypes and in the no less complex MAPMAKER analysis for the new maps obtained.

Finally and most owingly I would like to thanks my wife Carolina and my small daughter Claudia whose understanding, support, and affection have been essential throughout these years.

TABLE OF CONTENTS.

LIST OF FIGURES.

LIST OF TABLES.

LIST OF ABBREVIATIONS AND SYMBOLS.

AII	Angiotensin II.
ACE	Angiotensin converting enzyme.
AGT	Angiotensinogen.
AME	Apparent mineralocorticoid excess.
ANOVA	Analysis of variance.
ANP	Atrial natriuretic peptide.
BAC	Bacterial artificial chromosome.
BC	Back cross.
BN	Brown Norway rat.
BP	Blood pressure.
BW	Body weight.
cDNA	Complementary DNA.
cM	centiMorgan.
cR	centiRays.
Dahl R	Dahl salt resistant rat.
Dahl S	Dahl salt sensitive rat.
DBP	Diastolic blood pressure.
dNTP	Deoxinucleotide.
ddNTP	Dideoxinucleotide.
DNA	Deoxyribonucleic acid.
FHH	Fawn-hooded hypertensive rat.
F1	First filial progeny.
F2	Second filial progeny.
g	grams.
GH	Genetically hypertensive rat.
GRA	Glucocorticoid remediable aldosteronism.
HW	Heart weight.
LEW	Lewis rat.
LH	Lyon hypertensive rat.
LN	Lyon normotensive rat.
LV	Left ventricle.
LV+S	Left ventricle + septum.
MAP	Mean arterial pressure.
Mg^{+2}	Magnesium.
mg	Miligrams.
MHS	Milan hypertensive rat.
MNS	Milan normotensive rat.
mRNA	Messenger RNA.
PAC	Phage P1 clones.
PCR	Polymerase chain reaction.
QTL(s)	Quantitative trait locus (loci).
RFLP	Restriction fragment length polymorphism.
RH	Radiation hybrid.
RNA	Ribonucleic acid.
Sabra H	Sabra hypertensive rat.
Sabra N	Sabra normotensive rat.
SBP	Systolic blood pressure.

SD	Standard deviation of the mean.
SEM	Standard error of the mean.
SHR	Spontaneously hypertensive rat.
SHRSP	Stroke-prone spontaneously hypertensive rat.
WKY	Wistar-Kyoto rat.
YAC	Yeast artificial chromosome.
θ	Probability of breakage.

SUMMARY.

Human essential hypertension is a complex, multifactorial, quantitative trait under polygenic control. Several strategies have been developed over the last decade to dissect genetic determinants of hypertension. Of these, the most successful have been studies identifying rare Mendelian syndromes in which a single gene mutation causes high blood pressure (BP). The attempts to identify multiple genes, each having a small contribution to the common polygenic form of hypertension, have been less successful. Experimental models of genetic hypertension have been used to develop paradigms for the study of human essential hypertension in order to remove some of the complexity inherent in studying human subjects. Several laboratories, using diverse crosses between hypertensive and normotensive strains, identified several quantitative trait loci (QTLs) for BP regulation. The strategy used to identify BP QTLs is known as a genome scan and involves the determination of the BP in a large segregating F2 population derived by crossing contrasting inbred rat strains, and the genotyping of a large panel of polymorphic microsatellite markers with a thorough coverage of the entire rat genome. The next step is the production of congenic strains and substrains to confirm the existence of the BP QTLs and to narrow down the chromosomal region of interest.

The investigations reported in this thesis incorporate the use and validation of a "speed" congenic strategy to dissect two BP QTLs identified previously on rat chromosome 2. We produced 4 congenic strains through introgression

of various segments of chromosome 2 from the WKY_{Gla} strain into the recipient $SHRSP_{Gla}$ strain, and vice versa. Transfer of the region of rat chromosome 2 containing both BP QTLs from WKY_{Gla} into an $SHRSP_{Gla}$ genetic background lowered both baseline and salt-loaded systolic BP by ~20 and ~40 mmHg in male congenic rats compared with the SHRSP parental strain ($F=53.4$, $p<0.005$; $F=28.0$, $p<0.0005$, respectively). In contrast, control animals for stowaway heterozygosity presented no deviation from the BP values recorded for the $SHRSP_{Gla}$, indicating that if such heterozygosity exists, its effect on BP is negligible. Reciprocal congenic strain in which one QTL was transferred from $SHRSP_{Gla}$ onto the WKY_{Gla} background resulted in statistically significant but smaller BP increase. This implicated region contains different candidate genes including the Na^+-K^+ATPase α_1 isoform (*Atp1a1*), natriuretic peptide receptor A/Guanylate cyclase A (*Gca*), angiotensin II receptor type 1B (*Agtr1b*), and calcium/calmodulin-dependent protein kinase II delta subunit (*Camk2d*). Sequencing analysis showed no differences in the coding regions of the *Atp1a1* gene between the WKY_{Gla} strain and the published sequence. Two transitions were found between WKY_{Gla} and $SHRSP_{Gla}$ resulting in silent mutations. Therefore, the *Atp1a1* gene was not supported as a candidate gene for the BP QTL on rat chromosome 2.

Radiation hybrid mapping was performed along with fluorescence *in situ* hybridisation of rat chromosome 5 due to the discrepancies between our genetic map and other genetic maps of rat chromosome 5. We successfully constructed a radiation hybrid map of rat chromosome 5 using 35

microsatellite markers covering a genetic distance of 78 cM, corresponding to a physical distance of approximately 1,304 cR (17 cR/cM) and comparable to reports from other laboratories. The *Anf* microsatellite marker was mapped between *D5Rat48* and *D5Rat47* located at the telomeric end of rat chromosome 5. Fluorescence *in situ* hybridisation confirmed that the *Anf* gene is localised to the 5q36.3, which corresponds to the telomeric end of the chromosome 5. Two different physical mapping methods have therefore given identical results and are in agreement with genetic maps published by other groups. We also produced a high resolution radiation hybrid map of the telomeric end of rat chromosome 2 between markers *D2Mit6* and *D2Mgh12*. The physical to genetic distance conversion gave us an estimate of 20.8 cM to 31.2 cM for this region and facilitated fine mapping of the two BP QTLs on rat chromosome 2. We constructed congenic substrain SP.WKY.Gla2c* where a small segment of approximately 20 cM was transferred from the normotensive WKY_{Gla} strain into the hypertensive $SHRSP_{Gla}$. Phenotyping of the congenic substrain is currently ongoing and will determine if the BP QTL was successfully trapped. Additionally, we produced a high resolution radiation hybrid map of this segment, which will help in the identification of the gene(s) involved in this BP QTL.

It follows that we clearly demonstrated the applicability of a reciprocal speed congenic strategy in the rat. We began further dissection of the BP QTL by constructing congenic sub-lines and performing physical mapping of the region of interest. Careful phenotyping of these substrains will narrow down and refine the location of the BP QTL to a size where substitution

mapping, microarrays, positional cloning, and comparative mapping to human chromosomes will permit the identification of the causal genes. A better understanding of the gene(s) and their pathophysiologic pathways will pave the way for more specific treatments and more importantly, for earlier prevention of hypertensive cardiovascular disease.

CHAPTER 1

INTRODUCTION.

1.1 HYPERTENSIVE CARDIOVASCULAR DISEASE AS A GENETIC COMPLEX TRAIT.

A single gene can be contributing to a preventable or treatable disease. If the gene can be identified, then the structure, function, and ultimate role of that gene in influencing the disease can be revealed, thus leading to better ways of future detection, prevention, and treatment of the pathology (Shimkets and Lifton, 1996). However, for hypertensive cardiovascular disease this process has not yet been achieved for the most common of its representatives, essential hypertension. Hypertensive cardiovascular disease is an entity where, unfortunately, the individual susceptibilities to it and its sequelae are known to be mediated by a large number of genetic and non-genetic factors (Lander and Schork, 1994).

The key feature of genetically complex traits such as essential hypertension is that the disease "*per se*" can be attributed to intricate inter-relationships between genes and environmental factors (Figure 1.1). It follows that complex traits do not show classical Mendelian inheritance attributable to a single gene, and therefore their inheritance can be described as polygenic and multifactorial (Schork, 1997).

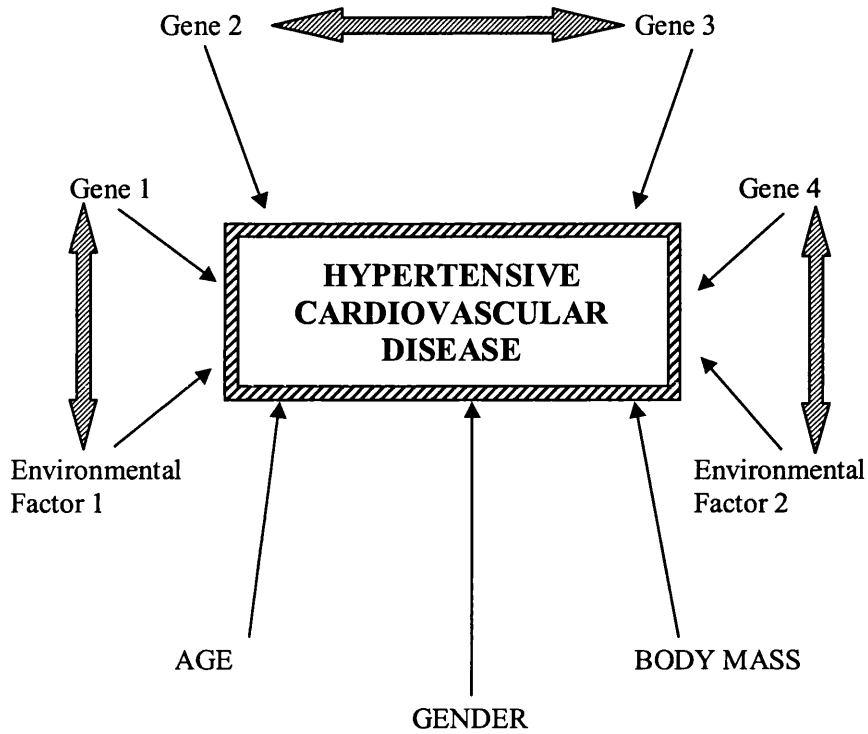

Figure 1.1. Multifactorial model of hypertensive cardiovascular disease, demonstrating the potential influence of genes, environmental factors, and demographic factors. The interactions between these determining factors is represented by arrows linking them. Modified from Lifton (Lifton, 1995).

1.1.1 HUMAN ESSENTIAL HYPERTENSION.

In mechanical and haemodynamic terms, the level of blood pressure is determined by the amount of blood that is pumped out by the heart and by resistance to flow in the peripheral arterial tree. The resistance to flow is found mainly in the small arterioles that are highly contractile and are at all times constricted to some degree; therefore the amount of constriction determines the level of blood pressure. Maintaining a blood pressure level guarantees adequate tissue perfusion, however for its regulation several systems have to work in conjunction, with complicated and intricate inter-relationships (Schork *et al.* 1996).

A number of factors can influence blood pressure level within an individual. This level is influenced by interactions of a host of systems and sub-systems (neural, hormonal, and circulatory). Additional phenomena, such as development, growth, and ageing, can further complicate blood pressure regulation. Each system and sub-system may have a more or less pronounced effect on an individual's blood pressure level at different times in life. This complexity, multitude of systems and age dependencies create enormous potential for a variety of mutant genes to produce deleterious effects on the blood pressure level (Schork, 1997). Essential hypertension is the term used for those patients found to have arterial hypertension but with no evident cause for the disorder. It is also referred to as primary

hypertension or idiopathic hypertension (Hamet *et al.* 1998). Essential hypertension is a very common disease, characterised by devastating consequences such as stroke, myocardial infarction and end-stage renal disease (Hamet *et al.* 1998). Since there is no dividing line between normal and high blood pressure, an arbitrary level has been established to define those who have an increased risk of developing a morbid cardiovascular event and will clearly benefit from medical therapy. This level according to the WHO (Gordon, 1994)criteria is defined as systolic blood pressure of 140 mmHg or greater and/or diastolic blood pressure of 90 mmH or greater.

1.1.2 BLOOD PRESSURE AS A HERITABLE TRAIT.

During the 1960s there was a dispute regarding the distribution of blood pressure levels in the general population. Sir Robert Platt claimed the existence of bimodality in the distribution of blood pressure, and it was attributed to the segregation and effect of a single major gene (Platt, 1967). In contrast, Sir George Pickering supported the existence of a unimodal distribution in detailed, large surveys of the London population (Pickering, 1967). This type of distribution would suggest the existence of many genetic factors segregating independently. At present, we could say that both Sir George and Sir Robert were right: although several monogenetic forms of hypertension have been clearly identified, essential hypertension is now understood as being a polygenic disease with complexities such as "gene-gene" and "environment-gene" interactions (Shimkets and Lifton, 1996) (Figure 1.1).

All evidence suggests the existence of a genetic component in the aetiology of human essential hypertension, with 30-50% of variation in blood pressure between individuals being attributed to genetics factors. Different studies have calculated the percentage of the phenotypic variability in blood pressure attributable to genetic factors (Table 1.1) (Ward, 1990). Even though these studies used different designs, the results are remarkably similar. In general, the percentage of blood pressure variability is lower and more variable for diastolic blood pressure than for systolic blood pressure. This can be explained either because the definition of diastolic blood pressure values varies from one study to another or because diastolic blood pressure is more influenced by environmental factors than systolic (Mongeau, 1989).

The clustering of blood pressure observed within families revealed a highly significant aggregation according to several epidemiological studies. When the blood pressure measurements are compared in relatives, correlations increase with the degree of genetic relationship between the relatives. Identical twins who share all their genes demonstrate higher concordance for blood pressure than non identical twins who share 50% of their genes (McIlhany *et al.* 1975; Feinleib *et al.* 1977; Havlik *et al.* 1979; Rose *et al.* 1979; Heiberg *et al.* 1981; Levine *et al.* 1982). Similarly, correlations are higher for first-degree relatives such as sib-sib and child-parent pairs than

Reference	Location	Design	% of variance due to genetic factors.	
			SBP	DBP
(Annest *et al.* 1979)	Montreal	Adopted	34	30
(Moll *et al.* 1983)	Detroit	Family sets	26	___
(Morton *et al.* 1980)	Hawaii	Families	24	18
(Ward, 1990)	Tokelau	Families	28	25
(Longini *et al.* 1984)	Tecumseh	Families	42	30
(Iselius *et al.* 1983)	Pooled data	____	19.4-36	14.7-34.6

Table 1.1. Percentage of blood pressure variability attributable to genetic factors reported in several studies. SBP; Systolic blood pressure, DBP; Diastolic blood pressure. Adapted from Mongeau (Mongeau, 1989).

for more distant relatives (Zinner *et al.* 1971; Annest *et al.* 1979; Weinberger *et al.* 1981). As an important control, studies of families with adopted and biological children have shown a significant correlation in the blood pressure of parents and their biological children that is not seen when blood pressures are compared between parents and their adopted children (Biron *et al.* 1976). Individuals with essential hypertension are about twice as likely as normotensive individuals to have a parent who is hypertensive (Perera *et al.* 1972).

Blood pressure is recognised today as a quantitative trait, and the general consensus is that blood pressure distributions stratified by age and sex, are unimodal (Rapp, 1983). This mode of distribution suggests that the genetic contributions to blood pressure are polygenic in nature. There are many genetic loci influencing blood pressure and the effect of each gene at these loci is not readily discernible from the others. Since the aetiology of hypertension is being multifactorial, multiple combinations of genetic and environmental factors may lead to the same blood pressure level (Mongeau, 1989). The effects of individual genes can be independent, or can demonstrate greater complexity, characterised by phenomena such as epistasis and pleiotropy (Schork *et al.* 1996). Moreover, genes that mediate the response of blood pressure to environmental factors, such as stress and diet, are significant determinants of the hypertensive phenotype (Hamet, 1996). For example, hypertension-related phenotypes such as left ventricular hypertrophy, insulin resistance, obesity, dyslipidaemia and

sensitivity of blood pressure to stress are not consistently present in all hypertensive individuals (Williams *et al.* 1996).

1.2 GENETIC DISSECTION OF HUMAN ESSENTIAL HYPERTENSION.

Human essential hypertension has an important genetic component, however genes accountable for this variation, and their specific interaction with environmental and demographic factors, remain unknown. Human studies have been conducted in two main areas for gene recognition; firstly the in-depth analysis of rare Mendelian forms of hypertension, and secondly the equivalently intense interrogation of diverse candidate genes involved in blood pressure regulation.

1.2.1 MENDELIAN DISORDERS ASSOCIATED WITH ABNORMAL BLOOD PRESSURE.

Different monogenic disorders associated with altered blood pressure have been clearly identified (Table 1.2). These are characterised by Mendelian modes of inheritance attributable to a single-gene locus (Karet and Lifton, 1997). Although Mendelian forms of hypertension are rare, the understanding of their pathogenesis could bring a better knowledge of blood pressure regulation, which in turn will be useful in the study of the most common form of hypertension, essential hypertension. Moreover, the genes and pathways involved in these rare forms of hypertension are logical

Syndrome	Inheritance	Chromosome	Gene	Reference
Glucocorticoid remediable aldosteronism	AD	8q21	CYP11B1/ CYP11B2	(Lifton *et al.* 1992; Pascoe *et al.* 1992)
Apparent mineralocorticoid excess	AR	16q22	HSD11B2	(Mune *et al.* 1995)
Adrenal hyperplasia IV, Female pseudo-hermaphroditism	AR	8q21	CYP11B1	(White *et al.* 1991)
Adrenal hyperplasia V, Male pseudo-hermaphroditism	AR	10q24.3	CYP17	(Kagimoto *et al.* 1988)
Von Hippel Lindau syndrome	AD	3p26-25	VHL tumour suppressor	(Latif *et al.* 1993)
Isolated pheochromocytoma	AD	1p	?	(Moley *et al.* 1992)
Multiple Endocrine neoplasia type II A	AD	10q11.2	RET proto-oncogene	(Mulligan *et al.* 1993)
Multiple Endocrine neoplasia type II B	AD	10q11.2	RET proto-oncogene	(Hofstra *et al.* 1994)
Liddle's syndrome	AD	16p12-13	SCNN1B	(Shimkets *et al.* 1994)
			SCNN1G	(Hansson *et al.* 1995)

Table 1.2. Mendelian forms of altered blood pressure. Continued on next page.

Syndrome	Inheritance	Chromosome	Gene	Reference
Gordon's syndrome	AD	1q31-q42	?	(Mansfield *et al.* 1997)
		17p11-q21	?	
Hypertension and brachydactyly	AD	12p	?	(Schuster *et al.* 1996)
Pseudohypoaldosteronism type I	AD	4q31.1	MLR	(Geller *et al.* 1998)
	AR	12p13	SCNN1A	(Chang *et al.* 1996)
		16p13-p12	SCNN1B	(Chang *et al.* 1996)
Bartter's syndrome Type I	AR	15q11q22	NKCC2	(Simon *et al.* 1996)
Type II	AR	11q24	ROMK	(Simon *et al.* 1996)
Type III	AR	1p36	CLCNKB	(Simon *et al.* 1997)
Gitelman's syndrome	AR	16q13	NCCT	(Simon *et al.* 1996)

Table 1.2. Mendelian forms of altered blood pressure. AD, autosomal dominant mode of inheritance; AR, autosomal recessive mode of inheritance. Modified from Hamet *et al* (Hamet *et al.* 1998)

candidates for harbouring more common variants that contribute to less severe alterations in blood pressure. These disorders result in severe or resistant hypertension, making phenotypic selection easier and the effects of segregation of single alleles in families can be discerned by simplifying molecular genetic analysis.

The discovery of these rare hypertensive syndromes was due to not only a close collaboration between pathophysiological and genetic approaches but also to extremely good clinical observation of the cases (Shimkets and Lifton, 1996).

There are several Mendelian forms of human hypertension that have mutations (or at least its chromosomal localisation have been detected) imparting a large elevating effect on blood pressure levels.

1.2.1.1 GLUCOCORTICOID-REMEDIABLE ALDOSTERONISM (GRA).

GRA was first described by Sutherland and colleagues in the 1960s (Sutherland *et al.* 1966). GRA is characterised by autosomal dominant transmission of hypertension, variable elevated aldosterone levels with suppressed plasma renin activity and high levels of abnormal adrenal steroids, 18-hydroxy cortisol and 18-oxo cortisol. The aberrant steroids and aldosterone (which is normally under control of angiotensin II) are all under the control of the adrenocorticotrophic hormone (ACTH), and are consequently suppressible by exogenous glucocorticoids (Ulick *et al.*

1990). Elevation of 18-oxo cortisol is the most consistent and reliable biochemical marker of the disease. This steroid is an agonist for the mineralocorticoid receptor and has been shown to raise blood pressure in animal models (Hall and Gomez-Sanchez, 1986). Laboratory and clinical abnormalities are suppressed by treatment with glucocorticoids, whereas infusion of ACTH exacerbates these problems (Oberfield *et al.* 1981; Ganguly *et al.* 1984).

Hypertension in GRA patients is caused by constitutive secretion of aldosterone (and perhaps other adrenal mineralocorticoid hormones) but, critically this secretion is regulated by ACTH and not by angiotensin II (Figure 1.2). Cortisol is produced in the zona fasciculata and its secretion is regulated by ACTH (Lifton *et al.* 1992). The genes encoding 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) are 95% identical in nucleotide sequence and are both located on chromosome 8 (Mornet *et al.* 1989). Lifton *et al* (Lifton *et al.* 1992) performed a genetic analysis of GRA kindreds finding that the chromosome 8 carries normal copies of CYP11B1 and CYP11B2 but, in addition, have a novel, chimaeric gene absent in normal subjects (Figure 1.2). This gene arises by unequal crossing over between the CYP11B1 and CYP11B2 genes, fusing 5' regulatory sequences from 11β-hydroxylase gene into coding sequences of the aldosterone synthase gene. Therefore, aldosterone synthase gene expression and enzymatic activity is brought under control of ACTH by the abnormal 5' regulatory region of the 11β-hydroxylase gene, and aldosterone becomes ectopically secreted by the zona fasciculata (Lifton *et al.* 1992).

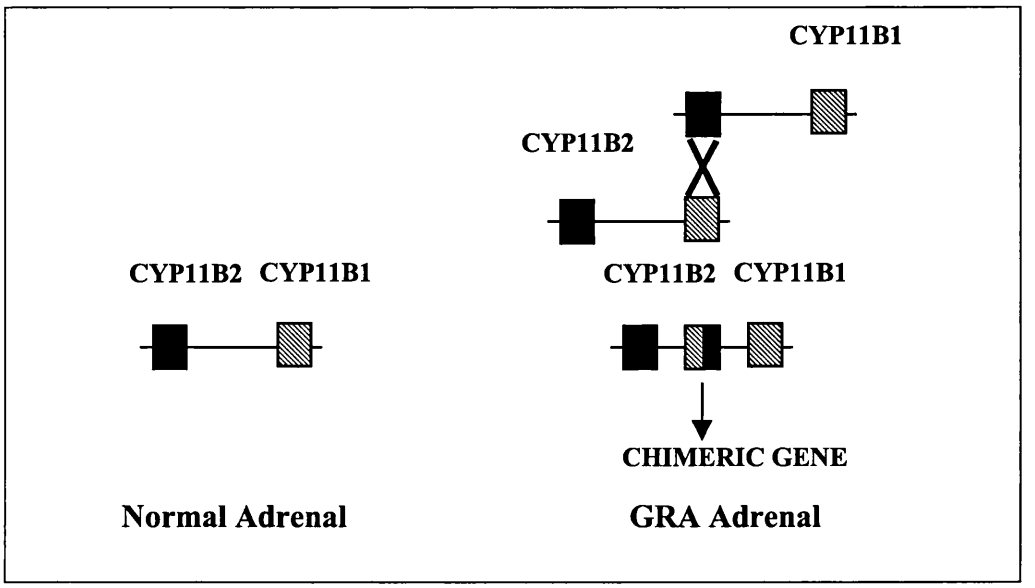

Figure 1.2.a. GRA mutation. Unequal crossing over producing the chimera between 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) genes.

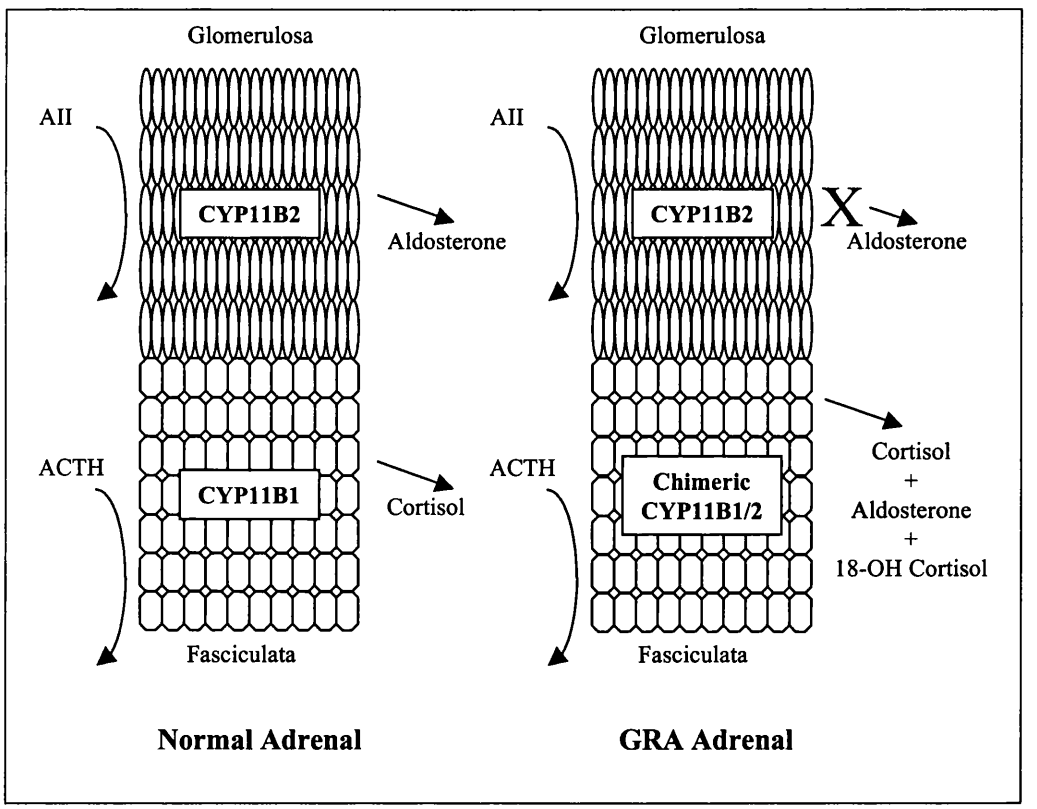

Figure 1.2.b. Pathophysiology of the adrenal cortex in GRA. In the normal adrenal gland aldosterone is secreted from the glomerulosa in response to angiotensin II (AII). In GRA adrenal gland, unequal crossing over generates a chimeric CYP11B1/2 gene that has the aldosterone activity but is expressed in the zona fasciculata under control of ACTH. Modified from Lifton *et al* (Lifton *et al.* 1992).

Even though GRA is a monogenic disease following Mendelian inheritance, there is evidence that particular conditions can modify its phenotypic expression. Genetic analysis of large kindreds reveal that the presence of the chimaeric gene does not always result in severe hypertension, and that kallikrein levels may affect the development of hypertension in this disorder (Dluhy and Lifton, 1995). One study found that blood pressure in patients with GRA is higher when the disease is inherited from the mother than when it is paternally inherited raising the possibility that the gene is imprinted (Jamieson *et al.* 1995).

1.2.1.2 LIDDLE'S SYNDROME.

Liddle's syndrome is characterised by an early onset of moderate to severe hypertension, suppressed plasma aldosterone and renin activity. Interestingly the biochemical values in these patients appear like if they were hyperaldosteronaemic (Liddle *et al.* 1974). In the original description of Grant Liddle, hypokalemia was included, however this is not a universal finding. Also, renal transplantation in Liddle's original proband corrected the defect (Botero-Velez *et al.* 1994) suggesting that the abnormality was intrinsic to the kidney. These patients respond to the administration of triamterene and amiloride which inhibit the amiloride-sensitive epithelial sodium channel. It is thought that in normal subjects the regulated absorption of sodium through this channel by aldosterone appears to be the major determinant of net renal sodium reabsortion (Figure 1.3). Canessa *et al* (Canessa *et al.* 1994), described the structure of the epithelial sodium

channel receptor. Being a heteromultimer of three subunits, each having two transmembrane domains and intracytoplasmatic amino and carboxyl terminal segments. The α-, β-, and γ-subunits are all necessary for normal channel function and are encoded by separate genes, with the α subunit on chromosome 12 and β- and γ- subunits close together on chromosome 16.

Genetic analysis demonstrated that Liddle's Syndrome is attributed to a mutation in the β-subunit gene.: specifically, the introduction of a premature stop codon (R564X) that truncates the cytoplasmatic carboxyl terminal segment of the encoded protein (Shimkets *et al.* 1994). Similar mutations have been reported in the β- and γ- subunit genes, all removing the carboxyl terminal amino acids of the protein (Hansson *et al.* 1995).

Heterologous expression of the epithelial sodium channel with mutant subunits in *Xenopus* oocytes demonstrated that these mutations result in constitutive activation of the channel (Schild *et al.* 1995). The over-activity is not explained just by alteration of single channel conductance and/or open probability, but rather by an increased number of channels inserted into the plasmic membrane. The mutations indicate that the cytoplasmatic carboxyl terminus of the β- and γ- subunits have elements required for the normal negative regulation of channel activity. The two channel subunits share a

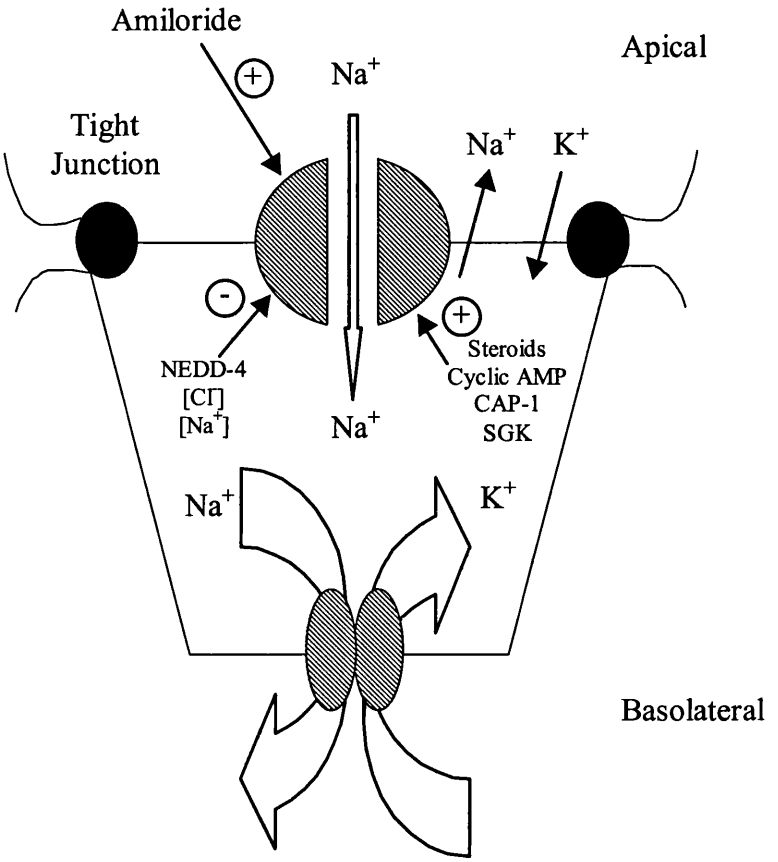

Figure 1.3. Mechanism of active sodium reabsorption across tight epithelia showing the amiloride-sensitive epithelial sodium channel. Passive electrodiffusion of sodium through the apically expressed epithelial sodium channel is coupled to an active extrusion of sodium through the basolateral sodium/potassium ATPase. Positive and negative effectors of sodium absorption are indicated with arrows. Adapted from Barbry (Barbry, 1999).

short proline-rich domains that are altered by the mutation and are often mediators of protein-protein interaction. It is proposed that the alteration of this segment may lead to defective endocytosis and results in the accumulation of the channels at the apical membrane. Such a mechanism has also been described for the LDL receptor, the lisosomal acid phosphatase and the β-adrenergic receptor (Snyder *et al.* 1995).

Another explanation has been proposed recently. Staub *et al* (Staub *et al.* 1996) have identified the rat NEDD-4 protein (a binding partner for the proline rich regions of the β- and γ- subunits) which initiates the removal of the channel from the apical membrane (Figure 1.3). Abriel *et al* (Abriel *et al.* 1999) discovered that NEDD-4 acts as a negative regulator of the wild-type epithelial sodium channel, but is inactive on the Liddle form.

No Liddle's syndrome mutations have been recognised so far in the human α-subunit of the channel, and the effects of α-subunit *in vitro* mutations on sodium channel function are still unclear (Snyder *et al.* 1995; Schild *et al.* 1996).

1.2.1.3 SYNDROME OF APPARENT MINERALOCORTICOID EXCESS (AME).

AME is an autosomal recessive disorder characterised by early onset of moderate to severe hypertension, hypokalaemia, low renin activity, and low levels of aldosterone in contrast to GRA. A low salt diet or blockade of

mineralocorticoid receptors with spironolactone ameliorates the hypertension, whereas ACTH and glucocorticoids exacerbate it. These findings suggest that cortisol acts as a stronger mineralocorticoid than is normally the case. In AME patients cortisol half-life is prolonged from approximately 80 to 120-190 minutes, and very low levels of cortisone metabolites are excreted in the urine as compared with cortisol metabolites (Ulick *et al.* 1979).

Cortisol has been shown to bind to and activate the mineralocorticoid receptor. This interaction *in vivo* is normally prevented by conversion of cortisol to cortisone by the enzyme 11β-hydroxysteroid dehydrogenase in the kidney (Arriza *et al.* 1987). Patients with AME are deficient in this enzymatic activity and normal circulatory levels of cortisol lead to profound mineralocorticoid excess. Cloning of the renal isoenzyme of 11β-hydroxysteroid dehydrogenase permitted a search for mutations in this gene (HSD11B2), and 18 different mutations in the HSD11B2 gene have been published (Mune *et al.* 1995; Wilson *et al.* 1995; Stewart *et al.* 1996; Kitanaka *et al.* 1997; Li *et al.* 1997; Dave-Sharma *et al.* 1998; Wilson *et al.* 1998). All of these mutations affect the enzymatic activity of pre-mRNA splicing. Similar but milder abnormalities occur with liquorice intoxication, where the active component of the liquorice, glycyrrhetinic acid, inhibits the activity of the 11β-hydroxysteroid dehydrogenase (Stewart *et al.* 1987; Monder *et al.* 1989).

Most patients with AME are homozygous for single mutations, with only three published cases being compound heterozygotes for two different mutations. This could suggest that the prevalence of AME mutations in the general population is low and the disease is found in limited populations with high inbreeding (Shimkets and Lifton, 1996). In patients with essential hypertension a prolonged half-life of cortisol in plasma has been found suggesting that mild 11β-hydroxysteroid dehydrogenase deficiency might contribute to essential hypertension (Walker *et al.* 1993). However, heterozygotes for mutation in the HSD11B2 gene have shown neither abnormalities in conversion of cortisol to cortisone nor an increased prevalence of hypertension (Shimkets and Lifton, 1996).

1.2.1.4 PSEUDOHYPOALDOSTERONISM TYPE II OR GORDON'S SYNDROME.

Type II pseudohypoaldosteronism was the designation used by Schambelan *et al* (Schambelan *et al.* 1981) for a syndrome in which chronic mineralocorticoid-resistant hyperkalemia with hypertension was noted. This syndrome shows autosomal dominant inheritance with variable expression. Mansfield *et al* (Mansfield *et al.* 1997) performed linkage analysis in 8 families with Gordon's Syndrome, indicating locus heterogeneity for the trait, with a multilocus lod score of 8.1. Linking the Gordon's Syndrome gene to 1q31-q42 and 17p11-p21, possible candidate genes are angiotensinogen at 1q42-q43 and the $CL^-HCO_3^-$ anion exchanger at 17p21-

p22. However, careful analysis of all the exons of both genes in 15 patients with the disease revealed no novel mutations altering the encoding products.

1.2.1.5 HYPERTENSION AND BRACHYDACTYLY.

This syndrome follows an autosomal-dominant mode of inheritance and co-segregates 100% with short stature and type E brachydactyly (Toka *et al.* 1998). Schuster *et al* (Schuster *et al.* 1996) undertook a linkage analysis study in a Turkish kindred localising the responsible gene to chromosome 12p in a region defined by markers D12S364 and D12S87. Bahring *et al* (Bahring *et al.* 1997) studied a Japanese child with type E brachydactyly and hypertension finding a *"de novo"* chromosomal deletion at 12p11.2-p12.2 that overlapped the segment to which the hypertension and brachydactyly gene had been mapped. This allowed the region of interest to be narrowed down to a 4 million base-pair segment, which is, however, still too large for positional cloning of the gene.

A putative mechanism explaining hypertension in these families has been proposed by Bahring *et al* (Bahring *et al.* 1997), involving the posterior inferior cerebellar artery at the ventrolateral medulla. Magnetic resonance imaging of the posterior fosa showed a tortuous, looping vessel, and this structure might cause neurovascular compression responsible for the hypertension.

1.2.1.6 CONGENITAL ADRENAL HYPERPLASIA DUE TO 11-BETA-HYDROXYLASE DEFICIENCY OR ADRENAL HYPERPLASIA IV.

Eberlein and Bongiovanni (Bongiovanni *et al.* 1967) first described this disease. The enzyme 11-β-hydroxylase (encoded by the CYP11B1 gene) catalyses the hydroxylation of 11-deoxycortisol to cortisol in the glucocorticoid pathway. The block of the 11 carbon results in the accumulation of 11-deoxycortisol and deoxycorticosterone, the latter being a potent salt-retaining hormone that causes hypertension rather than salt loss (Mornet *et al.* 1989). The CYP11B1 gene has been mapped to 8q 21 (Chua *et al.* 1987) and a number of different mutations have been found. The more characteristic is a missense mutation (ARG448HIS) found in Jewish subjects of Moroccan origin (White *et al.* 1991).

1.2.1.7 CONGENITAL ADRENAL HYPERPLASIA DUE TO 17-ALPHA-HYDROXYLASE DEFICIENCY OR ADRENAL HYPERPLASIA V.

The pathology is produced by mutations in the 17-alpha-hydroxylase gene or CYP17. The 17-alpha-hydroxylase enzyme catalyses both 17-alpha-hydroxylation of pregnenolone and progesterone and 17, 20-lysis of 17-alpha-hydroxypregnenolone and 17-alpha-hydroxyprogesterone. The accumulation of corticosterone and deoxycorticosterone results in both hypertension and alkalosis.

A 4-base duplication in exon 8 of the CYP17 was discovered by Kagimoto *et al* (Kagimoto *et al.* 1988) which produces a protein with an altered C-terminal amino acid sequence which resulted in loss of both enzymatic activities. However, many other mutant allelic variants have been found to yield a significant reduction in the activity of the enzyme (Yanase *et al.* 1989; Lin *et al.* 1991; Miura *et al.* 1996).

1.2.1.8 MENDELIAN FORMS OF HYPOTENSION.

The identification of mutations causing recessive forms of severe hypotension could have implications in the wider population; heterozygous carriers of these mutations could be protected from development of hypertension. Hypotension in these rare syndromes is due to the alteration of the renal sodium-handling mechanisms. This is a powerful argument for the further dissection of these pathways, both to investigate how far protective alleles mitigate against the development of hypertension in the wider population and to consider the possibilities of therapeutic intervention at this end of the spectrum of human blood pressure variation.

1.2.1.8.1 Autosomal dominant pseudohypoaldosteronism type I (ADPHA1).

ADPHA1 syndrome is characterised by neonatal renal salt wasting with hyperkalaemic acidosis, high levels of aldosterone in plasma, and clinical improvement with age (Chang *et al.* 1996). Patients with the disease do not have mutations in the epithelial sodium channel. Geller *et al* (Geller *et al.*

1998) found 4 different mutations in the mineralocorticoid receptor gene (MLR) in these patients with sequence analysis revealing two variants introducing frameshift mutations resulting in a gene product lacking the entire DNA binding and hormone binding domains, as well as the dimerisation motif.

1.2.1.8.2 Autosomal recessive pseudoaldosteronism type I (ARPHA1).

This pathology is distinguished by life-threatening dehydration and hyperkalaemia in the neonatal period, marked hypotension, salt wasting, metabolic acidosis, and high aldosterone levels and plasma renin activity. Chang *et al* (Chang *et al.* 1996) performed a genetic analysis of affected sibs demonstrating linkage of the disease to segments of chromosomes 12 or 16, with each segment containing genes encoding different subunits of the epithelial sodium channel. The same channel is mutated in Liddle's syndrome, however in ARPHA1 the mutations result in loss of function of the epithelial sodium channel. The mutations found in ARPHA1 have been located in the α- and β- subunit introducing frameshift, premature termination or missense mutations (Chang *et al.* 1996).

1.2.1.8.3 Gitelman's syndrome.

Gitelman's syndrome is an autosomal recessive disorder characterised by hypokalemic alkalosis, salt wasting, hypotension, hypomagnesemia and hypocalciuria (Gitelman *et al.* 1966). There is a marked similarity between

the clinical manifestations of this disease and the electrolyte abnormalities induced by thiazides diuretics, which motivated a candidate gene approach to detect the causal gene (Figure 1.4). Simon *et al* (Simon *et al.* 1996) cloned the gene for the thiazide-sensitive Na^+/Cl^- co-transporter (NCCT) in humans. They subsequently carried out a linkage analysis and found a very high co-segregation of marker alleles within the NCCT gene with the Gitelman's syndrome phenotype. Seventeen different mutations were recognised and mutant alleles have been shown to result in loss of normal NCCT function, leading to defective sodium and chloride reabsorption in the distal convoluted tubule. This defect is expected to result in NaCl wasting, hypovolaemia, low blood pressure and metabolic acidosis, with a subsequent elevation of renin and aldosterone levels. The elevated aldosterone levels produce increased electrogenic sodium reabsorption via the epithelial sodium channel in the cortical collecting tubule. The voltage strongly favours potassium and hydrogen excretion down the electrochemical gradient at this site, thereby inducing hypokalemia and metabolic alkalosis (Simon and Lifton, 1998).

1.2.1.8.4 Bartter's syndrome.

Bartter's syndrome is an autosomal recessive disorder that has the same features as Gitelman's apart from hypercalciuria, and normomagnesemia (Bettinelli *et al.* 1992). Three different channels have been found to be altered and produce identical Bartter's phenotype.

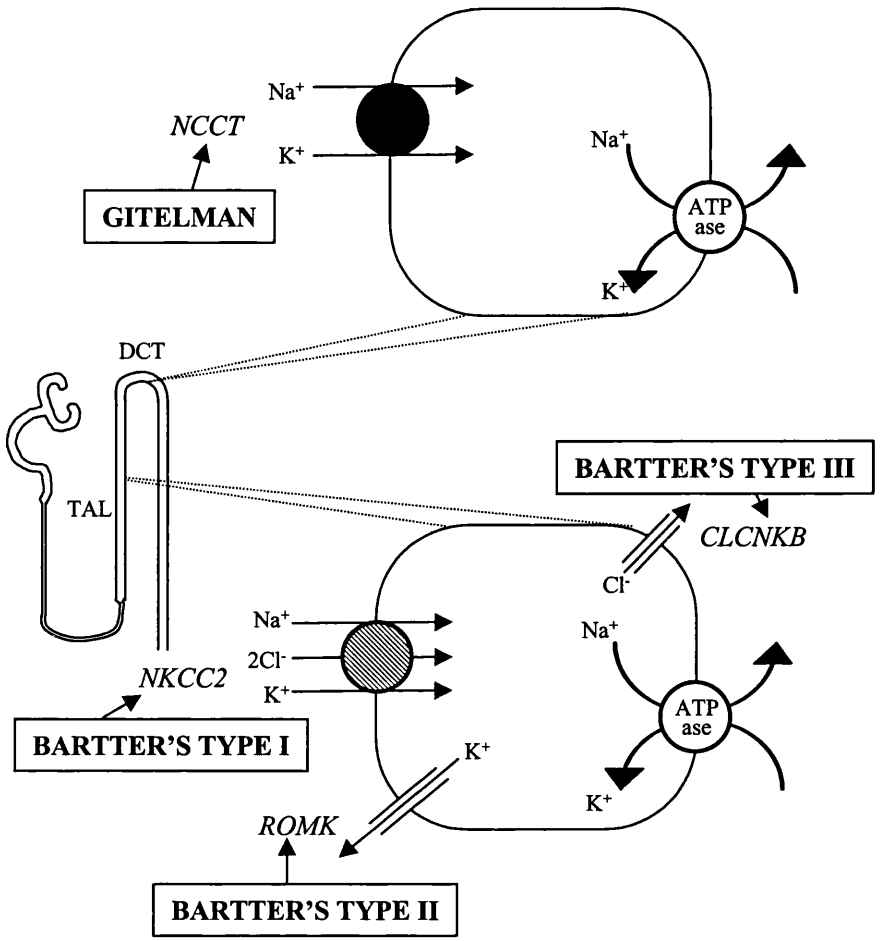

Figure 1.4. Schematic representation of representative cells from 2 different nephron segments and the channels involved in the development of Gitelman's and Bartter's syndrome. DCT, distal convoluted tubule; TAL, thick ascending limb. Modified from Simon and Lifton (Simon and Lifton, 1998).

Bartter's syndrome type I is produced when mutations in the NKCC2 (encoding for the $Na^+/K^+/2Cl^-$ co-transporter of the thick ascending limb) occur and result in loss of function of the channel. This markedly reduces NaCl reabsorption in the thick ascending limb, thereby producing salt wasting, hypovolaemia, low blood pressure, and activation of the renin-angiotensin-aldosterone system. Normal function of the $Na^+/K^+/2Cl^-$ co-transporter is required to generate the lumen positive transepithelial voltage in the thick ascending limb which drives calcium reabsorption into the bloodstream. Therefore, loss of the channel function will produce severe hypercalciuria (Simon *et al.* 1996).

Simon *et al* (Simon *et al.* 1996) provided evidence of genetic heterogenity in Bartter's syndrome patients, finding that mutations in the apical ATP-sensitive K^+ channel (ROMK) can produce identical Bartter's phenotype (Bartter's syndrome type II). The ROMK channel is a channel which recycles potassium back into the lumen of the thick ascending limb and is critical for continued co-transporter activity.

The same group (Simon *et al.* 1997) found that mutations in the CLCNKB gene can cause Bartter's syndrome type III. CLCNKB has been proposed to mediate chloride reabsorption across the basolateral membrane of renal tubular cell into the bloodstream (Figure 1.4). Patients with mutations in the CLCNKB do not have nephrocalcinosis. This clearly distinguishing this group from those with type I and type II syndrome. The loss in function of the CLCNKB channel results in increased intracellular Cl^- that inhibits

further Cl^- entry via the apical $Na^+/K^+/2Cl^-$ co-transporter, reproducing the Bartter's syndrome.

1.2.2 GENETIC STUDIES IN HUMAN ESSENTIAL HYPERTENSION.

Genetic studies of human essential hypertension were initially performed using the candidate gene strategy. However, enough genomic resources have now become available to progress to total genome-wide scanning methods (Koike and Jacob, 1998).

The candidate gene approach focuses on a single gene of interest, and the main assumption is that the hypertensive disease is caused by altered expression or structure of a gene product which in turn is reflected by changes in the gene sequence. These studies are used when the pathophysiology of a trait is at least partly understood and a number of genes are suggested as mutations within them that could result in the trait of interest (Khoury and Wagener, 1995). The extensive study of the physiology of blood pressure regulation has identified many systems that affect its regulation suggesting a number of candidate genes. For example, genes encoding enzymes and peptides of the renin-angiotensin system, the adrenergic nervous system, genes involved in sodium homeostasis, intracellular calcium regulation, and those involved in lipid metabolism. An additional source of candidate genes comes from genetic studies of animal hypertension identifying novel genes or chromosomal regions (Lifton and Jeunemaitre, 1993).

The candidate gene strategy works well for Mendelian diseases. However, the genetic background of essential hypertension is both polygenic and heterogeneous and is likely to involve epistatic (gene-gene) and ecogenetic (gene-environment) interactions with incomplete penetrance as well as the confounding phenomenon of a pronounced sexual dimorphism. It follows that, with current technology, the candidate gene strategy is not an efficient way to study hypertension owing to the hundreds or thousands of combinations of candidate genes within the population (Karet and Lifton, 1997). An additional limitation of the candidate gene approach is that the candidates are selected from a pool of previously identified genes, which represent a small portion of the estimated total. This number in the human genome has been estimated to be between 30,000 to 120,000 (Ewing and Green, 2000; Roest *et al.* 2000; Liang *et al.* 2000), and if the essential hypertension causative genes are, as is likely, among the unknown majority, it will be nearly impossible to find them using this strategy (Dominiczak and Lindpaintner 1994). Moreover, the reproducibility is poor between different populations, and different outcomes have been obtained depending on whether a linkage study or an association study was used for the analysis (Table 1.3.) (Kunz *et al.* 1997; Brand *et al.* 1998).

A genome-wide scanning strategy was first proposed by Lander and Botstein (Lander and Botstein, 1986). It has since become a strategy of choice as the numbers of genetic markers have increased, and quantitative trait loci in animal models have been studied successfully. In this strategy, a complete genetic linkage map has to be available, and the total genome can

then be scanned simultaneously to find genetic markers that cosegregate with the blood pressure phenotype (Falconer, 1989). The advantage of this approach is that the region containing the gene responsible for hypertension could be mapped without any "*a priori*" knowledge and with the potential to identify novel genes. However, identification of the chromosomal location is only the first step towards the discovery of the causal gene, and so far this strategy has only been applied successfully in three studies designed to find QTLs for blood pressure regulation (Krushkal *et al.* 1999; Xu *et al.* 1999; Hsueh *et al.* 2000).

There are three widely used genetic epidemiological approaches for localising hypertension susceptibility genes in the population: linkage analysis (pedigree analysis), allele-sharing analysis (affected sib-pairs study), and association analysis (case control study) (Lander and Schork, 1994). Classical linkage analysis involves proposing a model to explain the inheritance pattern or phenotypes and genotypes observed in a pedigree (Risch, 1990). This parametric analysis is ideal for Mendelian disorders, but in essential hypertension heterogeneity, incomplete penetrance (individuals with the susceptibility gene but without hypertension), and phenocopy (individuals with hypertension but not the susceptibility gene), make this model more complicated and less powerful (Lander and Schork, 1994).

To address the limitations of classical linkage analysis, non-parametric allele-sharing methods have been developed (O'Connor *et al.* 1996). Information regarding the mode of inheritance and penetrance of the disease

is not required and the analysis is based on detecting a significant increase in sharing of alleles between affected relatives at the loci apparently involved with the disease. This method is less powerful than a correctly specified linkage model, as the relatively late onset of hypertension leads to a shortage of sib and parent-offspring pairs (Risch and Merikangas, 1996).

Association studies are the most commonly used strategy for genetic analysis. These are based on a comparison of unrelated affected and unaffected individuals from a given population, and test whether a particular allele is observed at a higher frequency in affected individuals (Lander and Schork, 1994). Association studies give a positive result if alleles chosen for genotyping are the cause of the disease, or if these alleles lie very close to the susceptibility gene (are in linkage disequilibrium with it). However, positive associations can be observed even in the absence of linkage disequilibrium because of undetected heterogeneity in the population, when population subgroups differ systematically both in alleles frequencies and in disease incidence. For example, the presence of an association can arise as an artefact when any trait is present at a higher frequency in a subgroup and the allele studied also happens to be more common in this group (Mitchell *et al.* 1997).

Multiple candidate genes have been studied thus far (Table 1.3) and a few judged to be most relevant will be described in more detail (Section 1.2.2.1). It is important to consider the phenotype used in studies of candidates genes in essential hypertension. Most of the patients studied could be receiving

Chromosome	Localisation	Locus	Description	Strategy (A, L)	Significant result	Reference
1	1p36.2	ANP	Atrial natriuretic peptide	A	No	(Barley *et al.* 1991)
	1p36.1-35	ANPH	Na^+/H^+ antiporter	A	No	(Lifton *et al.* 1991)
	1q21.3-32.3	REN	Renin	A	Yes	(Barley *et al.* 1991)
				A	No	(Zee *et al.* 1991)
				L	No	(Jeunemaitre *et al.* 1992)
				L	No	(Naftilan *et al.* 1989)
	1q23-25	AT3	Antithrombin III	A	No	(Zee *et al.* 1991)
	1q42-43	AGT	Angiotensinogen	L	Yes	(Jeunemaitre *et al.* 1992)
				L	Yes	(Caulfield *et al.* 1994)
				L	No	(Atwood *et al.* 1997)
3	3q21-25	AT2R1	Angiotensin II type I receptor	A	Yes	(Bonnardeaux *et al.* 1994)
				L	No	(Lesage *et al.* 1997)
4	4p16.3	ADD1	Adducin α subunit	A	Yes	(Cusi *et al.* 1997)
				A	Yes	(Barlassina *et al.* 2000)
				A	No	(Alam *et al.* 2000)
				A	No	(Kato *et al.* 1998)
		EDNRA	Endothelin receptor type I	A	No	(Stevens and Brown, 1995)
5	5q31-32	ADRB2	β2-adrenergic receptor	A	Yes	(Svetkey *et al.* 1996)
				L	Yes	(Svetkey *et al.* 1997; Svetkey *et al.* 1997)
6	6p23-24	EDN1	Endothelin-1	A	Yes	(Stevens and Brown, 1995)
7	7q35-36	NOS3	Endothelial nitric oxide synthase	A	No	(Bonnardeaux *et al.* 1995)
8	8p22	LPL	Lipoprotein lipase	L	Yes	(Wu *et al.* 1996)
		NEFL	Neurofilament light polypeptide	L	No	(Wu *et al.* 1996)

Table 1.3. Candidate genes studied in human essential hypertension using association or linkage approaches. Continued on next page.

Chromosome	Localisation	Locus	Description	Strategy (A, L)	Significant result	Reference
	8p12-11.2	ADRB3	β3-adrenergic receptor	A	No	(Fujisawa *et al.* 1997)
10	10q24-26	ADRA2A	α2-adrenergic receptor	A	Yes	(Svetkey *et al.* 1996)
		ADRB1	β1-adrenergic receptor	A	No	(Svetkey *et al.* 1996)
		ADRA2C10	α2c10-adrenergic receptor	A	No	(Svetkey *et al.* 1996)
11	11p15.5	IGF2	Insulin-like growth factor II	A	No	(Morris *et al.* 1993)
		INS	Insulin	A	No	(Nabika *et al.* 1995)
12	12p13	GNB3	G-Protein subunit-β3	A	Yes	(Siffert *et al.* 1998)
				A	Yes	(Beige *et al.* 1999)
				A	Yes	(Hegele *et al.* 1998)
				A	Yes	(Schunkert *et al.* 1998)
				A	No	(Brand *et al.* 1999)
				A	No	(Kato *et al.* 1998)
16	16p13.11-12.3	SA	SA gene	L	No	(Nabika *et al.* 1995)
				A	No	(Zee *et al.* 1997)
	16q22.1	HP	Haptoglobin	A	Yes	(Robinson *et al.* 1996)
17	17q23	ACE	Angiotensin I converting enzyme	A	Yes	(Morris *et al.* 1993)
				A	No	(Fornage *et al.* 1998)
				A	No	(Schmidt *et al.* 1993)
				L	No	(Jeunemaitre *et al.* 1992)
19	19q13.3-13.4	KLK 1	Renal kalikrein gene	A	No	(Morris *et al.* 1993)
	19p13.2	INSR	Insulin receptor	A	Yes	(Morris *et al.* 1993)
	19p13.1-13.2	LDLR	Low-density lipoprotein receptor	A	No	(Morris *et al.* 1993)

Table 1.3. Candidate genes studied in human essential hypertension using association or linkage approaches. A, association strategy; L, linkage strategy. Modified from Hamet *et al* (Hamet *et al.* 1998)

treatment and their pre-treatment blood pressure might be unknown. In addition, many variables that may affect blood pressure should be taken into account. These include the age and sex of the patient, age at the time of diagnosis, presence or absence of a documented family history of hypertension, and environmental factors (smoking, obesity, alcohol consumption, oestrogen treatment, etc.). Better clinical and biochemical description of hypertensive populations would allow studies to be performed on more homogeneous populations and thereby increase their statistical power (Corvol *et al.* 1999).

1.2.2.1 ANGIOTENSINOGEN (AGT).

Human angiotensinogen (AGT) is a glycoprotein which is cleaved in its N-terminal part by renin to generate the inactive decapeptide angiotensin I, angiotensin I is then cleaved by the angiotensin-converting enzyme (ACE) producing the active hormone angiotensin II which has vasoconstrictor properties among others. Plasma-AGT concentration is approximately 1,000 fold higher than renin, and in addition AGT limits the amount of angiotensin I generated because its plasma concentration is not far from the Km of renin. AGT is mainly synthesised and present in the liver but also in other tissues such as the brain, large arteries, kidneys, and adipocytes. Therefore, modest changes in AGT could play a major role in controlling blood pressure through the generation of angiotensin I and angiotensin II (Corvol and Jeunemaitre, 1997).

The role of AGT in human hypertension was first suspected from an epidemiological study, in which a strong correlation was found between plasma AGT concentration and blood pressure (Walker *et al.* 1979), and from another study where the offspring of hypertensive patients had elevated plasma AGT levels (Fasola *et al.* 1966). Positive linkage has been found between the AGT locus and human hypertension in different studies (Jeunemaitre *et al.* 1992; Caulfield *et al.* 1994; Caulfield *et al.* 1995) when a highly polymorphic AGT microsatellite marker located in the 3' region of the gene was used for the screening. Mutations were found, and the most commonly occurring one, the M235T (or 235T variant) was linked to hypertension (Jeunemaitre *et al.* 1992; Jeunemaitre *et al.* 1993; Schmidt *et al.* 1995). Subjects carrying the 235T variant of the AGT gene have a 20% increase in plasma levels of AGT (Jeunemaitre *et al.* 1993). Moreover, Kimura *et al* (Kimura *et al.* 1992) generated transgenic mice over expressing the rat AGT gene and this resulted in hypertension. Kim *et al* (Smithies and Kim, 1994; Kim *et al.* 1995) used targeted gene disruption and duplication to generate mice having one, two or four copies of the mouse *agt* gene. Blood pressure and plasma AGT concentrations increased progressively, according to the *agt* copy number.

Despite all this evidence, no linkage has been found between the AGT locus and hypertension in others studies (Atwood *et al.* 1997; Brand *et al.* 1998; Niu *et al.* 1998). A recently published meta-analysis (Staessen *et al.* 1999) found, pooling all the available data, a weak but significant association between the 235T variant and hypertension. The association found in this

meta-analysis increased modestly when positive family history of hypertension and severity of hypertension were considered together. Finally, a guanine for adenosine substitution at position –6 upstream of the initial transcription of the AGT gene was found to be in linkage disequilibrium with the 235T variant. Moreover, this substitution caused an increased basal rate of AGT transcription when the promoter activity was evaluated *in vitro* (Jeunemaitre *et al.* 1997; Inoue *et al.* 1997).

1.2.2.2 ANGIOTENSIN I CONVERTING ENZYME (ACE).

The ACE gene is an interesting candidate for the regulation of blood pressure because it plays a key role in the generation of angiotensin II and inactivation of bradykinin. It has been shown that the insertion/deletion (I/D) polymorphism within the ACE gene can modulate plasma ACE levels (Jeunemaitre *et al.* 1992). This I/D polymorphism has been used in several association studies in humans with conflicting results as illustrated in Table 1.3 (Schmidt *et al.* 1993; Summers *et al.* 1993; Morris *et al.* 1994; Maguchi *et al.* 1996; Frossard *et al.* 1997).

It is interesting that in 5 different rat crosses BP QTLs on rat chromosome 10 have been identified containing the angiotensin I converting enzyme locus (Table 1.6). Moreover, this region has been transferred in congenic strains displaying important changes in blood pressure (Dukhanina *et al.* 1997). This region on rat chromosome 10 has been found to be homologous to human chromosome 17 using homology mapping between species. Two

studies looked at areas in the human genome homologous to BP QTLs detected on rat chromosome 10. These studies, performed with a number of highly informative microsatellite markers on chromosome 17, found evidence of linkage with a region 18 cM from the ACE locus (Julier *et al.* 1997; Baima *et al.* 1999). This region on 17q has also been linked to Gordon's syndrome. Therefore, it is possible that the human chromosome 17 carries more than one blood pressure locus.

1.2.2.3 α-ADDUCIN.

Adducin is an α/β heterodimer protein thought to regulate cell-signal transduction through changes in the actin cytoskeleton regulating the tubular absorption of ions (Hughes and Bennett, 1995). By cross-immunisation Salardi *et al* (Salardi *et al.* 1989) found differences in the adducin proteins between Milan hypertensive and normotensive strains, and point mutations in the α and β adducin subunits account for up to 50% of the difference in blood pressure between these two strains (Bianchi *et al.* 1994).

Cusi *et al* (Cusi *et al.* 1997) found significant linkage between mutated α-adducin and essential hypertension in two different populations. Moreover, a Gly460Trp mutation was found to be more frequent in hypertensive patients with increased sensitivity to changes in sodium balance, suggesting that this polymorphism may identify hypertensive patients who will benefit from diuretic treatment. In a recent paper Manunta *et al* (Manunta *et al.* 1999) tested the proximal renal tubular function by the fractional excretion

of lithium in patients carrying the 460Trp variant and found increased sodium reabsorption as compared to those with the wild type α-adducin. More recently, lack of association between the α-adducin mutation and hypertension has been reported in the Japanese and Australian populations (Kato *et al.* 1998; Ishikawa *et al.* 1998; Alam *et al.* 2000; Ranade *et al.* 2000). However, Barlassina *et al* (Barlassina *et al.* 2000) found positive association of the α-adducin gene in black South Africans, reminding us of the important role of ethnic variation in the nature of genetic susceptibility loci (Cusi *et al.* 1997).

1.3 EXPERIMENTAL MODELS FOR THE STUDY OF HYPERTENSIVE CARDIOVASCULAR DISEASE.

Historically, one of the first attempts to develop an animal model for hypertension research was that of Tigerstedt and Bergman (Tigerstedt and Bergman, 1898) when the injection of a kidney extract into the blood of dogs produced an increase in blood pressure. This experiment led to generation of several models of experimental hypertension in which some kind of lesion was induced in the vasculature, kidney, or other organs of otherwise healthy animals. This resulted in a hypertensive state that could resemble the hypertensive disease in humans. Harry Goldblatt developed the most classical model, which is based on clipping the renal artery of a dog to produce secondary hypertension (Goldblatt *et al.* 1934). Although these experimentally induced models of hypertension provided insight into the regulation of blood pressure, they were not always consistent with respect to

producing equivalent levels of hypertension. However, animal models, and in particular the rat, offer several advantages for genetic research. Their low cost, easy handling and breeding, access to sophisticated physiological measurements, short generation time, and large litters all help to remove some of the complexity inherent in studying human subjects and families (Lovenberg, 1987). Many aetiologies of human hypertension (primary or secondary) can be mimicked in rat models and these have been developed to investigate further different aetiologies known or suspected to occur in human hypertension (Figure 1.5).

Genetic hypertensive animal models were developed to provide reliable and reproducible forms of high blood pressure. The creation of inbred, genetically homogeneous hypertensive stains from outbred colonies by selective breeding of animals showing elevated blood pressure avoids the confusing heterogeneity of the human disease (Dominiczak and Lindpaintner, 1994). To establish a hypertensive inbred rat colony, the strategy most commonly used has been to measure blood pressure in a large number of outbred animals and then selectively breed those animals having the highest blood pressure. In each successive generation the offspring with the highest blood pressure are then brother-sister mated to fix the strain. After 20 generations of brother-sister mating the offspring should be homogeneous at more than 99% of loci and therefore all animals within the strain are nearly isogenic. In the inbred hypertensive rat strains the variance of blood pressure of the selected lines is lower than in the starting

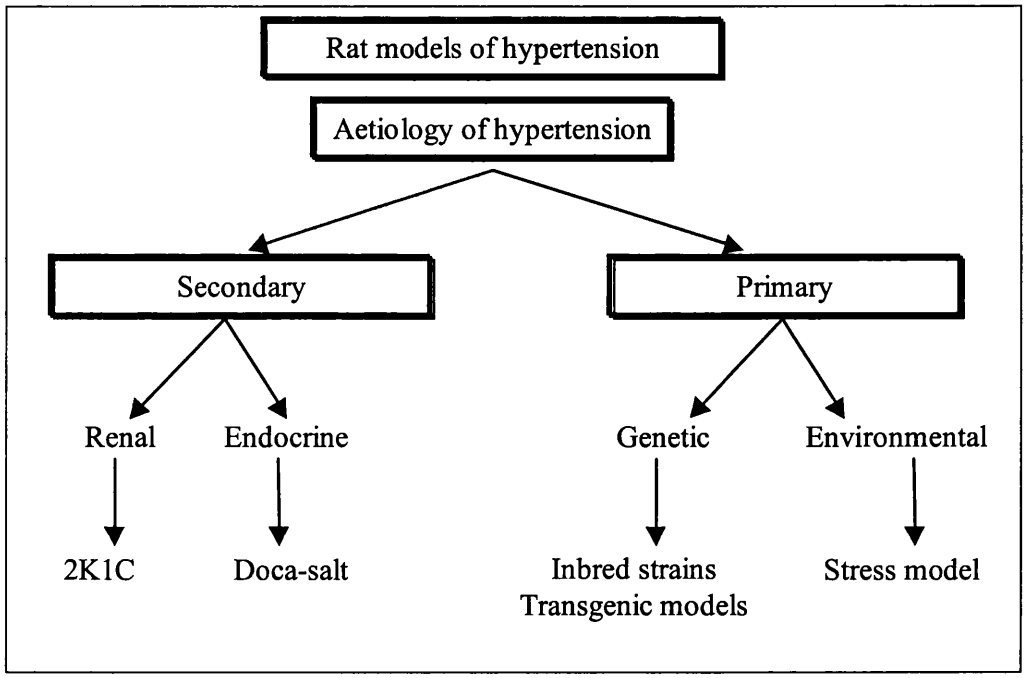

Figure 1.5. Simplified version of rat models of hypertension grouped according to their aetiology. 2K1C, 2-kidneys 1-clip or Goldblatt-hypertension model where one of the renal arteries is clipped to produce an increase in blood pressure; DOCA-salt, deoxycorticosterone in combination with a high salt diet is given to these animals to raise blood pressure. Modified from Pinto *et al* (Pinto *et al.* 1998).

population, since repeated selective breeding concentrates the hypertensive alleles in one strain in the homozygous state (Rapp, 1983).

Each of the rat strains selectively bred for blood pressure (Table 1.4) has unique pathophysiological features linked to the development of the disease. These features are similar to the hypertension-related phenotypes such as left ventricular hypertrophy, stroke, renal failure, salt sensitivity, and stress-induced hypertension (Williams *et al.* 1996) found in subgroups of hypertensive patients but not consistently present in all individuals.

For many years these rats have been used for comparative studies contrasting them with their control strain constructed from the same outbred stock but selecting against hypertension (Lovenberg, 1987). In these studies, differences at any level (biochemical, anatomical, physiological, etc.) between the two strains were wrongly considered to be a causal factor for hypertension (Dominiczak and Lindpaintner, 1994). Moreover, most of this work is difficult to analyse given the confounding presence of many differences unrelated to blood pressure between the two strains compared (genetic drift) and the innate inability of association studies that use intermediate phenotypes (rather than direct genetic information) to determine casuality (Rapp, 1983).

There is little genetic diversity in the different hypertensive strains. Some of them have been derived from Sprague-Dawley stocks and some from

Strain	Location	Lines	Original stock	Year first reported references
Genetically hypertensive rat (GH)	New Zealand (Dunedin)	H, C	Wistar	(Smirk and Haller, 1958)
Dahl Salt-sensitive (S) and Dahl Salt-resistant (R)	USA (Brookhaven)	H, L	Sprague-Dawley	(Dahl *et al.* 1962)
Spontaneously hypertensive rat (SHR)	Japan (Kyoto)	H	Wistar	(Okamoto and Aoki, 1963)
Stoke-prone spontaneously hypertensive rat (SHRSP)	Japan (Kyoto)	H	Wistar	(Okamoto *et al.* 1974)
DOCA salt-sensitive (Sabra hypertensive) and DOCA salt-resistant (Sabra normotensive)	Israel (Jerusalem)	H, L	Unknown	(Ben-Ishay *et al.* 1972)
Lyon hypertensive (LH), normotensive (LN), and low blood pressure (LL) rats	France (Lyon)	H, C, L	Sprague-Dawley	(Dupont *et al.* 1973)
Milan hypertensive (MHS) and Milan normotensive (MNS) rats	Italy (Milan)	H, C	Wistar	(Bianchi *et al.* 1974)
Fawn-hooded hypertensive (FHH) and Fawn-hooded low blood pressure (FHL) rats	The Netherlands (Utrecht)	H, L	Greman Brown x White Lashley	(Kuijpers and Gruys, 1984)
Inherited stress-induced arterial hypertensive rats	Russia (Novosibirsk)	H	Wistar	(Markel, 1985)
Prague hypertensive (PHR) and Prague normotensive (PNR) rats	Czech Republic	H, L	Wistar	(Heller *et al.* 1993)

Table 1.4. Rodent strains selectively bred for blood pressure. H, C, and L refer to the kind of lines developed in each model as follows: H, line selected for high blood pressure; C, control line, unselected, or random bred; L, line selected for low pressure. Modified from Rapp (2000).

Wistar-related stocks, but both Sprague-Dawley and Wistar rats have a common origin (Lindsey, 1979). This low genetic diversity is important in terms of genetic tools that can be applied for a more refined dissection of high blood pressure in the rat (Dietrich *et al.* 1996).

1.3.1 THE STROKE-PRONE SPONTANEOUSLY HYPERTENSIVE RAT (SHRSP).

Forty years ago, Okamoto and Aoki (Okamoto and Aoki, 1963) examined the blood pressure of several hundreds of rats from the Wistar colony of the animal centre at Kyoto University. The blood pressure in these Wistar rats averaged 120 to 140 mmHg measured with tail plethysmography, but one of the male rats examined exhibited blood pressures of 145 to 175 mmHg. This male was mated with a female rat with blood pressures of 130 to 140 mmHg four times, and the offspring of this mating exhibiting hypertension for over 1 month were used for further brother-sister matings. Successive generations of hypertensive animals were obtained by brother-sister matings of animals selected for higher blood pressure. The mean blood pressure of the succeeding generations increased rapidly, with a plateau being approached at the sixth generation (F6). This procedure was continued until a colony of rats was produced with blood pressure uniformly higher than 180 mmHg by 20 weeks of age. These animals were called spontaneously hypertensive rats (SHR) and the inbred strain was obtained in 1969 after successive brother-sister matings (Okamoto and Aoki, 1963). The typical hypertensive lesions often associated with human essential hypertension

such as cardiac hypertrophy, cardiac failure, renal dysfunction, and depressed endothelium dependent relaxations were frequently observed in these rats suggesting that they might be good models for the human disease (Pinto *et al.* 1998).

Before full inbreeding was reached, a substrain of SHR with exceptionally high blood pressure was found to be more susceptible to stroke than other SHR substrains when the SHR strain was separated into three groups in 1971. The incidence of spontaneous stroke was different in the three groups, being high in substrain A (80%) and low in B and C (50%) despite almost identical blood pressure. Speculations regarding the existence of independent genetic factors in the pathogenesis of stroke led to the animals from substrain A being selected and bred. Okamoto *et al* (Okamoto *et al.* 1974) selected and maintained the offspring only from the SHR which died of stroke, resulting in the production of the stroke-prone spontaneously hypertensive rats or SHRSP. The SHRSP is characterised by an early onset of hypertension and by 20 weeks of age adult males have systolic blood pressures of 230 mmHg or higher. Moreover, 80% of the animals have a stroke by 9 to 13 months of age (Okamoto *et al.* 1974), with cerebrovascular lesions localised in the anteromedial and occipital cortex and the basal ganglia (Shibota *et al.* 1976). The SHRSP strain is also characterised by salt sensitivity (Yamori *et al.* 1981).

The SHRSP develops many features of the hypertensive cardiovascular disease seen in humans, such as cardiac hypertrophy, cardiac failure, renal

dysfunction, sexual dimorphism in blood pressure and fibrinoid necrosis of intracerebral arteries (Yamori *et al.* 1979; Conrad *et al.* 1991), although macroscopic atherosclerosis or vascular thrombosis is not developed (Frohlich, 1986). Interestingly, the most common location for cerebrovascular lesions in the SHRSP is the cortical region (69.8%), the next highest being the basal ganglia (24.5%); these percentages are very similar in human stroke (Yamori *et al.* 1976).

The SHR and SHRSP were of significant interest to cardiovascular research and in 1960s they and their control reference strain WKY were sent to the National Institutes of Health in the United States, before some of the strains were fully inbred. This brought the major problem of genetic heterogeneity between colonies of SHRSP, SHR and WKY from commercial suppliers in the United States and the colonies originating directly from Japan (Kurtz and Morris, 1987; Kurtz *et al.* 1989; Samani *et al.* 1989; Matsumoto *et al.* 1991). This problem does not affect the utility of these animal models, but it is difficult to compare experimental results obtained in different laboratories due to the lack of standardisation (St.Lezin *et al.* 1993).

To conclude, the SHRSP exemplified a good animal model of human hypertensive and cerebrovascular disease with which to commence the exploration of the specific genetic determinants of hypertension and stroke, their interaction, and the impact of environmental factors.

1.3.1.1 BLOOD PRESSURE PHENOTYPE IN THE SHRSP.

There are a variety of methods to measure blood pressure in the rat that differ in the degree of restraint that is applied. Great effort has been made to provide error free, high fidelity and continuous blood pressure recordings. Stressful methods that induce increases in blood pressure (additional to the genetic hypertensive background) could lead to different results because not only would genes involved in the spontaneous hypertension be detected, but also those stress-induced hypertensive genes (Kurtz *et al.* 1994). It follows that an ideal phenotypic method to show small genetic components altering blood pressure should be able to give coverage of the blood pressure continuously (be able to show diurnal variations in blood pressure), should provide total freedom for the animal, and eliminate human contact.

1.3.1.1.1 Animal restraint.

There are two main types of animal restraint: chemical and physical. Chemical restraint refers to the use of anaesthesia for acute studies of blood pressure lasting no more than a few hours. However, anaesthesia itself produces physiological changes that can affect blood pressure recording such as increased cardiac output, reduced arterial pressure, modifications to total peripheral resistance, and modifications to blood flow to the brain, gastrointestinal tract, myocardial muscle, and liver (Smith and Hutchins, 1980; Seyde *et al.* 1985).

Physical restraint is used in both acute studies lasting only a few hours and in long-term studies that require intermittent monitoring over the course of years. Restraint itself affects the physiological behaviour of the animals, and as a consequence measurement error and variability are introduced to the data. Diurnal variation can be studied using this procedure, however it is very labour intensive and expensive. Immobilisation stress is one of the best-documented problems associated with physical restraint producing an increase in circulating adrenaline and noradrenaline (Kvetnansky *et al.* 1978). Immobilisation also causes elevation of blood pressure to a new level for a long period of time, frequently resulting in its inability to return to the pre-immobilisation level (Lamprecht *et al.* 1973).

1.3.1.1.2 Indirect techniques for measuring blood pressure.

All indirect systems are based on the original work described by Byrom and Wilson (Byrom and Wilson, 1938) and Williams *et al* (Williams *et al.* 1939). An inflatable cuff is applied to the proximal portion of the rat tail while the distal portion of the tail is enclosed in the plethysmographic chamber. The pressure in the pneumatic cuff, connected to a mercury manometer, is raised above the systolic pressure and then released slowly. The increase in volume of the tail due to the inflow of blood when the pressure of the occluding cuff reaches the systolic blood pressure can be observed in the plethysmographic manometer. These methods require the animal to be restrained and the tail artery must be dilated sufficiently to allow sensors to detect pulsations of blood as the cuff is deflated. Rats are

warmed up to assure adequate dilation of the tail artery, as rats thermoregulate by modifying caudal artery blood flow (Bunag, 1983). The advantage of this technique is that it is non-invasive and permits repeated measurements on large numbers of animals. However, the measurement can be unreliable if the animal moves, is subjected to loud noises or others stressors, or the tail artery is vasoconstricted (Borg and Viberg, 1980; Bunag, 1983).

For the detailed genetic analysis of blood pressure, this method has several limitations as it is unable to provide long-term continuous monitoring and only systolic blood pressure and heart rate can be obtained. It will miss any genetic component producing alteration in the diurnal variation or in diastolic or pulse pressure.

1.3.1.1.3 Direct techniques for measuring blood pressure.

Tethering systems allow continuous monitoring of several different parameters from chronically instrumented conscious animals, but they require surgical cannulation of a major artery. The cannula is connected to a pressure transducer that has been calibrated with a recording device. Trauma and anaesthesia during implantation in acute experiments can profoundly modify blood pressure data, and in chronic experiments maintenance of infection-free postoperative animals and patent cannulas create major problems (Stanton, 1971). Stagnation of blood flow after arterial cannulation encourages the formation of blood clots and fibrous outgrowths

which can obstruct the catheter. Furthermore, emboli from carotid catheters can result in stroke and those from femoral or iliac catheters can result in hind-leg paralysis (Bunag, 1983). Despite the above limitations this approach provides a more accurate measure of both systolic and diastolic pressures than tail plethysmography. One study which analysed an F2 intercross between the genetically hypertensive rat (GH) and the Brown Norway rat measured blood pressure by two different methods, direct intra-arterial catheters and the tail-cuff method. The hypothesis was that measurements of blood pressure by different methods but under the same conditions would result in the detection of the same quantitative trait loci (QTLs). However, they detected regions affecting blood pressure on chromosomes 2, 6, and 18 by the tail-cuff method, and only one QTL on chromosome 6 by the direct catheter method, suggesting that these two methods represented different blood pressure phenotypes with the stress-inducing tail-cuff method probably detecting additional stress-related genetic components (Koike and Jacob, 1998).

Radio-telemetry systems provide an alternative means of obtaining measurements from freely moving, conscious animals. The scientific literature documents the use of non-implantable (backpacks attached to the animal and percutaneous catheters inserted in blood vessels) telemetry back in 1960 (Franklin *et al.* 1964) and implantable telemetry in 1965 (Deboo and Fryer, 1965). Implantable telemetry, where there are no percutaneous catheters, has a number of potential benefits such as eliminating a potential source of infection, providing total freedom for the animal to move about its

cage, and providing the ability to collect data around the clock without the stress from human contact (Brockway and Hassler, 1993). The recent evolution of electronics, packaging sensors, and battery technology has made it feasible to produce reliable, miniaturised implantable devices for rats. This system is capable of simultaneously monitoring several cardiovascular variables such as systolic, diastolic, pulse, and mean arterial pressures, heart rate and motor activity (Rubenson *et al.* 1984). The Dataquest IV system (Data Science International, St Paul, MN) consists of 5 basic elements:

i) an implantable miniature transmitter, which continuously senses, processes, and transmits information from within the animal;

ii) a receiver located near the cage which detects the signal from the implantable transmitter and converts it to a form readable by the computer;

iii) a consolidation matrix or multiplexer that combines the signals from a number of receivers together;

iv) an ambient pressure monitor which measures atmospheric pressure to be converted to a gauge pressure; and

v) a computer-based data acquisition system which collects, displays, and stores the telemetered pressure data and corrects it according to changes in atmospheric pressure (Figure 1.6).

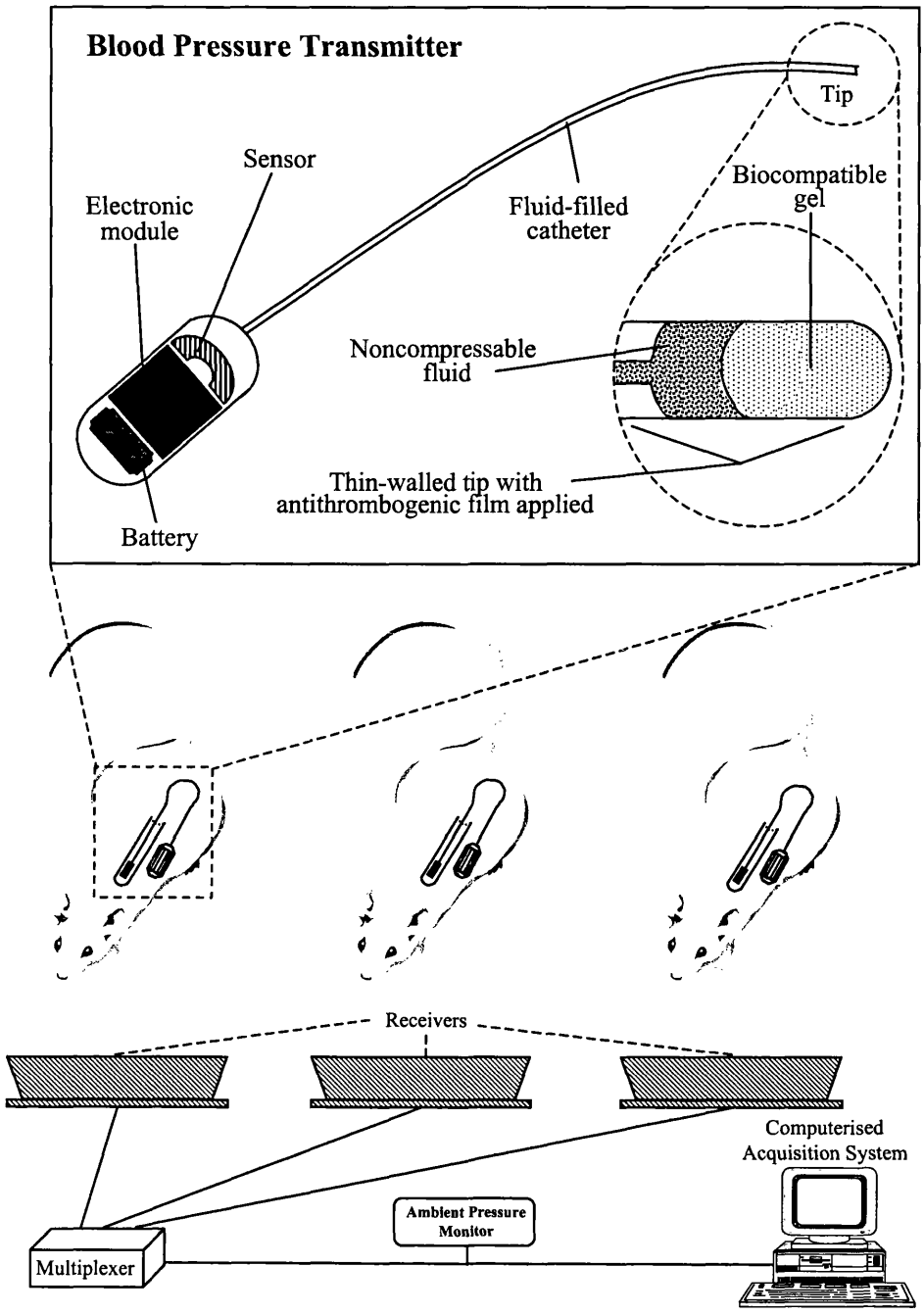

Figure 1.6. Diagram illustrating the components of the telemetry system and the blood pressure transmitter. The ambient pressure monitor is an electronic barometer required to compensate telemetered pressures for changes in barometric pressure. The blood pressure transmitter employs fluid-filled catheters that refer pressure from a specially designed tip to a sensor located in the body of the implant. The gel at the tip prevents blood from entering the lumen of the catheter. Modified from Brockway and Hassler (1993).

The implantable pressure transmitter for rats consists of four key components:

i) a sensor;

ii) a micro-power electronic module to process and digitise the information from the sensor;

iii) a battery; and

iv) a radio frequency transmitter.

It has a built-in magnetic switch that allows the device to be turned on and off either *in vivo* or *ex vivo* to maximise battery life. The pressure transmitter has a fluid-filled catheter attached to the sensor located in the body of the transmitter composed of two sections. The distal 1 cm consists of a thin-wall thermoplastic membrane, while the reminder of the catheter is composed of a thick-wall, low-compliance urethane. The distal 2 mm of the thin-wall tip is filled with a blood compatible gel that prevents blood from entering the catheter lumen and retains the low-viscosity fluid that fills the non-compliant section of the catheter (Brockway *et al.* 1991) (Figure 1.6).

Whilst implantation of the transmitter requires invasive surgery, the capacity for long-term monitoring allows time for blood pressure and heart rate to stabilise post-operatively. Several studies have indicated that radio-telemetry is an accurate and reliable method of determining cardiovascular parameters in long-term studies, yielding chronic measurements that are repeatable and free of stress-induced artefacts (Brockway *et al.* 1991; Guiol *et al.* 1992; Bazil *et al.* 1993; Davidson *et al.* 1995; Clark *et al.* 1996; St.Lezin *et al.* 1997)

1.3.2 GENETIC APPROACHES TO THE STUDY OF EXPERIMENTAL HYPERTENSION.

Hypertensive inbred rats are animal models that provide a useful resource to dissect human hypertensive disease. Tanase *et al* (Tanase *et al.* 1970) used estimates of heritability to perform a genetic analysis of hypertension. This was completed by crossing the SHR hypertensive strain with three different normotensive strains and calculating that between 2 and 6 loci are responsible for high blood pressure. The number of loci originally involved in this biometric analysis, the ability to control environmental factors, and the availability of high fidelity phenotyping methods (as described in Section 1.3.1.1.3) indicate the practicability of the genetic identification of these loci (Samani and Lodwick, 1993). Inbred rat models overcome the limitations inherent to the search for genes in humans because we can produce genetic crosses with a large number of progeny, thus increasing the statistical power for linkage analysis (Broeckel *et al.* 1998).

Although the genomic resources available for the rat have been limited until recently, collaborative efforts from several laboratories around the world have now achieved an appropriate level of genomics for the dissection of complex traits. For example, there is a major difference when comparing the number of genetic markers available between the mouse and the rat, where the former has been more extensively studied in the genetic field. While scientific work in the mouse has traditionally maintained a strong focus on genetics, inherited-disease models in the rat were used primarily in

physiological research, and the genetic aspect received little attention. However, the focus on physiological research with the rat has generated a wealth of experience and methodological sophistication for the accurate determination of blood pressure measurements. Furthermore, much of our current understanding of integrative physiology is based on rat studies (James and Lindpaintner, 1997).

It follows that genetic studies in the rat have gone through different phases. In the first phase (Section 1.3.2.1), the inbred hypertensive rats were used solely for comparative studies contrasting them with the control strain. These studies found a wide variety of biochemical, anatomic, and histological differences as putative causal factors for the difference in blood pressure (Dominiczak and Lindpaintner, 1994). Not surprisingly, most of these differences have subsequently proved unrelated to the pathogenesis of hypertension or its sequelae. These contrasting phenotypes have arisen as a consequence of genetic drift during the fixation of the different inbred strains (Rapp, 1983). After this first attempt, genetic studies progressed using candidate gene strategies, genome-wide scanning and finally, fine mapping of the QTLs with the use of congenic strains described in detail in Section 1.3.2.2.4.

1.3.2.1 CO-SEGREGATION ANALYSIS.

Early cosegregation studies in the rat consisted of experiments in which the co-inheritance of elevated blood pressure and another phenotypic trait

distinguishing hypertensive from normotensive strains was investigated. The weakness of this strategy is that it infers *"a priori"* a causal relationship between blood pressure and the intermediate phenotype, thus pre-supposing that the latter is genetically controlled (the intermediate phenotype represents an "indirect" genotype of blood pressure in this approach) (Rapp, 1991). Cosegregation studies emerged because before them, any phenotypic or genotypic differences between the contrasting strains were considered to be a causal factor of hypertension. Rapp (Rapp, 1983), who was very critical of this genetically faulty approach, formulated a set of 4 criteria to be fulfilled before any differences between contrasting strains could be considered as pathogenetically important:

i) a difference in a biochemical or physiological trait between the two strains must be demonstrated;

ii) the trait under study must be shown to follow Mendelian inheritance;

iii) the genes identified in criterion 2 must cosegregate with an increment in blood pressure significantly different from zero in an F2 or backcross population of rats derived from a cross of the hypertensive and control strains; and

iv) there must be some logical biochemical and/or physiological link between the trait and blood pressure.

The most important criterion is the requirement for cosegregation as it allows one to distinguish between random association or causal relationship of the intermediate phenotype measured and blood pressure (Dominiczak and Lindpaintner, 1994). In a wide variety of studies blood pressure has been shown to cosegregate with different physiological characteristics such

as increased vascular smooth muscle cell responses to cations (Rapp, 1982) and ouabain (Bruner *et al.* 1986), enhanced oscillatory activity of mesenteric resistance arteries (Mulvany, 1986) and sympathetic nerve activity (Judy and Farrell, 1979; Judy *et al.* 1979), decreased renal blood flow and glomerular filtration rate (Harrap and Doyle, 1988), increased heart rate response to stress (Casto and Printz, 1988), augmented noradrenalin-induced oscillatory activity in tail arteries (Bruner *et al.* 1986), increased lymphocyte potassium efflux (Furspan *et al.* 1987), and red blood cell Na^+/K^+ co-transport (Ferrari *et al.* 1987). Cosegregation of blood pressure has been found with some biochemical markers such as increased adrenal production of 18-hydroxy-11-deoxycorticosterone (Rapp and Dahl, 1976), renin isoforms pattern (Sessler *et al.* 1986) and esterase-4 isoforms pattern (Yamori *et al.* 1972).

However, without the genetic tools that could provide an insight at the gene level it was not possible to prove that a cosegregating "intermediate" phenotype trait was inherited and causally related to hypertension.

1.3.2.2 MOLECULAR GENETIC STUDIES.

The development of various molecular genetic tools and their application for the study of hypertension in inbred rats has shed light on the generation of different but complementary genetic approaches. In contrast to studies described in Section 1.3.2.1, these powerful tools permit the dissection of

intermediate phenotypes up to genomic level and assess if there is a relationship between blood pressure and the genotype.

1.3.2.2.1 Genetic tools.

The first genetic monitoring in inbred rat strains was limited to different biochemical measurements, immunological markers, and different coat colour (Bender *et al.* 1984; Hedrich *et al.* 1990). This problem has been resolved with the generation of a variety of DNA-based markers characterised by polymorphism between strains (Serikawa *et al.* 1992). Being polymorphic means that the marker is genetically different between the hypertensive and normotensive strains in the sequence of DNA. This allows the measurement of the genotype in a cosegregation experiment and its correlation with the phenotype. The first DNA-based markers developed resulted from the recognition of restriction fragment length polymorphisms (RFLPs). Digestion of DNA with restriction enzymes results in cleavage at specific sites (a candidate gene sequence) different between two strains resulting in DNA fragments of different size. These fragments can be visualised using hybridisation with radio-labelled molecular probes after electrophoretic separation (Jeffreys *et al.* 1979; Jeffreys, 1979). Rapp *et al* (Rapp *et al.* 1989) were the first to show cosegregation of blood pressure with a polymorphism of the renin gene. This was done in a cross between salt-sensitive (SS/Jr) and salt-resistant Dahl rats (SR/Jr) using the RFLP within the first intron of the renin gene. They concluded that a structural alteration in the renin gene, or a closely linked gene, may be a pathogenetic

determinant of the increased blood pressure observed in this experimental model.

RFLP analysis is limited to sequenced and well-characterised genes. However, the identification of variable DNA sequences composed of randomly organised stretches of the same sequence repeated over and over throughout the genome made possible genetic analysis without any knowledge about the gene of interest. These repeated sequences are numerous, scattered throughout the noncoding DNA of eukaryotes, and highly polymorphic with regard to length. The first generation of this kind of DNA-based marker was the minisatellite, a repetitive element of 10-100 base pairs in length. These markers can be visualised as a specific banding pattern by autoradiography if restriction enzyme digested-DNA is electrophoretically separated and hybridised against a radiolabelled probe complementary to the minisatellite sequence. The distance of the minisatellite to the neighbouring enzymatic cleavage sites will vary between different strains, thus producing a highly specific and characteristic banding pattern which can be used as a genetic finger-print to differentiate between strains (Jeffreys *et al.* 1985).

The second generation of genetic marker is a short tandem repeat sequence of DNA consisting of mono-, di-, tri-, or tetranucleotide repeated sequences called microsatellites. These are also referred to as short tandem repeats (STR), simple sequence length polymorphism markers (SSLP), or simple sequence repeats (SSR). Microsatellites are easily genotyped using

polymerase chain reactions (PCR) (Beckman and Weber, 1992). They are the most useful genetic marker at present due to their large number and random distribution throughout the genome (O'Brien *et al.* 1999). The use of microsatellite markers creates a powerful tool to screen the genome for loci which cosegregate with the blood pressure phenotype without *"a priori"* knowledge of the candidate gene involved in the pathogenesis. However, limitations of this approach were visible back in 1992 when the number of polymorphic markers was limited and coverage was found to be poor on the chromosomal linkage map in certain regions (Lindpaintner, 1992). Nevertheless, in recent years, the number of DNA-based markers has increased very substantially making feasible high resolution linkage maps for fine localisation of blood pressure QTLs (http://www.genome.wi.mit.edu; http://www.well.ox.ac.uk/pub/genetic/ ratmap) (Jacob *et al.* 1995; Bihoreau *et al.* 1997; Cai *et al.* 1997; Steen *et al.* 1999).

A supplementary technique for preparing genetic maps utilises radiation hybrid-cell panels (RH) (Walter and Goodfellow, 1993). Any DNA-sequence amplified by PCR can be localised and mapped using this technique, and the markers do not need to be polymorphic. Data on the presence or absence of a rat marker detected by PCR in the RH panel can be used to construct more dense genetic maps. An extensive radiation hybrid map of the rat genome containing 5,255 markers has recently been published (Watanabe *et al.* 1999) (http://www.well.ox.ac.uk/rat-mapping-resources) and a complementary set of radiation hybrid and linkage maps

for the rat are also available (See Section 5) (Steen *et al.* 1999) (http://goliath.ifrc.mcw.edu/lgr/research/rhp /index.html).

1.3.2.2.2 Candidate gene strategy.

The candidate gene strategy in the rat is identical to the strategy in humans (Section 1.2.2), where the choice of a candidate gene is based on *"a priori"* knowledge of the pathogenesis of high blood pressure (Khoury and Wagener, 1995). Among the candidate genes for which cosegregation studies have been reported are the genes coding for: renin (Rapp *et al.* 1989; Kurtz *et al.* 1990; Dubay *et al.* 1993), atrial natriuretic peptide, angiotensin converting enzyme (Deng and Rapp, 1992), 11β-hydroxylase (Cicila *et al.* 1993), kallikrein (Pravenec *et al.* 1991), the heat shock protein *Hsp70* (Hamet *et al.* 1992), phospholipase C-delta 1 (Katsuya *et al.* 1992), the *Sa* gene (Iwai *et al.* 1992; Iwai and Inagami, 1992; Lindpaintner *et al.* 1993; Samani *et al.* 1993), tumor necrosis factor alpha (*Tnf α*) (Harris *et al.* 1998), and neuropeptide Y (Katsuya *et al.* 1993).

The only study which identified a mutation within the candidate gene under investigation was by Cicila *et al* (Cicila *et al.* 1993) that investigated a cross between the DS/Jr and the DR/Jr rats. They cloned and sequenced full-length cDNAs for P450c11β from the adrenals of these inbred rats. The *Cyp11b1* gene encodes the P450c11β enzyme which has 11β-hydroxylase activity, that converts 11-deoxycorticosterone (DOC) into 18-hydroxy-11-deoxycorticosterone (18-OH-DOC). It has been proposed that 18-OH-DOC,

in rats fed large amounts of NaCl, may act as the principal steroid that promotes sodium retention even though its mineralocorticoid effects are much weaker than those of aldosterone. In this study, the predicted protein sequence of *Cyp11b* in the DS/Jr rat was found to be identical to the sequence reported in the outbred Sprague-Dawley rat, and five amino acid substitutions were found in the sequence of the DR/Jr rat with two of them located near the putative steroid binding site of the enzyme. They also demonstrated that an intragenic RFLP for the *Cyp11b1* gene cosegregated with significant decrease in the adrenal synthesis of 18-OH-DOC and BP. Matsukawa and colleagues (Matsukawa *et al.* 1993) found significantly lower conversion of DOC to 18-OH-DOC in heterologous COS-7 cells expressing P450c11β from the DS/Jr rat, suggesting that the amino acid substitutions are responsible for the altered P450c11β activity of the DR/Jr rat.

As explained in Section 1.2.2., candidate gene approaches lack precision. This strategy can provide misleading results as the significant correlation for a candidate gene marker and the blood pressure phenotype might actually reflect an effect of another gene located in close proximity to the candidate. Moreover, the limited power of candidate gene strategies in polygenic experimental crosses is in contrast to the considerable power of similar studies in humans. The meiotic recombination is limited to one generation in cosegregation experiments in animals, and linkage disequilibrium remains robust across relatively large stretches of chromosomal segments. It follows that the power of these studies to discriminate between

neighbouring genes within a chromosomal segment is comparatively poor to that achieved in an outbred population, for example in human populations where linkage disequilibrium is much more limited yielding higher localisation power (Hamet *et al.* 1998).

Two candidate genes, renin and the *Sa* gene, deserve special mention because they have been extensively studied and information from several independent experimental crosses is available.

1.3.2.2.2.1 Renin.

As described in Section 1.3.2.2.1 the renin gene RFLP was the first DNA-based polymorphic marker ever used in the rat. Rapp *et al* (Rapp *et al.* 1989) reported linkage of the *BglII*-RFLP in the first intron of the renin gene with blood pressure in an F2 cross between the DS/Jr and the DR/Jr rats. Interestingly, Kurtz *et al* (Kurtz *et al.* 1990) performed an F2 cross between the SHR and inbred normotensive Lewis rats and used the same RFLP. Elevated blood pressure was shown in those animals heterozygous for the renin allele, but similar and not significant difference in blood pressure was observed for those homozygous for either the SHR or the Lewis allele. Positive linkage was found in a cross between the Lyon hypertensive and normotensive strains (Dubay *et al.* 1993), but no linkage in an F2 cross of SHRSP and WKY rats (Lindpaintner *et al.* 1990).

These findings indicate that depending on the particular inbred strains chosen, linkage to a gene may or may not be detectable. Moreover, no sequence differences within the coding region and the 5' untranslated region were found between DS/Jr and DR/Jr rats (Alam *et al.* 1993). This suggests that another locus close to the renin gene may play a role in the pathogenesis of hypertension in some of these strains.

1.3.2.2.2.2 The Sa gene.

Sa is a gene of unknown function identified during screening for genes with increased expression in the kidney of the SHR as compared with the normotensive reference strain WKY (Iwai and Inagami, 1991). The *Sa* gene exhibited a 10-fold difference in expression in kidneys isolated from SHR and WKY. Its potential contribution to rat hypertension has been suggested based on the demonstration of cosegregation of the *Sa* gene RFLP polymorphism with blood pressure in F2 crosses derived from SHR x WKY (Iwai *et al.* 1992; Iwai and Inagami, 1992; Samani *et al.* 1993), SHRSP x WKY (Lindpaintner *et al.* 1993), and DS/Jr x Lewis normotensive rats (Harris *et al.* 1993). However, in DS/Jr x WKY, and MHS x MNS rat crosses no linkage could be found (Harris *et al.* 1993; Lodwick *et al.* 1998), inferring that the *Sa* gene or a closely linked gene contributes to the high blood pressure but this is dependent on the genetic background used. These discrepancies reflect the genetic differences between strains and the necessity for precise definition of the strains by laboratory or breeder for genetic and phenotypic comparisons between different studies (Kurtz and

Morris, 1987; Kurtz *et al.* 1989; Samani *et al.* 1989; Matsumoto *et al.* 1991). Recently, the *Sa* gene has been ruled out as a candidate for high blood pressure by performing a congenic experiment between the WKY and SHR rat strains (for further details see Section 1.3.2.2.4) (Hubner *et al.* 1999).

1.3.2.2.3 Genome-wide scanning.

In this strategy a genome is scanned to find QTLs that cosegregate with the blood pressure phenotype without any previous assumptions regarding genes responsible for the disease (Section 1.2.2). A QTL is defined as a broad chromosomal region containing a gene, or set of genes, having an effect on a quantitative trait such as blood pressure (Hamet *et al.* 1998). The genome-wide scanning approach is based on the principle of linkage analysis, using a large number of genetic markers that are polymorphic between the strains used to create the cross and distributed as evenly as possible across all chromosomes. These markers are genotyped on a large segregating F2 population constructed from two inbred strains (one hypertensive and one normotensive or hypotensive). The statistical power of such linkage analysis depends on the number of microsatellite markers available and the number of progeny in the F2 cross examined (Lindpaintner, 1992).

The F2 cross is produced by mating a hypertensive with a normotensive rat to produce a first filial (F1) progeny. F1 rats are genetically identical, since

they inherit one chromosome from each parent. The F1 progeny is then brother and sister mated to produce a second filial generation (F2). The F2 cohort is a very powerful medium through which to detect QTLs statistically by linkage analysis, as many meiotic events are available for study (Lander and Schork, 1994).

Genetic linkage occurs when the BP QTL and a DNA-based marker are physically close together on the same chromosome. During prophase I of meiosis, pairs of homologous chromosomes exchange segments. Each chromosome consists of two sister chromatids and chiasma forms between homologous chromatids. Crossing over or recombination and exchange of genetic material takes place generating recombinant chromosomes. Due to the meiotic recombination events, the association between the marker and the BP QTL will not be a perfect one. As the frequency of recombination events occurring between two genetic loci is a function of their physical distance on the chromosome, the degree of genetic linkage between the marker and the BP QTL represents a measure of distance between the two loci. The closer the marker and the gene of interest are on a given chromosome, the less chance that a recombination event will occur between them and the higher the linkage. If the marker and the gene of interest are far away from each other or on different chromosomes, they will recombine frequently and segregate independently (Lindpaintner, 1992).

The genetic linkage between the marker and the BP QTL is a measure of distance between the two loci, this recombination distance is measured by

the recombination fraction or θ (the number of recombinants observed divided by the total number of meiotic events). If loci are unlinked, whether on the same or different chromosomes, approximately half of the progeny will be recombinant and $\theta = 0.5$. If the loci are linked $\theta < 0.5$ and parental haplotypes will predominate. If the loci are very close and no recombination event ever takes place between them $\theta = 0$. As the frequency of recombination events occurring between two genetic loci is a function of their physical distance on a chromosome, the recombination fraction can be converted into distance expressed in centiMorgan units (cM). One cM represents a 0.01 probability of recombination, which, incidentally corresponds to a distance of approximately 1×10^6 base pairs in the human genome and 2×10^6 in the rat (Jacob, 1999).

For BP QTL detection, unambiguous genotyping at the genetic marker is required to place the locus on genetic maps and to perform the linkage analysis. If an F2 segregating population is produced between the hypertensive SHRSP strain (carrying ss homozygote alleles at every single locus due to its inbred status) and the normotensive WKY strain (ww homozygote alleles), any DNA-based polymorphic marker has three potential genotypes and frequencies as follows: 25% ss homozygous (carrying both alleles from the SHRSP strain), 25% ww homozygous (having both alleles from the WKY), and 50% ws heterozygous (having one allele from each strain), with the genotypes being in Hardy-Weinberg equilibrium. Each animal is scored for the genotype at specific markers spread throughout the genome and also phenotyped for its blood pressure.

Alleles at loci not genetically linked to the blood pressure causative gene will segregate independently and these will have no statistical association with the phenotype. However, if there is a significant difference in the blood pressure levels between the genotype groups at the marker, one can infer the presence of a QTL.

Traditionally, marker loci across the genome have been analysed using one way analysis of variance (ANOVA) to calculate the contribution of the alleles to the phenotypic changes observed in the F2 population and a significance level of $p < 0.05$ was indicative of linkage (Soller and Brody, 1976). Once blood pressure linkage to a single marker is found, however, this type of analysis fails to localise the BP QTL. Significant linkage of a single marker and blood pressure can mean either that the marker is very closely linked, or identical, to the BP QTL or that there is a BP QTL with a larger effect located at a greater distance from the marker (Rapp and Deng, 1995). It is possible to study all the available markers along a chromosome using one way ANOVA to correlate them with the phenotype, but without precise information on the location of the markers it is impossible to identify the BP QTL.

To solve the problem of proving the existence of a QTL and localising its map position sophisticated computer packages such as MAPMAKER (Lander *et al.* 1987), have been developed to undertake maximum-likelihood linkage analysis using an interval mapping approach (Lander and Botstein, 1989).

1.3.2.2.3.1 MAPMAKER.

MAPMAKER is a computer program which was developed by Lander *et al* (1987) to facilitate the construction of linkage maps using any number of DNA-based polymorphic markers and, at the same time, interrogate all those markers for the phenotype measured in the F2 segregating population. The first function of MAPMAKER is the construction of a genetic linkage map with the aid of a mapping function. This function uses the equivalence between the recombination fraction and the genetic distance, converting θ into cM distances (the mapping function is needed in multilocus mapping to convert the raw data of the recombination fraction into a genetic map), Haldane's mapping function is widely used and is given as $\theta = 0.5\ (1\text{-}e^{-2\omega})$, where ω is equal to the map distance in Morgans (Haldane, 1919). Haldane's mapping function does not take into account interference, and assumes crossovers occur at random along the chromosome with no influence on one another. However, the presence of one chiasma inhibits the formation of another, thereby the assumption that crossovers occur at random is not true. Alernatively, Kosambi's mapping function can be used as a more realistic formula, which takes into account the interference, $\theta = 0.5\ [2^{(4\omega)\text{-}1}] / [e^{(4\omega)+1}]$ (Kosambi, 1943).

After the genetic linkage map has been constructed, the second operation that MAPMAKER carries out is the QTL analysis using maximum-likelihood techniques, where each marker is examined for linkage to the measured phenotype and is compared with adjacent markers. The

significance of linkage between given markers and the measured phenotype is determined by the LOD score (which is the Log of the ratio of likelihood of there being a QTL present versus the likelihood of no QTL being present at a particular map position). The LOD scores are calculated at many selected points in an interval between markers and then are plotted versus map position. The LOD score is described as $Z = Log_{10} [L(\theta) / L(0.5)]$ and is calculated at various levels of θ to identify the value at which Z is greatest (Zmax) and Zmax is considered to be the best estimate (Lander *et al.* 1987).

Genetic analysis of Mendelian traits assumes that a LOD score of 3.0 is significant to declare linkage for a putative QTL, i.e. when Z is equal to or greater than 3.0, which corresponds to 1000:1 odds in favour of linkage. In contrast, a LOD score of –2 or less indicates evidence of exclusion of linkage (McKusick, 1975). The peak of the LOD plot gives the most likely location of the QTL, and the height of the peak is a measure of statistical significance (Figure 1.7). However, more stringent levels of statistical significance are required for non-Mendelian polygenic or oligogenic traits where factors such as the size of the genome, the assumed genetic model of inheritance for the QTL (additive, dominant, recessive, codominant, or free), and the breeding paradigm (backcross or F2 population) influence the interpretation of the LOD plot (Paterson *et al.* 1988). For example, for an additive mode of inheritance, an F2 intercross is preferred, but in some cases the phenotypes from the F1 progeny are closer to one of the parental inbred strains; in this case a backcross (in which F1 animals are mated with one of the parental strains) would be preferred.

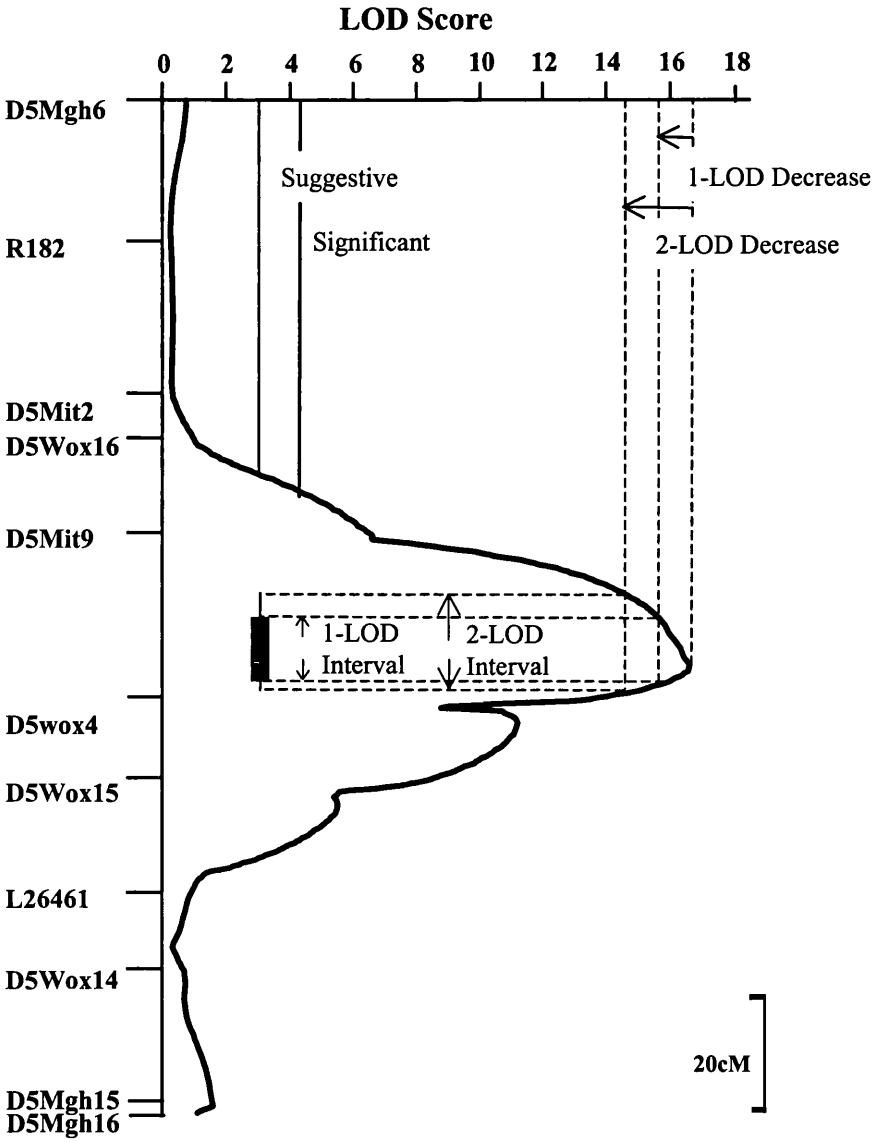

Figure 1.7. Logarithm of the odds favouring linkage of the phenotype to genetic markers (LOD plot) and construction of the 1-LOD and 2-LOD support intervals for localisation of the QTL. Suggestive and significant statistical linkage for the trait are represented with solid lines. Modified from Jeffs (1997).

Lander and Kruglyak (Lander and Kruglyak, 1995) have made calculations accounting for such factors and proposed stringent LOD thresholds to distinguish between suggestive linkage (statistical evidence for the existence of a QTL would be expected to occur once at random in a genome scan) and significant linkage (statistical evidence for a QTL would be expected to occur 0.05 times at random in a genome scan) (Table 1.5). Whilst suggestive linkage results may be incorrect, they are worth reporting if accompanied by the appropriate level of significance; it also follows that as a LOD score of 3.0 occurs by chance 5% of the time, the greater the LOD value the greater the confidence in the linkage result.

The localisation of the QTL is supported by an interval defined by the range needed to contain a drop of one LOD unit around the peak, giving an approximate confidence interval for the QTL localisation (Figure 1.7). It has been calculated that one-LOD unit around the peak gives an interval with a 60-95% probability of containing the QTL (Mangin *et al.* 1994). However, simulation studies suggested that support intervals based on two-LOD units should be used to increase the probability of finding the QTL (Van Ooijen, 1992). An important factor that can influence the success of QTL-finding using the MAPMAKER program is the variance of the phenotype measurements which correlates inversely with the likelihood of linkage due to the introduction of excessive noise (Dominiczak and Lindpaintner, 1994). It follows that high quality phenotyping is required for fine mapping of blood pressure QTLs (Section 1.3.1.1.3).

Mapping method (QTL mapping in rat.)	Suggestive Linkage P value (LOD)	Significant linkage P value (LOD)
Backcross (1 d.f.)	3.4×10^{-3}(1.9)	1.0×10^{-4}(3.3)
Intercross (1 d.f.., additive)	3.4×10^{-3}(1.9)	1.0×10^{-4}(3.3)
Intercross (1 d.f., recessive)	2.4×10^{-3}(2.0)	7.2×10^{-4}(3.4)
Intercross (1.d.f., dominant)	2.4×10^{-3}(2.0)	7.2×10^{-4}(3.4)
Intercross (2 d.f.)	1.6×10^{-3}(2.8)	5.2×10^{-5}(4.3)

Table 1.5. Thresholds for mapping loci underlying complex traits. d.f., degrees of freedom. From Lander and Kruglyak (Lander and Kruglyak, 1995).

1.3.2.2.3.2 Blood pressure QTLs.

The first genome-wide scan for the dissection of BP QTLs was applied to an F2 cosegregating population derived from the $SHRSP_{Heidelberg}$ and WKY strains (Hilbert *et al.* 1991; Jacob *et al.* 1991). These studies demonstrated the presence of three chromosomal loci which showed LOD scores in excess of 3.0 and thus fulfilled the accepted criteria for significant linkage according to the particular conditions of the experiment (Lander and Kruglyak, 1995). These pioneering experiments were carried out screening a panel of 240 mini- and microsatellites and both mapped a locus on rat chromosome 10 which contributed significantly to the difference in blood pressure between the two strains. This locus was called *BP1* by Jacob *et al* (1991) and *BP/SP-1* by Hilbert *et al* (1991) and contained a putative candidate gene encoding for the angiotensin I converting enzyme (ACE gene). Jacob *et al* (1991) identified another BP QTL (*BP2*) on chromosome 18 linked to diastolic blood pressure whilst Hilbert *et al* (1991) mapped an X-linked locus contributing to the difference in blood pressure between the two strains. The discrepancies between the two set of results were entirely due to the different statistical analysis used as the genetic cross and its phenotyping were one and the same in both studies.

As can be seen in Table 1.6, despite great variability between the hypertensive strains and the segregating populations used in QTL localisation, several QTLs have been present reproducibly in many independent experiments (Figure 1.8). These common or reproducible QTLs

have become important candidate loci for human essential hypertension. This new strategy which aims to achieve a direct transfer of rat QTLs to homologous human chromosomal regions has been successful for the QTL on rat chromosome 10 (Julier *et al.* 1997; Baima *et al.* 1999). The BP QTL on rat chromosome 10 has been reported several times in the literature using different F2 segregating populations (Hilbert *et al.* 1991; Jacob *et al.* 1991; Rapp and Deng, 1995; Harris *et al.* 1995; Garrett *et al.* 1998). Based on comparative mapping data, Julier *et al* (Julier *et al.* 1997) used this BP QTL to investigate the homologous region of conserved synteny on human chromosome 17. Analysis of affected sib-pairs with essential hypertension gave significant linkage to two closely linked microsatellite (D175183 and D175934) on human chromosome 17q. Another study conducted by Baima *et al* (Baima *et al.* 1999) confirmed the linkage of this region on human chromosome 17q using a different population and refined the localisation of the human blood pressure QTL to markers D17S1814 and D17S800, which are 0.7 cM apart. These two studies (Julier *et al.* 1997; Baima *et al.* 1999) provide an elegant proof of the concept that it is feasible to directly translate the QTLs discovered in the rat to human essential hypertension and pave the way for similar studies of different rat BP QTLs (Dominiczak *et al.* 2000).

Localising BP QTLs to chromosomal regions of approximately 30 cM is only the first step towards the identification of the causal gene or genes. Further strategies include construction of congenic strains and sub-strains and finally cloning by position. There strategies are described below.

Chr	Rat Strains	Central Gene/Markers	Lod Score	Reference
1	WKY x $SHRSP_{(Izm)}$	*Lsn, Myl2*	4.5	(Nara *et al.* 1993)
	Lewis x S	*D1Mco1, Cytp450*	3.4	(Gu *et al.* 1996)
	Lewis x S	*Sa*	2.5	(Gu *et al.* 1996)
	ACI x FHH	*Mt1pa*	4.2	(Brown *et al.* 1996)
	Sabra N x Sabra H	*D1Mit2, Sa*	4.9	(Yagil *et al.* 1998)
	Sabra N x Sabra H	*D1Mit1, Cytp450*	4.7	(Yagil *et al.* 1998)
	Donryu x SHR	*D1mit3*	4.3	(Innes *et al.* 1998)
2	LN x LH	*Cpb*	7.0	(Dubay *et al.* 1993)
	WKY x S	*ATP1a1*	3.4	(Deng *et al.* 1994)
	MNS x S	*Camk*	2.6	(Deng *et al.* 1994)
	RI (BN x SHR)	*D2N35*	n/a	(Pravenec *et al.* 1995)
	BN x SHR	*Mt1pb*	3.0	(Schork *et al.* 1995)
	BN x SHR	*Gca*	6.3	(Schork *et al.* 1995)
	BN x SHR	*R598*	3.0	(Schork *et al.* 1995)
	BN x GH	*Gca*	n/a	(Harris *et al.* 1995)
	WKY x $SHRSP_{(Gla)}$	*D2Mit6*	3.4	(Clark *et al.* 1996)
	WKY x $SHRSP_{(Gla)}$	*D2Mit14*	3.1	(Clark *et al.* 1996)
	WKY x SHR	*D2Wox7*	5.6	(Samani *et al.* 1996)
	Lewis x S	*D2Mco19*	2.9	(Garrett *et al.* 1998)
3	WKY x $SHRSP_{(Izm)}$	*D3Mgh16*	n/a	(Matsumoto *et al.* 1995)
	WKY x $SHRSP_{(Gla)}$	*D3Mgh16*	5.6	(Clark *et al.* 1996)
	WKY x $SHRSP_{(Izm)}$	*D3Mgh12*	6.2	(Matsumoto *et al.* 1996)
	Lewis x S	*D3Mgh6*	3.0	(Garrett *et al.* 1998)
	BN x S	*D3Mgh2*	3.92	(Kato *et al.* 1999)
4	RI (BN x SHR)	*Il-6*	n/a	(Pravenec *et al.* 1995)
	BN x SHR	*Npy2*	4.6	(Schork *et al.* 1995)
5	WKY x SHR	*Mitr1678, Anp, Bnp*	4.2	(Zhang *et al.* 1996)
	Lewis x S	*Edn2*	4.5	(Garrett *et al.* 1998)

Table 1.6. QTLs for blood pressure. Continued on next page.

Chr	Rat Strains	Central Gene/Markers	Lod Score	Reference
8	BN x SHR	*R850*	5.1	(Schork *et al.* 1995)
9	R x S	*Inha*	5.0	(Rapp *et al.* 1998a)
10	WKY x $SHRSP_{(Hd)}$	*Ace*	n/a	(Hilbert *et al.* 1991)
	WKY x $SHRSP_{(Hd)}$	*Ace*	5.1	(Jacob *et al.* 1991)
	WKY x S	*Nos2*	2.3	(Deng *et al.* 1995)
	MNS x S	*Nos2*	6.3	(Deng *et al.* 1995)
	MNS x S	*Ace*	4.8	(Deng *et al.* 1995)
	BN x GH	*Ace*	n/a	(Harris *et al.* 1995)
	Lewis x S	*D10Wox6*	5.5	(Garrett *et al.* 1998)
	MNS x S	*D10Wox13*	6.69	(Kato *et al.* 1999)
12	WKY x S	*D12Wox16*	n/a	(Kato *et al.* 1999)
13	LN x LH	*Renin*	5.6	(Dubay *et al.* 1993)
	WKY x SHR	*D13Mit2*	5.7	Samani *et al*, 1996
16	BN x SHR	*R220*	4.3	(Schork *et al.* 1995)
17	Sabra N x Sabra H	*D17Mgh5*	3.4	(Yagil *et al.* 1998)
18	WKY x $SHRSP_{(Hd)}$	*Rr1094*	3.2	(Jacob *et al.* 1991)
19	RI (BN x SHR)	*D19Mit7*	n/a	(Pravenec *et al.* 1995)
X	WKY x $SHRSP_{(Hd)}$	*Per-Ha-2/Per-Ha-7*	n/a	(Hilbert *et al.* 1991)
	Sabra N x Sabra H	*DxMgh9/DxMgh12/ DxRat2*	4.3	(Yagil *et al.* 1999)

Table 1.6. QTLs for blood pressure. The normotensive strain is cited first on all occasions. WKY, Wistar-Kyoto; $SHRSP_{izm}$, SHRSP from Izumo colonies; $SHRSP_{Hd}$, SHRSP from Heidelberg colonies; $SHRSP_{gla}$, SHRSP from Glasgow colonies; S, Dahl salt-sensitive; R, Dahl salt-resistant; ACI, AxC9935 Irish; FHH, Fawn-hooded hypertensive; Sabra H, Sabra hypertensive; Sabra N, Sabra normotensive; LN, Lyon normotensive; LH, Lyon hypertensive; MNS, Milan normotensive; RI (BN x SHR), recombinant inbred strains HXB and BXH derived from the normotensive Brown-Norway and SHR; GH, genetically hypertensive rat. The following represent markers within known genes; *Lsn*, leukosianin; *Myl2*, myosin short chain; *Cytp450*, cytochrome P450 cluster; *Mt1pa*, metallothionein 1, pseudogene a; *Cpb*, carboxypeptidase; *ATP1a1*, Na^+K^+ATPase α1-isoform; *Camk*, calmodulin-dependent protein kinase II-delta; *Mt1pb*, metallothionein 1, pseudogene b; *Gca*, guanylylcyclase A; *IL-6*, interleukin-6; *Npy2*, neuropeptide Y2; *Anf*, atrial natriuretic factor; *Bnf*, brain natriuretic factor; *Ace*, angiotensin-converting enzyme; *Nos2*, inducible nitric oxide synthase; *Inha*, inhibin alpha subunit gene; *Per-Ha-2* and *Per-Ha-7* are minisatellite bands. The remaining symbols represent anonymous microsatellite markers. n/a, LOD score not given in reference. Modified and updated from Dominiczak *et al* (Dominiczak *et al.* 1998)

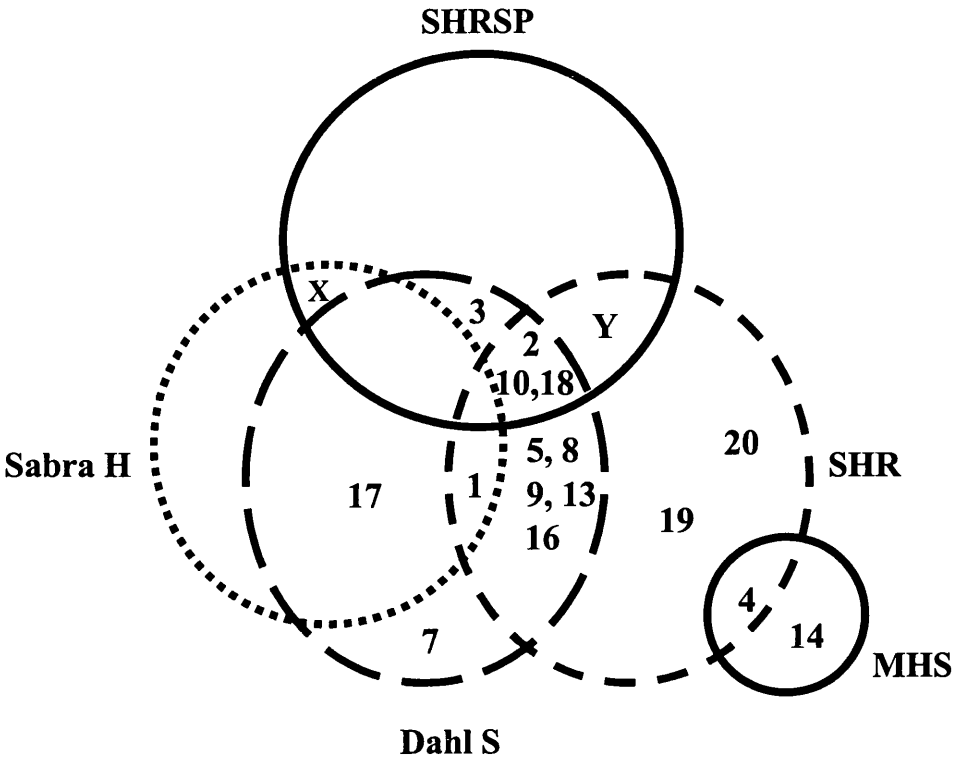

Figure 1.8. Common or reproducible blood pressure QTLs identified in several independent experiments. Only the hypertensive strain is indicated. SHRSP, Stroke- prone spontaneously hypertensive rat; SHR, spontaneously hypertensive rat; MHS, Milan hypertensive rat; Dahl S, Dahl salt-sensitive strain; Sabra H, Sabra hypertensive strain. Adapted from Dominiczak *et al* (2000).

1.3.2.2.3.2 QTLs influencing other cardiovascular phenotypes.

Genome-wide scanning experiments have also been applied to the elucidation of cardiovascular phenotypes other than blood pressure. Four cardiovascular phenotypes have received special attention: left ventricular hypertrophy (Pravenec *et al.* 1995; Clark *et al.* 1996; Hamet *et al.* 1996; Vincent *et al.* 1996; Innes *et al.* 1998; Sebkhi *et al.* 1999), ischemic stroke (Rubattu *et al.* 1996; Jeffs *et al.* 1997), renal failure (Brown *et al.* 1996), and insulin resistance (Aitman *et al.* 1997). In this section I shall discuss in detail mapping studies for LVH and stroke phenotypes, with the remaining studies being listed in Table 1.7.

BP QTLs are essentially always associated with effects on heart weight as the heart hypertrophies in response to chronically increased blood pressure. However, genome-wide scanning strategies in four different crosses between hypertensive and normotensive inbred rat strains demonstrated three different QTLs responsible for a proportion of left ventricular hypertrophy in a blood pressure independent manner (Clark *et al.* 1996; Hamet *et al.* 1996; Vincent *et al.* 1996; Innes *et al.* 1998). Moreover, Sebkhi *et al* (Sebkhi *et al.* 1999) found significant linkage for a left ventricular weight QTL on chromosome 3, centred around marker *D3Rat29* with a LOD score of 4.4 in a cross between two normotensive strains (Fisher 344 and Wystar-Kyoto rats), thus minimising the confounding effect of blood pressure. Innes *et al* (1998) reported a QTL for LVH in an F2 cross between SHR and Donryu rats. This QTL had a LOD score of 4.3,

Disease	Chromosome	Rat *strains* (crosses)	Central gene/marker	LOD score	Reference
Left ventricular Hypertrophy	2	Donryu x SHR	*D2Mgh15*	4.3	(Innes *et al.* 1998)
	12	WKY x SHR	*Hsp27*	n/a	(Hamet *et al.* 1996)
	14	WKY x $SHRSP_{Gla}$	*D14Mgh3*	3.1	(Clark *et al.* 1996)
	17	RI (BN.Lx x SHR)	*Drd1a*	n/a	(Pravenec *et al.* 1995)
	X	LN x LH	N/a	n/a	(Vincent *et al.* 1996)
	3	F344 x WKY	*D3Rat29*	4.8	(Sebkhi *et al.* 1999)
Stroke	1	SHR x $SHRSP_{Hd}$	D1Mit3	7.4	(Rubattu *et al.* 1996)
	4	SHR x $SHRSP_{Hd}$	*D4Mgh16*	3.0	(Rubattu *et al.* 1996)
	5	SHR x $SHRSP_{Hd}$	*Anf*	4.7	(Rubattu *et al.* 1996)
	5	WKY x $SHRSP_{Gla}$	*D5Wox4*	16.6	(Jeffs *et al.* 1997)
Renal impairment	1	ACI x FHH	D1Mit6	8.9	(Brown *et al.* 1996)
Metabolic syndrome	4	WKY x SHR	*D4Arb13, Ae2*	6.4	(Aitman *et al.* 1997)
	12	WKY x SHR	*D12Mit18*	6.3	(Aitman *et al.* 1997)

Table 1.7. Cardiovascular QTLs in genetic hypertension (at least partially blood pressure independent). The following are microsatellite markers: *D1Mit3*, *D4Mgh16*, *D5Wox4*, *D2Mgh15*, *D14Mgh3*, *D1Mit6*, *D4Arb13*, *D12Mit18*, and *D3Rat29*. Other symbols represent markers within known genes: *Anf*; atrial natriuretic peptide, *Drd1a*; dopamine 1a receptor, *Hsp27*; heat shock protein. RI(BN.Lx x SHR) denotes recombinant inbred strains derived from the SHR and the normotensive Brown Norway rats, LN and LH are Lyon normotensive and hypertensive rat strains, ACI are (AxC9935 Irish) normotensive rats from Harlan Sprague Dawley, FHH is the fawn-hooded hypertensive rat strain, and F344 is the Fisher 344 rat.

accounted for 29.5% of genetic variance and was BP independent. It is of interest that this QTL is localised in an area of a well established BP QTL (Rapp, 2000). It follows that there are several potential loci that might account for left ventricular hypertrophy, however further fine mapping techniques are required to further dissect this cardiovascular phenotype.

Three BP independent QTLs for susceptibility to spontaneous stroke were mapped to chromosome 1 (STR-1), 4 (STR-3), and 5 (STR-2) in a SHR_{Hd} x $SHRSP_{Hd}$ cross by Rubattu *et al* (Rubattu *et al.* 1996). Together they accounted for 28% of the overall phenotypic variance. The selection of two hypertensive strains was done to avoid any confounding effects of blood pressure on the stroke phenotype. The phenotype measured was the stroke latency phenotype which was estimated by the number of days until clinical evidence of stroke on a diet high in sodium and low in potassium and protein. Both STR-2 (LOD of 4.7, this QTL mapped closed to the *Anf* gene on rat chromosome 5) and STR-3 (LOD of 3.0) appeared to protect from stroke in the SHRSP strain, as F2 rats inheriting both alleles from the SHRSP strain had significantly greater latency to spontaneous stroke compared to SHR homozygotes for the same alleles. In contrast, STR-1 (LOD score of 7.4) strongly affected latency to stroke in a recessive mode with SHR alleles being protective.

Our group in Glasgow demonstrated the existence of a stroke severity locus in a cross between $SHRSP_{Gla}$ and WKY_{Gla}. This QTL was localised on chromosome 5 with a highly significant linkage (LOD of 16.6), accounted

for 67% of the phenotypic variance and was blood pressure independent (Jeffs *et al.* 1997). Jeffs *et al* carried out high fidelity phenotyping which included a surgical occlusion of the middle cerebral artery to measure infarct size. It is of interest that this stroke severity locus mapped in close proximity to the BP QTL observed in studies on Dahl salt-sensitive rats. It follows that there seem to be two different stroke QTLs on rat chromosome 5, explained perhaps by the different stroke phenotypes used in these studies (stroke latency versus infarct size after middle cerebral artery occlusion). Moreover, the SHRSP alleles at the locus reported by Jeffs *et al* confer increased susceptibility to stroke, whilst for the locus reported by Rubattu *et al* (1996) the same alleles confer protection (increasing the latency to stroke).

1.3.2.2.4 Congenic strains.

Several BP QTLs have been identified in experimental crosses (Section 1.3.2.2.3.2). However, the identification of large chromosomal regions of approximately 20-30 cM in size is only the first step in the ultimate goal of gene identification (Dominiczak *et al.* 1998). Indeed, simulation studies have shown that the best confidence intervals achievable by QTL mapping are 10 cM wide. Darvasi *et al* (Darvasi *et al.* 1993) demonstrated that for cloning by position it is necessary to localise the locus to 1-2 cM.The next step is the production of congenic strains and substrains containing progressively smaller chromosomal regions, with the final task being positional cloning of the causal gene(s) (Rapp and Deng, 1995).

The use of congenic breeding strategies is a standard procedure in mouse genetics that was pioneered by George D. Snell as a component of his Nobel prize-winning strategy for the dissection of mouse histocompatibility genes and tumour resistance (Snell, 1948). A congenic strain represents a genetic composite of the disease and the reference strains such that the congenic strain is identical to the reference strain except for a single chromosomal region of interest that is derived from the disease strain and has been introgressed into the reference strain background or *vice versa*.

The classic protocol for the production of congenic strains is to backcross serially the donor to the recipient strain, accompanied by selection for progeny carrying the desired disease-locus in each backcross generation (Figure 1.9). This essentially leads to a serial "dilution" of the donor genome into the recipient genome with continuous maintenance of the specified disease-locus (Markel *et al.* 1997). Following Mendelian laws, each backcross results in a reduction by a factor of 2 of the genetic material derived from the donor strain in favour of the recipient strain, so that after eight backcrosses > 99%

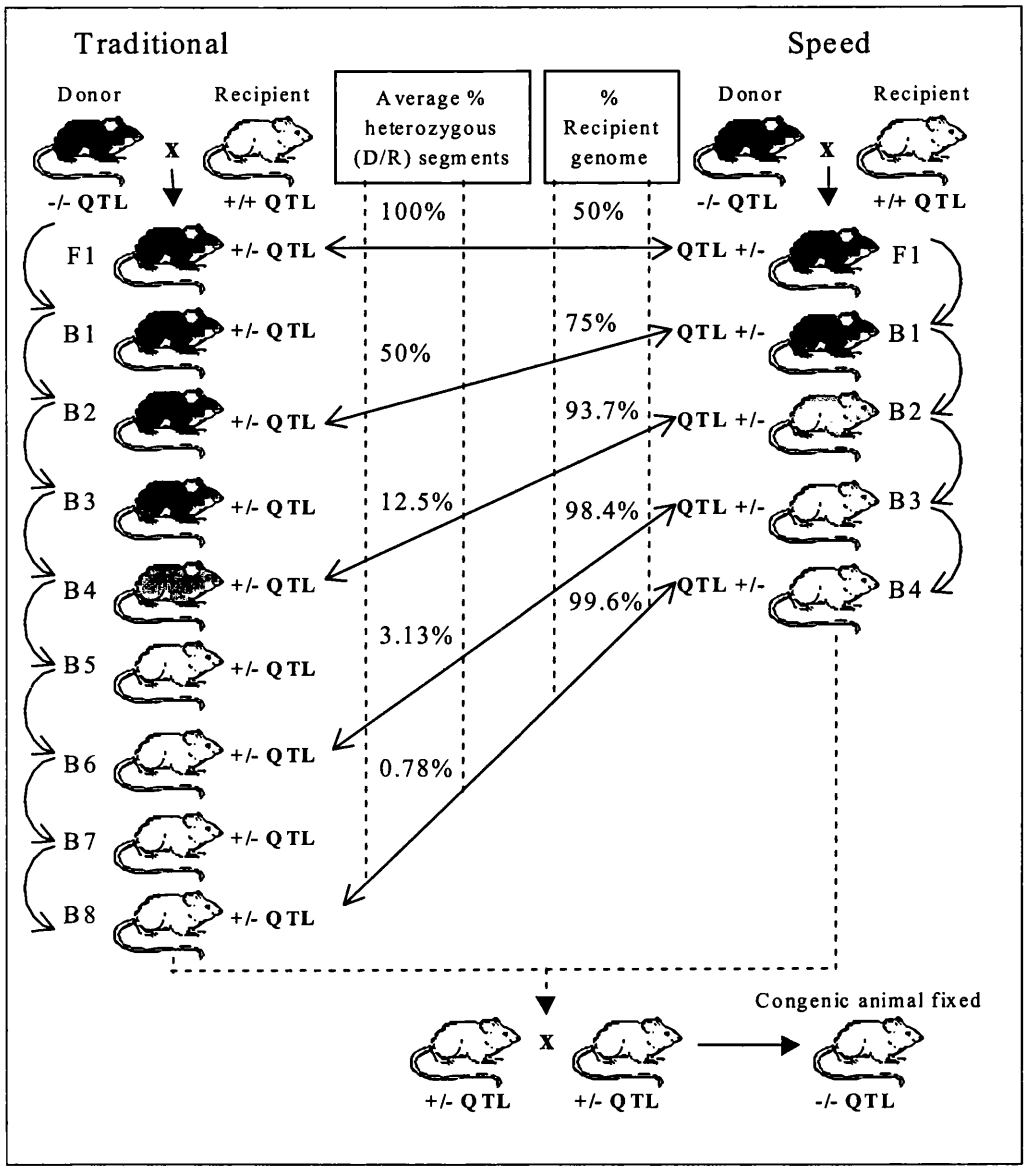

Figure 1.9. Congenic strain production using the traditional and speed congenic methods. Donor strain is represented with black colour and recipient strain with white colour. Decreasing shades of grey from black to white represent the increase in percentage genetic background that occurs with each backcross. D, donor strain alleles; R, recipient strain alleles; B, backcross; F1, first filial generation.

of the congenic strain will be from the recipient strain. After eight to twelve backcross generations have been bred, the protocol is completed by an intercross to produce homozygous congenic animals for the derived disease-QTL.

Several laboratories began the production of congenic lines between different hypertensive and normotensive inbred rat strain approximately 7-8 years ago. The lack of DNA-based markers specifically designed for the rat limited the congenic selection to a single marker (RFLP for example). It is known that regions linked to a selected marker that is examined to choose animals for the next backcross generation will be fixed by chance for the donor strain alleles (Rapp, 2000). In the single marker selection experiment for the congenic breeding paradigm, the number of backcrosses reduces proportionally the size of the region fixed by chance along with the marker. A congenic strain that results from selecting only one marker will have flanking donor chromosomal regions on average equal to 100/N cM on each side, where N is the number of backcrosses (Silver, 1995).

Congenic strains can be constructed in two ways with a given pair of parental strains, i.e. the normotensive strain can be the donor and the hypertensive strain can be the recipient, or vice-versa; the difference between the two types of congenic strain is the genetic background. This strategy is called reciprocal congenic breeding and allows investigation of how a single QTL may influence blood pressure on a permissive and a resistant genetic background, and thus providing a control for genome

background effects (Jacob, 1999). The generation of reciprocal congenic strains also provides a classic 2 x 2 study design, whereby physiological experiment and phenotypic measurement have appropriate controls for the QTL region and genetic background (Figure 1.10). One of the best examples of the use of reciprocal congenic lines has been published by Frantz *et al* (Frantz *et al.* 1998). A region of chromosome 1 (of 2.4 cM in size) around the *Sa* gene from the WKY strain was transferred to the SHR genetic background and *vice versa* using twelve backcrosses. The blood pressure was recorded at 25 weeks of age using tail-cuff and interestingly the magnitude of change in systolic blood pressure was similar between the two congenic strains (approximately 10 mmHg). Although Frantz *et al* (1998) concluded that the same QTL was introgressed and produced similar effects on contrasting backgrounds, high fidelity phenotyping of blood pressure (Section 1.3.1.1.3) is required to determine pleiotropic or epistatic effects due to the genetic background that can be easily missed with the tail-cuff method.

Several congenic lines that have been produced for the dissection of BP QTLs are summarised in Table 1.8; however, causal genes have not yet been identified. It is of interest to describe in some detail congenic strains constructed around the renin gene on rat chromosome 13. St. Lezin *et al* (St.Lezin *et al.* 1996) reported that in congenic Dahl R rats carrying the Dahl S renin gene and fed a high salt diet, the systolic blood pressure was significantly lower than in the progenitor Dahl R rats. These results were the opposite of what was predicted from the original linkage data

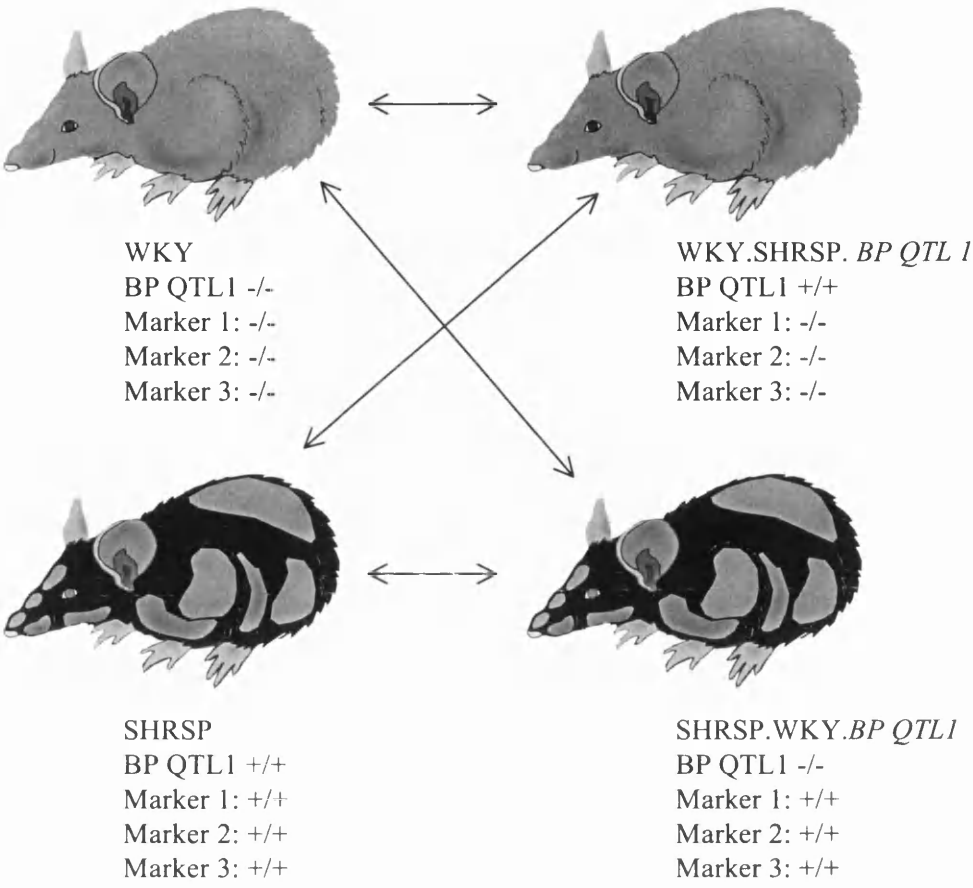

Figure 1.10. Diagram showing generation of congenic strains in a classic 2x2 study design. The QTL of interest can be studied in both normotensive and hypertensive backgrounds. Modified from Jacob (1999).

Chromosome	Congenic strain	Chromosomal region transferred (cM)	Blood pressure change (mmHg)	Reference
1	SHR.BN	22	-10	(St.Lezin *et al.* 1997)
	S.LEW	33	-26	(Garrett *et al.* 1998)
	WKY.SHR	26	+11	(Frantz *et al.* 1998)
	SHR.WKY	15	-11	(Frantz *et al.* 1998)
	WKY.SHR	13.5	+7	(Iwai *et al.* 1998)
	WKY.SHR	20	+5	(Iwai *et al.* 1999)
	WKY.SHRSP	22	+10	(Hubner *et al.* 1999)
	SHR.BN	22	-17	(St.Lezin *et al.* 2000)
	SHR.BN	8	-23	(St.Lezin *et al.* 2000)
2	S.WKY	38	-44	(Deng *et al.* 1997)
	S.MNS	78	-29	(Deng *et al.* 1997)
	SP.WKYGla2a	151	-40	(Jeffs *et al.* 2000)
	WKY.SPGla2c	99.7	+15	(Jeffs *et al.* 2000)
3	S.R	17	-21	(Cicila *et al.* 1999)
4	SHR.BN	36	-15	(Pravenec *et al.* 1999)
5	S.LEW	32	-15	(Garrett *et al.* 1998)
7	S.R	9.9-35.2	-40	(Cicila *et al.* 1997)

Table 1.8. Congenic strains for BP QTLs. Continued on next page.

8	SHR.BN (*Lx*)	31	-20	(Kren *et al.* 1997)
9	S.R	21	-19	(Rapp *et al.* 1998a)
10	WKY.SHRSP$_{Hd}$	6	+5	(Kreutz *et al.* 1995)
	S.MNS (10a)*	31	-42	(Dukhanina *et al.* 1997)
	S.LEW	27	-42	(Garrett *et al.* 1998)
13	R.S-*renin*	19	-8	(St.Lezin *et al.* 1996)
	S.R-*renin*	6	+27	(Jiang *et al.* 1997)
	S.R-*renin*	24	-24	(Zhang *et al.* 1997)
19	SHR.BN	12	+21	(St.Lezin *et al.* 1999)
Y	SHR.WKY	Whole	-15	(Turner *et al.* 1991)
	WKY.SHR	Whole	+35	(Turner *et al.* 1991)
2 and 10	Chr2/10 S.WKY/MNS	38+29	-47	(Rapp *et al.* 1998b)

Table 1.8. Congenic strains for BP QTLs. The recipient strain is cited first and the donor strain second. R and S are Dahl salt-sensitive and Dahl salt-resistant rats; LEW is Lewis strain and MNS is Milan normotensive strain. BN(Lx) is a donor strain originally derived by introgressing the mutant Lx gene of the polydactylous rat on to the normotensive Brown-Norway background. *, S.MNS (10a) represents the congenic which produced the greatest blood pressure reduction from the several constructed (10a-d); **, This blood pressure change refers only to the epistatic interaction between the loci in chromosome 2 and 10. Modified from Dominiczak *et al* (Dominiczak *et al.* 1998)

(Rapp *et al.* 1989). Jiang *et al* (Jiang *et al.* 1997) confirmed these results in a reciprocal congenic line for the same region in which the systolic blood pressure was increased compared to the Dahl S parental strain. Zhang *et al* (Zhang *et al.* 1997) found the predicted effect with the renin gene transfer from the Dahl R rat into the Dahl S background resulting in a significantly lower blood pressure. Moreover, St. Lezin *et al* (St.Lezin *et al.* 1998) constructed a congenic strain introgressing the renin gene from the normotensive Brown-Norway rat into the SHR rat and demonstrated no changes in blood pressure. The discrepancies in these studies may be due to different intervals flanking the renin gene being transferred resulting in different interacting loci being fixed in each congenic strain. Moreover, phenotyping protocols were not identical to the phenotyping protocol used to determine the initial linkage of renin to high blood pressure.

1.3.2.2.4.1 Speed congenic strategy.

Most of the congenic strains produced (Table 1.8) used the traditional or classical strategy that requires 8-12 backcross generations to achieve suffiecient dilution of the donor background. However, with the availability of a large number of molecular genetic markers and the complete genetic linkage and radiation hybrid maps for the rat (Jacob *et al.* 1995; Jacob *et al.* 1995; Bihoreau *et al.* 1997; Watanabe *et al.* 1999; Steen *et al.* 1999; Steen *et al.* 1999; Jacob *et al.* 1995), it is possible to reduce the time necessary to generate congenic animals by 50-60% using a speed congenic approach (Figure 1.9). This strategy uses marker-assisted selection to increase the rate

of introgression of target loci into the background by successive backcrosses (Lande and Thompson, 1990). Genetic markers spanning the genome are used to identify animals which have the greatest proportion of the desired background (Wakeland *et al.* 1997). The acceleration of congenic strain production is based on a genome-wide analysis of genetic polymorphism distinguishing the donor and the recipient strains. Selection at each generation is based not only on the presence of the desired loci, but also on the absence of contaminating donor genes from other parts of the genome. This is assessed through analysis of polymorphic marker loci distributed throughout the genome (Markel *et al.* 1997). This strategy has been applied successfully in mice (Yui *et al.* 1996; Morel *et al.* 1996; Markel *et al.* 1997). However, at the time of initiation of my Ph.D. project it had yet to be tested in rat genetics.

Superovulation has been also suggested for reducing the breeding cycle (Behringer, 1998). This technique, followed by embryo transfer, might shorten the time taken to produce a congenic line to 1 year, but it requires a very high level of technical expertise and has not yet been used in practise (Rapp, 2000).

1.3.2.2.5 Fine genetic mapping.

Once a congenic line is produced, the next stage is the refining and narrowing down of the location of the BP QTL using congenic strains with smaller and smaller donor fragments to localise the QTL for fine genetic

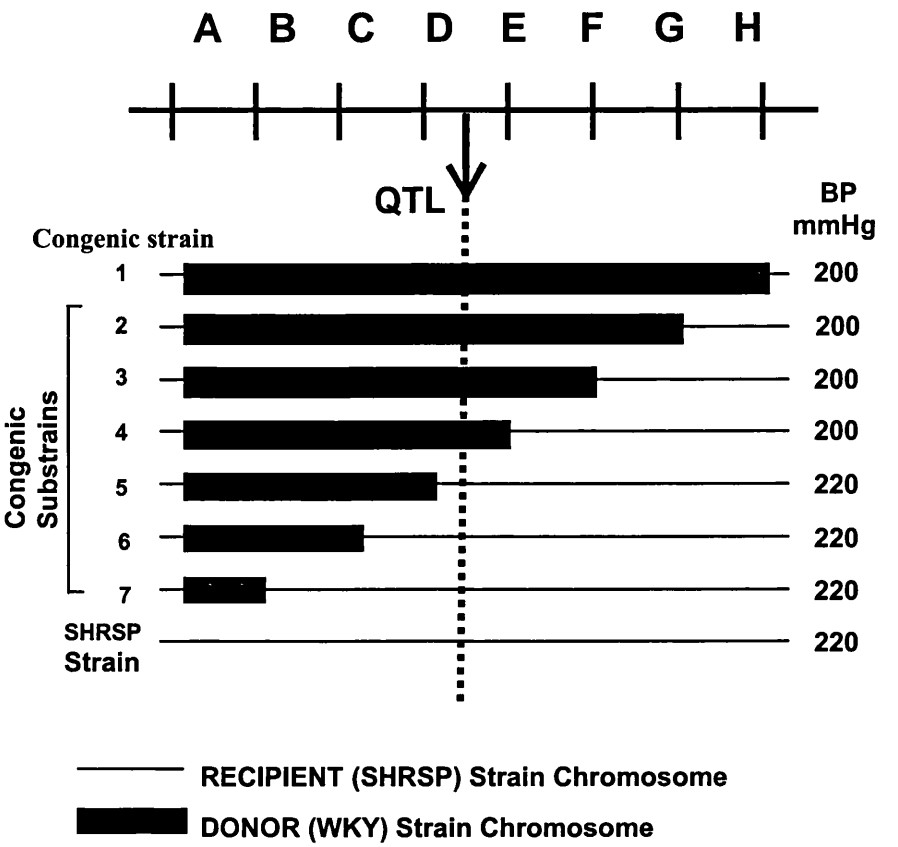

Figure 1.11. Substitution mapping of a QTL with the use of congenic strains. A relatively large QTL from the normotensive WKY strain defined by markers A through H is substituted into the genetic background of the hypertensive SHRSP strain resulting in the congenic strain 1. Strain 1 is then backcrossed to the SHRSP strain and the progeny genotyped at markers A through H to select rats with crossovers in various places throughout the region. Congenic substrains 2 to 7 are then produced and fixed in the homozygous state. The narrowed down QTL is between markers D and E as shown by a step change in blood pressure among the strains. Modified from Rapp & Deng (1995) and Dominiczak *et al* (1998).

substitution mapping (Figure 1.11). Fine genetic mapping has been applied to agricultural research (Paterson *et al.* 1990), however the size of the region to be narrowed down will depend on the density of polymorphic microsatellite markers within the segment of interest. Once the position of a QTL has been refined to a small candidate region, a physical map of the segment is needed. Such maps are constructed by using genetic markers from the candidate segment to screen recombinant DNA-libraries (typically yeast, bacterial, or P1-derived artificial chromosomes, known as YACs, BACs, and PACs, respectively) for a set of clones that cover the entire region forming a contig. The clones can then be used to discover what genes are in the region by exon trapping (Duyk *et al.* 1990), cDNA selection (Lovett, 1994), or direct DNA sequencing.

An alternative strategy is the use of cDNA microarrays that can help to provide a faster gene localisation without the need to shorten the introgressed regions (Aitman *et al.* 1999). Recently Aitman *et al* (1999) combined several new strategies inducing cDNA microarrays, congenic mapping, and radiation hybrid mapping to identify a defective SHR gene, the *Cd36* on rat chromosome 4, providing evidence for the role of this gene in insulin resistance, defective fatty acid metabolism, and hypertrigliceridemia in the SHR. However, *Cd36* genotyping performed in the SHRSP did not reveal the deletion variant carried by the SHR (Gotoda *et al.* 1999; Collison *et al.* 2000).

1.4 AIMS OF THE STUDY.

The aim of this investigation was to dissect two BP QTLs on rat chromosome 2, previously identified by Professor Dominiczak's group (Clark *et al.* 1996; Jeffs *et al.* 2000). This was accomplished by using a "speed" congenic strategy with the aid of high resolution physical mapping tools. This strategy will ultimately lead to the localisation of the gene(s) involved in the high blood pressure observed in the $SHRSP_{Gla}$ and their identification by positional cloning. The principal aims of this investigation were:

1. To develop a congenic strategy for the analysis of the BP QTLs identified on rat chromosome 2. This has been achieved by the development of an improved genetic linkage map for rat chromosome 2, the application of a speed congenic approach in the rat, and the development of reciprocal congenic strains.

2. To sequence one of the putative candidate genes, Na^+K^+ATPase contained in the chromosomal segment defined by the speed congenic strategy. This will be done to detect any possible mutations that might explain the contrasting blood pressure phenotype between the WKY_{Gla} and $SHRSP_{Gla}$ strain. However, for the candidate gene to be considered as a susceptibility gene for high blood pressure, it has to be shown to play a functional role in the pathogenesis of hypertension.

3. To construct a high resolution radiation hybrid map of the relevant segments of rat chromosome 2 and 5. Rat chromosome 5 radiation hybrid mapping has been performed in order to address discrepancies between our genetic map and other genetic maps available in the literature and in the databases deposited on the world wide web. This has been further refined by physical mapping using fluorescence in situ hybridisation (FISH).

CHAPTER 2

METHODS.

2.1 GENERAL LABORATORY PRACTICE.

Reagents, solutions, electrical, and computerised equipment used met the highest standard available. Routinely, laboratory white coat and gloves (light-powdered or powder-free depending on particular experiments) were used during all procedures, gloves were changed frequently to avoid cross-contamination. Laboratory spectacles, face mask, and fume hood (Holliday, Fielding & Hocking Ltd) used to meet the safety requirements when hazard labelling of the reagents indicated.

All items of glassware were washed in solutions of the detergent Decon 75, rinsed in distilled water and dried in a oven at 37°C. Measurements of pH were obtained using a CO720 digital pH meter (WPA Cambridge) which was regularly calibrated with a solution of pH 7.0 and pH 4.0 prepared from buffer tablets (Sigma). When centrifugation was needed for small samples (up to 2,000 μL) a centrifuge 5402 (Eppendorf) was used giving a maximum of 14,000 rpm, for larger samples a IEC centra-GP8R refrigerated centrifuge (Life Science International Ltd) was employed.

Volumes between 0.1-5,000 μL were transferred using the appropriate Gilson pipettes (Gilson Medical Instruments) and the appropriate tips (Alpha Laboratories Ltd). For aqueous solutions autoclaved glass-distilled water was used. For dissolving powder or solid reagents in the appropriate solution a HB502 stirrer/hot plate (Bibby Sterilin Ltd) was utilised. Two balances were used a Mettler AT250 (European Instrument Sales) balance

accurate up to five decimal places or a Mettler P2000 (European Instrument Sales) balance accurate to three decimal places.

2.2 EXPERIMENTAL ANIMALS AND GENETIC CROSSES.

Thirteen SHRSP and thirteen WKY (6 males and 7 females of each) were obtained from the colonies maintained in the University of Michigan, they were a gift made by Dr. D.F. Bohr of the Department of Physiology at the University of Michigan, USA. These colonies have been maintained as inbred for more than 15 years in Michigan and were originally obtained from the National Institutes of Health (Bethesda, MD) (Davidson *et al.* 1995). These rats have been brother and sister mated in Glasgow to provide an SHRSP colony and a WKY colony since 1991.

We performed two reciprocal genetic crosses (Figure 2.1) to obtain an F2 cohort. This ensured that the Y chromosome from both hypertensive and normotensive strains was fully represented in the F2 segregating population. One male SHRSP was mated with two WKY females (cross 1) and one male WKY with two SHRSP females (cross 2). From the F1 rats of each cross, 3 males and 6 females were brother and sister mated to generate F2 rats (60 in cross 1 and 80 in cross 2). All rats were housed under controlled conditions of temperature (21°C) and light (12 hours light/dark cycle; 7am to 7pm) and were maintained on normal rat chow (rat and mouse N° 1 maintenance diet, special diet service) and water *ad libitum*. Litters were

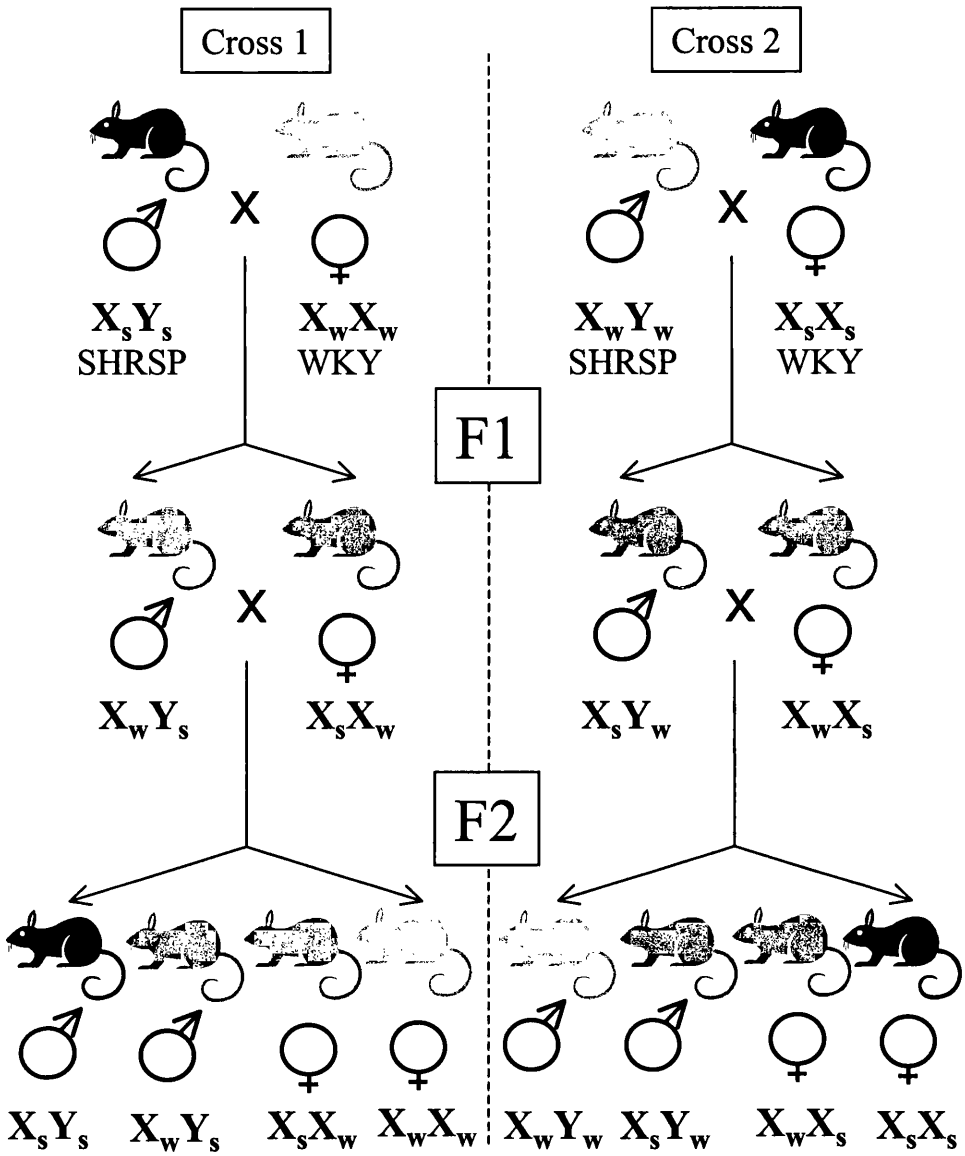

Figure 2.1. Diagram illustrating cross 1 and cross 2 with the origin of each sex chromosome in subscript; X_s denotes X chromosome of SHRSP origin, Y_s denotes Y chromosome originating from the SHRSP, X_w denotes X chromosome of WKY origin, and Y_w denotes Y chromosome originating from the WKY. Shades of grey in the rats from black to light grey represent the genotype status of the sex chromosomes, where light grey, WKY origin, dark grey, heterozygosity, and black, SHRSP origin.

weaned and sexed after 3 weeks and maintained by sibling group and sex thereafter. A maximum of three rats were kept in each cage.

2.2.1 CONGENIC CROSSES.

We used a speed or marker-assisted strategy for the development of congenic strains. This strategy is explained in detail in Section 1.3.2.2.4. The traditional strategy involves 8-12 backcrosses whereas a speed strategy reduces by half the number of backcrosses required (Figure 1.9). Reciprocal congenic strains were produced using the speed congenic strategy involving the transfer of different segments of rat chromosome 2 from WKY_{Gla} to the genetic background of the $SHRSP_{Gla}$ strain, and in the reciprocal direction from the $SHRSP_{Gla}$ to the genetic background of the WKY_{Gla} strain (Figure 2.2).

Two reciprocal F1 generations were produced by mating 1 SHRSP with two WKY females (cross 1) and 1 male WKY with two SHRSP females (cross 2) to allow the production of reciprocal congenic lines having not only all the autosomes of the recipient strain but also the sex chromosomes from the same origin (Figure 2.2). For the first backcross, males from the corresponding F1 generation were crossed with females from the recipient strain. Twelve to thirty seven microsatellite markers were genotyped throughout the desired QTL on chromosome 2. In addition we genotyped the first backcross at 83 genetic markers spanning the genome.

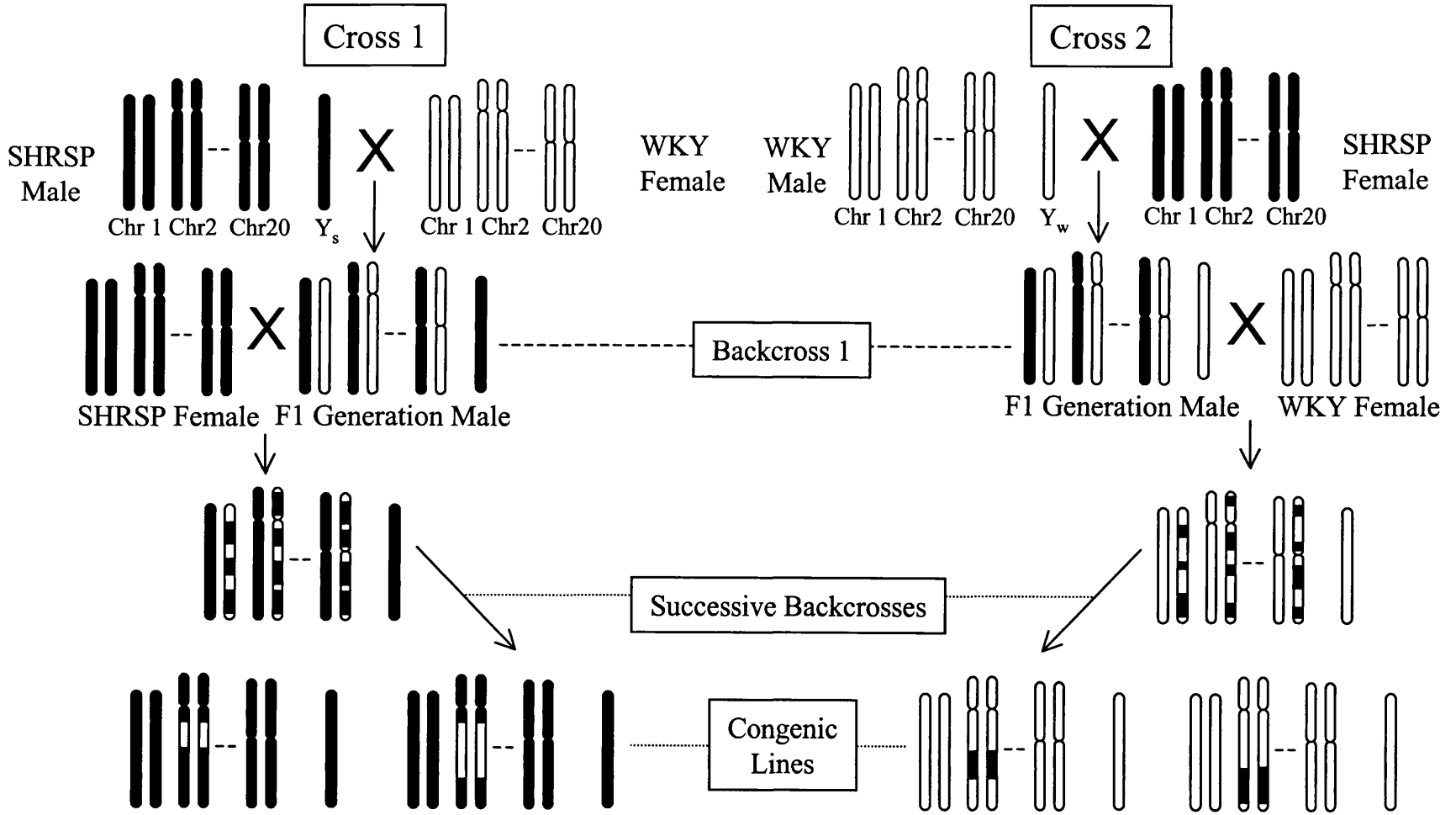

Figure 2.2 Diagram showing the strategy to construct reciprocal congenic lines. SHRSP alleles are represented in black, WKY alleles are represented in white. From the sex chromosomes only the Y is showed to represent the origin of both autosomes and sex chromosomes in each congenic strain.

This procedure allowed selection of "best males" for the next backcrosses. These "best males" were defined as those animals with heterozygosity at the QTL of interest but with less heterozygosity at markers in the genetic background or with less contamination of the background with donor alleles. This procedure was repeated in all offspring after every backcross until the donor's alleles were eradicated in the 83 background markers.

Background microsatellite markers for genotyping the backcross offspring were selected based on the need for as thorough as possible coverage of the entire rat genome, with special emphasis on the region harbouring BP QTLs previously identified by our group (Clark *et al.* 1996; Jeffs *et al.* 2000). Several rat genomic databases available on-line were used to fulfil this selection including: Rat Map, the Rat Genome Database (Goteberg University, Sweden; http://ratmap.gen.gu.se/); The Whitehead Institute Center for Genome Research Rat Mapping Project (http://www.genome.wi.mit.edu/rat/public/); and the Wellcome Trust Centre for Human Genetic Linkage Maps of the Rat Genome (http://www.well.ox.ac.uk/~bihoreau/).

When the contaminating donor's genome had been removed, one male and one female with heterozygosity at the QTL of interest were intercrossed to achieve homozygosity for the alleles of the donor strain at the QTL on chromosome 2. These fixed congenic strains were then maintained through successive brothers-sister matings.

A major criticism of the speed congenic approach is that there is increased risk of "passenger" loci being carried (regions of the donor genome different from that of the QTL being fixed). It follows that any phenotypic change might be due to these "passenger" loci and not due to the QTL introgressed. The transfer and carry over of "passenger" loci can be possible regardless of how complete the coverage of the genetic background achieved. A control congenic strain was constructed through introgression of a region of rat chromosome 2 abutting, but not overlapping, the chromosomal segment that include the BP QTL. These animals have been through the same selection processes as the true congenics, and although they do not contain the donor QTL, they will contain in their genetic background the same, if any, residual heterozygosity. Whether these animals go on to display a change in phenotype similar to that observed in the true congenic strain will determine not only the validity of the QTL but also the rationale for the speed congenic strategy.

2.3 PHENOTYPE.

The studies were approved by the Home Office according to regulations regarding experiments with animals in the United Kingdom. All experiments were undertaken following the safety guidelines set down by the Radiation Protection Service at the University of Glasgow.

2.3.1 RADIOTELEMETRY.

The Dataquest IV Telemetry System (Data Science International) was used for measurements of systolic pressure, diastolic pressure, mean arterial pressure, activity, and heart rate. The monitoring system consisted of a transmitter (radio frequency transducer model TA11PA-C40), receiver panel, consolidation matrix, and personal computer with accompanying software. Before the device was implanted, calibrations were verified to be accurate within 3 mmHg. Each implant was obtained in a sterile condition and used a maximum of three times following re-sterilisation in Cidex (activated glutaraldehyde solution).

Surgical implantation of each telemetry transmitter took place under standard sterile conditions in animals at 12 weeks of age within the peritoneal cavity. Rats were anaesthetised with halothane, and the abdominal cavity exposed by the temporary externalisation of the intestines. Silk sloops were placed around the aorta below renal arteries, and around both iliac arteries. While these sloops were tightened to reduce blood flow a small incision of approximately 1 mm of diameter was made with a 21 G needle in the abdominal aorta just above the point at which it begins to branch into the iliac arteries. The flexible catheter was then inserted pointing upstream (against the blood flow) and was secured with an sterilised cellulose patch using biological glue (VetBond, Scotch 3M). After this procedure all sloops were removed from the arteries, intestines returned

to the abdominal cavity, and the transmitter sutured to the abdominal wall with non-reabsorbable suture (Ethilon 4-0).

Rats were housed in individuals cages after the operation and each cage was placed over a receiver panel (RPC-1, Data Science) that was connected to the computer (Deskpro 333 PII, Compaq) for data acquisition with the dataquest acquisition and analysis system ART software (DQ ART Gold CM, Data Science). The rats were unrestrained and free to move within their cages. Haemodynamic data were sampled every 5 minutes for 10 seconds. To allow complete stabilisation of blood pressure after surgery, measurements used for further analysis were recorded from day 7 after surgery to day 42 and called "baseline haemodynamic measurements".

On day 43 after surgery rats on telemetry received 1% NaCl in their drinking water and this was continued for 3 weeks, until the rats were euthanised. Data collected from this period were considered "salt-loaded haemodynamic measurements".

2.3.2 EVALUATION OF BODY WEIGHT AND CARDIAC WEIGHT.

The body weight of each rat was obtained to within two decimal places using a CT200V portable top balance (Ohaus Corporation). Where necessary, a deduction of 9 grams was made to take account of the telemetry probe.

Immediately after euthanasia the thorax was opened and the heart was removed, blotted with tissue paper, and weighed using a Mettler AT250 balance accurate to within three decimal places. Both atria and the right ventricle were then removed surgically, and the left ventricle and septum weighed. The ratios of heart weight to body weight and left ventricle plus septum weight to body weight were then determined in order to correct for differences in body size.

2.4 GENETIC ANALYSIS.

2.4.1 TISSUE HARVEST FOR DNA.

To obtain DNA from the congenic backcrosses, the offspring were briefly anaesthetised at 4 weeks of age with halothane, and a 4 mm tip from the tail was removed and placed into a 1.5 mL microfuge tube. The wound was immediately sealed with an electrical cauteriser (Engel-Loter 1005) and tissue stored at –20°C. For genotyping F2 hybrids, livers and spleens were collected immediately after sacrifice and stored frozen at –70°C.

2.4.2 DNA EXTRACTION.

DNA extraction method was according to Laird *et al* (Laird *et al.* 1991) where the standard mammalian DNA isolation procedure was simplified. The aim of this simplification was to minimise the number of manipulations

required for each sample to yield enough high quality DNA for several polymerase chain reactions and/or Southern blot analysis.

Rat tails were defrosted in 1.5 mL microfuge tubes to which was added 700 µL of solution for extraction of DNA from rat tails (50 mM tris base, pH 8.0; 0,5% SDS; 100 mM EDTA, pH 8.0) and 35 µL of 10 mg/mL solution of proteinase K. The tubes were incubated and rotated in a hybridisation oven (Stuart Scientific) at 55°C overnight to ensure complete digestion. 700 µL of water-saturated phenol was added to the tube and the resulting solutions vortexed on a Fisons whirlmixer (Fisons Scientific Equipment). Then centrifuged for 3 minutes at 14,000 rpm, the aqueous phase was removed to a fresh tube and 700 µL of an equal volume (1:1; V/V) of water-saturated phenol and chloroform isoamyl alcohol (24:1; V/V) added and centrifuged at 14,000 rpm for two minutes. The aqueous layer was again removed to a fresh tube and 700 µL of chloroform isoamylalcohol added, vortexed and centrifuged for 2 minutes at 14,000 rpm. The aqueous layer was again removed to a fresh tube and 70 µL 3M sodium acetate, pH 6.0 and 700 µL 100% analar ethanol added to precipitate the DNA. To pellet the DNA the tubes were centrifuged at 14,000 rpm for 10 minutes, the supernatant was carefully removed and 1000 µL of 70% ethanol was added. This was done to wash the DNA and remove any traces of phenol and SDS. The tube was centrifuged to re-pellet the DNA and the supernatant discarded. The DNA pellet was allowed to dry in the inverted tube over tissue paper for 20 minutes. Finally, 100 µL of TE (10 mM tris base, pH

8.0; 1 mM EDTA, pH 8.0) was added to dissolve the pellet. The DNA solution was stored at 4°C.

The protocol for DNA extraction from livers and spleens is slightly different. Here 500 mg of defrosted tissue is placed in a sterile glass homogeniser and 4 mL of suspension buffer (100 mM EDTA, pH 8.0; 50 mM tris base, pH 8.0) is added and passed through 3 to 4 times. The homogenate was then transferred to a 50 mL Falcon polypropylene tube (Becton Dickinson & Co) and 224 µL 4 M NaCl, 60 µL of a 20 mg/mL solution of proteinase K, and 1.2 mL 10% SDS added, followed by incubation at 37°C overnight. The DNA was extracted using the water-saturated phenol, and chloroform isoamyl alcohol mixture described for rat tails (6 mL of each solution was added) and centrifuged for 20 minutes at 2,000 rpm at each step. The DNA was precipitated by addition of 12 mL 100% analar ethanol poured from a reasonable height. The DNA was removed from the Falcon tube with a heat-steriled sealed glass Pasteur pipette (Bilbate Ltd) and transferred to a small tube for subsequent washing with 5 mL 70% ethanol, drying, and resuspension and storage in 1 mL TE at 4°C as for the rat tails.

Due the large number of DNA extractions that were required for the congenic isolation, we used the Wizard Genomic DNA Purification Kit (Promega). This reduced the number of steps and tubes needed for the procedure, resulting in faster extractions and less possibility of cross-contamination. The protocol is similar for different tissues with the volumes

of the reagents adjusted for larger samples. 600 μL of a EDTA/nuclei lysis solution (100 μL 0.5 M EDTA, pH 8.0; 500 μL of nuclei lysis solution (Promega)) and 17.5 μL of 20 mg/mL solution of proteinase K were added to a 1.5 mL microfuge tube with gentle shaking. Alternatively, a 3-hour 55°C incubation (with shaking) and vortexing samples once per hour was done. When the tails were completely digested, 3 μL of RNase solution (Promega) was added and the tubes mixed by inverting them 2-5 times, followed by an incubation for 15-30 minutes at 37°C. When the samples were at room temperature, 200 μL of protein precipitation solution (Promega) was added and the mix vortexed vigorously for 20 seconds and centrifuged for 4 minutes at 13,000 rpm allowing the proteins to precipitate. The supernatant was carefully removed and transferred to a sterile 1.5 mL microfuge tube containing 600 μL of isopropanol, the solution was mixed by inversion until white thread-like strands of DNA form a visible mass. To pellet the DNA the tubes were centrifugated at 13,000 rpm for 1 minute and supernatant carefully decanted. 600 μL of 70% ethanol was added to wash the DNA as in the previous protocol, the tubes were centrifuged again to pellet the DNA and the ethanol aspirated using a Pasteur pipette (Alpha Laboratories Ltd). DNA was allowed to air-dry for 10-15 minutes and 100 μL of TE was added to store the DNA at 4°C.

All DNA extracted was quantified using a Ultrospec 2000 UV/Visible spectrophotometer (Pharmaco-Biotech). 5μL of the extracted DNA was added to 995 μL of sterile water (1 in 200 dilution) in a quartz cuvette and 10 μL of the extracted DNA was added to 990 μL sterile water (1 in 100

dilution) in another quartz cuvette. After mixing, the optical density of the sample was determined in triplicate at 260 nm and 280 nm against a blank and averages calculated for each dilution. From the mean absorbance value at 260 nm the amount of DNA in the sample was calculated as follows: [DNA]ng/μL = 200 x OD_{260} (for the 1 in 200 dilution) and [DNA]ng/μL = 100 x OD_{260} (for the 1 in 100 dilution). The ratio of the absorbance at 260:280 nm was a measure of the purity of the sample. A ratio of 1.8 was considered ideal and all such samples were diluted to a working concentration of 20 ng/μL and stored at 4°C. Ratios higher than 2.0 and below 1.0 indicated contamination (phenol and/or proteins) and such samples were discarded and re-extracted.

2.4.3 POLYMERASE CHAIN REACTION (PCR).

The technique was first described in 1985 by Saiki (Saiki *et al.* 1985). However its potential was not fully realised until 1988 (Saiki *et al.* 1988) coinciding with two key developments: a DNA polymerase that could be heated to a higher temperature without losing its activity; and robust machines that would quickly heat and cool samples repeatedly in a cyclic fashion. PCR is an *in vitro* method of nucleic acid synthesis by which a particular segment of DNA can be specifically replicated. It involves two oligonucleotide primers that flank the DNA fragment to be amplified and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase enzyme. These primers hybridise to opposite strands of

the target sequence and are orientated so that DNA synthesis by the polymerase proceeds across the region between the primers. Since the extension products themselves are also complementary to and capable of binding primers, successives cycles of amplification essentially double the amount of the target DNA synthesised in a previous cycle. The result is an exponential accumulation of the specific target fragment, approximately 2^n, where n is the number of cycles of amplification performed.

The molecular markers used in both the genome scan and congenic experiments consisted of polymorphic microsatellite markers typed by PCR. The PCR primers were either synthesised in-house from previously published sequences, some were custom synthesised in MWG-Biotech (Europe) or purchased from Genosys Biotechnology (Europe) or Research Genetics (Huntsville, Al). Some markers were obtained from Drs. Gauguier and Bihoreau of the Wellcome Trust Centre for Human Genetics in Oxford as part of the EURHYPGEN collaboration funded by the EU Grant.

The PCR reactions were carried out using 5 μL of the DNA template (100 ng of DNA) aliquoted in a Costar Thermowell 6511 96 well plate (Corning Incorporated) using a multichannel pipette to avoid cross-contamination. Amplification was carried out using a MJR thermal cycler (PTC 200, MJ Research) where the genomic DNA was amplified in a total volume of 20 μL containing (mM unless noted otherwise) 45 tris, pH 8.0; 11 $(NH_4)SO_2$, pH 8.0; 1.5 $MgCl_2$; 6.7 β-mercaptoethanol; 4.5 μM EDTA; 25 μM each dATP, dCTP, dGTP, and dTTP; 0.4U polymerase (Promega); and 0.25 μM

of each primer. The PCR program was as follows: 4 minutes at 94°C and 35 cycles of 1 minute at 94°C, 1 minute at 55°C or 60°C, and 30 seconds at 72°C. For some primers a modified "touch down" protocol was used, which involved a 0.5°C reduction in annealing temperature during the initial cycles (0.5°C drop). The final annealing temperature was then used for the last 30 cycles.

Using the PCR method described above, polymorphism of microsatellite markers can be read using a radioactive element such as ^{32}P followed by autoradiography (see Section 2.4.5). Due to the large number of microsatellites scanned within the region of interest and the large number of animals to be genotyped, a non-radioactive PCR protocol was developed. The 5'-end of the forward primer was labelled with a fluorescent dye (FAM, TET, and HEX dyes) and subsequently analysed with an automated ABI 377 DNA sequencer (Applied Biosystems/Perkin Elmer) (Section 2.4.5). The PCR reaction was carried out in a total volume of 20 μL as before but with the following reagents: 5 μL of DNA template (100 ng); 2 μL of 10X buffer (Qiagen) containing, tris-HCL, KCL, $(NH_4)_2SO_4$, and 15 mM $MgCL_2$ pH 8.7; 1 μL of polyoxyethylene (Life Technologies); 200 μM of each dATP, dCTP, dGTP, and dTTP; 0.5 μL of each primer; and 0.2 U HotStart Taq polymerase (Qiagen). PCR programs were identical except for a first 94°C for 15 minutes step to activate the HotStart Taq polymerase.

Further optimisation was required for some primers where annealing temperatures were varied from 50°C to 68°C in increments on 1°C and

concentration of $MgCl_2$ from 1.0 to 3.0 mM in increments of 0.25 mM to find best conditions for amplification.

The PCR-amplified products were tested on a 1% agarose gel. 1 g of agarose (Gibco) was mixed in 100 mL of 1X TBE (89 mM tris borate, pH 8.3; 20 mM EDTA, pH 8.0), heated, dissolved in a microwave oven for 1 minute 30 seconds, and stained with 1 μL of a 10 mg/mL solution of ethidium bromide. The gel was placed in a horizontal gel electrophoresis unit (Kodak International Biotechnologies, Inc) and 5 μL of several random samples of each marker amplified were mixed with 2X formamide loading buffer (90% formamide, 2.5% bromophenol blue (Xylene cyanol dye)). The samples were placed in the wells and ran against a 100 bp DNA ladder (Promega) for 40 minutes at 100 volts with the help of an electrophoresis power supply (Kodak International Biotechnologies, Ltd). Products were visualised either on a chromato-VUE TM-20 Transilluminator (VVP Inc) or a Fluor S-Multimager (Bio-Rad) and the actual size compared to that expected (Figure 2.3).

2.4.4 RESOLUTION OF POLYMORPHISM ON AGAROSE GELS.

When the differences between the WKY and SHRSP alleles where in the order of 10-15 bp for a given marker, high resolution Metaphor (FMC Bioproducts) agarose gels were prepared to resolve the products and genotype the animals at the same time.

100 mL of 1X TBE buffer was added to a 500 mL beaker and stirred rapidly with a teflon stir bar. Slowly 3 g of Metaphor agarose powder were sprinkled into the solution. The agarose was allowed to soak for 15 minutes in the buffer to reduce the tendency of the agarose solution to foam during heating and the weight of the beaker was calculated. The beaker was covered with cling film and a small hole pierced to allow the escape of steam. The agarose solution was heated in a microwave oven on medium power for 2 minutes. The beaker was swirled gently to resuspend any settled powder and gel pieces and then reheated on high power until the solution was boiling for at least 1 minute. The beaker was then removed from the microwave and sufficient hot distilled water added to obtain the initial weight. While the agarose solution was cooling, ethidium bromide was added to a final concentration of 0.2 μg/mL. When the solution was sufficiently cool (50-60°C) it was poured into a gel casting tray.

After the gel was set, the agarose was allowed to reach room temperature (between 45-60 minutes) and then was placed at 4°C for 20 minutes for maximum resolution and handling characteristics. 20 μL of the PCR amplification products were mixed with 2X formamide loading buffer and were loaded onto the gel. The samples were run against a DNA size ladder marker at 120 V for 2-4 hours according to the size difference

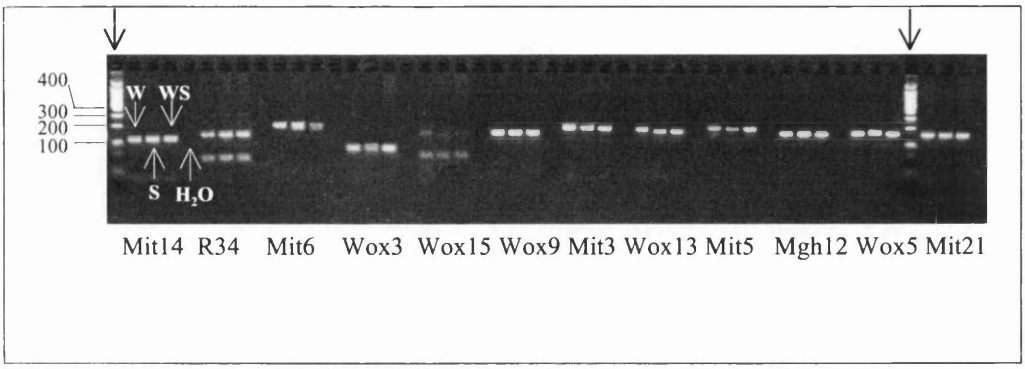

Figure 2.3. Image of a 1% agarose gel from the Fluor S-Multimager containing 12 different PCR amplified microsatellite markers. All markers are located on rat chromosome 2. Arrows indicate ladder marker loaded into the gel, sizes of the standard bands showed in bp on the left. Amplification of markers is tested with 4 controls, WKY genomic DNA, SHRSP genomic DNA, heterozygote DNA (obtained from F1 animals), and a water control.

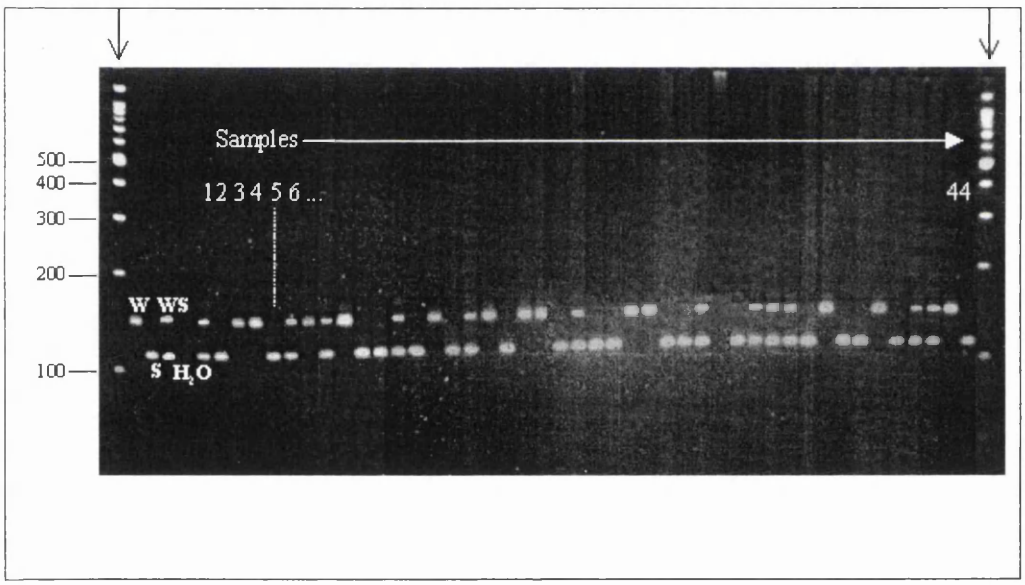

Figure 2.4. Image of a 3% Metaphor gel from the Fluor S-Multimager for resolution of polymorphism of marker D2Rat231. Arrows indicate the ladder marker loaded into the gel, sizes of the standard bands showed in bp on the left. Four different controls were loaded as described above. 44 samples corresponding to congenic genomic DNAs were loaded and the genotypes of them can be easily seen on the gel.

between the alleles to allow sufficient separation and resolution. The gels were scored by two independent observers (Figure 2.4).

2.4.5 POLYACRYLAMIDE GEL ELECTROPHORESIS, AUTO-RADIOGRAPHY AND ABI 377 DNA SEQUENCER GENOTYPES.

Samples from non-fluorescent PCR were prepared for resolution of polymorphisms on polyacrylamide denaturing sequencing gels electrophoresis, followed by southern blot and visualisation by autoradiography.

5 μL of each sample were transferred from the Costar Thermowell thin-walled polycarbonate 96 well plate (Corning incorporated) to a 96 well Falcon 3911 Microtest III titer-assay plates (Fred Baker Scientific) using a multichannel pipette. Once completed, 5 μL of 2X formamide loading buffer was added to each sample using an electronic multi-dispense pipette (Jencons). A Falcon 3913 Microtest III flexible lid (Fred Baker Scientific) was placed on the plate and samples were stored at 4°C until required. Different PCR products were pooled on the same plate providing they were of similar size because of the large number of markers utilised.

An 8% polyacrylamide gel was prepared using the SequaGel sequencing system solutions (National Diagnostic). Polymerisation was initiated with 60 μL of TEMED (Sigma) and 700 μL of 10% ammonium persulfate (APS). The gel was poured immediately. A small aliquot of the gel mixture

was left on the beaker to test for setting (approximately 1 hour). Once set, the gel was placed into a vertical STS-45 standard thermoplate sequencer gel electrophoresis unit (Kodak International Biotechnologies Inc) with both buffer reservoirs filled with 1X TBE. A 60 well comb (Kodak International Biotechnologies Inc) was inserted into the top of the gel and the gel pre-run at 2000 V (40 mA, 70 W) for approximately an hour to heat it to around the optimum temperature for loading of 55°C. Alternatively, a temperature probe was used and fixed into the glass plate to allow the gel reaching automatically the optimum temperature when a Power Pac 3000 power supply (Bio-Rad) was used.

The prepared PCR samples were denatured at 94°C for 4 minutes prior to loading and 7 μL of each sample was loaded in the gel with the aid of a 0.4 mm duck-billed pipette tip (Sorensen Bioscience Inc). The orientation was carefully annotated to enable genotyping. The time for the optimum separation between the different PCR products was calculated as follows: products of approximately 100 bp were run until the slowest component of the loading dye had ran down the gel 30 cm, products of approximately 150 bp 40 cm, and those of approximately 200 bp 50 cm. This allowed us to get the optimum use of a gel.

After electrophoretic separation was completed, the gel was allowed to cool by running cold water over the surface and then the glass plates split. A 28 x 28 cm Hybond-N^+ nylon membrane was placed on the gel, followed by two pieces of absorbing paper, the next glass plate without the gel, and weights

to allow southern blotting overnight. Once blotting was completed, the membrane was carefully removed and placed in a tray with 500 mL of 0.4 M sodium hydroxide for 20 minutes, followed by 2 x 10 minute washes in 2X SSC (300 mM sodium chloride; 30 mM sodium citrate, pH 7.0), during which the membrane was shaken. The membrane was placed in a hybridisation tube (Stuart Scientific). The forward primer was radiolabelled with [α-^{32}P]dCTP, and the reaction completed as follows: 12.5 µL of distilled water, 4 µL of terminal transferase buffer (Promega), 1µL of forward primer, 1 µL of [α^{32}P]dCTP, and 0.5 µL of terminal deoxynucleotidyl transferase (TDT) followed by incubation at 37°C for 30 minutes and 10 minutes at 68°C to stop the reaction. Once the reaction was completed, a pre-hybridisation solution was prepared (24 mL sterile water; 12 mL 20X SSC; 4 mL 10X SDS; 0.1 g powdered milk to prevent non-specific binding) and the radiolabelled primer added. This mixture was decanted into to the hybridisation tube containing the membrane for hybridisation at 55°C for 2 hours or at 42°C overnight. After hybridisation was completed, the membrane was washed 2 times with 1000 mL of post-hybridisation solution (0.2% SDS; 2X SSC), dried between two pieces of absorbent paper, wrapped in cling film, and directly exposed to autoradiography hyperfilm (Amersham) in a hypercassette (Amersham) for 2-14 hours at –80°C according to the strenght of the signal. The films were developed in a film processor (Kodak International Biotechnologies Inc). Alternatively probed-membrane was wrapped with Saran wrap (Dow Chemical Company) and placed in a special cassette (Bio-Rad) against an imaging screen K (Bio-Rad). This screen reduced ten times the exposure

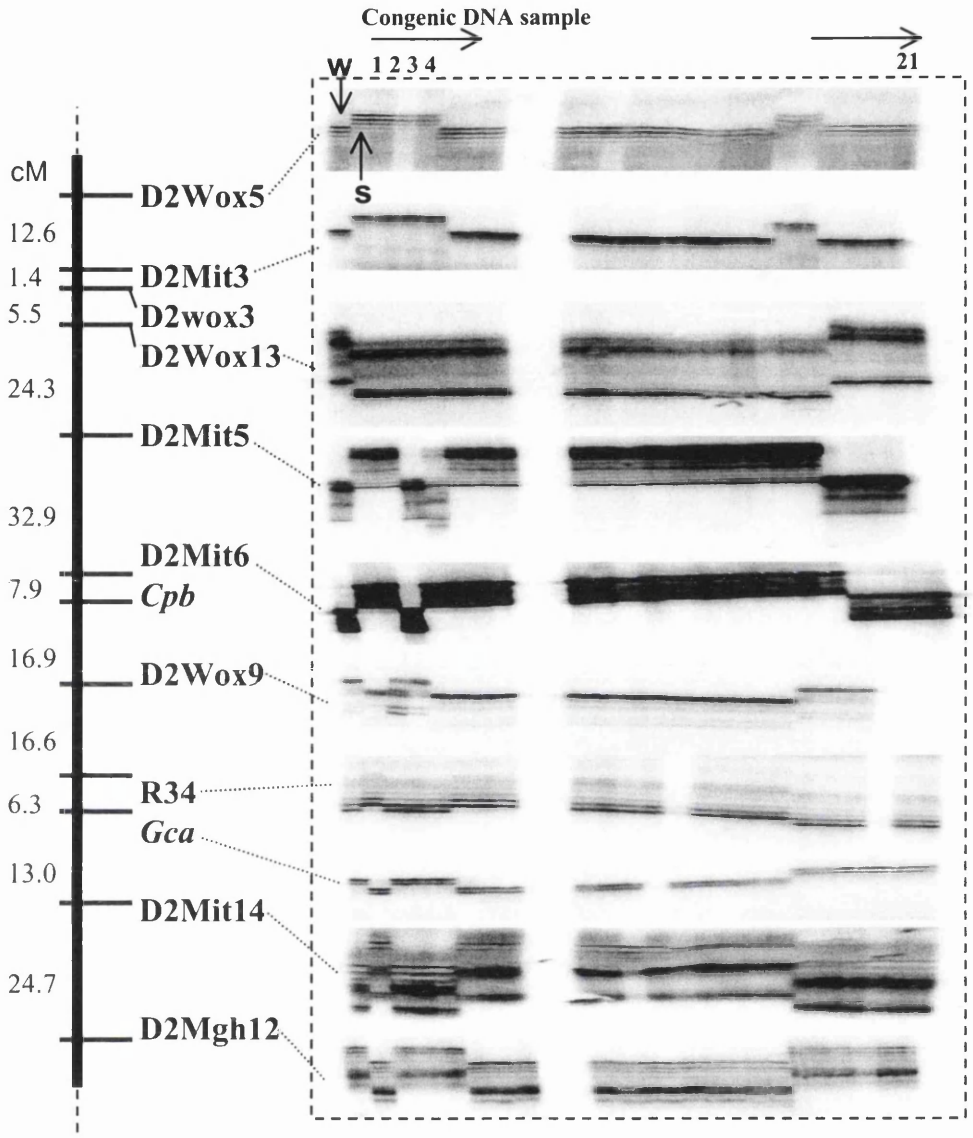

Figure 2.5 Composite figure showing a simplified linkage map of chromosome 2 and genotype of 21 congenic animals for different markers along the chromosome using a Molecular Imager FX System (Bio-Rad). W, represents WKY homozygote alleles and S, SHRSP homozygote alleles. Distances to the left of the map are in centiMorgans (cM).

required with normal film due to its increased sensitivity to ^{32}P. An image was then acquired with the aid of a Molecular Imager FX System (Bio-Rad) which scanned the screen with a dual wave-length laser (Figure 2.5). To reprobe the membrane with different primers it was washed two times in boiling 0.4% SDS followed by two washes in 2X SSC.

When fluorescent PCR were used products were pooled according to the size and fluorescent labels used, this led to 5 different pools (Appendix 3). Having three different fluorescent labels, three different markers with alleles of similar size were labelled with different fluorescent molecules and pooled because they could be differentiated according to their fluorescent tag by the DNA sequencer. The gels for the ABI 377 were prepared in a filter unit with a cellulose filter (Whatman). 18 g of urea, 5 mL of long ranger 50% stock gel solution (FMC Bioproducts), a small spatula of AG 501-X8 resin (Bio-Rad), and 45 mL of deionised water were mixed and stirred in a 100 mL beaker. Once the filtration was completed, 250 µL of 10% APS and 25 µL of TEMED (Amresco) were added to allow polymerisation of the acrylamide. A special cassette holding the glass-plates was prepared to pour the gel solution. After the gel was set it was placed on the ABI 377 DNA sequencer and the corresponding buffer tanks were filled with 1X TBE. A 96 well comb was placed on the top of the gel for loading the samples. Plates were checked in the sequencer verifying a baseline scan flat line indicating well cleaned plates. The instruments was pre-run for 10 minutes at 1000 V, 35 mA, and 50 W to warm it up and reach the optimum temperature for sample separation (51°C). 1.5 µL of the PCR products were

taken and mixed with 2 μL of loading cocktail consisting on ultra-pure-grade formamide (Amresco), GENESCAN-350 TAMRA, and loading buffer (50 mg/mL blue dextram, 25 mM EDTA) (1:2:2; v/v/v), the samples were denatured at 94°C for 3 minutes and placed on ice immediately after. A volume of 1.5 μL of each sample was loaded on each well using a GL/2000 channel gel loader (Kloehn) that allowed to load 8 samples simultaneously avoiding cross contamination.

The samples were run at 3000 V, 60 mA, 200 W, for 2 hours giving sufficient time for separation of alleles of different sizes. Gel image was then obtained with the Genescan 3.1 software (Perkin Elmer), this software allowed to track all the lanes in the gel with the corresponding fluorescent PCR product and, at the same time, analysed the sizes of the standard used.

If the standard sizes were correctly called, we used Genotyper 2.1 software (Perkin Elmer). This software allows determination of the genotype of different microsatellite markers loaded on the gel. If the standard sizes were incorrectly called or contamination was observed in the water blank the whole procedure was repeated (Figure 2.6).

2.4.6 SEQUENCING.

DNA was sequenced using an enzymatic method developed by Sanger (Sanger *et al.* 1977; Sanger *et al.* 1992) where single strand DNA is used as a template for synthesis of new DNA strands by a DNA polymerase in

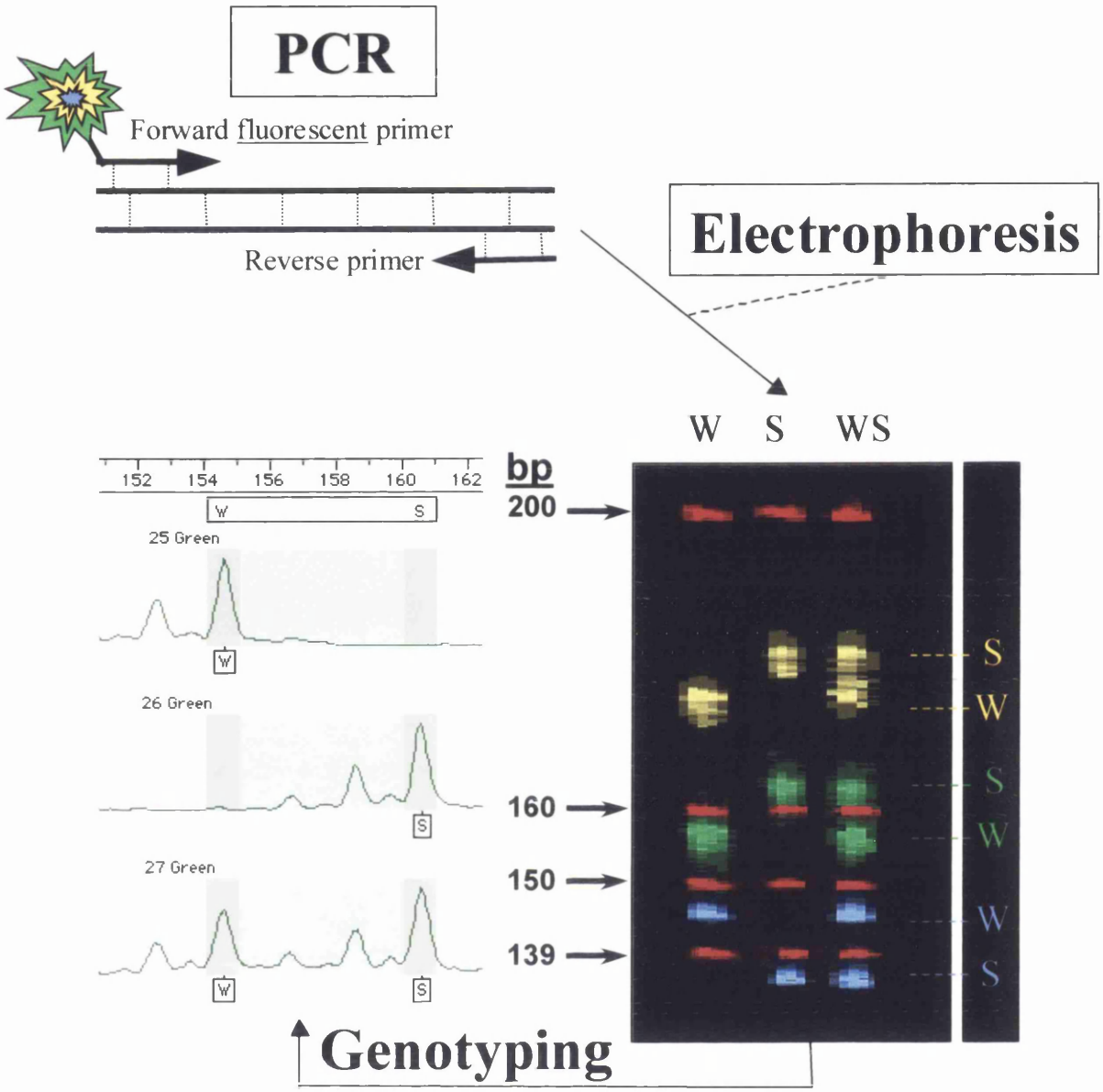

Figure 2.6 Schematic representation of the genotyping carried out using the ABI Prism 377XL DNA sequencer. Fluorescent PCR were done using a forward primer labelled, and then electrophoresed on the sequencer. To the right is the actual image of the gel with the colours yellow (HEX), blue (FAM), and green (TET) representing genotypes for three different microsatellites. The red bands on the gel represent the size standard (TAMRA 350) with the corresponding size in base pairs (bp) to the left. At the left of the gel image there is an screenshot of the genotype obtained using the Genotyper 2.1 software for the marker labelled with TET.

the presence of normal nucleotide precursors and base-specific dideoxynucleotides (ddNTPs). These ddNTPs can be incorporated by a DNA polymerase into a growing DNA strand through their 5'-triphosphate groups but, because these analogues lack a hydroxyl group at the 3'-carbon position, they cannot form phosphodiester bands with the next incoming dNTP and the chain extension terminates, whenever an analogue is incorporated. When a specific ddNTP is included along with the four different dNTPs normally required for DNA synthesis, the resulting extension products are a series of different length DNA chains that are specifically terminated at that dideoxy residue. To obtain sequence data, a separate reaction must be run for each of the four ddNTPs, all being run together on a simple polyacrylamide gel to provide a complete sequence information.

The AmpliCycle™ Sequencing Kit (Perkin Elmer), which is a modified version of Sanger's sequencing method, was used. Specific primers for the coding regions of genes of interest were synthesised in-house yielding products of 150-200 bp. Once PCR was completed, products were run on 1% agarose gel and the bands cut-out with a sharp scalpel. The DNA products were extracted from the agarose gel using a gel nebulizer inserted into a Microcon microconcentrator (Amicon) and centrifuged at 14,000rpm for 20 minutes at 4°C. After the gel was broken up, the filtrate was washed through the Microcon by adding 20 μL of sterile distilled water followed by centrifugation at 3,000 rpm for 24 minutes at 4°C. DNA concentration was determined using a DynaQuant fluorimeter (Hoefer). Before the sequencing

reaction was run each primer was radiolabelled with [γ-^{32}P]dATP to allow visualisation by autoradiography. The labelling reaction consisted of 1.1 μL of sterile distilled water, 0.6 μL of 10X kinase buffer, 0.5 μL of the sequencing primer (20 μM), 1 μL of [γ-^{32}P]dATP, and 3.0 μL T4 polynucleotyde kinase all incubated at 37°C for 2 hours. Sequencing reactions were done using 2 μL of each ddNTPs added to separated wells in a Costar plate followed by addition of 6 μL of a sequencing Master Mix containing 20 μL of sterile water, 4.0 μL cycling mix (Amplitaq DNA polymerase; 2.0 μL PCR template (100 fM); 1.0 μL dimethyl sulfoxide (DMSO)), and 1.0 μL of the radiolabelled primer mix. The plate was placed in the MJR thermocycler and ran at 94°C for 4 minutes followed by 35 cycles of 94°C for 1 minute, the annealing temperature of the determined primer for 1 minute, and 72°C for 1 minute (elongation step). The reactions were stopped by the addition of 4 μL stop solution and resolved on a standard 6% polyacrylamide gel running for 2 hours as described in Section 2.4.5. The gel was dried directly onto a 3 mm Whatman filter paper (Whatman International Ltd) using a slab Gel-Vac dryer (Hybaid) and then, directly exposed to film. Sequences were read by two independent observers.

Alternatively, automated fluorescent DNA sequencing was used (Wilson *et al.* 1990) where the ddNTPs were attached to fluorophores or chemical groups capable of fluorescing. The ABI 377XL sequencer was used, to detect the fluorescence from four different dyes that are used to identify the A, G, C, and T extension reactions. Each dye emits light at a different

wavelength when it is excited by laser light; thus, all four colours (and therefore all four reactions) can be detected and distinguished in a single gel lane improving sequencing accuracy and eliminating problems caused by variations in electrophoretic mobility from lane to lane. Moreover, the sequencer can increase the number of templates that can be analysed on a single gel. The sequencer's software stores automatically the information precluding transcription errors when an interpreted sequence is typed by hand into a computer file. PCR products were extracted from agarose gels as described above. As an alternative, a PCR product Pre-Sequencing kit (USB) was used for preparing the products for sequencing.

DNA was extracted and purified from agarose gels because the excess of dNTPs from the PCR reaction could affect the balance of the sequencing reaction which would result in decreased termination. Moreover, carryover of PCR primers or primer-dimmers could generate multiple sequence ladders interfering with the sequencing of the specific fragments. The PCR pre-sequencing kit consisted of two hydrolytic enzymes, shrimp alkaline phosphatase and exonuclease I, that removed dNTPs and primers. Exonuclease I removed residual single-stranded primers and any extraneous single-stranded DNA produced by the PCR. The shrimp alkaline phosphatase removed the remaining dNTPs from the PCR mixture that would interfere with the sequencing reaction. For the reaction 5 μL of the PCR product were mixed with 1 μL of exonuclease I (10 U/μL), and 1 μL of alkaline phosphatase (2 U/μL), and incubated at 37°C for 1 minute, the enzymes were inactivated by a final step at 80°C for 15 minutes.

For the sequencing reaction 3 μL of the enzymatic-treated-DNA was added to a well of a 96-well Costar thermowell 6511 plate (Corning Incorporated) with 3.2 pmol of the primer, 4 μL of Ready Reaction Mix (containing Amplitaq DNA polymerase; RTth pyrophosphatase; fluorescently labelled 2', 3'-ddNTPs; 2 mM $MgCL_2$; 80 mM tris-HCL, pH 9.0) (Perkin Elmer), 2 μL of 5X sequencing buffer (containing tris-HCL, pH 9.0; and $MgCL_2$) (Perkin Elmer), and the final volume made up to 20 μL with sterile distilled water. The reaction was overlaid with a drop of mineral oil (Sigma) and placed on the MJR thermal cycler for 25 cycles of 96°C for 30 seconds, 50°C for 25 seconds, 60°C for 4 minutes. To a 0.5 mL microfuge tube 50 μL of 95% ethanol and 2 μL of 3 M sodium acetate (pH 4.6) was added followed by 20 μL of the sequencing reaction and centrifugation at 14,000 rpm for 20 minutes for precipitation of the sequencing products. The supernatant was carefully removed and 250 μL of 75% of ethanol was added, the pellet was vortexed and centrifugated at 14,000 rpm for 6 minutes to re-pellet the products. The supernatant was removed and the tubes were placed on a PCR block at 90°C for 4 minutes and dried down. To each tube 6 μL of loading buffer containing formamide and blue dextran dye (V/V; 5:2) were added and vortexed to resuspend the DNA.

A 5.0% polyacrylamide gel was prepared as described in Section 2.4.5, the samples were denatured at 90°C for 2 minutes and 1.5 μL of each loaded onto a gel. Samples were electrophoresed in a field of 1680 V at a constant temperature of 51°C using 1X TBE as buffer on the ABI 377XL DNA sequencer. The labelled DNA fragments migrated down the gel entering the

"read region" 36-cm below the wells where an argon-ion laser scanned horizontally back and forth. The laser operated at 40 mW emitting two wavelengths and exciting the fluorescent dye on each DNA fragment which fluoresced, emitting light at different wavelengths. The 2', 3'-ddNTPs in the ready reaction mix were labelled with dichloro-rodamine fluorescent dyes as follows; ddGTP, dichloro R110; emitting light at 532 nm; ddATP, dichloro R6G, emitting light at 560 nm; ddTTP, dichloro TAMRA, with a wavelength of 594 nm; and ddCTP, dichloro R0X, with a wavelength of 260 nm. These different wavelengths were detected by the instrument, and the data was sent to a Power Macintosh G3 computer and analysed using the ABI Prism Sequencing Analysis Software v3.0 (Perkin Elmer).

2.5 STATISTICAL ANALYSIS.

Phenotypes, which were not normally distributed, were logarithmically transformed before inclusion in any statistical procedure as dependent variables. Phenotypes which were strongly bimodal due to sex differences, but the distribution for each sex separately were acceptably normal, were analysed separately by sex. Confirmatory analysis of QTLs was performed by one-way ANOVA with a conservative significance level of $p<0.01$. Additionally, a stepwise regression analysis was used to determine the QTL effects while controlling for possible confounding and covariate effects (Schork *et al.* 1995).

2.5.1 GENETIC LINKAGE MAPPING (MAPMAKER/EXP 3.0).

Multipoint linkage analysis was carried out using MAPMAKER/EXP 3.0 (Lander *et al.* 1987) which allowed markers to be mapped relative to each other using all the raw genotyping data. This was done to determine a multitude of possible maps consisting of a specific marker order and map distances. The software was specifically designed for the construction of primary genetic linkage maps from marker genotype data from F2 segregating populations. Distances were calculated from estimates of recombination fractions with the Haldane mapping function. Different maps were suggested by MAPMAKER/EXP 3.0 and for each map the level of probability that would explain the observed data was then computed. This probability is called the likelihood of the map. The best map was then selected as the one with the highest likelihood.

2.5.2 LINKAGE ANALYSIS (MAPMAKER/QTL 1.1).

QTLs affecting phenotypes were mapped relative to the microsatellite markers with MAPMAKER/QTL 1.1 (Lander *et al.* 1987). This software infers the genotype of each animal in the F2 cross at any chromosomal position from the available genetic map, and then models the phenotype in terms of the inferred genotype, additivity and heterozygosity, as well as any covariate information. The strength of evidence for the existence of a QTL at any location is provided by the maximum Log_{10} likelihood (LOD Score) of the model at that point. Moreover, the proportion of phenotypic variance

explained by the model is also calculated. A purely phenotypic model (genetic parameters set to zero) was used initially to assess the likelihood contribution of sex as the only explanatory variable, and the corresponding percentage of variance explained. The full genetic model, representing free inheritance of the trait after correction for sex, was then fitted, as were models for dominant, recessive, and co-dominant inheritance. The software calculated the most likely phenotypic effect of having the ss (SHRSP homozygote) or ww (WKY homozygote) genotype at a putative QTL and then calculated a LOD score. To correct for the effect of multiple-hypothesis testing, stringent thresholds were required for mapping loci underlying complex traits, with LOD scores between 3.3 and 4.3 to establish significant linkage (Lander and Kruglyak, 1995) (Section 1.3.2.2.3.1).

2.5.3 RADIO-TELEMETRY HAEMODYNAMIC DATA.

A total of 10,080 measurements of each blood pressure phenotype were made in each animal during the 5-week baseline period, and 6,048 measurements during the 3-week salt-loaded phase. Within each phase, haemodynamic measurements were separated into daytime (7 AM to 7 PM) and night-time (7 PM to 7 AM) periods. Summary statistics were provided for each combination of experimental phase and time of day through the calculation of overall mean and SEM values separately by sex and congenic strain. Repeated measures ANOVA was used for comparisons between each congenic strain and the corresponding background parental strain.

2.6 RADIATION HYBRIDS.

Rat whole genome radiation hybrid was obtained from Research Genetics (Huntsville, Ala). To create the panel, a rat donor cell line (Rat FR, a diploid fibroblast cell line derived from skin biopsy of a foetal Sprague-Dawley rat) was exposed to 3,000 rad of X-rays and then fused with non-irradiated thymidine kinase-deficient hamster recipient cells (A23). The panel consists of 106 clones and has an average locus retention of 28% to 30%. The presence or absence of each microsatellite marker was determined by PCR. Additionally, 4 other control samples underwent PCR: FR DNA, A23 DNA, WKY rat DNA, and a water blank. PCR reactions were done as previously described (Section 2.4.3). The PCR products were separated by electrophoresis on a 3% agarose gel containing ethidium bromide and visualised with a Fluor-S Multimager (Bio-Rad). PCR was done in duplicate for each microsatellite marker and scored by two independent observers.

2.6.1 RADIATION HYBRID MAPPING.

The radiation hybrid mapping programmes of the RHMAP package, version 3.0 (http://www.sph.umich.edu/group/statgen/software) were used to analyse the data. These programs assume that breakage is at random along the chromosome, with constant intensity and no interference (Boehnke *et al.* 1991; Lange *et al.* 1995). The RH2PT program was used to perform a 2-point analysis. A set of best orders with the fewest obligate chromosome breaks was defined with the use of stepwise locus ordering strategy with the

RHMINBRK program. The order of each marker on the map was determined with the RHMAXLIK program and a branch and bound strategy. Map distance estimates; D, were calculated with the mapping function, D= -Ln (1-θ), where θ is the breakage probability estimate between two markers. D was expressed in centiRays (cR), where a distance of 1 cR3000 corresponds to a 1% probability of breakage between 2 markers after exposure to 3,000 rad of X-rays.

Appendix 1 lists the suppliers of all chemicals and reagents used. Appendix 2 is a detailed list of all the protocols for the preparation of solutions.

CHAPTER 3

CONSTRUCTION OF SPEED CONGENIC STRAINS TO DISSECT TWO BLOOD PRESSURE QUANTITATIVE TRAIT LOCI ON RAT CHROMOSOME 2.

3.1 INTRODUCTION.

Human essential hypertension is a complex, multifactorial and polygenic disease (Lander and Schork, 1994), with estimates of heritability of 30-50% (Ward, 1990). Despite a very significant recent progress in genomic and statistical tools, the genetic dissection of human essential hypertension still provides a major challenge. As discussed in Section 1.2 the two major strategies developed for gene identification in human essential hypertension (search for single-causative genes in Mendelian forms of hypertension and the candidate gene approach), have significant limitations due to the complex nature of human essential hypertension (Dominiczak *et al.* 2000). It should be noted that the prevalence of these Mendelian syndromes represents a very small percentage of all the aetiologies of human hypertension.

Some of the complexity inherent to the study of human subjects and families can be overcome by the use of inbred rat models of genetic hypertension. The creation of inbred, genetically homogeneous hypertensive strains from outbred colonies has been completed by selective breeding of animals showing elevated blood pressure. This avoids the confusing heterogeneity of the human disease and hundreds of homogeneous progeny can be studied under controlled environmental conditions using high fidelity phenotyping.

The stroke-prone spontaneously hypertensive rat (SHRSP) is one of the best existing animal models of human essential hypertension and is characterised by a number of vascular complications. These include cardiac hypertrophy, cardiac failure, and stroke not dissimilar to those found in the human disease (Yamori *et al.* 1979; Conrad *et al.* 1991).

The SHRSP has been used in several studies aiming at identification of BP QTLs performing total genome scans (Hilbert *et al.* 1991; Jacob *et al.* 1991; Nara *et al.* 1993; Matsumoto *et al.* 1995; Clark *et al.* 1996; Matsumoto *et al.* 1996). Previous published data from Professor Dominiczak's laboratory identified 2 separate BP QTLs on rat chromosome 2 (Clark *et al.* 1996) (Figure 3.1). The most significant of these had its peak close to the microsatellite marker *D2Mit6* with a LOD score of 3.6. It was significant for baseline and salt-loaded systolic and diastolic blood pressures in both male and female F2 cohorts. The second QTL with a LOD of 3.1 was localised 73 cM from *D2Mit6* and contributed to salt-loaded blood pressure in the male F2 cohort only.

The identification of such BP QTLs is only the first step towards the ultimate goal of gene identification, which can be done through the genetic and physiological analysis of congenic lines (Rapp and Deng, 1995). Congenic strains provide a genetic test to confirm the existence of a BP

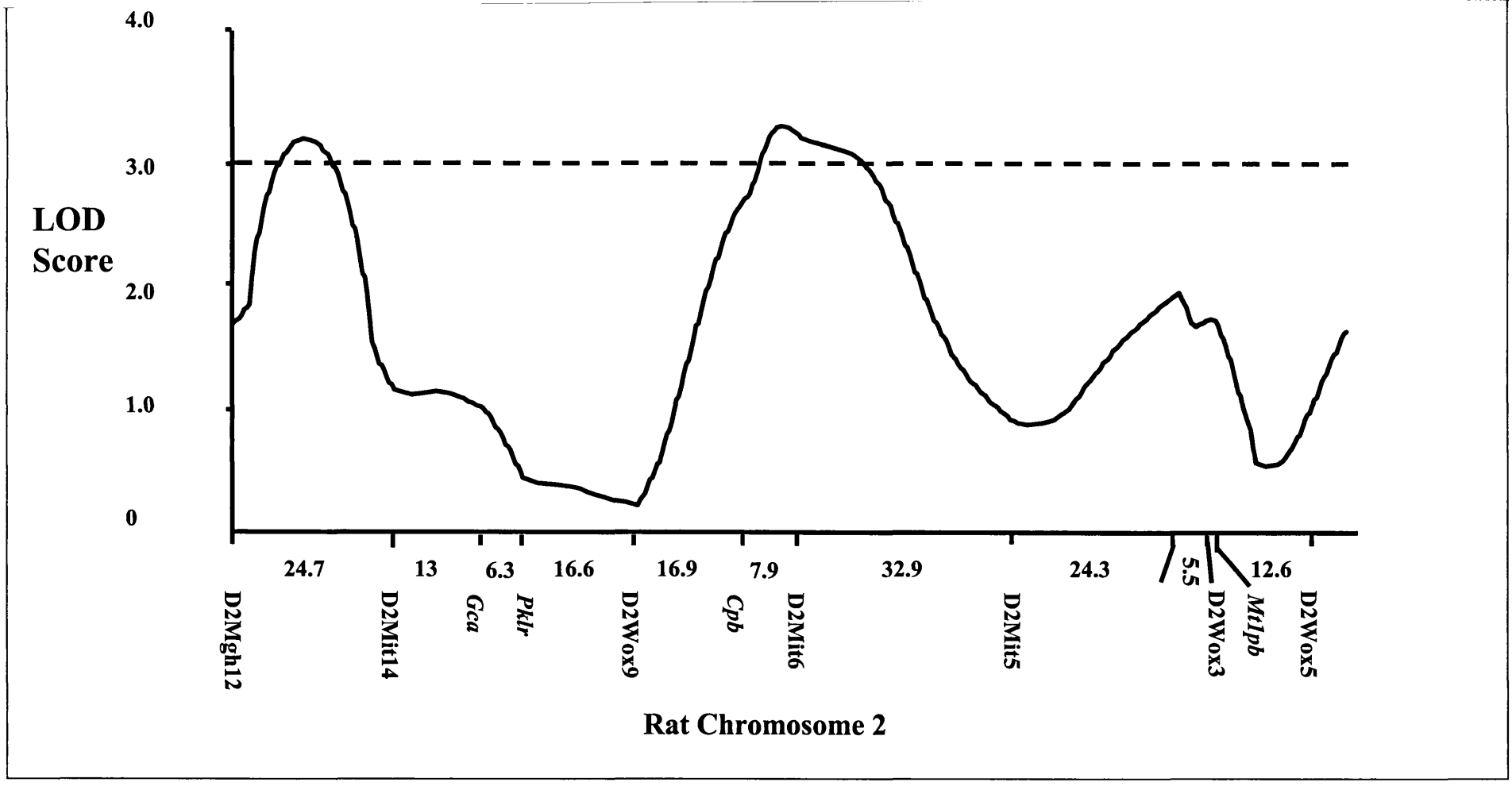

Figure 3.1 Rat chromosome 2 linkage map and salt-loaded diastolic blood pressure QTL localisation for a F2 male only population derived from SHRSP x WKY. Distances between markers are in centiMorgans (cM). Broken line indicates the LOD threshold for significant linkage. All markers are anonymous except *Gca*, guanylyl cyclase A; *Pklr*, pyruvate kinase L; *Cpb*, carboxypeptidase B; *Mt1pb*, metallothionein 1 pseudogene b; and *Fst*, follistatin. From Clark *et al* (1996).

QTL and simultaneously, provide a genetic tool to narrow down the chromosomal region of interest. A congenic strain is one in which the chromosomal region of interest in one strain (the recipient) has been selectively replaced by the homologous region from another strain (the donor). If the blood pressure of the congenic strain is significantly different from that of the recipient strain, it can be concluded that this particular chromosomal fragment does indeed capture a QTL that contributes to a difference in blood pressure between the donor and the recipient strain.

Congenic strains have been traditionally developed by serially backcrossing the donor strain that harbours the genomic region with the BP QTL with the recipient inbred strain, accompanied by selection for progeny heterozygous for the desired region in each backcross generation (Wakeland *et al.* 1997). According to Mendelian principles half of the donor genomic material will be transmitted to a subsequent backcross generation. It follows that after 8-12 backcrosses the genetic make-up of a congenic strain will consist of more than 99% of the genome unlinked to the BP QTL of the recipient strain. Once the backcrosses have been completed, the introgressed region is then made homozygous by brother-sister mating. The resulting congenic strain theoretically has a genetic background identical to that of the recipient strain with the exception of the introgressed region.

This strategy has resulted in the production of several congenic rat lines during the past 2 years that confirm the existence of QTLs involved in blood pressure on rat chromosomes 1, 2, 3, 4, 5, 7, 8, 9, 10, 13, and 19 (Table 1.8).

All the strains have been produced using the protocol outlined above, which requires 3-4 years to complete. The percentage of contamination or donor genome (different from the introgressed region) in these strains is based on theoretical considerations, without any experimental assessment of the background. With the availability of a large number of microsatellite markers and the complete genetic linkage and radiation hybrid map for the rat (Jacob *et al.* 1995; Bihoreau *et al.* 1997; Cai *et al.* 1997; Watanabe *et al.* 1999; Steen *et al.* 1999), it is possible to reduce the time necessary to generate congenic animals by 50-60% by testing the genetic background at each backcross generation.

The speed congenic strategy involves repeated screening of the polymorphic markers scattered throughout the entire genetic background, thereby allowing the specific selection of a male from each backcross with the least amount of donor alleles remaining in the genetic background. These "selected males" or "best males" would allow the rate of background donor elimination to be accelerated, thereby reducing the number of generations necessary to construct a congenic strain. Computer simulations have indicated that a relatively modest selection effort (60 background markers, 25 cM markers spacing, 16 males per generation) would typically reduce unlinked donor genome contamination to less than 1% by the 4^{th} backcross generation making the reduction of donor genome equivalent to that achieved by 8 backcrosses with the traditional strategy (Markel *et al.* 1997). Additionally, different mice speed congenic lines have been developed

recently using the strategy outlined above (Yui *et al.* 1996), providing results that closely parallel these predicted outcomes.

The BP QTLs reported by us have been confirmed in different genome-wide scans using diverse strains (Dubay *et al.* 1993; Deng *et al.* 1994; Schork *et al.* 1995; Harris *et al.* 1995; Pravenec *et al.* 1995; Samani *et al.* 1996; Garrett *et al.* 1998). Moreover, the QTL present between markers *D2Mit14* and *D2Mgh12* has been confirmed constructing two different congenic strains introgressing the relevant region from the WKY rat or the Milan normotensive rat into Dahl salt-sensitive background. The regions transferred were 38 cM and 78 cM, and the resulting congenic strains had blood pressure of 44 mmHg, and 29 mmHg lower, respectively, than Dahl salt-sensitive rats on a high salt diet (Deng *et al.* 1997). However, no reciprocal congenic strains have been produced for chromosome 2, and the studies carried out by Deng *et al* (1997) only allowed to investigate the effect of normotensive alleles introgressed to a permissive genetic background. Reciprocal congenic strains provide a control for genome background effect and a classic 2 x 2 study design, whereby physiological experiments and phenotypic measurements have appropriate controls. It follows that the purpose of the current study was to undertake a more comprehensive congenic analysis of rat chromosome 2 and the QTLs identified. This has been achieved by the development of dense genetic linkage map for rat chromosome 2 and the first application of a speed congenic approach in the rat.

3.2 METHODS.

3.2.1 RAT STRAINS.

Inbred colonies of $SHRSP_{Gla}$ and WKY_{Gla} rats have been established at the University of Glasgow since December 1991 as described in Section 2.2.

3.2.2 CONGENIC CROSSES.

The development of the speed congenic strains utilised in this study involved the transfer of various segments of rat chromosome 2 from WKY_{Gla} to the genetic background of $SHRSP_{Gla}$, and in the reciprocal direction from $SHRSP_{Gla}$ to the genetic background of WKY_{Gla}. This required the production of a F1 generation by crossing WKY_{Gla} and $SHRSP_{Gla}$. Male F1 hybrids were then mated to the desired recipient strain (WKY_{Gla} or $SHRSP_{Gla}$). Microsatellite markers throughout the desired QTL, and an additional 83 markers spanning the remaining genome (Table 3.1), were genotyped in the offspring from this first backcross. Selection of these markers was based on the need for a thorough coverage of the entire rat genome and location around the QTLs previously identified by our group (Clark *et al.* 1996). Databases utilised to fulfil this selection are detailed in Section 2.2.1. Markers polymorphic on rat chromosome 2 were used to

Marker	Chromsome	Marker	Chromsome	Marker	Chromsome
D1Mit14	1	D6Wox21	6	D13Wox4	13
D1Wox37	1	D6Mgh5	6	D14Mgh3	14
D1Mgh5	1	D7Wox27	7	D14Wox24	14
D1Mgh18	1	D7Mgh5	7	D14Wox5	14
D1Cebr6	1	D7Mgh1	7	D14Wox8	14
D1Wox32	1	D7Mit10	7	D15Mit2	15
D1Mit11	1	D7Mit7	7	D15Mgh3	15
D1Mit1	1	D8Wox16	8	D15Mgh6	15
D3Wox19	3	D8Mgh1	8	D16Wox1	16
D3Mgh6	3	D8Wox22	8	D16Mgh1	16
D3Mit4	3	D8Mgh7	8	D16Wox12	16
D3Wox14	3	D8Wox13	8	D16Wox10	16
D3Mgh8	3	D8Mgh10	8	D16Mit1	16
D3Mgh16	3	D9Mit3	9	D17Mit3	17
D4Mit2	4	D9Wox18	9	D17Wox21	17
D4Cebr3	4	D9Mit1	9	D17Wox13	17
D4Mgh16	4	D9Wox13	9	D17Wox10	17
D4Mgh7	4	D9Mit4	9	D18Wox1	18
D4Mit14	4	D10Wox2	10	D18Wox12	18
D5Mit8	5	D10Mgh11	10	D18Wox16	18
D5Mgh6	5	D10Wox3	10	D19Mit2	19
D5Wox16	5	D11Mgh6	11	D19Wox2	19
D5Mit9	5	D12Wox1	12	D19Wox8	19
D5Wox7	5	D12Mgh3	12	D20Wox3	20
D5Wox4	5	D12Wox2	12	D20Wox5	20
D5Wox15	5	D13Mgh4	13	DXMit4	X
D5Mgh15	5	D13Mgh16	13	DXWox3	X
D5Mgh16	5	D13Mgh1	13		

Table 3.1. Microsatellite markers (n=83) utilised to genotype the genetic background of all chromosome 2 congenic strains.

select the congenic strains, in the first selection only 12 markers were used (Figure 3.1), then 23 additional markers were added to chromosome 2 to improve the QTL dissection. Those rats identified as heterozygous for the marker alleles within the various segments of rat chromosome 2, but mostly homozygous for the recipient alleles throughout the remaining genome were selected as the "best" males for breeding. They were then backcrossed again to the recipient strain to produce a second backcross. This procedure was repeated in all offspring after every backcross until the donor's genetic background was reduced as indicated by the 83 background markers. Once a male and female were identified in which all detectable background heterozygosity had been removed, they were mated to obtain rats homozygous for the donor alleles throughout the chromosome 2 regions of interest. These congenic strains were maintained by brother-sister mating (for further details see Section 2.2.1).

3.2.3 GENOTYPING.

Genomic DNA was isolated as described in Section 2.4.2. Genotyping was performed by PCR amplification of DNA around the polymorphic microsatellite markers from the total genomic DNA using the appropriate PCR primer pairs custom made by either Research Genetics (Huntsville, AL) or Genosys Biotechnologies (Cambridge,UK) as previously described in Section 2.4.3. All samples were stored in 100 μL of TE solution (10 mM tris, pH 8.0; 0.1 mM EDTA, pH 8.0). Primer sequences and PCR conditions

for the microsatellites used to genotype the genetic background are given in Appendix 4.

3.2.4 BLOOD PRESSURE MEASUREMENT.

The Dataquest IV telemetry system (Data Sciences International, St. Paul, MN, U.S.A.) was used for the direct measurement of systolic, diastolic and mean arterial pressure as previously described in Section 2.3.1 (Davidson *et al.* 1995). Surgical implantation of each telemetry transmitter took place under standard sterile conditions at 12 weeks of age. Haemodynamic data were sampled every 5 minutes for 10 seconds. To allow for a full stabilisation of blood pressure post-operatively, experimental observations were collected from day 7 to day 42 after surgery as "baseline haemodynamic measurements". On day 43, rats on telemetry received 1% sodium chloride (NaCl) in their drinking water, and this was continued for two weeks until they were euthanased. Measurements collected during this period were considered "salt-loaded haemodynamic measurements".

3.2.5 STATISTICAL ANALYSIS.

Analysis of radio-telemetry data has been performed as described in Section 2.5.3. Comparisons of congenic strains to their corresponding background parental strains were made by repeated measures analysis of variance of daytime or night-time means for each individual week of the two phases,

reporting the F-statistics and p-values corresponding to the main effects for sex and strain.

3.2.6 IMPROVEMENT OF THE GENETIC LINKAGE MAP OF RAT CHROMOSOME 2.

To improve the chromosome 2 genetic map obtained by Clark *et al* (1996), DNA from the same F2 reciprocal crosses as described in Section 2.2 was used (57 animals in cross 1 with a male-to-female ratio of 28:29 and 83 in cross 2 with a male-to-female ratio 37:46). In a first attempt, 33 new microsatellite markers for rat chromosome 2 were screened and of these 27% were found to be polymorphic between $SHRSP_{Gla}$ and WKY_{Gla}. Genotyping was performed by PCR amplification of DNA around the microsatellites as previously described in Section 2.4.3. The genotypic results obtained were added to those previously collected by Clark *et al* (1996) and mapped relative to each other using the MAPMAKER/EXP 3.0 computer package with an error detection procedure (Lander *et al.* 1987) as described in Section 2.5. Genetic distances were calculated with the Haldane mapping function. On completion of the new map, the QTLs on rat chromosome 2 found to influence blood pressure phenotypes by Clark *et al* (1996) were re-mapped relative to the new genetic map by using the MAPMAKER/QTL 1.1 computer program (Figure 3.2). An additional 75 markers on chromosome 2 were tested and 22% of them were polymorphic between the parental strains. These markers were obtained from the Whitehead Institute Center for Genome Research Rat Mapping Project

(http://www.genome.wi.mit.edu/rat/public/). A genetic linkage map of rat chromosome 2 consisting of 37 markers polymorphic between the WKY_{Gla} and $SHRSP_{Gla}$ strain was constructed using the MAPMAKER/EXP 3.0 computer package (Figure 3.3). The resolution of the genetic linkage map of rat chromosome 2 was improved but the mapping effort focussed around the BP QTLs previously reported by our group (Clark *et al.* 1996), to allow for a better dissection of both genetic regions in the congenic strains.

3.3 RESULTS.

The raw genotypic data collected to construct the new genetic map are given in the Appendices 5 and 6 along with the optimum PCR conditions of each microsatellite marker utilised. The phenotypic and genotypic data used to re-analyse BP QTLs are given in the Appendices 7 and 8.

A total of 108 markers were tested, of there 25 were informative. These markers were genotyped using the DNA of the entire F2 cohort and added to the original map obtained by Clark *et al* (1996) (Figure 3.3). As a result the genetic map of chromosome 2 expanded from 12 markers spanning 136.6 cM (Clark *et al.* 1996), to 21 markers covering 179.5 cM, and then to 37 markers extending over 236 cM (Figure 3.3). An additional 21 markers were polymorphic between the strains but were excluded from the map by the MAPMAKER programme. Some of these markers were not in the strongest

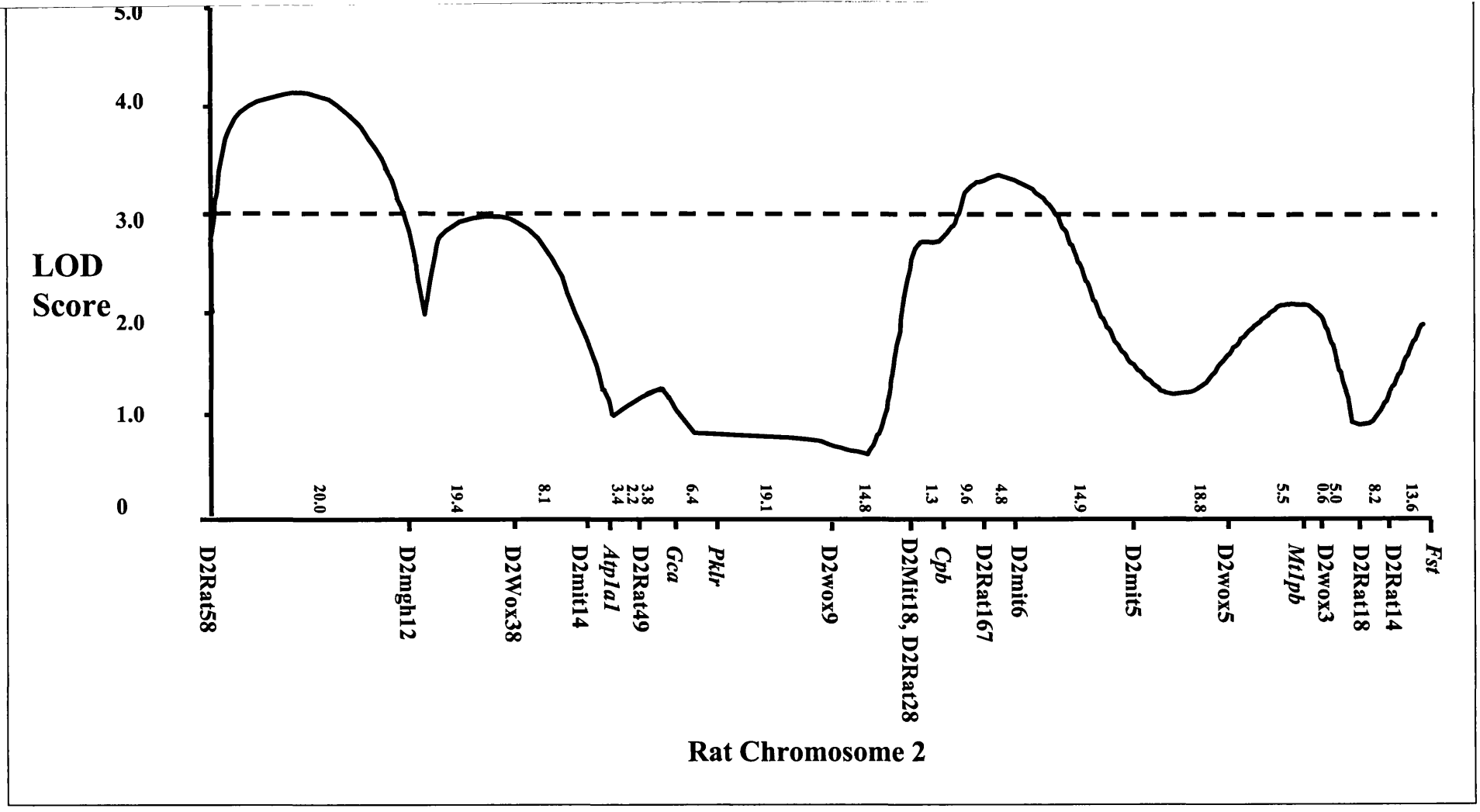

Figure 3.2 Rat chromosome 2 linkage map and salt-loaded diastolic blood pressure QTL localisation for a F2 male only population derived from SHRSP x WKY using 21 polymorphic markers. Map distances and symbols are the same as in Figure 3.1. *Atp1a1*, Na^+,K^+ ATPase α1 isoform. Adapted from Jeffs *et al* (2000).

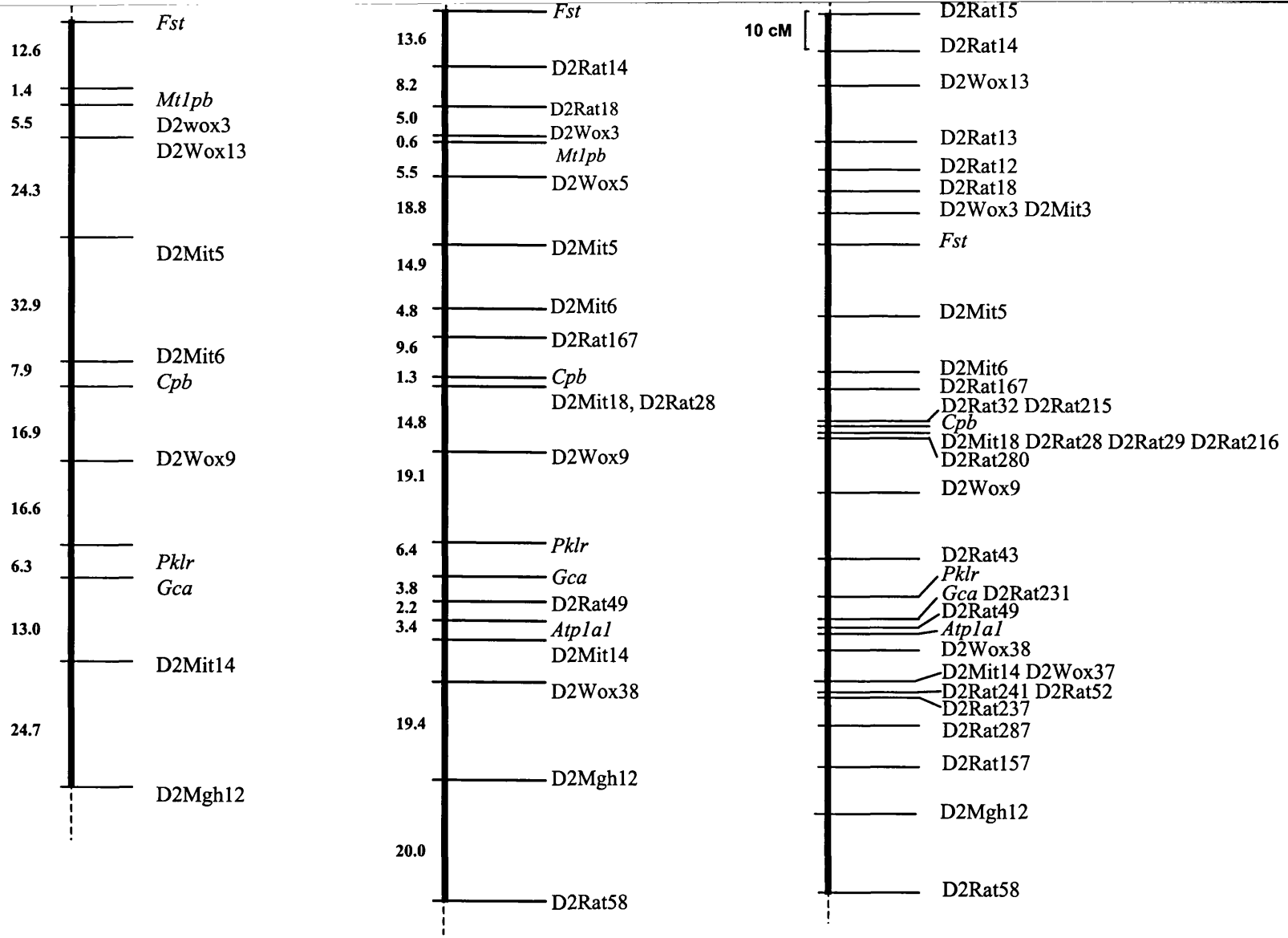

Figure 3.3 Comparison of genetic linkage map of rat chromosome 2 constructed by Clark *et al* (1996) (left), that achieved with the addition of 9 new markers (middle), and that constructed with the addition of 16 new markers (right). Map distances and symbols are the same as in Figure 3.1.

linkage group according to the Whitehead linkage map of rat chromosome 2, perhaps explaining the multiple placements obtained in our map and the inability to map them relative to other markers.

Using the new map and MAPMAKER/QTL 1.1 computer package, we re-analysed the significant QTL on rat chromosome 2 localised close to *D2Mit6* marker and the suggestive QTL between markers *D2Mit14* and *D2Mgh12.* The LOD score for the *D2Mit6* QTL did not differ from those recorded by Clark *et al* (1996). However, the LOD score for the suggestive QTL has now reached significance with a LOD score of 4.1 for salt-loaded diastolic blood pressure (Figure 3.2).

Two congenic strains (SP.WKYGla2a and 2b) were produced by introgressing regions of rat chromosome 2 from WKY_{Gla} into the recipient $SHRSP_{Gla}$ strain (Figure 3.4). Two congenic strains (WKY.SPGla2c, and 2d) were produced by introgressing regions of chromosome 2 from $SHRSP_{Gla}$ rats into the recipient WKY_{Gla} strain (Figure 3.5). In the nomenclature of the congenic strains, the first abbreviation refers to the recipient, the second to the donor. The number 2 refers to chromosome 2, and a, b, c, and d are arbitrarily assigned to each strain. The number of generations of backcrossing required for each strain to achieve complete homozygosity of the background genetic markers in a "best" male, which could then be utilised to fix the line is highlighted in Table 3.2. This varied between BC3 and BC4 for all strains examined. In total 300 progeny were necessary for screening in order to produce the congenic strains, an average

of 69 animals per strain, 19 animals per backcross. Two years and 6 months were required on average to produce the congenic lines (for SP.WKYGla2a, 2 years and 2 months; for SP.WKYGla2b, 2 years and 7 months; WKY.SPGla2c, 2 years and 9 months; WKY.SPGla2d, 2 years and 6 months).

The region of rat chromosome 2 transferred into the congenic strain SP.WKYGla2a incorporates both blood pressure QTL identified (Figure 3.4). Baseline and salt-loaded systolic blood pressure in the SP.WKYGla2a congenic strains are shown in Fig 3.6 and mean and diastolic blood pressures are given in tables 3.3, 3.4, and 3.5. All blood pressures sub-phenotypes either at baseline or salt-loaded are significantly lower in this congenic line as compared to the SHRSP parental strain. There are also highly significant sex differences within the strains (Figure 3.6 and tables 3.3 and 3.4). In comparison, the "control" congenic strain SP.WKYGla2b which contains no QTL, displayed no significant differences in any blood pressure phenotypes from those of the SHRSP (Figure 3.7).

The reciprocal congenic strains with the SHRSP strain as the donor and the WKY strain as the recipient were WKY.SPGla2c (containing both chromosome 2 BP QTLs), and WKY.SPGla2d (containing the QTL with a peak LOD score close to marker *D2Mit6*). Congenic strain WKY.SPGla2c displayed baseline but not salt-loaded systolic blood pressures which were significantly higher as compared to the WKY control (Figure 3.8). The magnitude of the difference for systolic blood pressure during baseline was

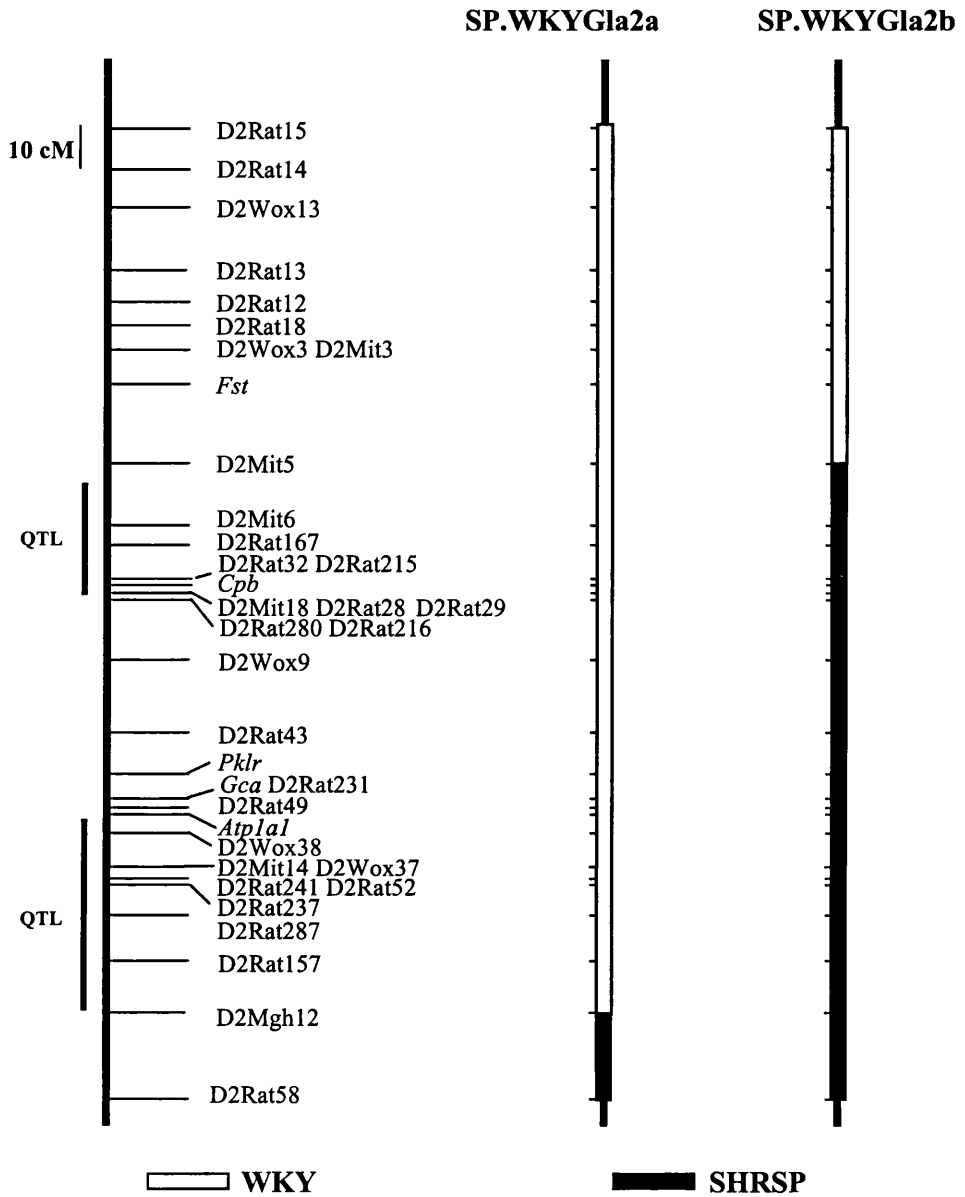

Figure 3.4 Rat chromosome 2 congenic strains SP.WKYGla2a and SP.WKYGla2b where SHRSP is the recipient strain and WKY the donor strain. The white bars represent the region transferred from the WKY into the SHRSP chromosome 2. Genetic linkage map of rat chromosome 2 is shown to the left. Solid bars to the left of the linkage map represent the position of the QTLs previously reported by Clark *et al* (1996). Distances between markers are in centiMorgans (cM).

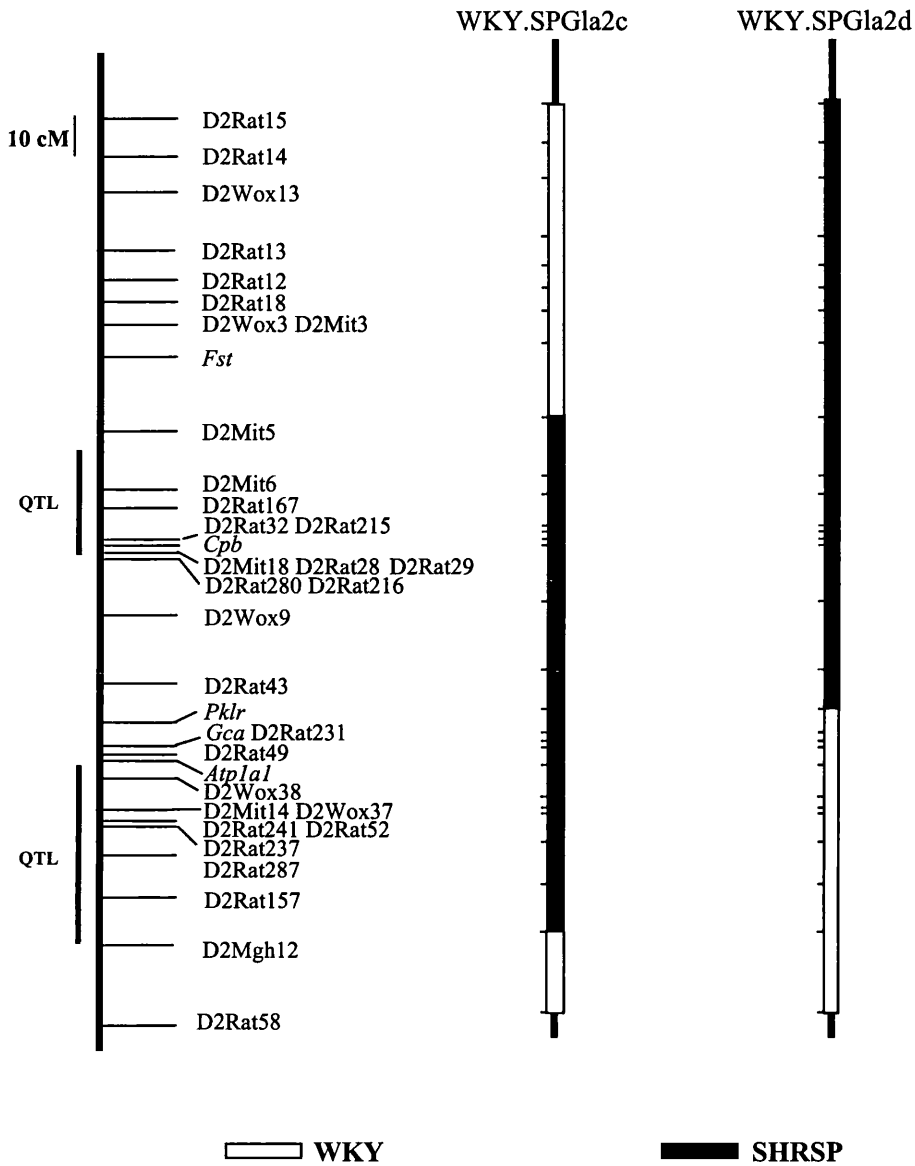

Figure 3.5 Rat chromosome 2 congenic strains WKY.SPGla2c and WKY.SPGla2d where WKY is the recipient strain and SHRSP the donor strain. The black bars represent the region transferred from the SHRSP into the WKY chromosome 2. Genetic linkage map of rat chromosome 2 is shown to the left. Solid bars to the left of the linkage map represent the position of the QTLs previously reported by Clark *et al* (1996). Distances between markers are in centiMorgans (cM).

Strain	Generation	Progeny Screened	% Background Marker Heterozygosity of "Best" Male
SP.WKYgla2a	BC1	56	28.3
	BC2	16	13.2
	BC3	23	1.9
	BC4	34	"zero"
SP.WKYgla2b	BC1	56	43.4
	BC2	15	24.5
	BC3	28	9.4
	BC4	27	"zero"
WKY.SPgla2c	BC1	28	17.0
	BC2	17	7.5
	BC3	14	"zero"
WKY.SPgla2d	BC1	28	17.0
	BC2	18	1.9
	BC3	5	1.9
	BC4	28	"zero"

Table 3.2 Rat chromosome 2 congenic strains. The actual number of progeny screened represents those genotyped for heterozygosity on chromosome 2 and/or residual heterozygosity. "Zero" heterozygosity denotes that all of 83 background markers tested were free of heterozygosity but it is not informative regarding other chromosomal regions which were not tested.

almost the same as for congenic strain SP.WKYGla2a. The congenic strain WKY.SPGla2d showed no blood pressure differences at baseline or following salt loading for any of the blood pressure sub-phenotypes studied (Figure 3.9). Weekly averages of systolic and diastolic blood pressure obtained for each animal are given in Appendices 9 and 10.

Overall the body weight values did not differ between the congenic lines and the parental controls (Table 3.6). An exception here was the strain WKY.SPGla2c where males had lower body weight as compared to the parental strain. Heart weight-to-body weight ratios (HW/BW) and left ventricle plus septum-to-body weight ratios (LV+S/BW) showed a trend to follow the blood pressure differences. Strain SP.WKYGla2b showed an increased HW/BW and LV+S/BW ratio compared to the parental strain. This finding was unexpected and we are currently verifying the heart weight data in the same strain using echocardiography. In the reciprocal lines, strain WKY.SPGla2c and strain WKY.SPGla2d did not differ from the parental strain. Raw data for body weight, heart weight, LV+S, HW/BW, and LV+S/BW ratios are given in Appendix 11.

3.4 DISCUSSION.

The presentation in this study of four congenic strains derived for rat chromosome 2 clearly demonstrates applicability of the speed congenic

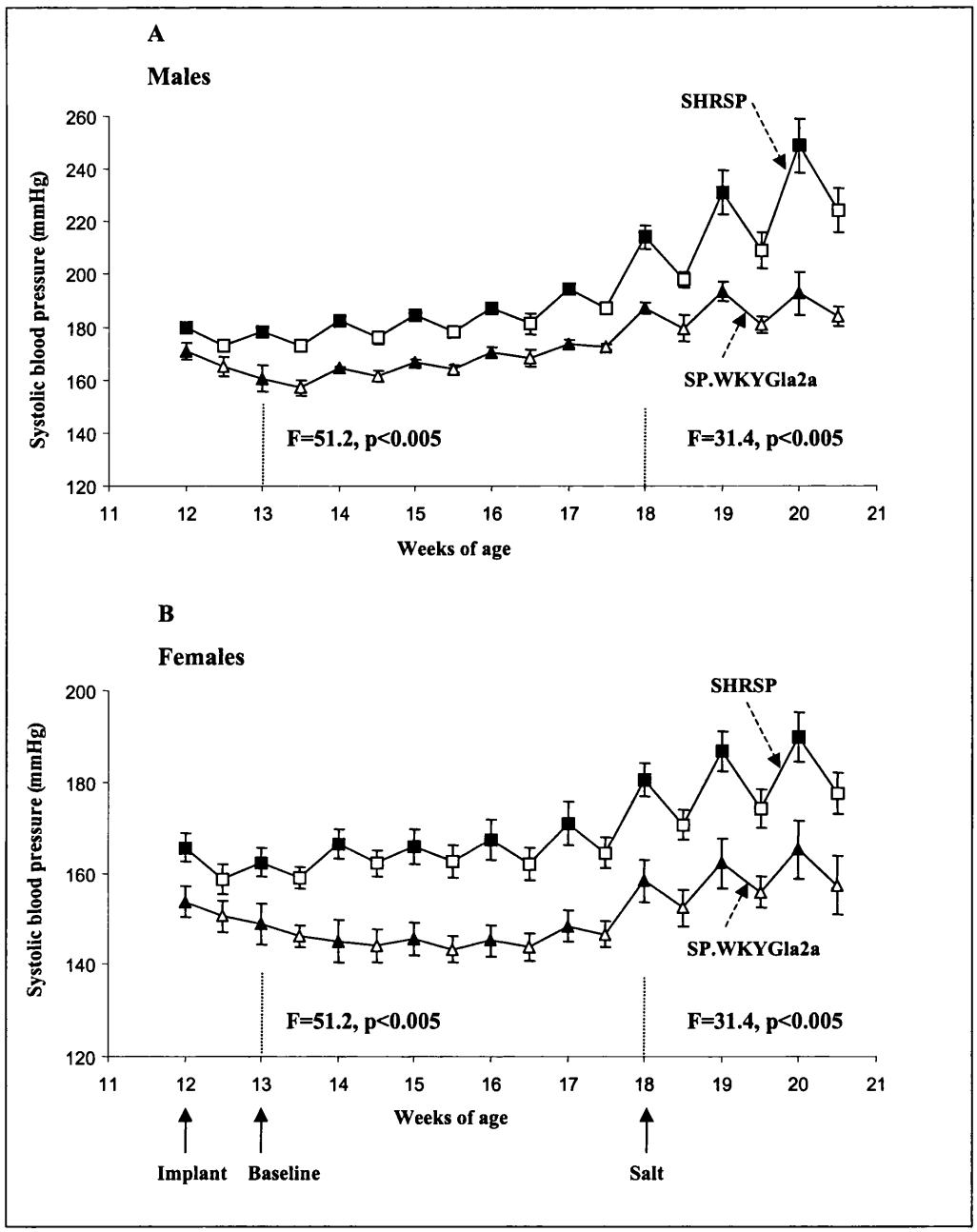

Figure 3.6 Daytime and night-time average systolic blood pressure (SBP) recorded by radio-telemetry over a 9-week period in $SHRSP_{(Gla)}$ and SP.WKYGla2a congenic strains. A, males, and B, females (n=3 for each group). Each data point represents weekly average daytime (open symbols) and night-time (closed symbols) SBP. Data points were calculated using 12-hour average daytime (7:00 a.m.-7:00 p.m.) and 12-hour average night-time (7:00 p.m.-7:00 a.m.). Arrows from left to right indicate the timing of the implantation of telemetry probe, the beginning of baseline blood pressure measurements, and the beginning of salt-loaded measurements. Values are mean ± SEM.

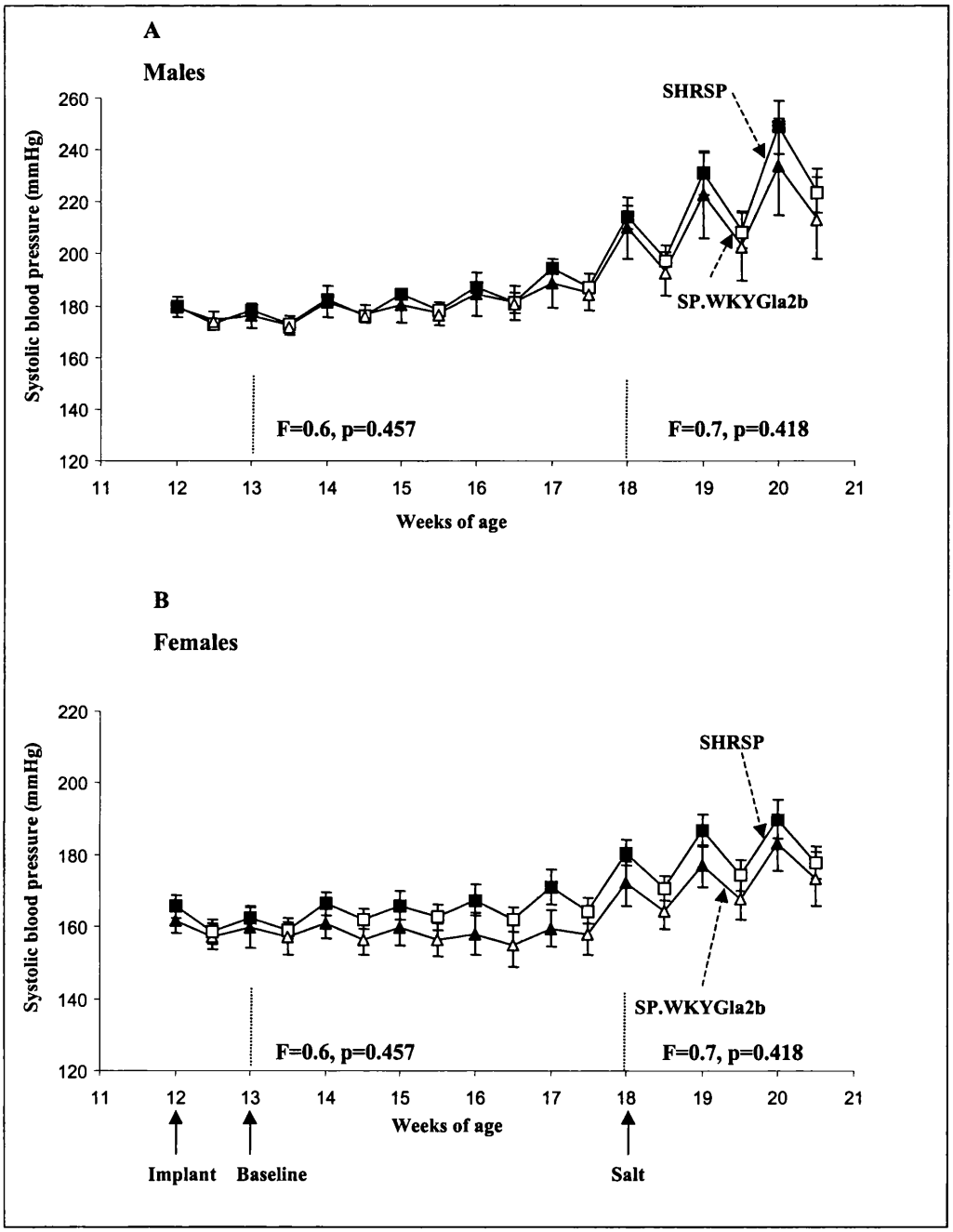

Figure 3.7 Daytime and night-time average SBP recorded by radio-telemetry over a 9-week period in $SHRSP_{(Gla)}$ and SP.WKYGla2b congenic strains. A, males, and B, females (n=3 for each group). Each data point represents weekly average daytime (open symbols) and night-time (closed symbols) SBP. Data points were calculated using 12-hour average daytime (7:00 a.m.-7:00 p.m.) and 12-hour average night-time (7:00 p.m.-7:00 a.m.). Arrows indicate the same time points as in Figure 3.6. Values are mean ± SEM.

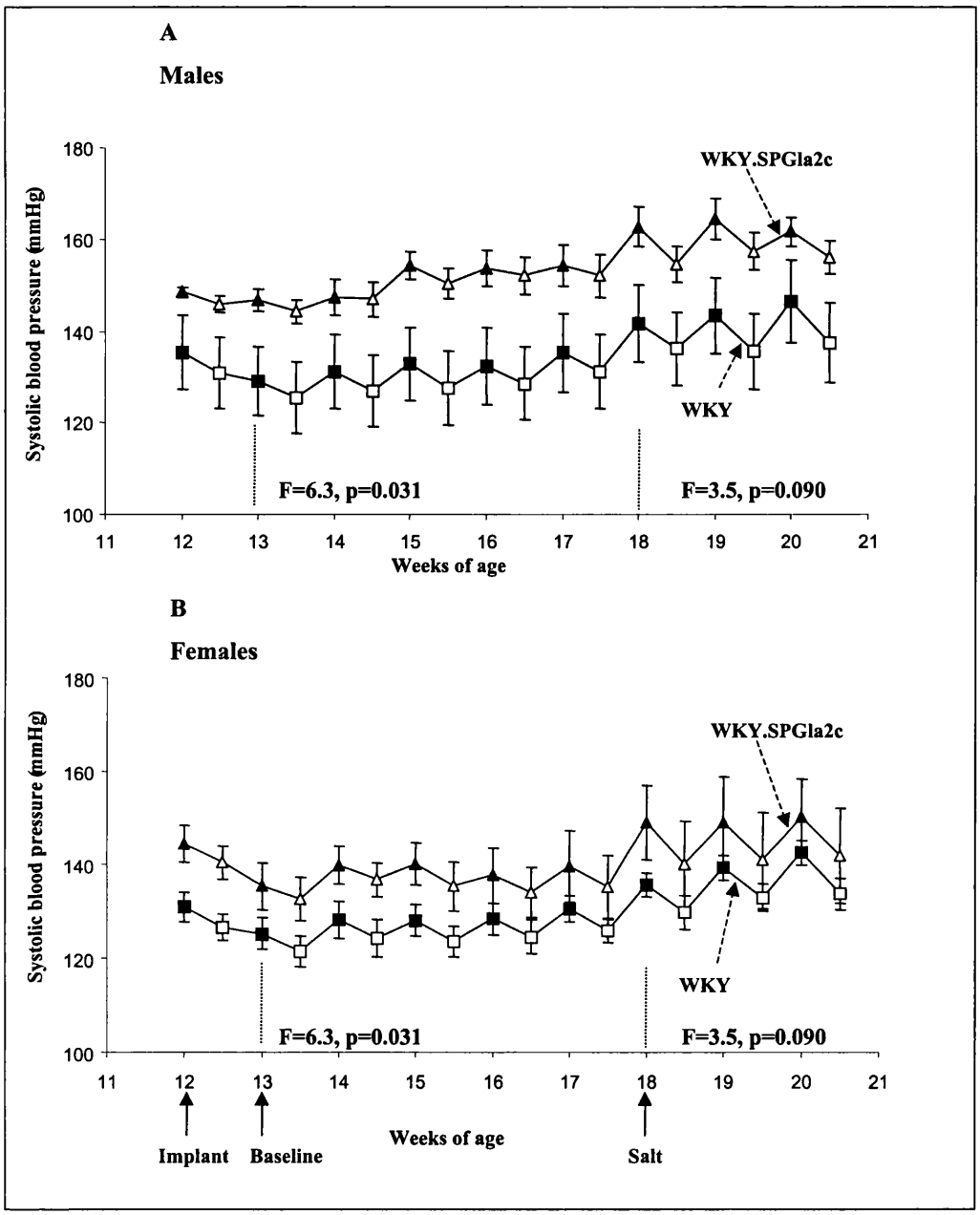

Figure 3.8 Daytime and night-time average SBP recorded by radio-telemetry over a 9-week period in $WKY_{(Gla)}$ and WKY.SPGla2c congenic strains. A, males, and B, females (n=3 for each group). Each data point represents weekly average daytime (open symbols) and night-time (closed symbols) SBP. Data points were calculated using 12-hour average daytime (7:00 a.m.-7:00 p.m.) and 12-hour average night-time (7:00 p.m.-7:00 a.m.). Arrows indicate the same time points as in Figure 2. Values are mean ± SEM.

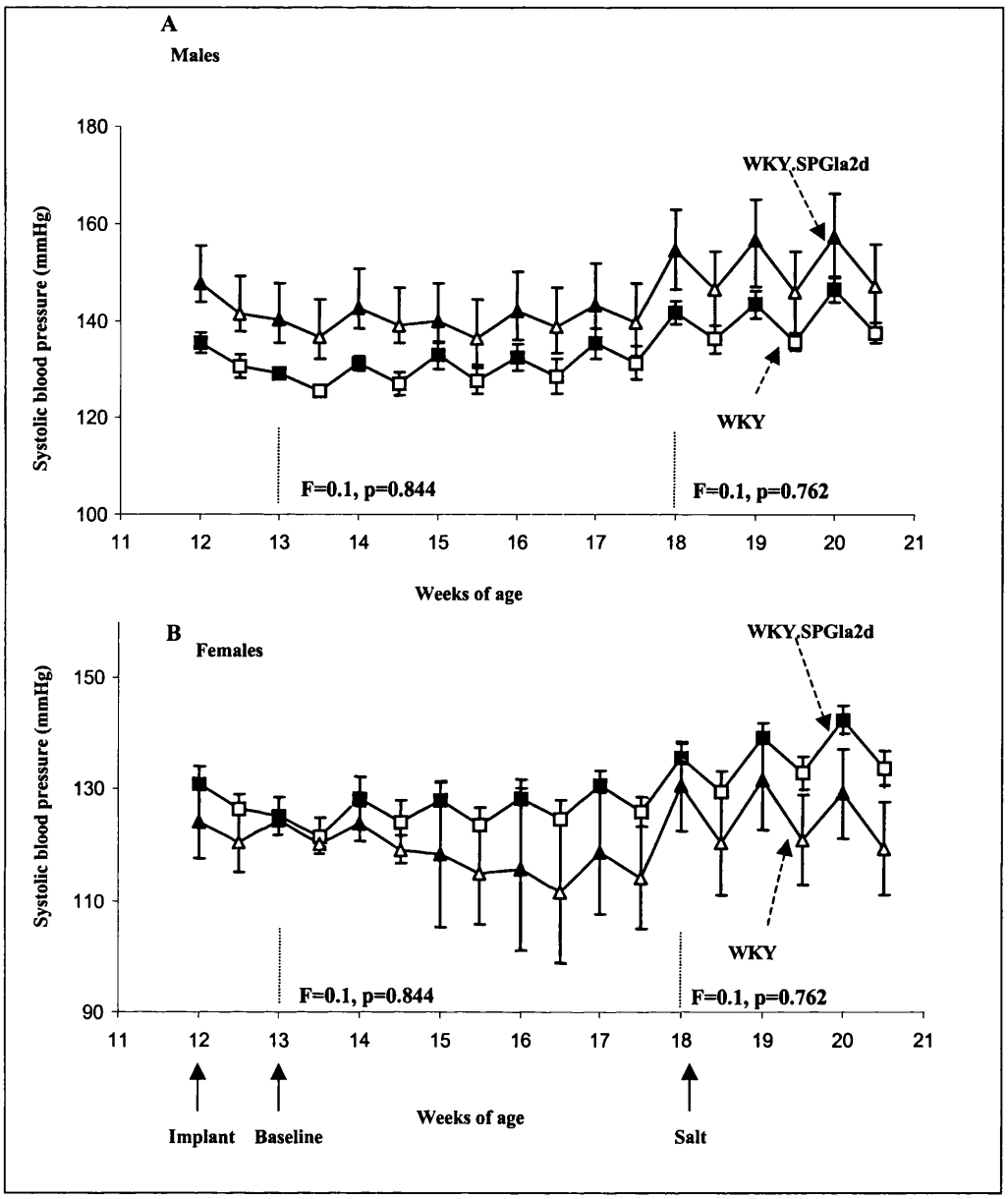

Figure 3.9 Daytime and night-time average SBP recorded by radio-telemetry over a 9-week period in $WKY_{(Gla)}$ and WKY.SPGla2d congenic strains. A, males, and B, females (n=3 for each group). Each data point represents weekly average daytime (open symbols) and night-time (closed symbols) SBP. Data points were calculated using 12-hour average daytime (7:00 a.m.-7:00 p.m.) and 12-hour average night-time (7:00 p.m.-7:00 a.m.). Arrows indicate the same time points as in Figure 2. Values are mean ± SEM.

		Baseline measurements							
		SHRSP				WKY			
		Males		Females		Males		Females	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
SBP	Day	178.4	2.1	161.8	3.0	127.8	7.9	123.8	3.4
	Night	185.1	1.1	166.5	3.8	132.1	7.9	128.0	3.3
DBP	Day	124.0	2.5	113.6	2.2	91.2	6.0	87.5	3.0
	Night	130.0	1.2	120.3	2.7	95.5	6.4	92.0	3.4
MAP	Day	142.1	2.3	129.6	2.5	103.4	6.7	99.6	3.1
	Night	148.4	1.0	135.7	3.1	107.7	6.9	104.0	3.4
		Salt phase measurements							
		SHRSP				WKY			
		Males		Females		Males		Females	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
SBP	Day	207.9	5.5	173.4	3.9	136.6	8.2	131.7	3.2
	Night	229.8	7.3	185.5	4.5	144.2	8.8	138.9	2.6
DBP	Day	152.9	5.2	121.7	3.1	96.8	6.4	92.0	2.9
	Night	167.2	5.2	134.6	3.5	104.4	6.9	98.8	2.8
MAP	Day	171.2	5.3	139.0	3.4	110.0	7.0	105.2	2.9
	Night	188.1	5.9	151.6	3.9	117.7	7.5	112.2	2.7

Table 3.3. Blood pressures for parental strains at baseline and in the salt phase. Data are means and standard errors of daytime (7am – 7pm) or night-time (7pm – 7am) periods for each blood pressure over 5 weeks for baseline and 3 weeks for the salt phase in mm Hg. SHRSP parental strain group consisted of 3 males and 3 females, and WKY parental strain group of 4 males and 3 females.

		Males		Females		Sex Comparison		Comparison to Control Strain	
Pressure	**Time**	**Mean**	**SE**	**Mean**	**SE**	**F**	**P**	**F**	**P**
SP.WKYGla2a									
SBP	Day	164.3	1.9	144.7	2.9	57.9	<0.0005	43.5	<0.0005
	Night	166.9	1.0	146.4	3.7	53.1	<0.0005	51.2	<0.0005
DBP	Day	117.4	2.8	100.2	2.4	26.9	0.001	13.7	0.005
	Night	118.9	2.6	103.5	3.0	22.2	0.001	26.4	0.001
MAP	Day	132.9	1.9	115.0	2.6	40.6	<0.0005	24.6	0.001
	Night	134.8	1.3	117.8	3.3	35.6	<0.0005	39.1	<0.0005
SP.WKYGla2b									
SBP	Day	178.0	5.0	156.3	4.9	25.4	0.001	0.6	0.457
	Night	182.0	6.9	159.4	5.0	21.0	0.001	1.3	0.290
DBP	Day	122.8	4.5	110.5	3.1	13.8	0.005	0.5	0.501
	Night	127.5	5.7	115.5	4.2	9.0	0.015	1.0	0.336
MAP	Day	141.2	4.7	125.8	3.3	19.3	0.002	0.6	0.467
	Night	145.7	6.1	130.2	4.1	13.6	0.005	1.2	0.308
WKY.SPGla2c									
SBP	Day	148.9	3.5	134.4	4.4	1.8	0.211	7.6	0.020
	Night	151.3	3.5	138.1	4.8	1.6	0.237	6.3	0.031
DBP	Day	106.3	3.6	94.1	3.8	2.2	0.173	5.4	0.042
	Night	108.9	3.7	98.3	4.2	1.6	0.239	3.9	0.076
MAP	Day	120.5	3.5	107.5	4.0	2.0	0.185	6.3	0.031
	Night	123.0	3.5	111.6	4.4	1.6	0.236	4.8	0.053
WKY.SPGla2d									
SBP	Day	137.9	2.6	116.3	6.4	3.6	0.089	0.1	0.793
	Night	141.5	2.0	120.4	8.1	3.2	0.104	0.1	0.844
DBP	Day	95.1	1.6	83.1	2.6	3.1	0.107	0.1	0.986
	Night	99.5	1.0	87.4	4.5	2.5	0.144	0.1	0.978
MAP	Day	109.3	1.9	94.1	3.8	3.4	0.095	0.1	0.917
	Night	113.5	1.3	98.4	5.7	2.8	0.123	0.1	0.948

Table 3.4. Blood pressures for congenic strains at baseline. Data are means and standard errors of daytime (7am – 7pm) or night-time (7pm – 7am) periods for each blood pressure over 5 weeks in mm Hg. Each congenic group consisted of 3 male and 3 female animals. F-statistics and p-values are for relevant main effects from repeated measures analysis of variance, with former being compared to F (1, total number of animals–3) in each analysis. First two congenic strains were compared to the SHRSP parental strain (3 males and 3 females); last two to the WKY parental strain (4 males and 3 females).

		Males		Females		Sex Comparison		Comparison to Control Strain	
Pressure	**Time**	**Mean**	**SE**	**Mean**	**SE**	**F**	**P**	**F**	**P**
SP.WKYGla2a									
SBP	Day	180.7	2.5	154.9	3.7	53.9	<0.0005	31.4	<0.0005
	Night	191.2	3.4	161.6	5.4	42.0	<0.0005	30.7	<0.0005
DBP	Day	131.2	5.5	108.5	3.6	36.3	<0.0005	15.4	0.003
	Night	139.0	8.1	115.4	4.4	26.3	0.001	19.1	0.002
MAP	Day	147.8	4.3	123.9	3.6	44.1	<0.0005	21.0	0.001
	Night	156.4	6.5	130.8	4.8	33.1	<0.0005	24.1	0.001
SP.WKYGla2b									
SBP	Day	202.1	12.4	167.6	5.5	23.7	0.001	0.7	0.418
	Night	221.3	15.4	176.8	6.3	25.5	0.001	1.0	0.347
DBP	Day	144.2	12.1	117.4	3.9	19.0	0.002	1.0	0.342
	Night	156.8	11.8	127.9	4.0	21.9	0.001	1.8	0.216
MAP	Day	163.5	12.2	134.1	4.2	20.9	0.001	0.9	0.364
	Night	178.3	13.0	144.2	4.5	23.7	0.001	1.4	0.260
WKY.SPGla2c									
SBP	Day	155.8	3.7	140.7	8.0	2.6	0.136	3.5	0.090
	Night	163.0	3.8	149.3	7.0	2.6	0.140	2.6	0.136
DBP	Day	112.0	5.6	97.8	6.8	3.0	0.114	2.8	0.127
	Night	118.2	5.6	105.4	6.3	3.1	0.111	1.9	0.203
MAP	Day	126.6	4.8	112.1	7.2	2.9	0.117	3.1	0.108
	Night	133.1	4.8	120.0	6.6	3.0	0.117	2.2	0.170
WKY.SPGla2d									
SBP	Day	146.1	2.2	120.6	8.5	4.1	0.071	0.1	0.982
	Night	156.0	2.5	130.6	8.2	3.9	0.076	0.1	0.762
DBP	Day	99.5	1.2	83.1	6.6	3.8	0.080	0.2	0.625
	Night	108.4	1.4	91.9	7.4	3.6	0.089	0.1	0.846
MAP	Day	115.0	1.5	95.6	7.1	4.0	0.074	0.1	0.760
	Night	124.3	1.6	104.8	7.5	3.8	0.080	0.1	0.999

Table 3.5 Blood pressures for congenic strains on salt diet. Data are means and standard errors of daytime (7am – 7pm) or night-time (7pm – 7am) periods for each blood pressure over 3 weeks in mm Hg. Each congenic group consisted of 3 male and 3 female animals. F-statistics and p-values are for relevant main effects from repeated measures analysis of variance, with former being compared to F (1, total number of animals–3) in each analysis. First two congenic strains were compared to the SHRSP parental strain (3 males and 3 females); last two to the WKY parental strain (4 males and 3 females)

	Parental WKY				Parental SHRSP			
	Males		Females		Males		Females	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
BW (g)	408	47.19	233.75	9.18	275.33	40.02	212	12.17
HW (mg)	1.34	0.17	0.83	0.05	1.2	0.1	0.94	0.05
LV+S (mg)	0.92	0.12	0.59	0.04	0.92	0.11	0.74	0.03
HW/BW (mg/g)	3.35	0.07	3.69	0.17	4.46	0.25	4.69	0.22
LV+S/BW (mg/g)	2.3	0.05	2.61	0.16	3.43	0.04	3.69	0.13

					Sex comparison		Comparison to control strain	
					p	95% C.I.	p	95% C.I.
SP.WKYGla2a								
BW (g)	336.67	18.58	204	11.14	<0.001	93,172	0.14	-48,171
HW (mg)	1.34	0.09	0.87	0.09				
LV+S (mg)	1.04	0.12	0.66	0.09				
HW/BW (mg/g)	4.1	0.12	4.46	0.32	0.21	-1.20,0.48	0.15	-0.33,1.01
LV+S/BW (mg/g)	3.22	0.15	3.39	0.48	0.62	-1.42,1.08	0.14	-0.17,0.57
SP.WKYGla2b								
BW (g)	258	57.86	189.33	14.19	0.18	-79,216	0.70	-147,112
HW (mg)	1.29	0.24	0.8	0.05				
LV+S (mg)	1.06	0.24	0.61	0.04				
HW/BW (mg/g)	5.21	0.24	4.44	0.1	0.035	0.13,1.40	0.03	-1.38,-0.12
LV+S/BW (mg/g)	4.25	0.13	3.38	0.15	0.005	0.50,1.23	0.009	-1.16,-0.47
WKY.SPGla2c								
BW (g)	319.33	13.32	211.33	3.65	0.001	-215,203	0.037	10,167
HW (mg)	1.06	0.04	0.8	0.04				
LV+S (mg)	0.77	0.03	0.59	0.05				
HW/BW (mg/g)	3.43	0.19	3.95	0.14	0.034	-0.90,-0.07	0.55	-0.58,0.41
LV+S/BW (mg/g)	2.48	0.14	2.91	0.22	0.045	-0.76,-0.01	0.16	-0.54,0.18
WKY.SPGla2d								
BW (g)	347.33	23	244	8.72	0.001	74,143.4	0.1	-128,17
HW (mg)	1.21	0.06	0.9	0.02				
LV+S (mg)	0.93	0.09	0.66	0.06				
HW/BW (mg/g)	3.59	0.39	3.85	0.12	0.23	-0.98,0.35	0.42	-0.45,0.82
LV+S/BW (mg/g)	2.79	0.44	2.82	0.24	0.7	-0.83,0.62	0.16	-0.29,1.12

Table 3.6. Body weight, heart weight-body weight ratio, and left ventricular septum-body weight ratio for congenic and parental strains. Data are means±SD, p values are obatined using an unpaired two-tailed *t* test. BW, body weight; HW, heart weight; LV+S, left ventricle plus septum weight; HW/BW, heart weight-body weight ratio; LV+S/BW, left ventricle plus septum weight-body weight ratio. 3 animals in each group.

strategy in the rat. The "best" male selection at each generation resulted in on average 19 rats per backcross. These were necessary to reduce all unlinked donor genome contamination to below 1% within 3 to 4 backcrosses. Transfer of the entire region of rat chromosome 2 containing both QTLs from WKY into an SHRSP genetic background significantly lowered all blood pressure phenotypes. Congenic lines designed as "controls" for potential "passenger" loci showed no deviation from the blood pressure values of the SHRSP or WKY, indicating that if such heterozygosity exists, its effect on blood pressure is negligible.

Some level of contaminating donor genome in the genetic background is unavoidable, even when utilising the traditional protocol for construction of a congenic strain. A potential problem with this is that during the process of fixing the chromosome region of interest for the donor strain into the homozygous state, an unlinked "passenger" QTL may become trapped in the residual donor strain. The presence or absence of the unknown QTL could therefore confound the interpretation that the blood pressure effect in the congenic strain. The present speed congenic strategy has provided the opportunity to monitor the presence of any passenger loci. Two control strains SP.WKYGla2b and WKY.SPGla2d were analysed and we found that the effect of any passenger loci is negligible. We were also able to observe several chromosomal "hot spots" at which donor alleles appeared to be preferentially conserved, including several loci implicated in blood pressure QTLs present in other rat strains. The utilisation of a speed congenic strategy enabled these loci to be monitored and "best" males without

heterozygosity in these regions to be selected. It follows that it may prove necessary in all congenic strategies, regardless of the breeding protocol utilised, to include at least some rudimentary assessment of the genetic background.

This study is the first to use congenic "control" strains to positively establish that the blood pressure change detected in the congenic strain is due to the target genomic region rather than the effect of any residual loci. Additionally, all the congenic strains were derived independently making it very unlikely that they will all by chance contain the same genomic regions of residual donor-strain genetic backgrounds that could potentially affect blood pressure.

Transfer of the entire region of rat chromosome 2, containing both QTLs from WKY_{Gla} into a $SHRSP_{Gla}$ genetic background significantly lowered both baseline and salt-loaded systolic blood pressure by approximately 20 and 40 mmHg, respectively as determined by continuous and direct recording by radio-telemetry. A previous study by Deng *et al* (Deng *et al.* 1997) also confirmed the existence of a blood pressure QTL on rat chromosome 2 in the region corresponding to that of the current study. They constructed two congenic strains introgressing the relevant region from the WKY rat or the Milan normotensive (MNS) rat into the Dahl salt-sensitive (S) background. These strains had blood pressure 44 mmHg and 29 mmHg lower, respectively, than Dahl S rats on a 2% NaCl diet (Deng *et al.* 1997), although the region introgressed from MNS was larger than the region from

WKY. Deng *et al* (Deng *et al.* 1997) hypothesised that this difference could have arisen: (i) if the QTL allele of the WKY rat was different from that of the MNS rat; (ii) if the WKY and MNS rats have the same QTL allele in the *D2Mgh12* region but the larger substitution in the MNS congenic strain also contained the *D2Mit6* locus which modified blood pressure effects of the former QTL; (iii) and/or if there is one or more additional blood pressure QTL located in this region of chromosome 2. These suggestions are partially supported by our data in the reciprocal congenic strains WKY.SPGla2c, and 2d. Small but significant blood pressure increase has been observed in strain WKY.SPGla2c, which contains both QTL on rat chromosome 2, as compared to the WKY control strain. However, the congenic strain WKY.SPGla2d, which contains only the QTL localised around D2Mit6, showed no blood pressure differences at baseline or after salt loading. These results confirm previous suggestions that the genetic background chosen for a given congenic strain might have profound effect on the phenotype (Rapp, 2000).

Additional and perhaps an important difference between the work of Deng *et al* (1997) and our data is the phenotyping methodology. Deng *et al* used traditional tail-cuff plethysmography whilst all our data have been generated by radio-telemetry. Radio-telemetry is capable of measuring all the components of the blood pressure phenotype, detecting small changes in blood pressure, and observing the diurnal variations of blood pressure. This can be seen in the difference between strains WKY.SPGla2c and SP.WKYGla2a which are reciprocal to each other. The former showed a

significant increase in blood pressure during the baseline period, but not during salt-loading. In contrast, the reciprocal strain (SP.WKYGla2a) displayed a significant reduction in blood pressure during both periods. Again subtle changes were detected with the use of radio-telemetry, with the genetic background of the reciprocal congenic strains having a profound effect on how the phenotype is expressed.

The chromosome 2 region introgressed in the present study and that of Deng *et al* (1997) harbours several genes that could be considered as candidates for the blood pressure QTL. These presently include the angiotensin type 1B receptor (*Agtr1b*) gene, both soluble and membrane-bound guanylate cyclases (*Gca*), the α1 isoform of the Na^+K^+ATPase gene (*Atp1a1*), and the calmodulin-dependent protein kinase II Δ (*Camk2d*) gene. Speculations as to the identity of the causal gene or genes are premature as the substituted region is still large and thus requires further investigation with the use of congenic substrains.

A recent study by Rapp *et al* (Rapp *et al.* 1998b) described the construction of a double congenic strain containing the low-blood pressure QTL alleles from chromosomes 2 (WKY) and 10 (MNS) on the Dahl S genetic background. The analysis of the blood pressure of this double congenic strain and comparisons with the relevant single congenic strains and the Dahl S strain provided evidence for a strong epistatic interaction on blood pressure of the QTLs on chromosome 2 and 10. It should be noted that this was the first epistatic interaction described on a quantitative trait in

mammalian genetics. The speed congenic strategy can be applied to address unknown aspects of blood pressure regulation by constructing congenic strains, sub-strains, and double congenic strains over fewer generations.

In conclusion, this study demonstrated, that the speed congenic strategy is applicable to the genetic dissection of experimental hypertension in the rat. Moreover, we confirmed the existence of a blood pressure QTL on rat chromosome 2, and we have begun its dissection. Our results suggest that the region between markers *D2Rat43* and *D2Mgh12* is the most important blood pressure QTL on rat chromosome 2. This region will undergo further analysis in order to proceed to cloning by position of the causative genes.

CHAPTER 4

SEQUENCE ANALYSIS OF GENES ENCODING SODIUM-POTASSIUM ATPASE α1 ISOFORM IN THE SHRSP AND WKY RAT STRAINS.

4.1 INTRODUCTION.

The sodium-potassium (Na^+-K^+) pump plays a key role in the regulation of intracellular concentration of monovalent cations (Geering, 1997). It provides hydrolysis of ATP coupled to the inward movement of K^+ and outward movement of Na^+. Due to its electrogenecity ($3Na^+/2K^+$), the pump also contributes to the regulation of membrane potential. The minimal functional unit of the Na^+-K^+ pump is composed of α- and β-subunits assembled in a 1:1 ratio (Blanco and Mercer, 1998).

Three isoforms of α- and β-subunits have been cloned in the rat (Peng *et al.* 1997). The α-subunit has 10 transmembrane domains with both NH_2 and COOH terminals in an endoplasmic position. All α subunits contain ATP, Na^+, K^+, and oubain-binding sites, exhibit Mg^{2+}-dependent ATP hydrolysis, and are able to provide movement of Na^+ and K^+ against their electrochemical gradients. The β-subunit consist of a short cytoplasmatic NH_2 terminus, one transmembrane domain, and a highly glycosylated extended ectodomain. This ectodomain is involved in regulation of the affinity of the enzyme for extracellular K^+ and for re-assembly of the Na^+-K^+ pump within the plasma membrane. The α1 isoforms are ubiquitous and a house keeping function is assumed in all cells. The α2 isoforms are expressed predominantly in the brain, skeletal muscle, and heart, whereas α3 isoforms are primarily expressed in the brain. Similar to the α1 isoform, the β1 isoform is also expressed ubiquitously. The β2 isoform is mainly expressed in muscle and brain, whereas the β3 isoform is found in a variety

of rat tissues (Adams *et al.* 1982; Maixent *et al.* 1987; Crambert *et al.* 2000).

The α1 Na^+-K^+-ATPase pump is the sole active Na^+ transporter in the renal basolateral epithelia throughout the nephron. Thus it can be considered as a logical candidate gene for the high blood pressure seen during salt-loaded diets in hypertensive rat strains (Herrera *et al.* 1998).

The gene encoding for the α1 isoform of the Na^+-K^+-ATPase is located on rat chromosome 2 (Watanabe *et al.* 1999) and different studies have shown QTLs for blood pressure around the locus defined by the *Atp1a1* gene (encoding the α1-Na^+-K^+-ATPase isoform) on chromosome 2 in distinct rat crosses (Deng *et al.* 1994; Clark *et al.* 1996; Herrera *et al.* 1998). Recently, congenic strains produced by introgressing the region containing the *Atp1a1* locus from the MNS or the WKY into the genome of the Dahl salt-sensitive rat showed a significant reduction in blood pressure compared to Dahl salt-sensitive parental strain (Deng *et al.* 1997). Additionally, Rapp *et al* (1998) showed epistatic interaction on blood pressure between the region on chromosome 2 containing the *Atp1a1* locus and a region on rat chromosome 10. Moreover, transgenic rats carrying the Dahl R rat wild-type *Atp1a1* allele inserted into the S rat genome showed a significant reduction in blood pressure compared to Dahl S rats (Herrera *et al.* 1998).

The above evidence suggests that *Atp1a1* is a strong candidate gene for blood pressure regulation. If *Atp1a1* is the gene causing the difference in

blood pressure between the SHRSP and WKY at this particular QTL on rat chromosome 2, there would have to be a nucleotide difference between the two strains. This mutation would then lead to a different function of the gene between the two strains. It was the aim of this study to ascertain whether the gene encoding the Na^+-K^+-ATPase $\alpha 1$ isoform was a likely candidate for the QTL on rat chromosome 2 by performing sequence analysis of its coding regions.

4.2 METHODS.

4.2.1 RNA EXTRACTION.

RNA was extracted from kidneys of 2 males WKY and 2 males SHRSP rats following the RNAzol^{TM}B protocol (Biogenesis Ltd.). For this, 90 mg of tissue was placed on a Biopulverizer tube (Hybaid) and 1,000 μL of RNAzol^{TM} solution added. Tubes were placed on the RiboLyser^{TM} Cell Disrupter (Hybaid) which utilised simultaneous shaking and twisting motion at very high speeds to provide efficient disruption of cell membranes. Following tissue homogenisation 200 μL of chloroform were added and samples centrifuged at 12,000 g (4°C) for 15 minutes. The aqueous phase was placed in a 1.5 mL tube and an equal volume of isopropanol added to precipitate the RNA. RNA precipitated as a white-yellow pellet at the bottom of the tube and it was washed with 800 μL of 75% v/v ethanol. The pellet was dried briefly and resuspended in 100 μL of diethylpyrocarbonate (DEPC)-treated water. A 5 μL aliquot was separated for quantification and

the remainder was stored in liquid nitrogen. The RNA was quantified using an Ultrospec 2,000 UV/Visible spectrophotometer (Pharmaco-Biotech) where 5 μL of extracted RNA was added to 995 μL of sterile water (1 in 200 dilution) in a quartz cuvette. The optical density of the sample was determined in triplicate at 260 nm and 280 nm against a blank and averages calculated. The ratio of absorbance at 260:280 nm was a measure of the purity of the sample. A ratio of 1.9 or higher was considered ideal. The amount of RNA was calculated from the mean absorbance value at 260 nm as follows: [RNA]ng/μL=200 x OD_{260}.

4.2.2 REVERSE TRANSCRIPTASE-PCR (RT-PCR).

Synthesis of cDNA from RNA was done using the 1^{ST}-strand ™ cDNA synthesis kit (Clontech). 1 μg of RNA was added to a 0.5 mL microcentrifuge tube and made up to a volume of 12.5 μL with DEPC-treated water. 1 μL of oligo $(dT)_{18}$ primer provided with the kit was added to the tube and the solution with the RNA was heated at 70°C for 12 min. Additionally, 4 μL of 5X reaction buffer, 1 μL of dNTP mix (10 mM), 0.5 μL recombinant RNase inhibitor, and 1.0 μL of Moloney-murine leukaemia virus reverse transcriptase (MMLV) were added to the tube and the content mixed by pipeting up and down. The reaction was then incubated at 42°C for 1 hour and heated at 94°C for 5 minutes to stop the cDNA synthesis and to destroy any DNase activity. The final volume was made up to 100 μL with 80 μL of DEPC-treated water and stored at –70°C.

4.2.3 PCR.

The sequence of the *Rattus norvegicus Atp1a1* gene was obtained from GeneBank, with accession numbers M28647, X53234, and X51461 (http://www.ncbi.nlm.nih.gov). The length of the coding sequence was calculated to be 3072 base pairs according to the published data. Primers were designed (Table 4.1) to amplify and clone the coding regions dividing them into two segments of approximately 1.8 kb in size with an overlap of 80 bp. The Clontech Advantage-HF 2 PCR kit was used to amplify the *Atp1a1* gene. The PCR reaction was completed in a total volume of 25 μL of mastermix consisting of 5 μL cDNA template (approximately 0.2 ng/μL), 13.5 μL PCR grade water, 1 μL of primer mix (10 μM of each), 2.5 μL of 10X HF 2 PCR buffer, 2.5 μL of 10X HF 2 dNTP Mix, 0.5 μL of 50X Advantage-HF 2 polymerase mix. The mix was transferred carefully to a well in a polycarbonate 96 well plate (Costar) and an oil drop added. The PCR amplification was performed in the MJ-Research PTC 225 thermocycler. The PCR cycle parameters were as follows: 94°C for 1 minute, 94°C for 30 seconds, 60°C and 68°C for 4 minutes.

The PCR reaction was cycled back to the 94°C at 30 seconds step for 25 cycles. The final extension temperature was held for 3 minutes. The product of PCR reaction was used immediately in the cloning reaction.

Prim	Sequence	Base pairs
F1	TAG TCT CCA GCC ACA GGA CCC G	22
F1A	CTG TCG TCA TCA TAA CTG GC	20
F1B	CAA CGT GCC GGA AGT TTT GC	20
R1	AGG AGG ATA GAA CTG CAT CG	20
R1A	AGG ATT CCA TGA TCT TGG AG	20
R1B	AGT GAG CGT CAG ACA TAC CG	20
F2	ACT GGA GCC TAA GCA CCT GC	20
F2A	ACA GAA GCT CAT CAT TGT GG	20
F2B	TGC CCT TTC ACC TGT TGG GC	20
R2	CTG GAT CCC CAC ATC CTT TG	20
R2A	TCA AAG CTG GAG AGT CAT TG	20
R2B	TCC ACA TCA TTG ATC CAG CG	20

Table 4.1. Primer sequences uses for PCR and internal sequencing of the *Atp1a1* gene. All primers were designed in house from sequences obtained in GeneBank (M28647, X53234, X51461). F, stands for forward primer and R, for reverse primer.

4.2.4 CLONING.

The tube containing the cloning vector pT-Adv was briefly centrifuged to collect all the liquid at the bottom. The following formula was used to calculate the amount of PCR product needed to ligate with 50 ng of pT-Adv,

x ng PCR product = (y bp PCR product)(50 ng pT-Adv)/3900 (size of pT-Adv)

The necessary volume was added to the following ligation reaction. PCR product X, 10X ligation buffer, pT-Adv Vector (25 ng/μL), T4 DNA ligase 1 μL and Sterile H_2O to make the volume 10 μL. this reaction was incubated overnight at 14°C on an MJ-PTC-225 thermocycler. This was then ready for transformation.

4.2.5 TRANSFORMATION.

The tubes containing the ligation reaction were placed on ice and a tube of 0.5 M β-mercaptoethanol (β-ME) was thawed along with one 50 μL frozen tube of TOP10F' *E.coli* competent cells for each ligation/transformation. 2 μL of 0.5 M β-ME was pipetted into each tube of competent cells and mixed by gently stirring with the pipette tip. The tubes were incubated on ice for 30 minutes. After this the tubes were then heat shocked for exactly 30 seconds in a 42°C water bath and placed on ice for 2 minutes. 250 μL of SOC medium (2% triptone, 0.5% yeast extract, 10 mM NaCl, 25 mM KCl,

10 mM $MgCl_2$, 20 mM glucose) were added to each tube at room temperature. The tubes were then shaken horizontally at 37°C for 1 hour at 225 rpm in a rotary shaking incubator. The tubes were then placed on ice, 50 μL and 200 μL of each tube were removed and spread on separate, labelled LB/X-gal plates containing 50 μL/ml of ampicilin. When the liquid was absorbed the plates were inverted and placed in a 37°C incubator for 18 hours. To allow blue/white colour development the plates were moved to 4°C for at least 2-4 hours.

4.2.6 ANALYSIS OF TRANSFORMATION.

To each plate a grid pattern was attached to ease identification of colonies. 20 white colonies supposedly containing insert and 5 blue colonies were picked and re-streaked on fresh LB/X-gal/IPTG plates. The plates were treated exactly as before, each toothpick after use was then dipped into 5 μL of distilled water to allow the remaining bacteria to enter suspension and lyse. This was then used as a DNA template to test for the presence of insert. The PCR was carried out as described earlier (Section 2.4.3) and the primers that were used had the forward primer annealing to the plasmid sequence and the reverse primer to the internal portion of the *Atp1a1* insert. Products were then visualised on a Fluor S-Multimager (Bio-Rad) (Figure 4.1). The blue colonies were used as a negative control. Five positive colonies were then grown up in 3 mL cultures and of these two were chosen to be used for 250 mL cultures. The culture media was L-Broth (LB) with ampicilin at a concentration of 50 μg/mL.

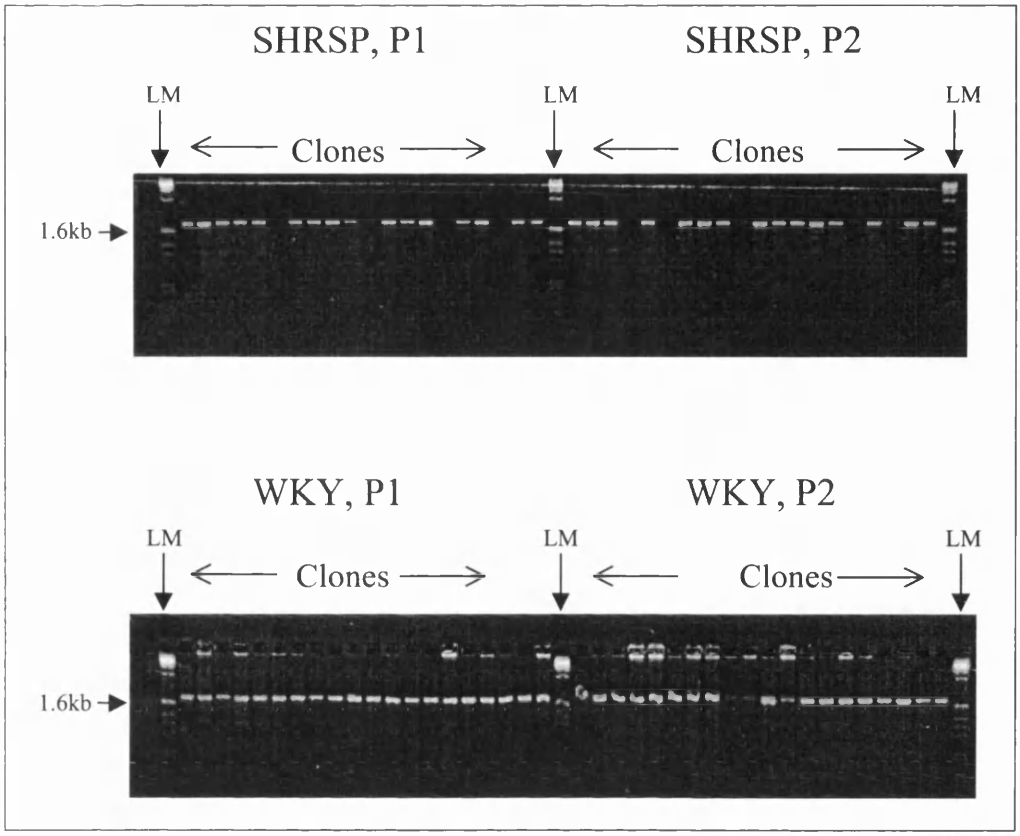

Figure 4.1. Agarose gels illustrating the analysis of transformation. Top panel, SHRSP; bottom panel, WKY. Product 1 and 2 were amplified using PCR with the specific primers represented in figure 4.2. Products of about 1.8 kb were expected after cloning. LM, ladder marker.

4.2.7 PLASMID PURIFICATION.

Qiagen-Tip 500 (QIAGEN), which uses an anion exchange resin for the purification of nucleic acids, was used for plasmid purification. Colonies were inoculated with 2 mL of LB medium and incubation was carried out for 8 hours at 37°C with vigorous shaking (300 rpm). 100 mL of LB medium were inoculated to the starter culture letting it grow at 37°C for 12 hours with shaking (300 rpm), bacterial cells were harvested by centrifugation at 6,000 g for 15 minutes at 4°C. After resuspension of the bacterial pellet in 4 mL of buffer P1 (50 mM tris-Cl, pH 8.0; 10 mM EDTA; 100 μg/mL Rnase A) 4 mL of buffer P2 (200 mM NaOH, 1% SDS) were added and mixed gently by inverting 4 to 6 times and incubated at room temperature for 5 minutes. 4 mL of P3 buffer (0.3 M potassium acetate, pH 5.5) were added to the tubes and the mixture inverted 4 to 6 times with incubation on ice for 15 minutes, centrifugation of the tubes was carried out at 20,000 g for 30 minutes at 4°C. The supernatant containing the plasmid DNA was removed promptly and re-centrifuged at 20,000 g for 15 minutes at 4°C. The Qiagen-tip (QIAGEN) was equilibrated by applying 4 mL of buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol; and 0.15% triton X-100) and the column was allowed to empty by gravity flow. The resulting supernatant was applied to the Qiagen-tip (QIAGEN) and it was allowed to enter the resin by gravity flow. The Qiagen-tip was washed 2 times with 10 mL of QC buffer (1.0 M NaCl; 50 m MOPS, pH 7.0; 15% isopropanol) and the DNA eluted with 5 mL of buffer QF (1.25 M NaCl; 50 mM tris-Cl, pH 8.5; 15% isopropanol).

The DNA was then precipitated by adding 3.5 mL of isopropanol at room temperature and the tube was centrifuged at 15,000 g for 30 minutes at 4°C. After decanting the supernatant the DNA pellet was washed with 2 mL of 70% ethanol and centrifuged at 15,000 g for 10 minutes. The supernatant was decanted, the pellet carefully air-dried for 5-10 minutes and the DNA was dissolved in 100 µL of TE (10 mM tris-Cl, pH 8.0; 1 mM EDTA) buffer. The yield was determined with an ULTROSPEC 2000 UV/visible spectrophotometer (Pharmaco-Biotech) as described in Section 2.4.2.

4.2.8 SEQUENCING.

Purified plasmid-DNA was sent to MWG Biotech (Germany) for double-strand sequencing. The plasmid-DNA was purified and concentrated by ethanol and ammonium acetate precipitation and an air-dried pellet in a 1.5 mL microcentrifuge tube was sent to the company for sequencing. Internal primers (Table 4.1, Figure 4.2) were sent for sequencing of the coding regions of the *Atp1a1* gene contained within the plasmid DNA. Additionally, we sequenced in-house 750 bp using the protocol described in section 2.4.6 in order to re-check any positive findings.

4.3 RESULTS.

Sequencing analysis revealed no difference in the coding region of the *Atp1a1* gene between the WKY strain and the published sequence for *Rattus norvegicus* (GeneBank accession number M28647, X53234, and X51461).

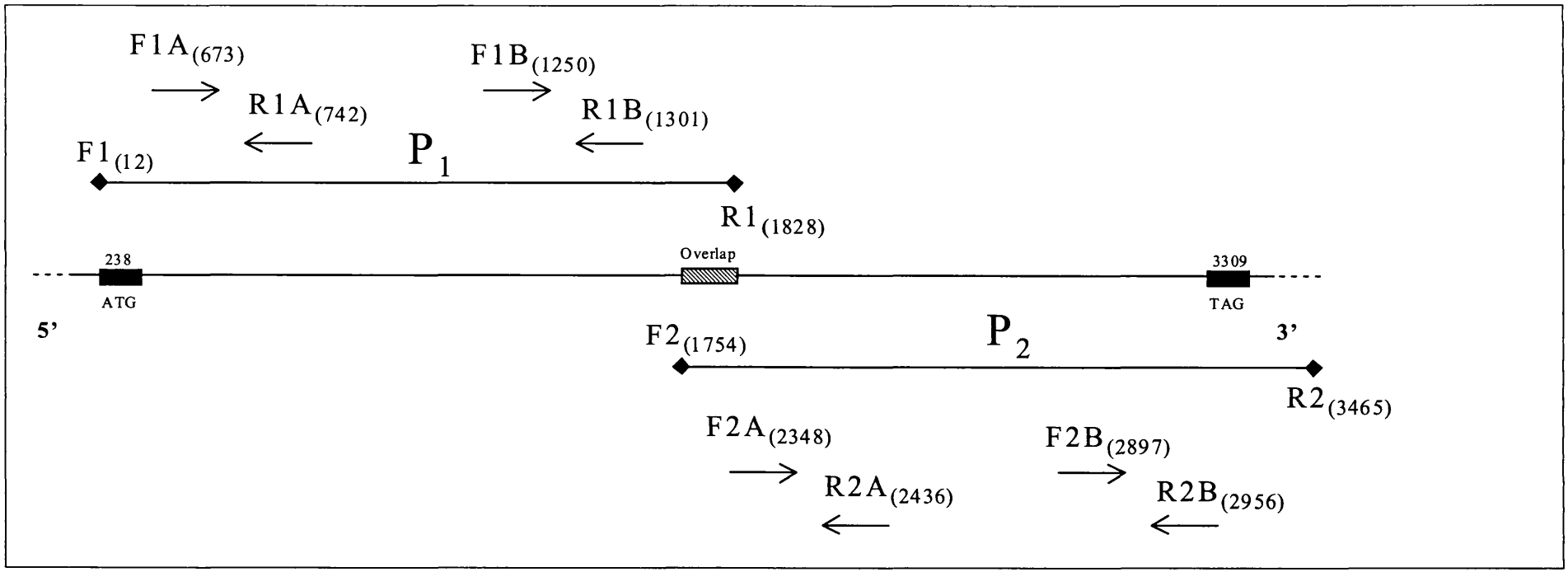

Figure 4.2. Diagram representing the structure of the *Atp1a1* gene and the strategy designed for its sequencing. P1 and P2 represent the gene coding sequences that were subsequently cloned after amplification with the corresponding primers as illustrated. The arrows represent the primers selected for internal sequencing of the gene. F, forward primer; R, reverse primer; 1-2 and A-B, designated arbitrarily.

Two transitions were found, the first at position 2121 (cytosine for thymine) and the second at position 2453 (adenine for guanine). Both transitions were silent as reflected in Table 4.2. Table 4.3 shows a comparison between the *Rattus norvegicus* sequence of the *Atp1a1* gene and the sequence of the two Glasgow rat strains in some specific areas.

4.4 DISCUSSION.

It has been suggested, both in animals and humans, that a decreased activity of the membrane sodium pump could be a pathogenetic determinant of increased blood pressure (Blaustein and Hamlyn, 1984). In the inbred strain of Dahl S rats, Herrera and Ruiz-Opazo (Herrera and Ruiz-Opazo, 1990) found a single nucleotide substitution in the gene encoding the α1 subunit isoform of the Na^+-K^+-ATPase. They proposed that this could impair ion transport and contribute to the pathogenesis of hypertension. In cloned *Atp1a1* cDNA from a single Dahl S rat they found a T for an A transversion at position 1079 that was predicted to cause an amino acid substitution at position 276 in a domain-important for the Na^+-K^+ transport. This group also found that in *Xenopus* oocytes injected with RNA that had been transcribed in vitro from the *Atp1a1* cDNA of Dahl S rat, the oubain-sensitive Rb^+ influx was significantly less than in oocytes injected with *Atp1a1* transcribed RNA from a Dahl R rat.

Simonet *et al* (1991) devised a PCR technique to screen the genomic DNA of multiple Dahl S rats for the T for A transversion reported in the cDNA of

the *Atp1a1* and could not find the mutation in eight Dahl S rats. They also carried out direct sequencing analysis of the gene in three Dahl S rats with negative results. Sequencing of the coding region of the *Atp1a1* gene in the current study revealed no significant nucleotide difference between the WKY strain and the one available at GeneBank. Two transversions were found between WKY and SHRSP sequences, both yielding silent mutations (Table 4.2). Therefore, sequencing of the *Atp1a1* gene in the SHRSP and WKY revealed no nucleotide differences that are potentially important to its function. Moreover, the A to T transversion found by Herrera and Ruiz-Opazo (1990) at nucleotide 1079 could not be identified in the current study.

To determine the role of the Dahl S Q276L α1 Na^+-K^+-ATPase gene variant, Herrera *et al* (1998) developed transgenic Dahl S rats bearing the Dahl R wild type of the *Atp1a1* gene. These trangenic rats exhibited less salt-sensitive hypertension, less hypertensive renal disease and a longer life span when compared with wild-type Dahl S controls.

Discrepancy between published sequences of the *Atp1a1*gene in Dahl S rats is very difficult to explain. Possible explanations are;

i) there is genetic diversity between the different inbred Dahl S rats and therefore the strain used by Simonet *et al* (1991) does not carry the T for A transversion, or

Transversions

Nucleotide		Amino acid	
WKY	SHRSP	WKY	SHRSP
GC**T**^{2121}	GC**C**^{2121}	Alanine (GCT)	Alanine (GCC)
TC**G**^{2453}	TC**A**^{2453}	Serine (TCG)	Serine (TCA)

Table 4.2. The two transversions identified within the coding region of the *Atp1a1* gene in the $SHRSP_{Gla}$ strain compared to the WKY_{Gla} sequence. The transversion at position 2121 and 2453 are silent.

Rattus Norvegicus	2041	gctgtgggca aatgccgcag cgctgggatt aaggtcatca tggtcacagg agaccatcca
WKY_{Gla}	2041	gctgtgggca aatgccgcag cgctgggatt aaggtcatca tggtcacagg agaccatcca
$SHRSP_{Gla}$	2041	gctgtgggca aatgccgcag cgctgggatt aaggtcatca tggtcacagg agaccatcca
Rattus Norvegicus	2101	atcacagcca aagccattgc t^{2121}aaggggtg ggcattatct cagaaggtaa cgagaccgtg
WKY_{Gla}	2101	atcacagcca aagccattgc t^{2121}aaggggtg ggcattatct cagaaggtaa cgagaccgtg
$SHRSP_{Gla}$	2101	atcacagcca aagccattgc **c**^{2121}aaggggtg ggcattatct cagaaggtaa cgagaccgtg
Rattus Norvegicus	2161	gaagacattg ctgcccgcct caacattcca gtgaaccagg tgaaccccag agatgccaag
WKY_{Gla}	2161	gaagacattg ctgcccgcct caacattcca gtgaaccagg tgaaccccag agatgccaag
$SHRSP_{Gla}$	2161	gaagacattg ctgcccgcct caacattcca gtgaaccagg tgaaccccag agatgccaag
Rattus Norvegicus	2221	gcctgtgtag tacatggcag tgacttgaag gacatgacct ctgaggagct ggatgacatt
WKY_{Gla}	2221	gcctgtgtag tacatggcag tgacttgaag gacatgacct ctgaggagct ggatgacatt
$SHRSP_{Gla}$	2221	gcctgtgtag tacatggcag tgacttgaag gacatgacct ctgaggagct ggatgacatt
Rattus Norvegicus	2281	ttgcggtacc acacggagat tgtctttgct aggacctctc ctcaacagaa gctcatcatt
WKY_{Gla}	2281	ttgcggtacc acacggagat tgtctttgct aggacctctc ctcaacagaa gctcatcatt
$SHRSP_{Gla}$	2281	ttgcggtacc acacggagat tgtctttgct aggacctctc ctcaacagaa gctcatcatt
Rattus Norvegicus	2341	gtggagggct gccagcggca gggtgccatc gtggctgtca caggggatgg tgtcaatgac
WKY_{Gla}	2341	gtggagggct gccagcggca gggtgccatc gtggctgtca caggggatgg tgtcaatgac
$SHRSP_{Gla}$	2341	gtggagggct gccagcggca gggtgccatc gtggctgtca caggggatgg tgtcaatgac
Rattus Norvegicus	2401	tctccagctt tgaaaaaggc agatattggg gttgccatgg ggattgttgg ctcg^{2453}gatgtg
WKY_{Gla}	2401	tctccagctt tgaaaaaggc agatattggg gttgccatgg ggattgttgg ctcg^{2453}gatgtg
$SHRSP_{Gla}$	2401	tctccagctt tgaaaaaggc agatattggg gttgccatgg ggattgttgg ctc**a**^{2453}gatgtg
Rattus Norvegicus	2461	tccaagcaag ctgctgacat gattcttctg gatgacaact ttgcctccat cgtgactgga
WKY_{Gla}	2461	tccaagcaag ctgctgacat gattcttctg gatgacaact ttgcctccat cgtgactgga
$SHRSP_{Gla}$	2461	tccaagcaag ctgctgacat gattcttctg gatgacaact ttgcctccat cgtgactgga
Rattus Norvegicus	2521	gtagaagaag gtcgtctgat atttgataac ttgaagaaat ccattgctta caccctaaca
WKY_{Gla}	2521	gtagaagaag gtcgtctgat atttgataac ttgaagaaat ccattgctta caccctaaca
$SHRSP_{Gla}$	2521	gtagaagaag gtcgtctgat atttgataac ttgaagaaat ccattgctta caccctaaca
Rattus Norvegicus	2581	agtaacattc cggaaatcac ccccttcttg atatttatta ttgcaaacat tccactgccc
WKY_{Gla}	2581	agtaacattc cggaaatcac ccccttcttg atatttatta ttgcaaacat tccactgccc
$SHRSP_{Gla}$	2581	agtaacattc cggaaatcac ccccttcttg atatttatta ttgcaaacat tccactgccc

Table 4.3. Comparison of the sequences of the coding regions of the *Atp1a1* gene between the Glasgow SHRSP, WKY, and the sequence for the *Rattus norvegicus* available at GeneBank (NM 012504). The transversions found in the SHRSP are highlighted in bold.

ii) the mutation found by Herrera and Ruiz-Opazo *et al* (1990) is due to a reverse transcriptase error during cDNA synthesis.

Ruiz-Opazo *et al* (Ruiz-Opazo *et al.* 1994) confirmed the mutation using a Taq DNA polymerase-independent approach based on polymerase allele-specific amplification and ligase chain reaction analysis of kidney mRNA subjected to RT-PCR. They claimed that the negative result obtained by Simonet *et al* (1991) was probably caused by a consistent Taq polymerase chain reaction error that selectively substituted adenine-1079 for thymidine. However, there are established differences between the Dahl S and the SHRSP rats as models of genetic hypertension. SHRSP rats are hypertensive without salt-loading whereas Dahl S rats need a high percentage of salt (4-8%) in the diet to become hypertensive (Dahl *et al.* 1962). The current data allow us to conclude that there is no difference in the sequence of the *Atp1a1* gene between the SHRSP and WKY. It seems, therefore, that the *Atp1a1* gene can be excluded as a candidate for BP QTL on the rat chromosome 2. Several other putative candidate genes are located in proximity to the *Atp1a1* locus and might be of interest for future studies. Polymorphic markers located within the locus for atrial natriuretic peptide receptor/guanylyl cyclase A (*Gca*) and for calmodulin-dependent protein kinase II-delta (*Camk2d*) have been found to cosegregate with blood pressure in different F2 populations (Deng *et al.* 1994).(Dubay *et al.* 1993; Schork *et al.* 1995; Harris *et al.* 1995) However, no sequence data are available for these candidate genes. Our study highlights the difficulties of the candidate gene approach and demonstrates that several candidate genes

would require detailed sequence analysis before any causal mutations might be identified.

To conclude, it is still probable that unidentified regions located either in the 5' end or the 3' end, or in the intronic regions of the *Atp1a1* gene could be potentially important in the regulation of its expression. However, the sequencing data obtained in the present study suggest that there is no difference in the coding region of the gene between the WKY and the SHRSP strains. Thus the current study suggests that the correct strategy towards the ultimate positional cloning of the gene(s) involved in the high blood pressure in the SHRSP is the construction of congenic sub-lines to narrow down the chromosomal region of interest (for details see Section 3).

CHAPTER 5

RADIATION HYBRID MAPPING OF RAT CHROMOSOME 2 AND 5 FOR THE LOCALISATION OF GENES AND DETERMINATION OF DISTANCES BETWEEN POLYMORPHIC MARKERS.

5.1 INTRODUCTION.

"If human cells are subjected to large doses of ionising radiation and then fused with rodent cells, hybrid clones are obtained in which linked human genes may be segregated. By determining the frequency with which pairs of linked genes are cotransferred after irradiation, it is possible to determine the linear order of groups of genes and to estimate the distances between them". This paragraph begins the article published in Nature by Stephen Goss and Henry Harris who originally pioneered the technique called radiation hybrid mapping (Goss and Harris, 1975). The original research was based on a former observation by Pontecorvo (Pontecorvo, 1975) who proposed that γ-irradiation of somatic cells would induce chromosome breakage, which might result in segregation of chromosomal fragments (Walter and Goodfellow, 1993). The only problem with this elegant approach is that irradiation causes not only chromosome breakage and segregation of genetic material but also cell death. The solution to this problem comes from the "irradiation and fusion gene transfer" experiments of Goss and Harris. They used as donor cells diploid male lymphocytes and as recipient Wg3-h hamster cells that had a deficient activity of the enzyme hypoxanthine phosphoribosyl transferase (HPRT). The HPRT enzyme acted like a gene marker for the X chromosome. It also allowed to recover the real hybrids after fusion because fused hamster cells that did not have the lymphocyte component, remained deficient in HPRT and were killed in hypoxanthine (HAT) medium; whilst man-hamster hybrid cells were able to proliferate (Goss and Harris, 1975). They were able to establish the order of

four markers (HPRT, glucose-6-phosphate dehydrogenase, α-galactosidase, and phosphoglycerate kinase) on the long arm of the X chromosome, to determine that the segregation of markers is a function of the dose of radiation used and to demonstrate retention of nonselected chromosome fragments (Goss and Harris, 1977).

In 1990, fifteen years after the original experiment, Cox and colleagues modified the original approach by using as the donor cell, not a diploid human cell, but a rodent-human somatic cell hybrid called CHG-3 that contained a single copy of human chromosome 21. They also quantified the X-rays irradiation to 8000 rads. Following the previous protocol they rescued the irradiated donor cells by fusing them with non-irradiated hamster recipient cells (GM459) deficient in HPRT. This resulted in the isolation of 103 independent somatic cell hybrid clones, each representing a fusion event between an irradiated donor cell and a recipient hamster cell. These clones were assayed for the retention of 14 DNA markers on human chromosome 21 by Southern blotting hybridisation analysis (Cox *et al.* 1990). This resulted in a map of the proximal area of the long arm of human chromosome 21.

Subsequently the radiation hybrid mapping technique has been used to map markers around disease loci in localised areas of the genome. For example: 44 loci ordered on human 5q related to acute lymphocytic leukaemia (Warrington *et al.* 1991; Warrington *et al.* 1992), 16 loci in 11q12-13 containing the multiple endocrine neoplasia type I locus (Richard *et al.*

1991), 16 loci on human chromosome 22 associated with the ataxia-telangiectasia locus (Frazer *et al.* 1992; Richard *et al.* 1993), and 17 markers around human 17q containing the BRCA1 locus (Abel *et al.* 1993).

Radiation hybrid mapping is a somatic cell hybrid technique that was developed to construct high-resolution, contiguous maps of mammalian chromosomes. The technique provides a method for ordering DNA markers spanning millions of base pairs of DNA at a resolution not easily obtained by other mapping methods (Cox *et al.* 1990; Burmeister *et al.* 1991). Another advantage of using radiation hybrid mapping is the ability to map non-polymorphic DNA markers that cannot be used for genetic linkage mapping. In this technique X-irradiation breaks the chromosomes of the donor cell line into numerous fragments. Chromosome fragments from the donor cell line are subsequently retained non-selectively after cell fusion with a recipient cell line. The resulting hybrid clones are then tested for the retention or loss of specific donor chromosome markers. Markers that are further apart on a chromosome are more likely to be broken apart by radiation and to segregate independently in the radiation hybrid cells than markers that are close together. By analysing the cosegregation of various loci in hybrid clones statistically, a map can be constructed giving information about the relative order and distance of markers (Cox *et al.* 1990; Burmeister *et al.* 1991; Warrington *et al.* 1991).

Cloning the gene underlying the BP QTL first requires refining the position of the QTL at much higher resolution than is possible with the mere

detection of the BP QTL (Darvasi, 1998). For positional cloning to be feasible, the size of the candidate region has to be typically reduced to about 1 million bases (Mb). Assuming an average frequency of recombination, 1Mb is only about 0.5 cM (Silver, 1995). Assuming an average gene density and 80,000 protein-encoding genes per genome, the potential number of candidate genes in 1Mb is approximately 27. The production of smaller congenic sub-lines as described in Section 1.3.2.2.4 and 1.3.2.2.4.1 is the logical way to follow to capture these 1 cM regions with the BP QTL, where cloning the gene by position becomes feasible. It should be noted that above calculations have been performed for mouse genome. Similar detailed calculations for rat genome are not as well developed (Rapp, 2000).

Comparison of the genetic maps of the mouse with the corresponding maps in the rat reveals large differences. Dense genetic maps of the mouse are available for a variety of genetic studies including:

i) Comparative mapping,

ii) Cloning of monogenic mutations,

iii) Complex trait analysis,

iv) Deletion mapping,

v) Physical map construction and,

vi) Marker-assisted congenic production (Markel *et al.* 1997).

In contrast, the genetic maps of the rat are so far not as dense as the mouse (Yamada *et al.* 1994; Jacob *et al.* 1995; Bihoreau *et al.* 1997; Brown *et al.* 1998; Steen *et al.* 1999; Gauguier *et al.* 1999). This situation has greatly limited the potential of the rat as a genetic model organism for human

disease. Construction of a genetic map in the rat is not as efficient as that of the mouse because of the low polymorphism rate in rat crosses. Some mouse crosses have a high polymorphism rate, more than 90% (Dietrich *et al.* 1996) allowing the mapping of thousands of markers without the need to derive a combined map from mapping data of different crosses from different laboratories. In contrast, most rat crosses have a polymorphism rate of about 50% or less (Jacob *et al.* 1995). As a consequence, a map of equal density as the mouse would require much greater effort to construct.

Radiation hybrid mapping can provide a much higher resolution of the BP QTLs than genetic maps, and this high resolution mapping is a requirement for the positional cloning of the gene or genes underlying the QTLs. With the current status of the microsatellite-based genetic maps available in the rat (Brown *et al.* 1998; Steen *et al.* 1999; Gauguier *et al.* 1999) it is possible to narrow down the BP QTLs. Radiation hybrid mapping provides a tool to place markers inside these regions and therefore enhance the resolution and information about the loci of interest for positional cloning.

Due to the discrepancies between our genetic map and other genetic maps of the rat chromosome 5 (Rubattu *et al.* 1996; Jeffs *et al.* 1997), it was the aim of this study to carry out radiation hybrid mapping and fluorescence in situ hybridisation (FISH) of rat chromosome 5. We also created a radiation hybrid map of the region between markers *D2Rat43* and *D2Rat58* on rat chromosome 2. This region was suggested by our speed congenic results as the chromosomal segment containing the BP QTL.

5.2 METHODS.

5.2.1 PCR.

PCR was performed using the rat whole genome radiation hybrid panel obtained from Research Genetics (Huntsville, Ala) as described in Section 2.6. The presence or absence of 41 microsatellite markers on rat chromosome 5 were determined by PCR reactions with rat primers purchased from either Research Genetics (Huntsville, AL) or Genosys Biotechnology (Cambridge, UK). PCR reactions were done in duplicate and scored by two independent observers to provide accurate consensus scorings. Table 5.1 list 36 markers out of 41 that we were able to amplify and map relatively to each other using radiation hybrid mapping along with the specific conditions used in the PCR reactions. Additionally, 10 markers were used to construct the radiation hybrid map of the telomeric region of rat chromosome 2, PCR conditions are listed in Table 5.2. The PCR products were separated by electrophoresis on a 3% agarose gel containing ethidium bromide and visualised with a FluorS-Multimager (Biorad, UK) (Figure 5.1).

5.2.2 STATISTICAL ANALYSIS.

The radiation hybrid mapping programs of the RHMAP package, version

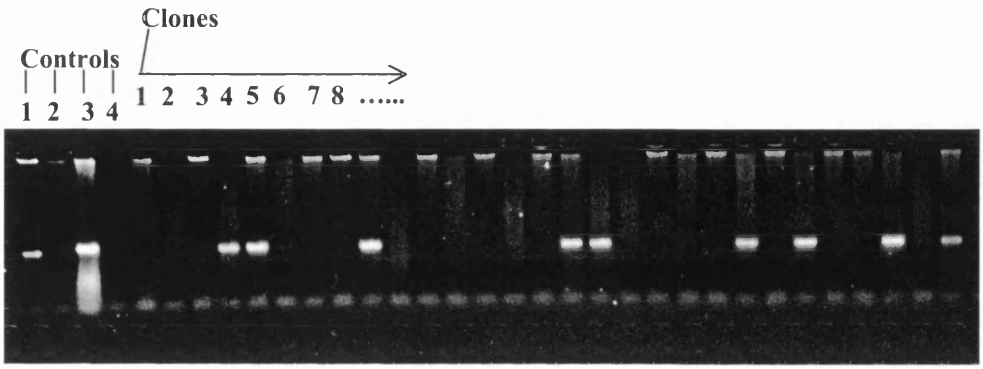

Figure 5.1. Agarose gel showing the PCR amplification of marker *D2Mgh12* in the radiation hybrid clones. Amplification is observed in clones number 4, 5, 9, etc. Control 1, Sprague-Dawley FR DNA; control 2, hamster A23 DNA; control 3, WKY_{Gla} DNA; and control 4, water blank.

Marker	Tm(°C)	Magnesium concentration (mM/L)
D5Rat49	55	1.5
D5Rat50	55	1.5
D5Mgh16	60-55	1.5
D5Mgh15	60-55	1.5
D5Rat48	55	1.5
Anf	55	1.5
D5Rat47	55	1.5
Ela2	55	1.5
D5Rat41	55	1.5
D5Wox14	55-50	1.5
D5Mgh8	50	1.5
D5Rat38	55	1.5
D5Wox15	60-55	1.0
D5Rat29	55	1.5
D5Rat32	55	1.5
D5Rat35	55	1.5
D5Mgh13	50	1.5
D5Wox4	60-55	1.0
D5Rat22	55	1.5
D5Mit3	55	1.5
D5Rat19	55	1.5
D5Wox16	55	1.0
D5Rat13	55	1.5
D5Wox5	55	1.0
D5Mgh11	50	1.5
D5Rat14	63-58	1.5
D5Rat16	55-50	1.5
D5Rat15	55	1.5
D5Rat84	55	1.5
D5Mit2	55	1.5
D5Mit9	55	1.5
D5Mgh6	55	1.5
D5Rat10	55	1.5
D5Mgh10	60-55	1.5
D5Rat8	55	1.5
D5Rat7	55	1.5

Table 5.1. Microsatellites and genes selected for radiation hybrid mapping on rat chromosome 5, annealing temperatures, and magnesium concentration used in the PCR reaction.

Marker	Tm(°C)	Magnesium concentration (mM/L)
D2Mit6	60-55	1.0
Pklr	50	1.5
Gca	55	2.0
D2Mit14	55-50	1.5
Atp1a1	60	1.0
D2Rat49	55	1.5
D2Rat287	55	1.5
D2Rat52	55	1.5
D2Rat157	55	1.5
D2Mgh12	55-50	1.5

Table 5.2. Microsatellites and genes selected for radiation hybrid mapping on rat chromosome 2, annealing temperatures, and magnesium concentration used in the PCR reaction.

3.0 were used to analyse the data as described in Section 2.6.1 (Boehnke *et al.* 1991; Lange *et al.* 1995). A more detailed explanation of the mathematical assumption taken by this programs is given in Appendix 12.

For the analysis of data on chromosome 5 we constructed 5 linkage groups using the two-point analysis and RHMINBRK best orders. Markers on chromosome 2 were analysed in a single linkage group. The orientation of the linkage groups was determined by reference to the genetic map. The order within each linkage group was calculated with the multipoint maximum likelihood method using the RHMAXLIK program and branch and bound strategy and then checked with a simulated annealing strategy using a random initial order. If the branch and bound algorithm could not be applied (too many loci under consideration), a set of best orders was established with the RHMINBRK program. The result was the checked with a stepwise ordering strategy and 10 simulated annealing runs starting with random orders. Map distances were expressed in centiRays (cR) as explained in Section 2.6.1., the maximum likelihood method was then used to calculate map distances of individual markers within linkage groups, and the 2-point analysis to calculate map distances between the linkage groups.

5.2.3 FLUORESCENCE IN SITU HYBRIDISATION (FISH).

FISH enables the visualisation of a marker of interest within a cytological preparation of chromosomal DNA on a microscope slide. Four polymorphic microsatellite markers and two genes on rat chromosome 5 were selected for

FISH and the experiments were completed by Dr. Claude Szpirer and colleagues at the Department of Molecular Biology, University Libre de Bruxelles (Belgium).

FISH was done as described previously, (Pinkel *et al.* 1988; Stephanova *et al.* 1996), with cultured vascular smooth muscle cells harvested from the $SHRSP_{Gla}$ and WKY_{Gla} (Dominiczak *et al.* 1991). The chromosome images were captured and treated with the ISIS imaging system (MetaSystem, D-68804 Althussheim, Germany). Only double spots (2 labeled sister chromatids) formed by the probes were taken into account, and 2 methods were used to determine the regional position of the signals. First, the fractional length distance of the fluorescent signal to the centromere relative to the total chromosome arm length was used to map the genes, with banded rat chromosomes as references (Levan, 1974). Second, the position of the fluorescent spots was superimposed on the image of 4,6-diamidino-2-phenylindole (DAPI)-banded chromosomes, with the use of ISIS system. The *Anf* probe was a 5.2 kb *Kpn*I fragment from the rat gene (JA200) (Argentin *et al.* 1985). The *Dsi*1 probe (Genbank accession number L26461) was the 1.8 kb *Sma*I-*Eco*RI fragment named dsI-SR (Vijaya). The primer sequences of the markers *D5Mgh16*, *D5Mit2*, *D5Mit9*, and *D5Wox15* were obtained from http://ratmap.gen.gu.se/and used to identify the P1 clones from the chromosome 5 fraction of a rat genomic library (Genomic Systems, St Louis, Mo). These 4 P1 clones were then used to localise the chromosomal position of these markers with the use of FISH.

5.3 RESULTS.

The presence or absence of each of the 35 rat microsatellite markers on rat chromosome 5 and 10 rat microsatellites on rat chromosome 2 in 106 radiation hybrid clones was determined by PCR screening with the primers shown in Tables 5.1 and 5.2. The mean retention frequency was 0.281 (range from 0.160 to 0.356) for the markers on rat chromosome 5 and 0.227 (range from 0.200 to 0.282) for markers on rat chromosome 2 (Table 5.3 and Table 5.4). For the analysis of the 35 microsatellites markers on rat chromosome 5, five linkages groups were constructed based on the 2-point analysis with the RH2PT program, with a LOD score of 8.0, followed by a stepwise locus ordering strategy with the RHMINBRK program. The orientation of these linkage groups was determined from the most comprehensive rat genetic linkage map available (Brown *et al.* 1998; Steen *et al.* 1999; Gauguier *et al.* 1999). The order within each linkage group was then determined with a branch and bound strategy (Boehnke *et al.* 1991) within the multipoint maximum likelihood method (RHMAXLIK program). The best order together with map distances calculated as described in Section 2.6.1 are show in Table 5.3 and a comparison with an integrated genetic map of the same region of rat chromosome 5 is shown in Figure 5.2. The 5 linkage groups covered a total distance of 1304 cR, corresponding to a genetic distance of 78 cM. The maximum likelihood analysis allows for the determination of the likelihood ratios, which are defined as the ratios of the overall maximum likelihood of the comprehensive map to the maximum likelihood of a given local order,

	Marker	Retention Frequency	θ	cR_{3000}
	D5Rat49	0.229		
	D5Rat50	0.248	0.094	9.9
	D5Mgh16	0.330	0.417	54.0
	D5Mgh15	0.356	0.236	26.9
	D5Rat48	0.340	0.192	21.3
	Anf	0.297	0.240	27.4
	D5Rat47	0.217	0.425	55.3
	Ela2	0.267	0.309	37.0
	D5Rat41	0.189	0.482	65.7
	D5Wox14	0.176	0.190	21.1
	D5Mgh8	0.292	0.458	61.3
	D5Rat38	0.286	0.056	5.9
2	*D5Wox15*	0.238	0.293	34.7
	D5Rat29	0.252	0.673	111.9
	D5Rat32	0.221	0.264	30.7
	D5Rat35	0.240	0.229	26.1
	D5Mgh13	0.274	0.356	44.0
	D5Wox4	0.245	0.647	104.1
	D5Rat22	0.231	0.258	29.8
	D5Mit3	0.160	0.512	71.7
3	*D5Rat19*	0.229	0.251	29.0
	D5Wox16	0.314	0.518	73.0
	D5Rat13	0.284	0.294	34.8
	D5Wox5	0.346	0.337	41.1
	D5Mgh11	0.333	0.102	10.7
	D5Rat14	0.346	0.252	29.1
	D5Rat16	0.330	0.205	23.0
4	*D5Rat15*	0.276	0.107	11.3
	D5Rat84	0.327	0.412	53.2
	D5Mit2	0.327	0.078	8.1
	D5Mit9	0.327	0.063	6.5
	D5Mgh6	0.283	0.078	8.2
	D5Rat10	0.305	0.357	44.1
	D5Mgh10	0.289	0.211	23.7
	D5Rat8	0.340	0.199	22.2
5	*D5Rat7*	0.337	0.398	50.7
		Average: 0.281		Total:1304.1

Table 5.3. Markers within linkage groups, retention frequencies, probabilities of breakage (θ), and calculated distances between the markers on rat chromosome 5. Distances were calculated using RHMAXLIK program, except for distances between linkage groups: *Ela2*-D5Rat41, D5Wox15-D5Rat29, D5Rat19-D5Wox16, D5Rat15-D5Mit2, which were calculated using the two-point analysis. The θ and cR3000 values are placed in line with the second marker of each pair.

Marker	Retention Frequency	θ	cR_{3000}
D2Mit6	0.282		
Pklr	0.217	0.520	73.5
Gca	0.214	0.116	12.3
D2Mit14	0.231	0.436	57.2
Atp1a1	0.235	0.072	7.5
D2Rat49	0.282	0.134	14.3
D2Rat287	0.226	0.304	36.2
D2Rat52	0.200	0.162	17.6
D2Rat157	0.219	0.355	43.9
D2Mgh12	0.214	0.392	49.8
	Average: 0.227		Total:312.4

Table 5.4. Markers within linkage groups, retention frequencies, probabilities of breakage (θ), and calculated distances between the markers on rat chromosome 2. Distances were calculated using RHMAXLIK program. The θ and cR3000 values are placed in line with the second marker of each pair.

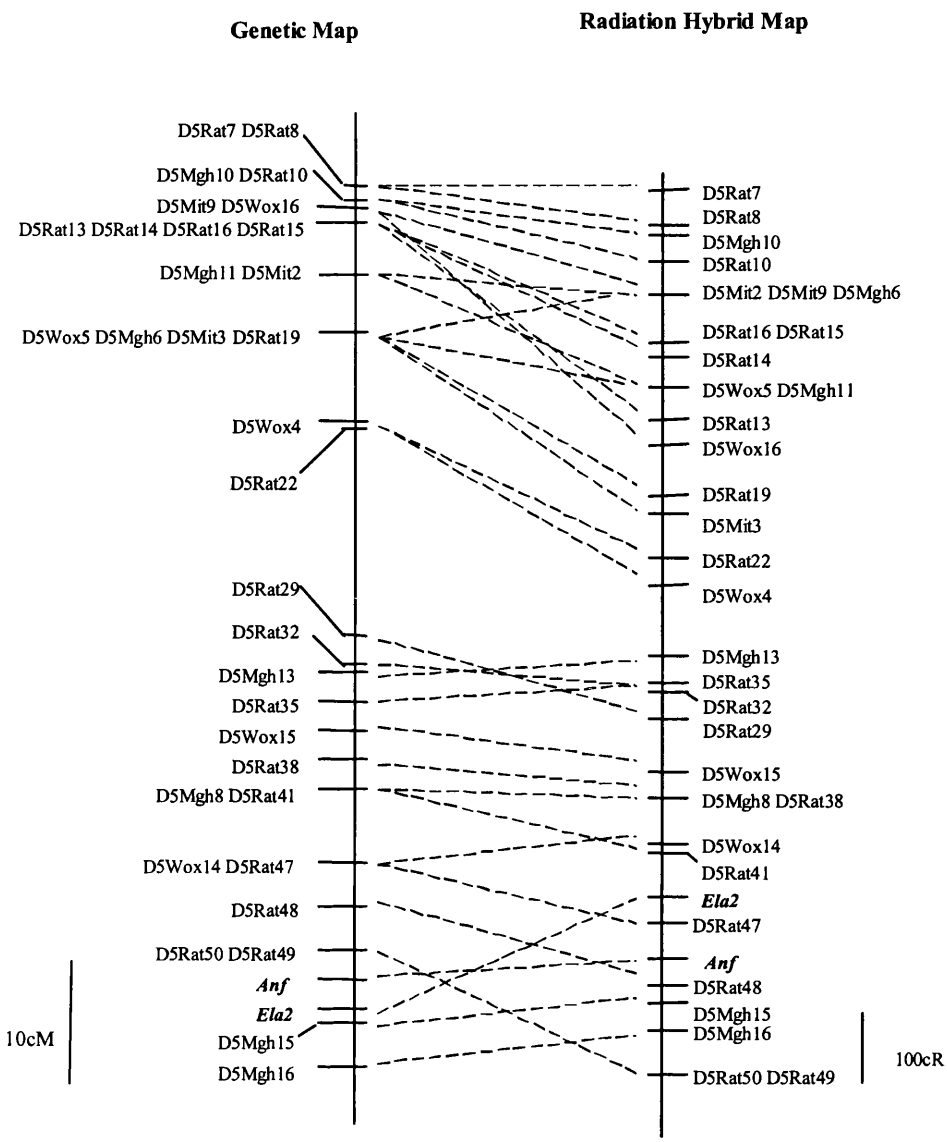

Figure 5.2. Radiation hybrid map of rat chromosome 5 from *D5Rat7* to *D5Rat49* to the right compared with an integrated genetic map of the same region to the left. Marker location on genetic map were based on a combination map between the Oxford map (http://www.well.ox.ac.uk/~bihoreau/key.html) and the Whitehead Institute map (http://www.genome.wi.mit.edu/).

the different orders and likelihood ratios are given in Table 5.5.

The 11 markers on rat chromosome 2 were analysed in the same linkage group because all markers were linked based on the 2-point analysis performed with the RH2PT computer program, with a LOD score threshold of 6.0. The RHMIMBRK program was used to determine a set of best orders using a branch and bound strategy, these orders were then confirmed with the RHMAXLIK program. The best order along with the distances between markers calculated with the RHMAXLIK program are shown in Table 5.4, the different orders and their likelihood ratios are given in Table 5.6. Figure 5.3 shows a comparison between our genetic linkage map of rat chromosome 2 (Section 3) and the radiation hybrid map of the region obtained. The 11 markers mapped on rat chromosome 2 covered 100 cM according to our genetic linkage map and comprised 312.4 cR according to the physical map performed on the region.

Four chromosome 5 microsatellite markers *D5Mit9*, *D5Mit2*, *D5Wox15*, and *D5Mgh16*, as well as the *Anf* gene and the *Dsi*1 locus, were unambiguously localised by FISH with either $SHRSP_{Gla}$ or WKY_{Gla} cells (identical results were obtained with the 2 cell types). *D5Mit9* maps at 5q24, *D5Mit2* maps somewhat more distally, namely in the 5q24-q31 interval, *D5Wox15*, and *Dsi*1 colocalise at 5q36.2, whereas *D5Mgh16* and *Anf* colocalise at the end of the chromosome, at 5q36.3 (Figure 5.4). These localisations thus establish several anchor points between the cytogenetic map and the genetic linkage map.

Linkage Group	Local Order	* Likelihood Ratio
1	*D5Rat49-D5Rat50-D5Mgh16-D5Mgh15-D5Rat48-Anf-D5Rat47-Ela2*	1.0
	D5Rat50-D5Rat49-D5Mgh16-D5Mgh15-D5Rat48-Anf-D5Rat47-Ela2	8.1
	Ela2-D5Rat47-D5Rat49-D5Rat50-D5Mgh16-D5Mgh15-D5Rat48-Anf	44.5
	D5Rat49-D5Rat50-D5Mgh15-D5Mgh16-D5Rat48-Anf-D5Rat47-Ela2	87.8
	D5Rat49-D5Rat50-D5Mgh15-D5Mgh16-D5Rat48-Anf- Ela2-D5Rat47	705.5
2	*D5Rat41-D5Wox14-D5Mgh8-D5Rat38-D5Wox15*	1.0
	D5Rat41-D5Wox14-D5Rat38-D5Mgh8-D5Wox15	2.1
	D5Wox14-D5Rat41-D5Wox15-D5Rat38-D5Mgh8	13.0
	D5Rat41-D5Wox14-D5Wox15-D5Rat38-D5Mgh8	33.0
	D5Wox14-D5Rat41-D5Mgh8-D5Rat38-D5Wox15	54.5
3	*D5Rat19-D5Mit3-D5Rat22-D5Wox4-D5Mgh13-D5Rat35-D5Rat32-D5Rat29*	1.0
	D5Rat19-D5Mit3-D5Rat22-D5Wox4-D5Rat29-D5Rat32-D5Rat35-D5Mgh13	1.5
	D5Rat19-D5Mit3-D5Rat22-D5Wox4-D5Rat29-D5Rat35-D5Rat32-D5Mgh13	2.6
	D5Rat29-D5Rat35-D5Rat32-D5Mgh13-D5Wox4-D5Rat22-D5Mit3-D5Rat19	2.6
	D5Rat29-D5Rat32-D5Mgh13-D5Rat35-D5Wox4-D5Rat22-D5Mit3-D5Rat19	7.6
4	*D5Wox16-D5Rat13-D5Wox5-D5Mgh11-D5Rat14-D5Rat16-D5Rat15*	1.0
	D5Wox16-D5Rat13-D5Mgh11-D5Wox5-D5Rat14-D5Rat16-D5Rat15	1.3
	D5Wox16-D5Rat13-D5Wox5-D5Mgh11-D5Rat14-D5Rat15-D5Rat16	5.4
	D5Wox16-D5Rat13-D5Mgh11-D5Wox5-D5Rat14-D5Rat15-D5Rat16	8.1
	D5Rat13-D5Wox16-D5Mgh11-D5Wox5-D5Rat14-D5Rat16-D5Rat15	30.8
5	*D5Mit2-D5Mit9-D5Mgh6-D5Rat10-D5Mgh10-D5Rat8-D5Rat7*	1.0
	D5Mgh6-D5Mit9-D5Mit2-D5Rat10-D5Mgh10-D5Rat8-D5Rat7	85.7
	D5Mit2-D5Mit9-D5Mgh6-D5Rat8-D5Mgh10-D5Rat10-D5Rat7	150.0
	D5Mit9-D5Mit2-D5Mgh6-D5Rat10-D5Mgh10-D5Rat8-D5Rat7	364.0

Table 5.5. Maximum-Likelihood marker orders within linkage groups for markers on rat chromosome 5. *The likelihood ratio represents the ratio of the overall maximum likelihood of the comprehensive map to the maximum likelihood of a given local order.

Local Order	* Likelihood Ratio
D2Mgh12-D2Rat157-D2Rat52-D2Rat287-D2Rat49-Atp1a1-D2Mit14-Gca-Pklr-D2Mit6	1.0
D2Mgh12-D2Rat157-D2Rat52-D2Rat287-D2Rat49-Atp1a1-D2Mit14-Pklr-Gca-D2Mit6	15
D2Mgh12-D2Rat157-D2Rat52-D2Rat287-D2Rat49-D2Mit14-Atp1a1-Gca-Pklr-D2Mit6	15.7
D2Mgh12-D2Rat157-D2Rat52-D2Rat287-D2Rat49- D2Mit14-Atp1a1- Pklr-Gca-D2Mit6	377.5
D2Rat157- D2Mgh12-D2Rat52-D2Rat287-D2Rat49-Atp1a1-D2Mit14-Gca-Pklr-D2Mit6	581.7

Table 5.6. Maximum-Likelihood marker orders within linkage groups for markers on rat chromosome 2. *The likelihood ratio represents the ratio of the overall maximum likelihood of the comprehensive map to the maximum likelihood of a given local order.

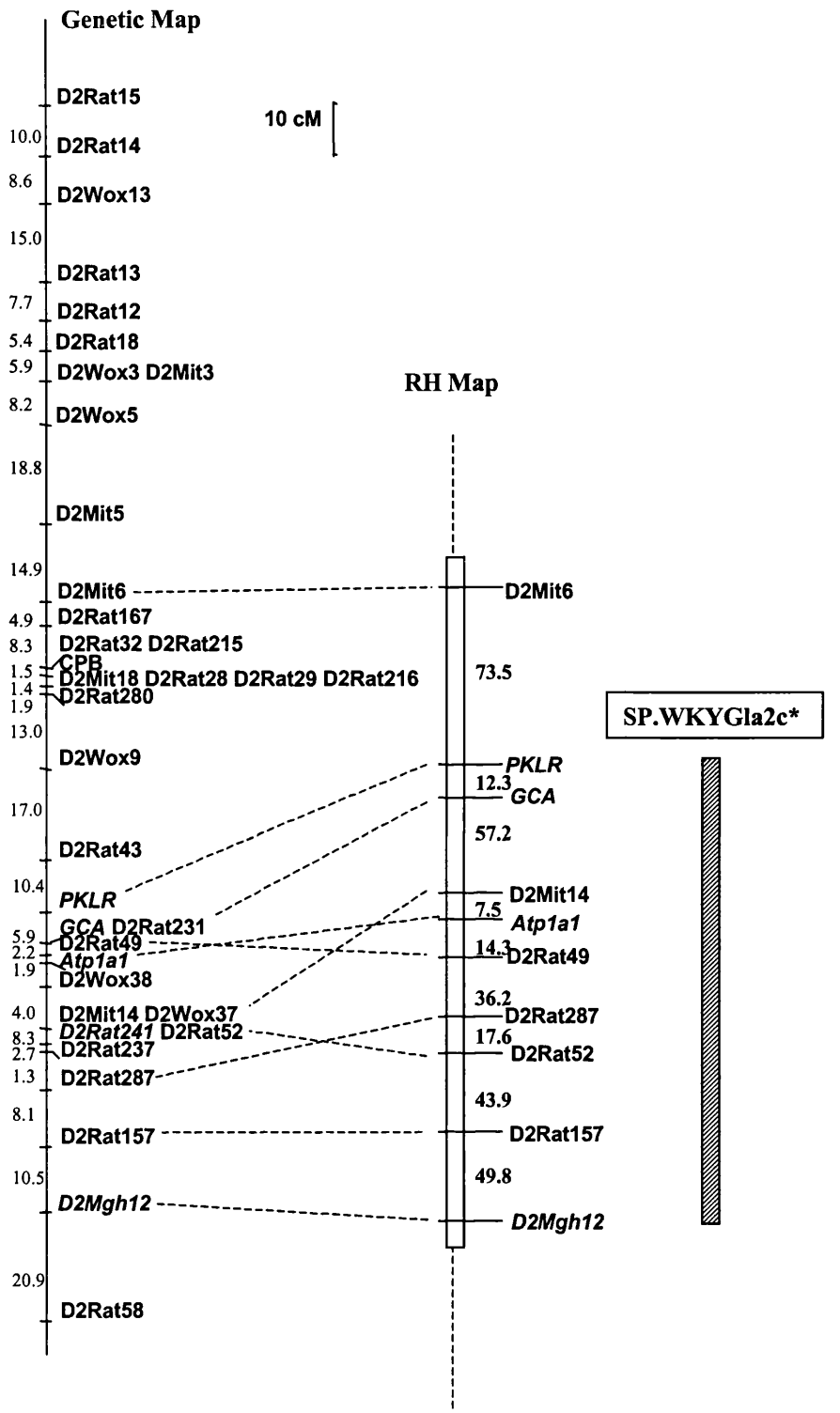

Figure 5.3. Radiation hybrid map of the telomeric region of rat chromosome 2 (between markers *D2Mit6* and *D2Mgh12*) and comparison to our genetic map of the same region. Distances between markers in the RH map are in cR. The shadowed rectangle to the right represents the region transferred in the congenic strain SP.WKYGla2c*.

5.4 DISCUSSION.

We have successfully constructed a radiation hybrid map of rat chromosome 5, using a combination of microsatellite markers and genes to confirm the orientation and localisation of the markers present in our genetic linkage map. We performed a physical map of rat chromosome 5 in view of the significant disagreement between the genetic map constructed in our F2 experiment (Jeffs *et al.* 1997) and other genetic maps of the region (Rubattu *et al.* 1996; Bihoreau *et al.* 1997). This map will facilitate the identification of genes underlying cardiovascular and cerebrovascular QTLs that map to these regions on rat chromosome 5. Radiation hybrids mapping was used, and we were able to map successfully 35 microsatellite markers covering 78 cM of the chromosome, corresponding to a physical distance of approximately 1,304 cR. We estimated that 17 cR on our radiation hybrid map correspond to approximately 1 cM on the genetic map. This is in agreement with published data (Watanabe *et al.* 1999; Steen *et al.* 1999). The resolution of the radiation hybrid map is considerably higher than that of existing genetic maps and is similar to that obtained by Steen *et al* (Steen *et al.* 1999) and Watanabe *et al* (Watanabe *et al.* 1999) where 10-15 cR equals approximately 1 cM, and lower than that obtained by Al-Majali *et al* (Al-Majali *et al.* 1999) were 49 cR equals approximately 1 cM. The *Anf* microsatellite marker was mapped between *D5Rat48* and *D5Rat47* (Figure 5.2 and Table 5.3), which corresponds to the telomeric end of rat chromosome 5 and therefore, it was misplaced in our previous genetic

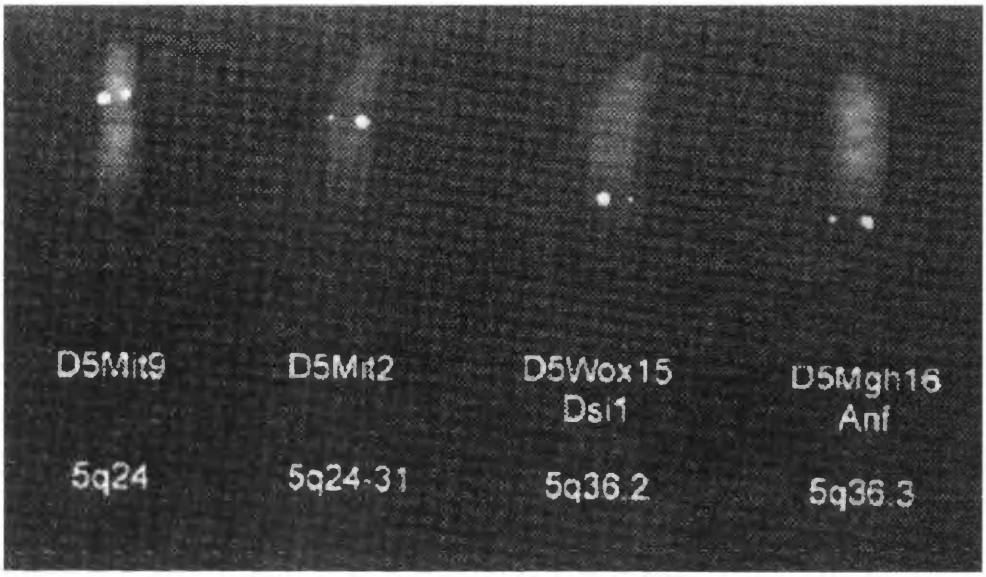

Figure 5.4. Regional localisation of *D5Mit9*, *D5Mit2*, *D5Wox15*, *Dsi1*, *D5Mgh16*, and *Anf* on rat chromosome 5. In each case, representative chromosome is shown, labelled by 2 fluorescent signals, and position of these signals is indicated below the corresponding loci. *D5Wox15* and *Dsi1* probes yielded identical results (fluorescent signals at 5q36.2); similarly, results with *D5Mgh16* and *Anf* probes were indistinguishable (signals at 5q36.3). Therefore, a single chromosome is shown in each of these 2 cytogenetic positions. Chromosomes were of 4,6-diamidino-2-phenylindole (DAPI)-counterstained; banding and probe signals were captured and treated with ISIS imaging system.

linkage map (Jeffs *et al.* 1997). Furthermore, we performed FISH on cells isolated from the $SHRSP_{Gla}$ and WKY_{Gla} and mapped the *Anf* marker at 5q36.3, which again corresponds to the telomeric end of the chromosome. Therefore, two different physical mapping methods have given identical results and are in agreement with the genetic maps published by other groups (Brown *et al.* 1998; Steen *et al.* 1999; Gauguier *et al.* 1999). The difference between our genetic map (Jeffs *et al.* 1997) and the physical maps described in this study remains unexplained but stresses the importance and the superior resolution of the physical mapping methods, which provide a firm base for the future positional cloning strategies.

The data presented in Chapter 3 suggest that the region between markers *D2Rat43* and *D2Mgh12* carries the BP QTL in our experimental model. We constructed a congenic strain called SP.WKY.Gla2c* (Figure 5.3) where a smaller region from the $SHRSP_{Gla}$ was transferred into the WKY_{Gla} background following the breeding protocol illustrated in Section 2.2.1. This congenic segment spans from markers *D2Wox9* to *D2Mgh12*. According to our improved genetic linkage map of rat chromosome 2, the genetic distance between markers *D2Mit6* and *D2Mgh12* corresponds to approximately 100 cM. However, according to the other genetic linkage maps published (Brown *et al.* 1998; Steen *et al.* 1999; Gauguier *et al.* 1999) and those available on-line at: http://goliath.ifrc.mcw.edu/LGR/research/rhp/index.html, and http://curatools.curagen.com/ratmap, the distance between these two markers (or other closely located to them) is approximately 45-57.2 cM. The physical to genetic distance conversion

obtained by Watanabe *et al* (1999) and Steen *et al* (1999), where whole genome radiation hybrid mapping was performed with thousands of markers, gives an estimate of 31.24 cM to 20.83 cM for the region between *D2Mit6* and *D2Mgh12*. This distance is therefore comparable to that achieved in different genetic linkage and radiation hybrid maps of the region (Brown *et al.* 1998; Watanabe *et al.* 1999; Steen *et al.* 1999).

Phenotyping of the congenic strain SP.WKY.Gla2c* is currently in progress and will determine whether the BP QTL was trapped in this region. The physical map of the transferred segment provides a region smaller than that obtained with the recombination map. The radiation hybrid mapping technique provides a way to place all markers regardless whether they are polymorphic or not, as well as genes, and expressed sequence tags (ESTs) quickly and accurately. This will in turn facilitate the identification of the gene(s) within this BP QTL.

In summary, we have constructed a radiation hybrid map of rat chromosome 5, which will facilitate identification of the candidate genes for the several QTLs that map to this chromosome. We have also developed a high-resolution map of the telomeric region of rat chromosome 2 which will lead to cloning by position the gene(s) within the BP QTL mapped to this area.

CHAPTER 6

GENERAL DISCUSSION.

Human essential hypertension is a classic example of a complex, multifactorial, quantitative trait under the regulation of multiple genes that interact with environmental factors. It is estimated that between 30-50% of the variation in blood pressure among individuals is due to genetic determinants (Ward, 1990). Several strategies have been developed over the last decade to dissect these genetic determinants and despite the very significant recent progress in genomic and statistical tools, the genetic dissection of human essential hypertension still provides a major challenge (Colhoun, 1999). Of these strategies, the most successful have been studies that identified rare Mendelian syndromes in which a single gene mutation causes high blood pressure. However, the attempts to identify multiple genes, each with a small contribution to the common polygenic form of hypertension, have been less successful. The heterogeneity of the human disease might be responsible for the limited progress that has been made towards the identification of the causal genes.

Experimental models of genetic hypertension are used to remove some of the complexity inherent in the study of human subjects. Studies of inbred rodent models of genetic hypertension have resulted in the identification of several BP QTLs using genome-wide scanning. This strategy involves the determination of blood pressure of a large segregating F2 population derived by crossing 2 contrasting rat strains, and the genotyping of a large panel of polymorphic microsatellite markers, with a thorough coverage of the entire rat genome. Using a genome-wide scan combined with high fidelity phenotyping of blood pressure, our group reported two BP QTLs on

rat chromosome 2 in an F2 cross between the WKY_{Gla} and $SHRSP_{Gla}$ strains (Clark *et al.* 1996).

The main difference from other genome scan studies was the use of high fidelity phenotyping to facilitate accurate characterisation of the BP QTLs. This investigation tested the applicability of a speed reciprocal congenic strategy to dissect BP QTLs in the rat (Jeffs *et al.* 2000). As a result of this strategy, the chromosomal region between markers *D2Wox19* and *D2Mgh12* was implicated as the important segment containing the BP QTL. We performed high resolution radiation hybrid mapping of this region to assist with the cloning by position of the causative gene located in this area. We also produced a congenic strain, SP.WKY.Gla2c*, which contains this segment introgressed from the WKY_{Gla} normotensive strain into the hypertensive $SHRSP_{Gla}$ strain. Further work will focus on genotyping and phenotyping this new congenic strain and then will proceed with production of relevant substrains for fine mapping of the region.

Several positional candidate genes are located on the BP QTLs identified by Clark *et al* (1996) and confirmed with the appropriate congenic lines by Jeffs *et al* (2000). These candidate genes include: *ATP1a1*, Na^+K^+ATPase α1 isoform; *Gca*, atrial natriuretic peptide receptor/guanylyl cyclase A; *Camk2d*, calmodulin-dependent protein kinase II-delta. We performed sequencing analysis of the coding regions of the *ATP1a1* gene located in the relevant area. Herrera and Opazo (Herrera and Ruiz-Opazo, 1990) found that a single nucleotide substitution in this gene could impair ion transport

and contribute to the pathogenesis of hypertension in Dahl S rats. The same group constructed transgenic Dahl S rats bearing the Dahl R wild type of the *ATP1a1* gene finding less salt sensitive hypertension (Herrera *et al.* 1998). However, Simonet et al (1991) could not find the same mutation in Dahl S rats. We found two transversions between the $SHRSP_{Gla}$ and WKY_{Gla} sequences, both yielding silent mutations revealing no nucleotide differences that are potentially important to its function. Moreover, the transversion originally found by Herrera and Opazo (1990) could not be detected. The evidence suggests that the *ATP1a1* gene can be excluded as a candidate gene for the telomeric BP QTL on rat chromosome 2, although mutations in regulatory regions or in intronic segments that were not screened in this study could be important in the regulation of its expression. The candidate gene approach demonstrates that concentrating on single candidate genes within large chromosomal regions is not the correct strategy towards the ultimate positional cloning of the gene(s) involved in high BP. Development of the appropriate congenic sub-lines will narrow down the chromosomal region of interest to a size at which cloning by position will be feasible.

We derived a RH map of the telomeric end of rat chromosome 2 according to the results provided by the reciprocal congenic, which suggested that the region between markers *D2Rat43* and *D2Mgh12* carries the BP QTL. The genetic distance between these two markers obtained by us was larger than that obtained by Brown *et al* (1998), and Gauguier *et al* (1999). The distance obtained by RH mapping yielded an estimate of 20.83 cM to 31.24

cM (equivalent to 312 cR) comparable to that achieved by others (Brown *et al.* 1998; Watanabe *et al.* 1999; Steen *et al.* 1999; Gauguier *et al.* 1999). RH mapping also provides a tool to place a variety of genetic markers regardless of whether they are polymorphic or not, which represents an indispensable step for homology mapping.

Based on the assumption that like many other genes, BP susceptibility genes are likely to be conserved between species during the course of evolution, common or reproducible QTLs have become important candidate loci for human essential hypertension. The best example is the BP QTL identified on rat chromosome 10, which is a classic reproducible QTL reported in the $SHRSP_{Hd}$ x WKY_{Hd} cross (Hilbert *et al.* 1991; Jacob *et al.* 1991), the Dahl S x MNS cross (Deng and Rapp, 1992; Deng and Rapp, 1995), and the Dahl S x LEW cross (Zhang *et al.* 1996). Julier *et al* (1997) used this BP QTL to investigate the homologous region of conserved synteny on human chromosome 17. In their study of 518 sib-pairs concordant for essential hypertension, there was evidence of significant linkage to human chromosome 17q between microsatellite markers, *D17S183* and *D17S934*. This region has also been linked to pseudohypoaldosteronism type II or Gordon's Syndrome in a subset of families (Mansfield *et al.* 1997). Baima *et al* (1999) confirmed these data in another population of patients with essential hypertension and refined the localisation of this BP QTL between markers *D17S1814* and *D17S800* which are only 0.7 cM apart. These 2 different studies provide a proof of the concept that it is feasible to directly translate BP QTLs discovered in the rat to human essential hypertension and

pave the way for the investigation of other reproducible BP QTLs in humans.

The newly identified region on rat chromosome 2 fulfils the criteria of a reproducible QTL, having been identified in different rat crosses originating from different genetic backgrounds (Dubay *et al.* 1993; Deng *et al.* 1994; Schork *et al.* 1995; Harris *et al.* 1995; Pravenec *et al.* 1995; Samani *et al.* 1996; Garrett *et al.* 1998). Moreover, Deng *et al* (1997) constructed two different congenic strains introgressing the relevant region from the WKY rat or the MNS rat into the Dahl S background. They observed lower blood pressure in both strains as compared to the parental strain. Homology mapping between rat, mouse, and human at this region identified conserved synteny segments on mouse chromosome 3 and human chromosome 1 (Gauguier *et al.* 1999). This area is an excellent candidate region for the genetic analysis of human hypertension.

Pharmacogenetic approaches can provide other links between human and experimental hypertension. For example, several studies have demonstrated that compared to white subjects, black hypertensives show a much poorer response to treatment with angiotensin converting enzyme (ACE) inhibitors or beta-blockers (Saunders *et al.* 1990; Materson *et al.* 1993). Therefore, heterogeneity in human hypertension extends to the blood pressure response to antihypertensive medications where differential responses are seen in some racial sub-groups to particular classes of drugs. Vincent *et al* (1997) studied whether genetic factors influence the acute cardiovascular responses

to pharmacological modulations of the renin-angiotensin system, the sympathetic nervous system, and the voltage-sensitive L-type calcium channels in Lyon hypertensive and normotensive rats. A backcross population was utilised and a QTL discovered on rat chromosome 2 for the hypotensive response of a dihydropyridine calcium antagonist, PY108-068. This locus had no effect on blood pressure responses to ganglionic blocking agents or angiotensin II receptor antagonists. These findings provide strong direct support for the paradigm that genetic factors may influence the response to antihypertensive drugs and suggest that the heterogeneity seen in the responses to different antihypertensive agents in human hypertension may have a significant genetic determination. Moreover, the congenic lines produced contained the region implicated by the cosegregation study outlined above and therefore provide a unique model to study pharmacogenetic interactions.

Finally, a further step for the goal of gene identification has been taken in the form of microarray technology. Aitman *et al* (1997, 1999) identified a QTL on rat chromosome 4 responsible of the defects in glucose and fatty acid metabolism in the SHR. Using a combination of new strategies including cDNA microarrays, congenic mapping, and radiation hybrid mapping they identified a defective SHR gene, *Cd36*, at the peak of the QTL on rat chromosome 4. Further work revealed multiple coding sequence variants in *Cd36* cDNA, with its protein product undetectable in SHR adipocyte plasma membrane. These data produced compelling evidence for the role of *Cd36* deficiency in insulin resistance, defective fatty acid

metabolism, and hypertriglyceridemia in the SHR. The congenic animal used by Aitman *et al* (1999) in the microarray experiment had a 38 cM region transferred from the BN into the SHR background. Congenic sub-line SP.WKY.Gla2c* has a transferred region of approximately 25 cM, making it ideal for cDNA microarray gene expression experiments which may lead directly to gene identification.

The outlined genome-wide study of hypertension in the SHRSP (Clark *et al.* 1996) has provided an initial glimpse into the complex interactions and the multiple loci that contribute to this disease. The congenic strains presented will permit detailed investigations of the means by which these loci exert their deleterious effects. This information, coupled with the forthcoming knowledge of the complete human and mouse genomes, will certainly allow the identification of the specific susceptibility genes, thereby leading to a better diagnosis, prognosis, and management of hypertension.

To conclude, we successfully used a speed congenic strategy to dissect two BP QTLs identified on rat chromosome 2. The construction of congenic sub-lines, radiation hybrid mapping of the region where the QTL has been trapped, and cDNA microarray technology will allow cloning by position of the causal gene. The use of comparative mapping between rat, mouse, and human will permit the translation of this information from the experimental model to human cardiovascular disease.

APPENDICES

APPENDIX 1.

Suppliers of all the chemicals and reagents used.

Chemical Reagents	Suppliers
1st-strandTM cDNA synthesis kit	Clontech
Advantage-HF 2 PCR kit	Clontech
Agarose	Gibco BRL
Ammonium persulphate	BDH
AmpliCycleTM Sequencing kit	Perkin-Elmer
Blue dextran	Perkin-Elmer
Bromophenol blue	Sigma
Buffer tablets	BDH
Cellulose patchs	Data Sciences Int.
Chloroform	Fisher Scientific
Cidex	Johnson & Johnson
Concentrated hydrochloric acid	BDH
Cresol Red	Sigma
Decon 75	Decon Laboratories Ltd.
dNTPs/deoxynucleoside triphosphates	Promega
ddNTPs/dideoxynucleoside triphosphates	Perkin-Elmer
Dimethyl sulfoxide	Sigma
EDTA	Bio-Rad
Ethanol	Sigma
Ethidium bromide	Sigma
Exonuclease I	USB
Formamide	BDH
GENESCAN-350 Tamra	Perkin-Elmer
Glacial acetic acid	Fisher Scientific
Halothane	Zeneca Ltd.
HotStartTaq DNA polymerase	Qiagen
Hybond-N+ membrane	Amersham
Isoamylalcohol	BDH

Isopentane	Fisher Scientific
10X kinase buffer	Promega
Long Ranger 50% stock gel solution	FMC
Magnesium chloride/MgCl2	Promega
Metaphor agarose	FMC
Mineral Oil	Sigma
Nuclei Lysis Solution	Promega
[α-^{32}P]dCTP	Amersham
[β-^{32}P]dATP	Amersham
10X PCR buffer	Qiagen
Phenol, water-saturated in Tris buffer	Life Sciences
Powered milk	Premier Beverages
Polyoxyethylene	Life Technologies
Protein Precipitation solution	Promega
Proteinase K	Sigma
Ready Reaction mix	Perkin-Elmer
Resin AG 501-X8	Bio-Rad
RNase solution	Promega
RNAzolTM B	Biogenesis Ltd.
0.9% Saline	Baxter Scientific
20X SSC	National Diagnostics
SDS (sodium dodecyl sulphate) powder	Bio-Rad
SequaGel Sequencing System	National Diagnostics
Shrimp alkaline phosphatase	USB
Sigmacote	Sigma
Sodium acetate	Sigma
Sodium chloride	Fisher Scientific
Sodium hydroxide pellets	Fisher Scientific
Sucrose	Fisher Scientific
Taq DNA polymerase	Promega
Tartrazine	Sigma
10X TBE	National Diagnostics
TEMED	Sigma

TEMED	Amresco
Terminal deoxynucleotidyl transferase/TdT	Promega
TdT buffer	Promega
10X Thermophilic buffer	Promega
Tris base	Bio-Rad
Ultra-pure formamide	Amresco
Urea	Sigma
VetBond	3M Animal Care Products
Xylene cyanol	Sigma

APPENDIX 2.

List of all the recipes for the preparation of solutions.

10% Ammonium Persulphate (10 mL).

1 g APS dissolved in 10 mL sterile distilled water. Store at 4°C.

Buffer P1.

Dissolve 6.06 g Tris base, 3.72 g EDTA in 800 mL of sterile distilled water. Adjust the pH to 8.0 with HCL. Adjust the volume to 1 L with sterile distilled water. Add 100 mg RNase A per litre of P1. Store at 4°C.

Buffer P2.

Dissolve 8.0 g NaOH pellets in 950 mL of sterile distilled water, 50 mL 20% SDS solution. The final volume should be 1 litre. Store at room temperature.

Buffer P3.

Dissolve 294.5 g of potassium acetate in 500 mL of sterile distilled water. Adjust the pH to 5.5 with glacial acetic acid (~110 mL). Adjust the volume to 1 litre with sterile distilled water. Store at room temperature.

Buffer QBT.

Dissolve 43.83 g of NaCl, 10.46 g MOPS (free acid) in 800 mL of sterile distilled water. Adjust the pH to 7.0 with NaOH. Add 150 mL pure

isopropanol and 15 mL 10% Triton X-100 solution. Adjust the volume to 1 litre with sterile distilled water. Store at room temperature.

Buffer QC.

Dissolve 58.44 g NaCl and 10.46 g MOPS (acid free) in 800 mL of sterile distilled water. Adjust the pH to 7.0 with NaOH. Add 150 mL pure isopropanol. Adjust the volume to 1 litre with sterile distilled water. Store at room temperature.

Buffer QF.

Dissolve 73.05 g NaCl and 6.06 Tris base in 800 mL sterile distilled water and adjust the pH to 8.5 with HCl. Add 150 mL pure isopropanol. Adjust the volume to 1 litre with sterile distilled water. Store at room temperature.

Chloroform Isoamylalcohol mixture (250 mL).

240 mL chloroform added to 10 mL isoamylalcohol. Store at 4°C wrapped in light resistant bottle.

dNTPs (200 μL, 25 mM).

5 μL of each dGTP, dATP, dTTP, and dCTP (provided in 100 mM stock solutions) added to 180 μL sterile distilled water. Store frozen.

dNTPs (1,000μL, 1 mM).

10 μL of each dGTP, dATP, dTTP, and dCTP (provided in 100 mM stock solutions) added to 960 μL of sterile distilled water. Store frozen.

0.5M EDTA, pH 8.0 (1 L).

186.1 g EDTA dissolved in 1 L sterile distilled water, pH adjusted with sodium hydroxide pellets. Sterilised by autoclave. Store at room temperature.

70% Ethanol (100 mL).

70 mL of 100% ethanol mixed with 30 mL sterile distilled water.

90% Ethanol (100 mL).

90 mL of 100% ethanol added to 10 mL of water.

2X Formamide Loading Buffer (10 mL).

9 mL formamide, 160 μL bromophenol blue, 160 μL xylene cyanol, 680 μL sterile distilled water. Store at 4°C.

LB medium.

Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 800 mL of sterile distilled water. Adjust the pH to 7.0 with 1 NaOH. Adjust the volume to 1 litre with sterile distilled water. Sterilise by autoclaving.

PCR Master Mix (15 μL, n=1) fluorescent.

Contains 2 μL of 10X buffer (Quiagen), 1 μL of polioxyethylene, 4 μL of dNTPs (1 mM), 6.96 μL of sterile distelled water, 0.04 μL of HotStartTaq polymerase (Qiagen), and 0.5 μL of each primer (20 μM). the preparation is ready for PCR in a thermal cycler.

PCR Master Mix (10 μL, n=1) non-fluorescent.

Contains 4 μL 5X Red, 2 μL 10X Promega thermophilic buffer, 1.2 μL 25 mM $MgCl_2$, 1.1 μL 2.5 mM dNTPs, 1.6 μL sterile distilled water and 0.08 μL *Taq* polymerase. The *Taq* was added immediately prior to the PCR reaction to avoid denaturation. Can be stored frozen until required without the polymerase enzyme.

Phenol choroform isoamylalcohol (200 mL).

100 mL water saturated phenol mixed with 100 mL chloroform isoamylalcohol mixture. Store at 4°C in light resistant bottle.

8% Polyacrylamide Gel Mix (100 mL).

32 mL SequaGel concentrate, 58 mL SequaGel diluent, and 10 mL SequaGel buffer mixed together.

Post-Hybridisation Wash Solution (1 L).

10 mL 20% SDS, 20 X SSC and 890 mL sterile distilled water mixed together.

Primer Solution (500 μL, 20 μM) for fluorescent PCR.

100 μL of each primer (forward and reverse) mixed with 300 μL of sterile distilled water. Store frozen.

Primer Solution (5 μL, n=1) for non-fluorescent PCR.

0.8 μL of each 6 μM primer (forward and reverse) mixed with 3.4 μL sterile distilled water. Final addition of a few particles of tartrazine to aid identification. Can be stored frozen until required.

5X Red (10 mL).

6.5 g sucrose dissolved in 10 mL sterile distilled water. Addition of 1 μL cresol red for colour. Store at 4°C.

0.4% SDS (500 mL).

2 g SDS powder dissolved in 500 mL sterile distilled water by heating to 68°C in microwave.

10% SDS (1 L).

500 mL 20% SDS mixed with 500 mL sterile distilled water.

3M Sodium Acetate, pH 6.0 (1 L).

408.1 g sodium acetate dissolved in 1 L of sterile distilled water. pH adjusted with glacial acetic acid. Sterilised by autoclave.

1% Sodium Chloride (1 L).

10 g sodium chloride dissolved in 1 L sterile distilled water.

4M Sodium Chloride.

117 g sodium chloride dissolved in 500 mL sterile distilled water. Sterilised by autoclave.

0.4M Sodium Hydroxide (1 L).

16 g sodium hydroxide pellets dissolved with stirring in 1 L sterile distilled water.

2 X SSC (1 L).

100 mL 20 X SSC mixed with 900 mL sterile distilled water.

Suspension Buffer (500 mL).

25 mL 1M Tris (pH 8.0), 100 mL 0.5M EDTA (pH 8.0), and 375 mL sterile distilled water mixed together.

1X TBE (1 L).

100 mL 10X TBE mixed with 900 mL sterile distilled water.

TE (20 mL).

200 µL 1M Tris (pH 8.0), 40 µL 0.5M EDTA (pH 8.0), and 19.76 mL sterile distilled water mixed together.

1M Tris, pH 8.0 (1 L).

121.1 g Tris base dissolved in 1 L sterile distilled water. pH adjusted with concentrated hydrochloric acid. Sterilised by autoclave.

Solution for extraction of DNA from rat tails (20 mL).

1 mL 1M Tris (pH 8.0), 100 mL 0.5M EDTA (pH 8.0), 1 mL 10% SDS and

14 mL sterile distilled water mixed together.

APPENDIX 3.

Chromosome 2 fluorescent microsatellite markers, the different pools constructed to genotype them and the size of each product. 1, forward; 2, reverse primer; F, Fam fluorescent label; H, HEX label; and T, TET label.

Pool 2.1

Marker Number	Name	Sequence	Volume (μL)	Size (bp)
1	*D2Wox13*-**H**	1-CCT CCT CCA GAG CCT TCA	3	182
		2-GAG GAA CAT CCA CTT CAG TCC		
2	*D2Mit3*-**T**	1-ACA GAC AGA CAA GCA AGG AC	1.1	200
		2-CCA AAA TGA GGC TTC TGC AA		
3	*D2Wox19*-**H**	1-CCT TTC TAT GAG GAT GTT CCC	2.3	133
		2-CAC CCC CAG TAC AGA GGA AG		
4	*D2Wox3*-**F**	1-GCC AGC AGG GTT AGA GAG A	1.1	92
		2-CTA AGA AAG AAT ATG TGA AGG TTG		
5	*D2Wox9*-**F**	1-CTG AGG ACC AAT CAT GTT CAC	2.1	146
		2-CCA GGT TAC AGT GAG TTC C		
6	*D2Mit14*-**T**	1-ATG AGA GGT CAA AGC TTC TCA	1.4	102
		2-AGA CCT GGG ACA GGG TCC T		
7	*D2Mit6*-**F**	1-TGT CAA AGG CAG GAA TCA AC	1.4	190
		2-ACC CCT TTT GAG ATA GCG CT		
8	*D2Mgh12*-**T**	1-AAT AAC CAA TAA AGG ACA TGC TCC	1.1	156
		2-AGG AGG AAA AGA GTT GAG TCC C		

Pool 2.2

9	*D2Mit5*-**H**	1-CAG CAG GTG GAA ACA AGT CA	2.7	184
		2-GGG AGG GAT TTG ATG GAG AT		
10	*D2Mit21*-**H**	1-GTT GAG TTG ATC CTC TGG CTG	0.6	156
		2-GGG AGG GAG TGT CTG TCC CA		
11	*D2Wox5*-**T**	1-CAC TGC TTT TCT CAC CAA ACC	1.5	160
		2-CTC TTC TGT TCT CTG TGA GCG		
12	*D2Wox15*-**F**	1-GGT GCT AGT AGA CAA TAG GAT AGA	4.8	145
		2-TTC ATG AGT TTT CAC TGT TTG C		

13	*D2Rat280*-**T**	1-CTG TCC AGC CTC TCA TCC TC 2-GTC CCA TTA TGG CTT CCA TG	0.6	145

Pool 2.3

14	*D2Arb18*-**H**	1-ATA TGC CTT TAG CGG TGG TGG C 2-TCT CGT CCA ACC TAC AGA TGG G	1.8	250
15	*D2Rat14*-**H**	1-AAA CCA AAG TCT GTG GCT CC 2-GCT AAA TGG GAG ACC TGG AA	3	147
16	*D2Rat18*-**F**	1-CTT CTC TTC CTC ACC CTC CC 2-GGA GTG ATC TGT TTC GTA TAA ATG C	1.2	172
17	*D2Rat167*-**F**	1-GGT AAC CTG ACA GAT CAC CTC C 2-TCC AAA TTT TCC TTT TCG TTT T	1.2	215
18	*D2Rat28*-**F**	1-CCA CAT GCC TTT CAG TTT CC 2-GAG TGC AAA GCC CAG TCT TC	1.2	294
19	*D2Rat49*-**F**	1-ATC CCT GGT GAG GGA AAA GT 2-CTT ATT GTA TTT ACA TGT GCA CGC	1.2	127
20	*D2Rat58*-**T**	1-TGA AGA CCT TTC GGT GTG TG 2-ACT GTA TTC CAC CAC CAC CC	1.2	124
21	*D2Mit18*-**T**	1-GGG-GAT-GTT CTG CTG GTA GA 2-TTC CAA TTC TGG AGG ACC TG	1.2	190

Pool 2.4

22	*D2Rat215*-**T**	1-CTT GGC AAC CCT GTG TTC TT 2-GTC TGA ATA TCA CTG CTT CTT TTG	0.5	330
23	*D2Rat29*-**H**	1-GCA CAT CTG TTA CAT ATA AGC ATG C 2-GGG GAT ATC AGA GAA TGA AAC A	2	137
24	*D2Rat216*-**H**	1-TTT TTA GGT CTG GAT ATG TTT GGA 2-AGA GAT GGC TCC AGC AGG TA	1	154
25	*D2Rat43*-**T**	1-CTAACACATGTAACAGATGTTCCAC 2-CCAACCATTTCTTCTGGTCA	0.5	200
26	*D2Rat231*-**F**	1-TTG GTC TTA TTG GGT CTT TCG 2-CCC ACC TAC TCA ACT CCA TGA	1	142
27	*D2Rat241*-**T**	1-CTT ACA GAG GCA CAT GCA CC 2-CCA GAC TGG TCT CAG ACT TCA A	0.5	174
28	*D2Rat287*-**H**	1-TCA TCA GTC CCA GCA ATC CT 2-GGG AAG TAC ATC CTT GAC CTT TT	2	200
29	*D2Rat157*-**F**	1-AGC AGG AAC ATG GAG GAA TG 2-GTC CCA CCA TTG AGG CAT AG	0.5	213
30	*D2Rat12*-**F**	1-GCA ACC ACA TTT TCA GAA TTG A 2-CCA GTC CCT CAG AAG GAA CA	0.5	154

Pool 2.5

31	*D2Rat13*-**T**	1-TGG ACT AGC CCA ACC ATA GC	0.5	132
		2-AAG GGC ATC ATG TTT CCA TC		
32	*D2Wox37*-**T**	1-TGA AAG GGC TAA CTG GGA A	0.5	151
		2-AGG CCA CTG GGA TAC ATA GAC		
33	*D2Rat32*-**H**	1-AACCTACTGACTAAAAACAACAGCA	1	132
		2-GTCTCCACAGTGCACAGCAT		
34	*D2Rat52*-**F**	1-CAC ACA CAA ACC TCC TGA AAA	2	166
		2-TAG GAC TCT ATG CCC ATC CG		
35	*D2Rat237*-**H**	1-CCC TTG TTT GGT TGT ATG TTC A	2	149
		2-CAA ATA ATA GTG CTC CAT GGC TT		

APPENDIX 4.

Background microsatellite markers. 1, forward; 2, reverse primer; Tm, annealing temperature in centigrade degrees.

Marker	Primer Sequences	Tm (°C)	Size (bp)	Mg^{2+} (mM)
D1Mit14	1- TCT GCC TTC TCA CAT GAA CA 2- TTC CAT CTA CTG CTG TTT AGG G	55-50	112	1.5
D1Cebr6	1- TCT TCC TTT TCT AGC ACT CCA 2- TGT AGG TTG TAT TCG TGT ATG C	60-55	141	1.5
D1Wox32	1- GCT ACT GCC TTG CCC TCA 2- TCA CAT TTA CCT GTA GTT GGA A	55-50	188	1.5
D1Mit11	1- ATA AGC CAGCCCCCATTC 2- CCT ACT GAA AGT GAA ATG TCT GG	58-53	275	1.5
D1Mit1	1- GGG GAA GTTTTAGGAAGTCCC 2- AGG GAA TGA AAA TAC AAC ACG C	55-50	146	1.5
D1Wox37	1- TAG TGG GGA CAA TGC TAT CTC 2- ACT CAG ATG TAT AAT TGA CTG ACT G	55-50	146	1.5
D1Mgh5	1- CCT TTG CTC TGA GCC TGG 2- AGA GAA AAA GAA AAG GGA AAA CG	55-50	155	1.5
D1Mgh18	1- ATT TAC AAA CAG GGA AGA AAG TGT 2- CCA ATT CTT CCC AAG AAT AGT CC	55-50	111	1.5
D3Mit4	1- AAA AAA CCA ACC CCC TTC C 2- GCA AAG AGA TGC AAC ATC TGG	63-58	191	1.5
D3Wox14	1- GCA TTG CCT GAG TAG GAT GT 2- GTT TGG CTG TAA TTG GCG	55-50	262	1.5
D3Mgh8	1- GCT GAA CGG ATG CTG AGA G 2- GGG ATT CCT AAG ACA GTT GCC	55-50	156	1.5
D3Mgh16	1- AGT CAG GGC TAT GTA TTG AGA ACC 2- CCT CTG ACC CCT ACA TGG G	55	120	1.5
D3Wox19	1- TTC TAC TTG GGG ATT TCT TGA T 2- ACT TTG ACG TTG AAT GGT TGA	55-50	172	1.5
D3Mgh6	1- CCT TTA CTT CAT CTC CAT TCA AA 2- GCA GAC AAA TGT TCA ATC AAG G	55-50	110	1.5
D4Cebr3	1- GGA GCA TGT GCT TTT AAT ACT T 2- CAG GGG CTG TGG CTG TAA	60-55	100	1.5
D4Mgh16	1- CAG GAG CTG TCT GGG ACT TC 2- GAA CAC TAG AGA AAC TAG GCA GGC	60-55	259	1.5
D4Mgh7	1- GAT CCA GCT CAC ATC TAA TCC C 2- CCA AAT GCT CTT GCA GTC AA	60-55	145	1.5
D4Mit14	1- AGG ACA GGT TTT TGG GCT TT 2- TCT GCC GCC ACC TTA GAG	55-50	150	1.5
D4Mit2	1- TTCTGTATTAACCACAGAAAGAAGC 2- AAGCCAGCCCAAAGTAAATG	55-50	215	1.5
D5Wox14	1- TGG GTA GGT CGT GTC TTC TC 2- CCT GGT TAG AGG AGG GAG TC	55-50	227	1.5
D5Wox15	1- GGT AGA GGT GAG TGG AAT GAA 2- CCT CCT CAG CTC TGC TAG TC	60-55	118	1.5
D5Mgh15	1- CAC CTC GAC CAA CAC CAA C 2- TTA ATC CCA ACT GTG ACA TTC G	60-55	159	1.5

D5Mgh16	1- GCA TAC AGC TTT ACA GTG CTG C 2- AGA CAA GGG ACA TGC TCG AG	60-55	133	1.5
D5Mit8	1- TGA CAG ACA GAC AGG TGC G 2- ATT ACA AAG TTT CCA GGA TGC C	55-50	128	1.5
D5Mgh6	1- GCA TAC AGC TTT ACA GTG CTG C 2- AGA CAA GGG ACA TGC TCG AG	60-55	133	1.5
D5Wox16	1- CAG CCT TCA TTC TCA CAC 2- TGA CTT CTG TGA GCT CCT AC	55-50	160	1.5
D5Mit9	1- CTA CTG GCC GTA GTG TTT GC 2- CCA CTG TGG TTG CTG TTC AG	63-58	127	1.5
D5Wox7	1- TTG GTG GTG CTG CAT CTA TTG 2-CTT AAG GCA ATA TAG GAA ACT AC	55	169	1.0
D5Wox4	1- CCT GCT GTT TCT GAC TCC C 2- AGC ATC CAA GAC TGG GTG C	60-55	157	1.5
D6Wox21	1- TTG AGA AGC GTT AAA ATA TGT G 2- TTG GTT TAC AGG GTG AGA A	55-50	120	1.5
D6Mgh5	1- CAG TTA GCA TAG AAA GCA AAG GG 2- ATA GGA ATA AAG AGT GCA CGT TTG	55-50	104	1.5
D7Mit10	1- TGC CCC AAA AAG GAA AAA C 2- TCA GCT TCA TAC GGA AGC AA	60-55	171	1.5
D7Mit7	1- ACA GCT GGA ATC CTC TGG G 2- GAG CTA GCC ATG CAG GAA AC	60-55	260	1.5
D7Wox27	1- TGT AGG TAT ACA TGC TAT ACA CCG 2- CAT ACT CCT CAT CAC CTA AGA TAG	55-50	189	1.5
D7Mgh5	1- TCC CAA CTC TCC CTT ACC G 2- TGT CTT GGA AGA AAG AAA GAA GG	55-50	229	1.5
D7Mgh1	1. CCC AAT TTG GAA TTT TTT AAA GG 2. CGT AGG CAT CTT TGC ATG AA	55-50	135	1.5
D8Wox22	1- GCA GTG TGA GAG GAA AGT GTC 2- GAA GTC CTC ACC TGT GTT CAG	60-55	181	1.5
D8Mgh7	1- TGA AGA GTT TTA CTG GGT AGC TCC 2- TGG ACC AGG CAA GTT CTC TT	60-55	191	1.5
D8Wox13	1- CAT CTG GGT CTG TGG TAA GG 2- TCT GGG AAG GAC TCT TGG A	60-55	174	1.5
D8Mgh10	1- CTT TGA TAC TGT ACC AAC AGC ACC 2- AAT GTC AGG ATG GCA GAG AC	55	144	1.5
D8Wox16	1- GAA GGG TAC AGT CTG GGA AAG 2- AAG GCT CCT ACT CCA GGT CTA	55-50	208	1.5
D8Mgh1	1- TTG TCT GTA AGT ATG CAC ATG TGG 2- GAT GAG CAG GGG CAT GTC	55-50	153	1.5
D9Mit1	1- GCT TTC AAA CAC CAC AGG GT 2- ACA ACT CCC ATC TCT TGA GAG G	55-50	130	1.5
D9Wox13	1- CCT TTG CGG GGT GTT GTA 2- ACC AAC AAT GCG ACA GAG AAT	55-50	278	1.5
D9Mit4	1- GCA TAA TGG AAG AAG ACA ACT ACC 2- TCC ATG CAT GTG TAT CTG CA	55	200	1.5
D9Mit3	1- TGA GAC TTG TAT TCA CTC CTC CC 2- CTA TCC CTG TCT CTG TGT CTA CCA	55-50	156	1.5
D9Wox18	1- GCC AGA TAT AAG ATG ATT AGT CTG 2- CCACAGTCATTGAGTTATTGGT	55-50	178	1.5
D10Wox3	1- GAA GTC TTC ACT TTT ACT TGT GG 2- GAC CCT TTT GAG AGA ACT TTT G	60	176	1.5
D10Mgh11	1- GGT GTA GGT TCG TCT GTC AGG 2- CGG TGC TCA TAA AAG GGA AA	55-50	140	1.5
D10Wox3	1- GAA GTC TTC ACT TTT ACT TGT GG 2- GAC CCT TTT GAG AGA ACT TTT G	60	176	1.5
D11Mgh6	1- AAC AGT CAA AAG AGA TAT CCA GGG 2- AAA CAA ATG ATG TAC ATG CAT ACA	60-55	100	1.5
D12Wox2	1- TAA CCT CCA AAG GAC CTC TC 2- CTA GAT AAG GTG TAT GTG GCT C	60	194	1.0

D12Mgh3	1- TTC AAC AAC CAC CTC ACT TCC 2- GGA TTG AGG GTG GGG TAA GT	60-55	124	1.5
D12Wox1	1- GAC ATT AAG GGG TCT TCC TAA G 2- TAT CTT TGC AAC GCT GAG G	63-58	401	1.5
D13Mgh1	1- AGA GAA AAT ATG TGG ACA GAA GCC 2- CAC TTC CCA AAT GCT AGC GT	60-55	149	1.5
D13Wox4	1- CCT GGA CAC TAA TCC TAT CTT G 2- GGG TAG GTC TGA GGG AAG G	55	180	1.0
D13Mgh4	1- CAT ATT TCC CCC AAT CCT GT 2- CGG ATC TCC ATA TGT ACT TGT ACA	55-50	143	1.5
D13Mgh16	1- TCA CAG AGA CCA TGG AGC AG 2- GAC CCT TAT TCT CTC CCT CCC	55	220	1.5
D14Wox24	1- AAG CCT GCT CCA CTC CAC 2- GCA GAG GGG AGA GGT AAT AA	60-55	257	1.5
D14Wox5	1- CAG GAA GAG AAA GGG AGT TGG 2- GTC TGA AGT GGT TGT GAG TTC C	63-58	154	1.5
D14Wox8	1- AAG AAT AGC AGT GAA TTG GTG 2- TTC ATC ATC CTT TCA TAA AGG C	60	151	1.0
D14Mgh13	1- CAAGCACACCGTAGTAGAGGG 2- AATGGCTAGCTATCTATTGTGCG	55-50	120	1.5
D15Mit2	1- TAC ATG GAA GCC AAA TGC AA 2- TAC TGA GAA AAT GGG TCT GC	55-50	140	1.5
D15Mgh3	1- AGA CCC AGG GTA GGC ATT TT 2- GAT TAC AAT TCT GTC CAA GTC ACG	55-50	146	1.5
D15Mgh6	1- AGC AGC GGT ATC TCC AGT GT 2- GGG TGA CGG AGC AGA GAA A	55-50	216	1.0
D16Wox12	1- TAG CAG GAT GTT GTA GGT GC 2- CCA GGG TAT TAA GGT CGG AC	60	230	1.0
D16Mit1	1- GGC TTG TGT GGA CAC CTG TA 2- AAA GAG CAG GGA AGA GAC AGG	55-50	164	1.5
D16Wox10	1- AGG CTT TGA TTG CAG GAA G 2- AAA GAG CTG TCG TCC ACA AC	60	118	1.5
D16Wox1	1- CCT GGG AAT TTC ATT CTT GG 2- AGC TGT CCT CTG ACC TTC ACA	60-55	122	1.5
D16Mgh1	1- GAC CTC CAG GAT TGG TGA GA 2- ACA ACC CAT GAG GCA GAC AG	55-50	237	1.5
D17Wox21	1- TAA GGA CCC CTG ATA CTC TGG 2- AGA TCT TTG TCA AAT TCA TGG C	60-55	153	1.5
D17Wox13	1- AGT AGA CAG GAG TGG GAA GGA 2- CTC TTT GGG CAG CTT ACA TT	60	134	1.0
D17Wox10	1- ATC TGT GTG CGA GTG CGT 2- CTG GCG AAG TGA CGT GAG	60	147	1.0
D17Mit3	1- TAA GGT CCC TCC AGA CTC CA 2- TGG GCA GAG AAC AGC AGT C	55-50	186	1.5
D18Wox12	1- CAC ATG TTT ACT TTC TAA GCA TTT G 2- CCC CTC TTC TGG ACT TCA TAG	60	148	1.5
D18Wox16	1- TCA CAA TAA AAA ACT CCT CCA AC 2- AGT CTG TGC CCT GTT CCC A	60-55	152	1.5
D18Wox1	1- CAA ACA ATT CTA AAA CAA AAA GTG 2- CAC CTA CTA CTG ACG GCA GGG A	55-50	101	1.5
D19Wox2	1- GGT ATG GGA GTA ACA TGA CCT C 2- GGA CAC ATA CGG TAA GCA CAT GC	63-58	129	1.5
D19Wox8	1- TGC CCG TCT CTG TTA CTC AT 2- CAA GAA CCC TGA GGC AAT AA	60	111	1.0
D19Mit2	1- AAG GTT GGC AGT TTC CCA G 2- ACC ATT TAT GTG CCC AGA TG	55-50	200	1.5
D20Wox5	1- GAA AAA TAC TTC CAC ACA CTA ATG 2- AAA GTC AAG CCC TGG AGT G	60-55	259	1.0
D20Wox3	1- AGG AAA TGG GTT TCA GTT CC 2- CAG GAT TCT GTG GCA ATC TG	55	125	1.0

DXMit4	1- ACT CCA ACA CCC AGT CAA CC 2- GCC AAA GCA TCT CCC TAT CA	55-50	188	1.5
DXWox3	1- GAT CGT CCA GCA TCG TGG 2- GTT GGT GCT ACT CAA GAT CGG	60	130	1.5

APPENDIX 5.

PCR conditions of microsatellite markers used to construct the genetic map of rat chromosome 2 and to genotype the congenic lines.

Polymorphic marker	Annealing temperature (°C)	Magnesium concentration (mM/L)
D2Wox13	50	1.0
D2Mit3	50	1.5
D2Wox19	55-50	1.75
D2Wox3	60	2.0
D2Wox9	55-50	1.0
D2Mit14	55	1.5
D2Mit6	55	1.5
D2Mgh12	55	1.5
D2Mit5	55	1.5
D2Mit21	55	1.5
D2Wox5	55-50	1.0
D2Wox15	55	1.5
D2Rat280	55	1.5
D2Arb18	60	2.0
D2Rat14	55	1.5
D2Rat18	55	1.5
D2Rat167	55	1.5
D2Rat28	55	1.5
D2Rat49	55	1.5
D2Rat58	55	1.5
D2Mit18	60	1.25
D2Rat215	55	1.5
D2Rat29	55	1.5
D2Rat216	55	1.5
D2Rat43	55	1.5
D2Rat231	55	1.5
D2Rat241	55	1.5
D2Rat287	55	1.5
D2Rat157	55	1.5
D2Rat12	55	1.5
D2Rat13	55	1.5
D2Wox37	55-50	1.0
D2Rat32	55	1.5
D2Rat52	55	1.5
D2Rat15	55	1.5
D2Wox38	55-50	1.0
D2Rat237	55	1.5

APPENDIX 6.

Raw genotypic data of polymorphic markers on rat chromosome 2. 140 F2 animals were studied. Codes are as follows: cross G refers to those F2 hybrids with an SHRSP grandfather; cross H refers to those with a WKY grandfather; H is heterozygote, B SS homosygote; A a WW homozygote; -, indicates no genotype recorded for that animal.

Cross	Sex	*D2Wox19*	*D2Mit21*	*D2Mit6*	*D2Wox15*	*D2Mit3*	*D2Mit5*	*D2Wox13*	*D2Wox3*	*D2Mgh12*	*D2Mit18*	*D2Wox9*	*D2Wox5*	*D2Mit14*	*D2Wox37*	*D2rat287*	*D2Rat241*	*D2Rat280*	*D2Rat216*
	F	H	H	H	H	A	H	H	A	H	-	H	H	-	H	H	H	H	H
G2	F	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
G3	F	A	A	B	B	H	B	H	H	A	B	H	H	A	A	A	A	B	B
G4	F	H	A	A	A	-	A	A	A	A	A	A	A	A	A	A	A	A	A
G5	F	B	B	H	H	H	H	H	H	B	H	H	H	B	B	B	B	H	H
G6	M	H	H	H	H	-	H	H	H	H	H	H	H	H	H	H	H	H	H
G7	F	A	A	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
G8	F	H	H	A	H	-	A	H	H	H	H	H	H	H	H	H	H	H	H
G9	F	A	A	H	H	A	-	H	A	A	H	A	H	A	A	A	A	H	H
G10	M	B	B	H	H	B	H	B	B	H	B	B	H	B	B	B	B	B	B
G11	M	B	B	-	H	B	B	B	B	B	H	B	H	B	B	B	B	H	H
G12	M	H	H	A	A	A	A	A	A	H	-	A	H	H	H	H	H	A	A
G13	M	B	B	B	B	B	B	B	B	B	-	B	B	B	B	B	B	B	B
G14	M	A	A	A	A	H	A	H	H	A	-	A	H	H	A	A	A	A	A
G15	M	B	B	B	B	H	B	B	H	B	-	B	B	B	-	B	B	B	B
G16	M	H	H	B	B	B	B	B	B	H	-	B	B	H	H	H	H	B	B
G17	F	A	A	A	A	A	A	A	A	A	-	A	A	A	A	A	A	A	A
G18	F	B	B	H	H	H	H	H	H	H	-	B	H	H	H	H	H	H	H
G21	M	H	A	A	A	A	A	A	A	A	-	A	H	A	-	A	A	B	A
G22	M	A	A	H	H	A	A	H	A	A	-	A	A	A	A	A	A	H	H
G43	F	B	B	H	B	H	A	H	H	H	-	B	H	H	H	H	H	B	B
G44	F	-	H	B	B	-	-	H	H	B	-	B	H	B	B	B	B	B	B
G26	F	H	B	A	A	A	B	A	A	H	-	B	A	H	H	H	H	A	A
G27	F	B	B	B	B	H	A	H	H	B	-	A	H	B	B	B	B	B	B
G28	F	B	A	H	H	H	B	H	H	A	H	A	H	A	A	A	A	A	A
G30	F	H	A	H	H	H	H	H	-	A	H	H	H	A	A	A	A	H	H
G32	F	A	B	H	H	A	H	A	A	B	H	B	H	B	B	B	B	H	H
G33	F	B	B	H	-	H	H	H	H	B	B	H	H	B	B	B	B	B	B
G34	M	B	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
G35	M	H	H	B	H	B	H	B	B	H	H	B	B	A	A	H	H	H	H
G36	M	H	B	B	B	H	B	H	H	B	B	H	H	B	B	B	B	B	B
G37	M	B	H	A	H	A	B	H	A	H	H	H	A	H	H	H	H	H	H

G39	M	H	H	H	B	H	A	H	H	H	B	A	H	H	H	H	H	B	B
G40	M	H	H	A	A	A	H	A	A	-	A	B	A	H	H	H	H	A	A
G41	M	H	B	B	B	H	A	B	H	B	B	H	B	B	B	B	B	B	B
G45	F	B	H	H	H	H	B	H	H	H	-	H	H	H	-	-	-	-	-
G46	F	H	H	H	H	H	H	H	H	A	H	B	H	A	A	A	A	H	H
G48	M	B	B	B	B	H	H	-	-	B	B	H	H	B	B	B	B	B	B
G49	M	B	B	B	-	B	H	B	B	B	H	A	B	B	B	B	B	H	H
G50	M	H	A	A	-	H	B	H	H	A	A	H	H	A	A	A	A	A	A
G51	F	B	H	H	-	H	H	H	H	-	-	H	H	H	-	-	-	-	-
G52	F	B	B	A	-	H	H	H	H	B	-	H	H	B	B	B	B	H	H
G53	F	B	H	A	-	A	A	A	A	B	A	H	A	H	H	-	H	H	A
G54	M	H	H	H	-	H	A	H	H	H	H	H	H	H	H	H	H	H	H
G55	M	H	A	B	-	B	H	B	B	A	-	H	B	A	-	A	A	H	H
G57	F	B	B	B	-	B	B	B	B	H	-	B	B	B	-	B	B	B	B
G58	F	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
H1	M	H	H	B	B	A	H	A	A	H	B	B	-	H	H	H	H	B	B
H2	M	H	H	A	A	A	A	A	A	A	A	H	H	H	H	H	H	A	A
H3	F	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
H4	M	H	H	A	H	A	A	A	A	H	H	H	H	H	H	H	H	H	H
H5	M	H	H	H	H	B	B	B	B	B	H	H	H	H	H	H	H	H	H
H6	M	A	A	A	A	A	A	A	A	H	A	A	H	A	A	A	A	A	A
H7	M	H	H	A	A	B	H	B	B	A	A	H	B	H	H	H	H	A	A
H8	M	H	H	H	H	H	H	H	H	-	A	H	H	H	H	H	H	H	H
H9	F	-	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
H10	M	H	H	H	H	A	-	A	A	H	H	H	A	H	H	H	H	H	H
H11	M	H	H	B	B	B	B	B	B	H	B	B	B	H	H	H	H	B	B
H12	M	H	H	H	H	H	H	H	H	A	H	H	H	H	H	H	H	H	-
H13	F	H	H	H	H	H	H	H	H	H	H	H	B	H	H	H	H	H	H
H14	F	B	B	H	B	H	H	H	H	B	B	B	H	B	B	H	B	B	B
H41	M	H	H	H	H	H	-	H	-	H	H	H	H	H	H	B	H	H	H
H42	M	B	B	B	B	H	B	H	H	B	B	B	H	-	B	H	B	A	B
H17	F	H	B	H	H	H	H	A	H	H	H	H	H	B	B	B	B	H	H
H18	F	H	H	H	A	H	-	H	H	H	A	A	H	H	H	B	H	A	A
H19	F	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
H20	M	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
H21	M	A	A	H	-	A	A	B	A	A	H	H	A	A	A	H	A	H	H
H22	F	H	H	H	H	H	H	A	H	H	H	-	H	H	H	A	H	H	H
H23	F	H	H	A	A	H	A	H	H	B	A	A	H	B	B	H	B	A	A
H24	F	A	A	A	A	H	A	B	H	-	A	A	H	-	A	B	A	A	A
H25	F	H	H	H	H	H	H	B	H	H	H	H	H	H	H	A	H	H	H
H28	M	H	H	H	H	B	-	B	B	-	-	H	B	H	-	H	H	H	H
H29	M	H	H	H	H	H	-	H	H	H	H	H	H	H	-	H	H	H	H
H30	M	B	B	B	B	B	B	-	B	A	B	B	B	A	A	H	A	B	B
H31	M	A	A	A	A	H	A	H	H	A	A	A	A	A	A	A	A	A	A
H32	F	B	B	H	B	A	H	H	A	B	B	B	H	B	B	A	B	B	B
H33	F	A	A	H	H	H	-	A	H	A	H	H	A	A	A	B	A	H	H
H34	M	A	A	H	H	H	H	H	H	A	H	H	H	A	A	A	B	B	B
H35	F	B	B	H	H	H	H	H	H	B	H	H	-	B	B	B	B	H	H
H36	F	B	B	B	B	B	B	A	B	-	B	B	B	B	B	B	B	B	-
H37	F	B	-	B	B	B	B	B	B	-	B	B	B	B	B	-	-	-	-
H38	F	-	H	A	H	A	-	A	A	-	H	H	A	H	H	H	H	H	-
H39	F	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
H40	F	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	-
H43	M	H	H	H	B	B	B	B	B	H	B	H	B	H	H	H	H	B	B
H44	F	-	H	-	-	-	-	-	H	H	B	B	H	H	H	H	H	B	B

H45	F	-	H	-	-	H	H	-	H	H	H	H	H	-	H	-	-	-	-
H46	F	H	H	-	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
H47	F	H	H	H	H	H	H	H	H	B	H	H	H	H	B	B	B	H	H
H48	M	-	-	A	-	A	-	-	A	H	H	H	A	H	H	H	H	H	H
H49	M	-	H	A	-	H	H	A	H	H	A	H	H	H	H	H	H	A	A
H50	F	H	H	-	A	A	A	A	A	H	A	H	A	A	H	H	H	H	A
H51	F	H	H	-	A	H	A	H	H	H	A	A	H	H	H	H	H	A	A
G59	F	A	A	H	H	H	H	H	H	H	-	A	H	A	-	A	A	H	H
G60	F	A	A	A	A	A	A	A	A	B	-	A	A	A	-	A	A	A	A
G61	F	A	A	A	A	A	A	A	A	H	A	A	A	A	-	-	-	-	-
C 2	F	H	H	B	H	H	B	H	H	H	H	H	H	A	-	-	-	-	-
G64	M	B	B	H	B	-	B	B	-	H	B	B	B	B	-	-	-	-	-
G65	M	H	H	B	B	H	B	-	B	H	-	-	B	H	-	-	-	-	-
G66	M	H	H	H	H	H	H	H	H	H	H	H	H	B	-	-	-	-	-
G67	M	H	H	-	H	H	H	H	H	B	A	H	H	B	-	-	-	-	-
G68	M	B	B	H	H	H	H	H	H	-	H	H	H	B	-	-	-	-	-
G71	M	B	B	B	B	B	B	B	B	A	B	B	B	A	-	-	-	-	-
H52	M	A	A	A	A	-	-	-	A	H	A	A	H	H	-	-	-	-	-
H53	M	H	H	H	H	B	B	B	B	-	H	H	B	H	-	-	-	-	-
H55	F	H	H	A	H	A	-	A	A	A	B	H	A	A	-	-	-	-	-
H56	F	A	A	A	H	A	A	A	A	H	H	H	A	H	-	-	-	-	-
H57	F	H	H	B	B	B	B	B	B	B	B	H	B	H	-	-	-	-	-
H58	F	A	A	H	A	H	H	H	H	-	A	A	H	A	-	-	-	-	-
H59	F	H	A	H	H	A	H	A	A	H	H	A	A	A	-	-	-	-	-
H60	M	A	A	A	A	-	-	H	H	B	A	A	H	A	-	-	-	-	-
H61	M	A	A	H	A	H	-	H	H	H	-	A	H	A	-	-	-	-	-
H62	M	-	-	H	-	-	-	B	B	-	-	H	B	A	-	-	-	-	-
H63	F	H	A	A	A	A	-	A	A	B	-	A	A	H	-	-	-	-	-
H64	F	H	H	H	H	A	A	A	A	H	-	H	A	H	-	-	-	-	-
H65	M	H	H	H	H	A	A	A	A	H	-	H	A	H	-	-	-	-	-
H66	F	H	-	-	-	-	-	H	H	H	-	H	H	H	-	-	-	-	-
H67	F	H	H	A	A	A	A	A	H	A	-	H	A	H	-	-	-	-	-
H68	M	H	H	B	B	B	B	B	B	-	-	B	B	H	-	-	-	-	-
H69	M	H	H	H	H	H	H	A	H	-	-	H	H	B	-	-	-	-	-
H70	M	B	B	B	B	B	H	B	B	-	-	B	H	H	-	-	-	-	-
H71	M	H	A	A	A	H	-	H	H	A	-	H	H	H	-	-	-	-	-
H72	M	H	H	H	H	H	-	A	A	A	-	H	H	B	-	-	-	-	-
H73	M	B	B	H	H	H	H	H	H	A	-	H	H	H	-	-	-	-	-
H80	F	H	H	H	H	B	B	A	B	H	-	H	B	H	-	-	-	-	-
H81	F	H	H	H	H	H	A	H	H	H	-	H	H	H	-	-	-	-	-
H82	F	H	H	A	A	A	A	A	A	H	-	A	A	H	-	-	-	-	-
H88	F	H	H	B	H	B	B	-	B	H	-	H	B	H	-	-	-	-	-
H74	M	H	H	H	H	H	H	H	H	B	-	H	H	H	-	-	-	-	-
H75	F	H	H	A	A	H	A	H	H	H	-	A	H	H	-	-	-	-	-
H76	F	A	A	H	A	H	H	A	H	A	-	A	H	A	-	-	-	-	-
H77	F	A	A	H	-	B	H	H	B	H	-	A	H	A	-	-	-	-	-
H78	F	H	H	A	A	A	A	H	A	B	-	A	A	H	-	-	-	-	-
H79	F	B	B	B	B	H	B	A	H	H	-	B	H	B	-	-	-	-	-
H84	F	A	A	A	A	A	A	H	A	H	-	A	A	-	-	-	-	-	-
H86	F	-	-	B	-	-	B	A	B	B	-	B	B	H	-	-	-	-	-
H91	F	H	H	H	H	A	H	B	A	A	-	H	A	H	-	-	-	-	-
H89	M	H	H	B	H	B	B	A	B	A	-	H	B	H	-	-	-	-	-
H90	M	H	H	-	H	H	B	B	A	A	-	H	H	H	-	-	-	-	-

Cross	D2Rat13	D2Rat15	DRat28	D2Rat12	D2Arb18	D2Wox38	D2Rat14	D2Rat49	D2Rat18	D2Rat58	D2Rat167	D2Rat32	D2Rat237	D2Rat231	D2Rat157	D2Rat215	D2Rat52	D2Rat29	D2Rat43
G1	-	-	-	-	-	-	-	-	-	-	-	H	-	H	H	-	H	H	-
G2	B	B	B	B	B	H	B	B	B	B	B	B	B	B	A	B	B	B	B
G3	H	H	B	H	A	A	H	A	H	A	-	B	A	A	A	B	A	B	A
G4	A	A	A	A	A	A	A	A	A	A	-	H	A	A	A	A	A	A	A
G5	H	H	H	H	B	B	H	B	H	B	H	H	B	B	B	H	B	H	B
G6	H	H	H	H	H	H	H	H	H	B	H	H	H	H	H	H	H	H	H
G7	A	H	B	A	A	A	A	A	H	A	A	A	A	A	A	A	A	A	A
G8	H	H	H	H	H	H	H	A	H	B	A	H	H	H	A	B	H	H	H
G9	A	A	H	A	A	A	A	A	A	A	-	H	A	A	A	H	A	H	A
G10	H	B	B	H	B	B	B	B	B	H	H	H	B	B	H	H	B	B	B
G11	B	B	H	B	B	B	B	B	B	B	H	H	B	B	B	H	B	H	B
G12	-	-	-	-	-	-	-	-	-	-	-	A	H	H	H	A	H	A	-
G13	-	-	-	-	-	-	-	-	-	-	-	B	B	B	B	B	B	B	-
G14	-	-	-	-	-	-	-	-	-	-	-	A	A	A	A	A	A	A	-
G15	-	-	-	-	-	-	-	-	-	-	-	B	B	B	B	-	B	-	-
G16	-	-	-	-	-	-	-	-	-	-	-	B	H	H	H	B	H	B	-
G17	-	-	-	-	-	-	-	-	-	-	-	A	A	A	A	A	A	A	-
G18	-	-	-	-	-	-	-	-	-	-	-	H	H	B	H	H	H	H	-
G21	-	-	-	-	-	-	-	-	-	-	-	A	A	A	A	A	A	A	-
G22	-	-	-	-	-	-	-	-	-	-	-	H	A	A	A	H	A	H	-
G43	-	-	-	-	-	-	-	-	-	-	-	B	H	B	H	B	H	B	-
G44	-	-	-	-	-	-	-	-	-	-	-	B	B	H	B	B	B	B	-
G26	-	-	-	-	-	-	-	-	-	-	-	A	H	B	H	A	H	A	-
G27	-	-	-	-	-	-	-	-	-	-	-	B	B	B	B	B	B	B	-
G28	H	H	H	H	A	A	H	A	H	A	H	H	A	A	A	H	A	H	A
G30	H	H	H	H	A	H	H	A	H	-	H	H	A	A	A	H	A	H	A
G32	A	A	H	A	B	B	A	B	A	H	H	H	B	B	B	H	B	H	B
G33	H	H	B	H	B	B	H	B	H	H	H	H	B	B	B	-	B	B	B
G34	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
G35	B	B	H	B	H	H	B	H	B	H	B	H	H	-	H	H	H	H	H
G36	H	H	B	H	B	B	H	B	H	B	B	B	B	B	B	B	B	B	B
G37	A	A	H	A	H	H	A	H	A	H	A	H	H	H	H	H	H	H	H
G39	H	H	B	H	H	B	H	H	H	H	H	B	H	H	H	B	H	B	H
G40	A	A	A	A	H	B	A	H	A	H	A	A	B	H	H	A	H	A	H
G41	H	A	B	H	B	-	H	B	H	H	B	B	B	B	B	B	B	B	H
G45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G46	H	H	H	B	A	-	H	A	H	A	H	H	A	A	A	-	A	H	H
G48	H	H	B	H	B	-	H	B	H	B	B	B	B	B	B	B	B	B	B
G49	B	B	H	B	B	-	B	B	B	B	B	B	B	B	H	B	B	H	B
G50	B	H	A	B	A	A	H	A	B	A	A	A	A	A	A	A	A	A	A
G51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G52	H	H	H	H	B	B	H	B	H	B	A	A	B	B	B	B	B	H	B
G53	H	A	A	H	H	B	A	H	A	B	A	A	B	H	B	A	H	A	H
G54	H	H	H	H	H	B	H	H	H	B	H	H	H	H	H	H	H	H	H
G55	-	-	-	-	-	-	-	-	-	-	-	H	A	A	A	H	A	H	-
G57	-	-	-	-	-	-	-	-	-	-	-	B	B	B	H	-	B	B	-
G58	A	H	H	A	H	A	H	H	H	B	H	H	H	H	H	H	H	H	H
H1	A	A	A	A	H	H	A	H	A	H	B	B	H	H	A	B	H	B	H
H2	A	A	B	A	H	H	A	B	A	H	A	A	H	H	H	A	H	A	H
H3	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H

H4	A	A	H	A	H	H	A	H	A	B	A	H	H	H	B	H	H	H	-
H5	H	H	H	B	H	H	B	H	B	H	H	H	H	H	H	H	H	H	H
H6	A	H	A	A	A	A	H	A	A	A	A	-	A	A	A	B	A	-	A
H7	B	B	B	H	A	A	B	H	B	H	A	A	H	H	H	B	H	A	H
H8	H	H	H	B	H	H	H	H	H	A	H	H	H	H	H	H	H	H	H
H9	H	H	H	H	H	H	H	-	H	H	H	H	H	H	H	H	H	H	H
H10	A	H	-	A	H	H	H	-	A	H	H	H	H	H	H	H	H	H	H
H11	B	B	B	B	H	H	B	H	B	H	B	B	H	H	H	B	H	B	B
H12	H	H	H	H	H	H	H	H	H	B	H	H	H	H	H	H	H	H	H
H13	H	H	H	H	H	H	H	H	H	H	H	H	A	H	H	H	H	H	H
H14	H	H	B	H	B	-	H	B	H	B	H	-	B	B	B	B	B	B	B
H41	H	A	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
H42	H	H	B	H	B	B	H	B	H	B	H	B	B	B	B	B	B	B	H
H17	H	H	H	H	B	B	H	B	H	B	H	H	B	B	B	H	B	H	H
H18	H	H	B	H	H	H	H	H	H	H	H	A	H	H	H	A	H	A	H
H19	A	A	H	A	H	H	-	H	A	B	H	H	H	H	H	H	H	H	-
H20	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
H21	A	-	H	A	A	-	H	A	A	H	H	H	A	A	B	-	A	H	B
H22	H	H	H	H	H	-	B	H	H	H	H	H	H	H	H	H	H	H	H
H23	H	H	B	H	B	B	-	B	H	B	A	A	B	H	A	A	B	A	H
H24	B	H	B	B	A	A	H	A	B	H	A	A	A	A	B	A	A	A	B
H25	-	H	H	H	H	-	H	H	H	B	H	H	H	H	H	H	H	H	B
H28	-	-	-	-	-	-	-	-	-	-	-	H	H	H	H	H	H	H	-
H29	H	H	H	H	H	H	H	H	H	B	H	H	H	H	H	H	H	H	H
H30	A	B	A	B	H	A	B	A	B	A	B	B	A	B	A	B	A	B	B
H31	H	H	A	H	A	A	H	A	H	A	A	A	A	A	A	A	A	A	-
H32	-	A	A	A	B	B	H	B	A	B	B	B	B	B	H	B	B	B	B
H33	H	H	H	H	A	A	A	A	H	A	-	H	A	A	A	H	A	H	H
H24	B	H	H	-	A	A	A	A	B	A	H	B	B	B	B	B	B	-	H
H35	-	H	H	H	B	B	A	B	H	B	H	H	B	B	B	H	B	H	B
H36	H	H	B	B	B	-	B	B	B	H	H	B	H	B	A	B	H	B	B
H37	H	A	B	B	B	-	B	H	B	B	H	-	-	-	-	-	-	-	B
H38	A	H	H	A	H	A	A	H	A	B	A	H	H	H	B	H	H	H	H
H39	B	H	H	H	H	H	H	H	H	H	B	H	H	H	H	H	H	H	H
H40	H	H	H	B	H	-	-	H	-	H	H	H	H	H	H	H	H	H	H
H43	H	B	B	B	H	H	B	H	H	H	B	B	H	H	H	B	H	B	H
H44	B	B	B	H	H	-	B	H	H	H	B	B	H	H	H	B	H	B	A
H45	B	B	H	H	H	H	B	H	H	H	H	-	-	-	-	-	-	-	H
H46	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
H47	-	H	H	H	H	A	H	B	H	B	H	H	B	H	B	H	B	H	H
H48	A	A	H	A	H	H	A	H	A	H	H	H	-	H	H	H	H	H	H
H49	H	A	A	H	H	H	H	H	H	H	H	A	H	H	H	A	H	A	H
H50	A	H	-	A	H	H	A	H	A	H	A	A	H	H	H	A	H	A	H
H51	H	H	A	H	H	H	H	H	H	H	A	A	H	H	H	A	H	A	H
G59	-	-	-	-	-	-	-	-	-	-	-	H	-	A	A	H	A	H	-
G60	-	A	-	-	B	-	-	-	-	-	-	A	A	A	A	A	A	A	-
G61	A	-	-	A	A	-	A	A	A	A	H	-	-	-	-	-	-	-	A
G62	H	-	-	H	A	A	H	H	A	H	-	-	-	-	-	-	-	-	-
G64	H	-	-	B	B	B	B	B	B	B	B	-	-	-	-	-	-	-	H
G65	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G66	H	H	-	H	H	-	H	-	H	H	H	-	-	-	-	-	-	-	B
G67	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G68	B	H	-	B	B	B	H	B	-	B	H	-	-	-	-	-	-	-	H
G71	B	B	-	B	B	B	B	-	-	B	B	-	-	-	-	-	-	-	H
H52	H	H	-	B	A	A	H	A	H	H	A	-	-	-	-	-	-	-	A

H53	H	H	-	H	H	H	H	H	H	B	H	-	-	-	-	-	-	-	H
H55	B	-	-	H	B	B	B	B	B	A	H	-	-	-	-	-	-	-	B
H56	A	A	-	A	A	-	A	A	A	A	A	-	-	-	-	-	-	-	-
H57	B	B	-	H	H	-	B	H	B	B	H	-	-	-	-	-	-	-	H
H58	H	H	-	H	A	-	H	A	H	A	H	-	-	-	-	-	-	-	A
H59	A	A	-	A	A	A	A	A	A	A	H	-	-	-	-	-	-	-	A
H60	H	H	-	H	A	A	H	A	H	A	A	-	-	-	-	-	-	-	A
H61	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H62	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H63	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H64	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H65	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H66	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H67	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H68	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H70	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H71	-	-	-	-	-	-	-	-	B	-	-	-	-	-	-	-	-	-	-
H72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H73	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H81	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H82	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H88	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H74	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H75	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H76	-	-	-	-	-	-	-	-	H	-	-	-	-	-	-	-	-	-	-
H77	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H78	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H79	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H84	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H86	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H91	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H89	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H90	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

APPENDIX 7.

Raw phenotypic data used to map the BP QTLs during the baseline telemetry period. Codes are as follows: Cross G refers to those F2 hybrids (n = 140) with a SHRSP grandfather; Cross H a WKY grandfather. LV/Bod is the left ventricular weight to body weight ratio; mean SBP, Mean baseline systolic blood pressure; Mean DBP, Mean baseline diastolic blood pressure; Mean PP, Mean baseline pulse pressure.

Cross	Sex	LV/Bod	Mean SBP (mmHg)	Mean DBP (mmHg)	Mean PP (mmHg)
G1	F	4.8	140.529	91.24	49.289
G2	F	2.66	169.981	119.743	50.238
G3	F	2.79	164.097	113.548	50.549
G4	F	2.81	152.583	105.167	47.416
G5	F	2.71	165.155	113.537	51.618
G6	M	2.72	175.056	121.365	53.691
G7	F	3.12	203.779	142.876	60.903
G8	F	2.82	165.54	115.223	50.317
G9	F	2.73	150.992	103.305	47.687
G10	M	2.37	163.951	112.611	51.34
G11	M	2.61	178.064	124.031	54.033
G12	M	3.33	188.875	129.279	59.596
G13	M	3.16	195.794	137.513	58.281
G14	M	3.3	189.094	127.583	61.511
G15	M	3.22	200.162	137.854	62.308
G16	M	2.79	194.039	134.614	59.425
G17	F	3.3	161.892	110.885	51.007
G18	F	3.5	171.188	117.245	53.943
G21	M	3.22	167.955	116.322	51.633
G22	M	2.76	177.665	122.825	54.84
G43	F	3.68	148.515	97.573	50.942
G44	F	3.07	223.01	159.222	63.788
G26	F	3.69	177.619	130.556	47.063
G27	F	2.8	148.149	96.428	51.721
G28	F	4.05	193.332	136.594	56.738
G30	F	2.74	165.606	115.688	49.918
G32	F	2.92	164.57	113.713	50.857
G33	F	3.51	171.58	115.293	56.287
G34	M	2.7	183.223	127.571	55.652
G35	M	2.69	171.942	116.903	55.039
G36	M	3.22	220.776	154.033	66.743
G37	M	3	196.762	135.377	61.385
G39	M	2.77	176.501	120.947	55.554
G40	M	2.6	170.627	115.756	54.871
G41	M	2.65	200.02	140.754	59.266
G45	F	3.66	164.143	111.548	52.595
G46	F	3.33	178.954	122.833	56.121

G48	M	3.14	182.102	122.334	59.768
G49	M	3.08	193.839	129.9	63.939
G50	M	2.58	172.842	120.126	52.716
G51	F	3.4	162.15	109.812	52.338
G52	F	3.43	176.358	117.459	58.899
G53	F	2.73	151.384	104.639	46.745
G54	M	3.27	196.231	134.634	61.597
G55	M	2.79	181.365	122.2	59.165
G57	F	3.06	156.847	108.152	48.695
G58	F	2.91	162.218	110.997	51.221
H1	M	2.4	175.138	116.155	58.983
H2	M	2.66	163.485	109.363	54.122
H3	F	3.1	164.929	112.311	52.618
H4	M	2.42	166.223	116.655	49.568
H5	M	2.64	162.11	111.837	50.273
H6	M	2.99	169.667	115.429	54.238
H7	M	2.7	177.056	124.311	52.745
H8	M	2.52	168.499	117.469	51.03
H9	F	3	163.641	117.987	45.654
H10	M	2.62	170.924	119.264	51.66
H11	M	2.83	194.4	132.619	61.781
H12	M	3.28	174.857	114.641	60.216
H13	F	2.83	153.607	102.79	50.817
H14	F	2.82	162.77	110.228	52.542
H41	M	2.57	166.125	112.044	54.081
H42	M	2.57	175.795	119.43	56.365
H17	F	2.79	163.417	111.344	52.073
H18	F	2.77	161.924	112.928	48.996
H19	F	2.4	146.758	102.109	44.649
H20	M	2.31	161.779	112.84	48.939
H21	M	2.68	189.375	127.667	61.708
H22	F	2.58	152.44	106.008	46.432
H23	F	2.33	139.103	99.83	39.273
H24	F	2.46	153.123	107.029	46.094
H25	F	2.59	149.674	101.538	48.136
H28	M	2.68	172.812	116.788	56.024
H29	M	1.97	168.912	114.672	54.24
H30	M	2.7	179.686	118.398	61.288
H31	M	2.31	167.444	114.588	52.856
H32	F	2.29	165.352	110.368	54.984
H33	F	2.43	151.852	105.494	46.358
H34	M	2.34	165.26	114.598	50.662
H35	F	2.7	153.97	108.864	45.106
H36	F	2.83	172.308	120.128	52.18
H37	F	2.36	162.784	113.349	49.435
H38	F	2.57	154.069	104.574	49.495
H39	F	2.76	158.429	108.678	49.751
H40	F	2.81	175.422	118.955	56.467
H43	M	2.22	156.564	109.059	47.505
H44	F	3.04	165.143	114.003	51.14
H45	F	2.67	161.449	110.438	51.011
H46	F	2.32	160.098	112.843	47.255
H47	F	2.48	166.739	122.047	44.692
H48	M	2.79	142.924	101.536	41.388
H49	M	2.73	182.634	109.728	72.906

H50	F	2.36	143.913	95.709	48.204
H51	F	2.42	157.125	108.221	48.904
G59	F	*	248.267	176.407	71.86
G60	F	3.1	153.734	105.29	48.444
G61	F	3.313	130.495	82.905	47.59
G62	F	2.866	168.27	113.923	54.347
G64	M	2.906	169.076	117.183	51.893
G65	M	3.208	232.824	169.005	63.819
G66	M	2.775	182.906	125.76	57.146
G67	M	2.938	191.575	131.605	59.97
G68	M	2.927	184.447	127.404	57.043
G71	M	3.448	195.156	132.841	62.315
H52	M	2.25	169.574	115.77	53.804
H53	M	2.48	178.854	122.496	56.358
H55	F	2.39	154.118	107.185	46.933
H56	F	2.95	162.815	111.437	51.378
H57	F	2.66	159.821	109.215	50.606
H58	F	2.74	150.137	102.08	48.057
H59	F	2.77	176.087	123.598	52.489
H60	M	2.42	167.897	116.801	51.096
H61	M	2.61	164.32	111.959	52.361
H62	M	2.48	182.776	129.292	53.484
H63	F	2.62	183.297	130.656	52.641
H64	F	2.93	184.825	130.322	54.503
H65	M	2.4	162.673	112.652	50.021
H66	F	2.33	149.872	104.099	45.773
H67	F	2.71	163.227	113.364	49.863
H68	M	2.73	178.693	124.238	54.455
H69	M	3.15	169.048	121.784	47.264
H70	M	2.52	161.442	114.849	46.593
H71	M	2.85	150.024	102.825	47.199
H72	M	2.43	142.993	101.079	41.914
H73	M	2.51	153.985	106.843	47.142
H80	F	2.69	181.118	128.443	52.675
H81	F	3.04	191.857	129.553	62.304
H82	F	2.82	179.667	119.43	60.237
H88	F	2.62	177.049	123.127	53.922
H74	M	2.79	185.923	126.973	58.95
H75	F	2.86	157.866	109.264	48.602
H76	F	2.52	144.867	102.647	42.22
H77	F	2.59	147.605	102.333	45.272
H78	F	2.9	165.105	113.988	51.117
H79	F	2.8	166.289	113.328	52.961
H84	F	2.66	137.874	96.994	40.88
H86	F	2.52	153.019	107.574	45.445
H91	F	1.94873	151.7	104.55	47.15
H89	M	2.60888	163.74	112.19	51.55
H90	M	2.59057	151.7	114.84	36.86

APPENDIX 8.

Raw phenotypic data used to map the BP QTLs during the salt-loaded phase. Codes are as follows: Cross G refers to those F2 hybrids (n = 140) with a SHRSP grandfather; Cross H a WKY grandfather; salt MSBP, mean salt-loaded systolic blood pressure; salt MDBP, mean salt-loaded diastolic blood pressure; salt MPP, mean salt-loaded pulse pressure.

Cross	Sex	Salt MSBP (mmHg)	Salt MDBP (mmHg)	Salt MPP (mmHg)
G1	F	147.93	100.07	47.86
G2	F	173.3	120	53.3
G3	F	168.56	115.73	52.83
G4	F	163.95	112.8	51.15
G5	F	171.57	117.94	53.63
G6	M	181.96	126.13	55.83
G7	F	211.33	146.46	64.87
G8	F	173.61	119.66	53.95
G9	F	154.98	105.26	49.72
G10	M	168.69	116.4	52.29
G11	M	182.11	125.52	56.59
G12	M	194.9	132.74	62.16
G13	M	209.57	146.95	62.62
G14	M	193.91	130.44	63.47
G15	M	196.82	132.08	64.74
G16	M	204.7	141.06	63.64
G17	F	181.66	126	55.66
G18	F	203	147.03	55.97
G21	M	182.6	128.11	54.49
G22	M	183.01	126.24	56.77
G43	F	159.54	104.61	54.93
G44	F	235.43	166.98	68.45
G26	F	178.47	134.1	44.37
G27	F	153.41	105.07	48.34
G28	F	229.25	162.76	66.49
G30	F	165.65	112.18	53.47
G32	F	167.62	112.33	55.29
G33	F	175.99	115.19	60.8
G34	M	193.36	134.83	58.53
G35	M	177.07	120.62	56.45
G36	M	223.34	155.43	67.91
G37	M	199.14	132.27	66.87
G39	M	177.33	119.71	57.62
G40	M	173.83	117.53	56.3
G41	M	206.23	143.5	62.73
G45	F	170.2	114.64	55.56
G46	F	181.56	122.72	58.84
G48	M	199.53	134.46	65.07
G49	M	218.61	147.9	70.71
G50	M	170.99	115.65	55.34

G51	F	173.22	116.54	56.68
G52	F	194.44	131.1	63.34
G53	F	159.68	110.63	49.05
G54	M	244.01	170.6	73.41
G55	M	189.85	127.71	62.14
G57	F	166.2	114.01	52.19
G58	F	162.63	108.55	54.08
H1	M	182.448	121.36	61.088
H2	M	173.9	116.38	57.52
H3	F	170.983	114.61	56.373
H4	M	167.894	116.83	51.064
H5	M	171.023	118.01	53.013
H6	M	174.969	118.2	56.769
H7	M	180.502	125.24	55.262
H8	M	177.21	123.59	*
H9	F	166.44	115	*
H10	M	*	*	*
H11	M	197.24	131.1	66.14
H12	M	180.05	118.27	61.78
H13	F	154.6	99.64	54.96
H14	F	170.43	115.4	55.03
H41	M	171.41	115.38	56.03
H42	M	184.29	126.14	58.15
H17	F	171.58	116.29	55.29
H18	F	171.92	116.88	55.04
H19	F	151.35	105.22	46.13
H20	M	167	116.39	50.61
H21	M	199.74	135.84	63.9
H22	F	160.85	109.01	51.84
H23	F	142.83	99.82	43.01
H24	F	161.88	111.93	49.95
H25	F	158.58	106.15	52.43
H28	M	189.71	129.97	59.74
H29	M	183.31	124.1	59.21
H30	M	196.18	132.32	63.86
H31	M	181.19	125.53	55.66
H32	F	169.3	112.14	57.16
H33	F	163.61	112.33	51.28
H34	M	170.37	117.78	52.59
H35	F	164.09	114.46	49.63
H36	F	180.34	124.34	56
H37	F	168.41	117.22	51.19
H38	F	159.35	108.25	51.1
H39	F	163.24	109.41	53.83
H40	F	187.25	126.51	60.74
H43	M	171.48	116.49	54.99
H44	F	170.51	115.89	54.62
H45	F	169.07	115.24	53.83
H46	F	157.02	107.12	49.9
H47	F	168.8	120.5	48.3
H48	M	148.13	103.54	44.59
H49	M	189.18	113.11	76.07
H50	F	147.89	99.01	48.88
H51	F	157.84	107.68	50.16
G59	F	279.44	208.54	70.9

G60	F	165.24	113.75	51.49
G61	F	140.8	88.89	51.91
G62	F	169.88	113.32	56.56
G64	M	176.35	121.12	55.23
G65	M	234.68	169.95	64.73
G66	M	187.52	130.55	56.97
G67	M	205.54	143.4	62.14
G68	M	192.62	135.62	57
G71	M	209.08	143.05	66.03
H52	M	170.12	115.17	54.95
H53	M	183.13	123.7	59.43
H55	F	157.99	106.39	51.6
H56	F	160.72	105.85	54.87
H57	F	161.68	109.37	52.31
H58	F	149.04	98.2	50.84
H59	F	170.84	115.6	55.24
H60	M	172.21	118.8	53.41
H61	M	174.57	119.1	55.47
H62	M	185.04	130.09	54.95
H63	F	195.45	136.94	58.51
H64	F	180.21	123.68	56.53
H65	M	172.51	119.77	52.74
H66	F	154.33	106.87	47.46
H67	F	162.85	111.92	50.93
H68	M	187.56	130.49	57.07
H69	M	174.53	123.98	50.55
H70	M	163.64	113.47	50.17
H71	M	159.64	108.61	51.03
H72	M	160.68	114.12	46.56
H73	M	160.14	108.87	51.27
H80	F	188.28	131.87	56.41
H81	F	208.65	140.74	67.91
H82	F	185.9	120.91	64.99
H88	F	194.02	134.96	59.06
H74	M	197.82	136.73	61.09
H75	F	165.66	113.89	51.77
H76	F	148.57	99.68	48.89
H77	F	150.9	101.94	48.96
H78	F	166.16	111.04	55.12
H79	F	183.02	124.58	58.44
H84	F	141.48	96.49	44.99
H86	F	156.51	106.03	50.48
H91	F	156.05	106.43	49.62
H89	M	172.27	118.99	53.28
H90	M	172.92	118.58	54.34

APPENDIX 9.

Weakly systolic blood pressure values (SBP) obtained by radio-telemetry for each animal in mmHg. First weekly value corresponds to the night-time period, second to the day-time.

Weeks	WKY males				WKY females		
Animal number	A1283	A1284	A1287	A1529	A1274	A1276	A1534
0.5	147.046	111.94	143.197	139.171	134.175	133.935	124.458
1	143.461	108.618	138.177	133.262	129.499	128.877	120.944
1.5	141.477	107.906	136.662	130.037	129.288	127.931	118.467
2	137.51	103.81	135.259	125.283	125.618	123.797	115.086
2.5	140.585	107.709	143.059	132.994	132.746	131.389	120.403
3	137.83	104.652	137.982	127.185	128.874	127.357	116.298
3.5	144.565	113.208	146.235	127.254	131.207	131.652	121.181
4	141.18	105.774	138.991	124.34	127.595	125.886	117.098
4.5	145.134	111.337	146.048	126.773	132.504	130.874	121.493
5	142.19	107.872	139.853	124.311	128.907	127.304	117.342
5.5	149.279	114.086	149.222	128.384	132.477	133.981	125.074
6	144.605	110.424	143.806	126.175	128.424	128.465	120.793
6.5	155.069	121.086	156.149	134.321	137.926	138.392	130.407
7	149.774	116.052	147.306	131.431	134.345	132.039	122.669
7.5	156.371	122.62	157.055	137.14	142.027	141.879	133.914
8	150.299	114.536	147.326	130.02	136.895	134.52	127.229
8.5	159.862	122.514	161.142	142.044	145.556	144.606	137.204
9	151.781	113.768	148.136	136.173	137.628	136.332	127.005

Weeks	SHRSP males			SHRSP females		
Animal number	C2138	C2207	C2414	C2152	C2153	C2154
0.5	179.815	181.75	177.485	171.81	161.098	163.989
1	172.869	174.566	171.657	165.243	154.065	156.628
1.5	176.943	177.929	179.137	168.246	157.962	160.944
2	172.11	174.894	172.023	162.989	155.045	158.695
2.5	179.047	185.254	182.552	171.304	160.391	167.511
3	174.05	180.768	173.082	164.974	156.414	164.919
3.5	183.054	186.777	184.397	172.304	159.162	166.07
4	178.502	180.395	175.149	168.104	155.827	163.95
4.5	185.807	190.32	184.669	174.635	159.4	168.043
5	181.452	187.339	174.477	168.053	155.803	162.008
5.5	192.904	196.682	193.002	179.973	163.133	169.661
6	186.372	191.17	183.54	170.757	158.833	163.537
6.5	207.057	222.24	213.083	186.042	173.488	181.904
7	192.637	202.194	198.903	175.635	164.462	171.486
7.5	214.635	242.431	236.855	193.303	178.155	188.579
8	195.457	218.464	213.224	181.238	166.764	174.374
8.5	229.268	263.657	253.717	197.737	179.517	192.29
9	207.715	236.04	229.444	185.253	169.783	177.645

Weeks		WKY.SP.Gla2c males			WKY.SP.Gla2c female		
	Animal number	T560	T561	T562	T564	T566	T606
0.5		148.927	146.248	150.059	137.179	150.67	145.4723
1		147.415	142.47	147.754	134.64	147.076	139.5349
1.5		151.268	143.365	145.766	130.483	145.135	130.1527
2		149.213	141.095	142.486	129.308	141.527	126.8109
2.5		154.87	144.929	142.336	137.554	147.955	134.1203
3		153.969	145.218	141.646	135.784	143.334	130.8156
3.5		160.192	151.629	151.142	134.925	149.139	136.2796
4		156.788	146.698	147.388	128.397	145.413	131.9939
4.5		161.468	148.624	151.041	134.931	148.958	128.8065
5		160.244	148.305	147.549	131.003	144.339	126.7282
5.5		163.304	149.545	149.633	131.808	147.142	
6		161.313	147.661	147.096	128.201	141.923	
6.5		171.319	158.746	157.958	140.956	157.079	
7		162.411	150.433	150.919	130.719	149.252	
7.5		173.215	160.023	159.924	139.568	158.727	
8		165.207	153.756	153.082	130.536	151.27	
8.5		167.395	160.184	157.189	142.167	158.257	
9		163.134	153.882	151.321	131.714	152.103	

Weeks		WKY.SP.Gla2d males			WKY.SP.Gla2d female		
	Animal number	T497	T499	T555	T556	T544	T545
0.5		149.881	143.4	149.42	133.031	111.448	127.882
1		143.345	136.749	144.025	127.449	110.01	123.808
1.5		140.583	139.567	140.436	125.357	124.928	122.984
2		138.706	134.443	136.82	121.061	120.991	118.769
2.5		145.234	141.68	140.305	129.967	121.794	119.439
3		143.689	136.296	136.558	124.044	115.94	117.49
3.5		145.353	137.084	136.97	131.289	92.268	131.045
4		141.513	133.645	133.391	124.626	96.524	123.396
4.5		147.296	139.643	138.618	132.311	86.528	128.05
5		145.902	134.771	135.454	126.879	85.776	121.818
5.5		149.252	140.814	139.351	133.459	97.3	124.632
6		146.316	135.857	136.648	127.011	96.198	119.282
6.5		159.168	151.656	152.456	142.824	115.613	133.226
7		151.74	142.623	144.839	133.761	102.325	125.154
7.5		161.905	152.524	155.464	144.364	114.797	135.731
8		149.786	143.799	145.235	132.879	105.217	124.513
8.5		160.698	152.239	158.488	141.505	114.219	132.072
9		150.935	143.645	146.583	131.802	103.392	122.659

APPENDIX 10.

Weakly diastolic blood pressure values (DBP) obtained by radio-telemetry for each animal in mmHg. First weekly value corresponds to the night-time period, second to the day-time.

Weeks	WKY males				WKY females		
Animal number	A1283	A1284	A1287	A1529	A1274	A1276	A1534
0.5	108.6	81.9622	104.47	101.13	97.703	100.361	86.7993
1	105.15	78.1718	100.047	93.372	91.461	94.151	81.405
1.5	103.262	74.5851	98.611	95.54	93.067	94.437	84.4154
2	97.005	71.7017	95.983	89.926	88.238	89.614	78.4877
2.5	101.929	75.2942	103.231	97.223	94.409	96.334	85.752
3	97.663	72.5379	97.798	91.651	89.64	91.106	79.7141
3.5	104.286	77.2682	105.328	93.365	93.43	96.623	85.6406
4	100.792	72.6786	98.002	88.15	88.117	90.468	79.9379
4.5	104.354	77.8258	105.106	91.908	94.767	95.76	85.5741
5	100.859	73.7265	98.066	87.615	90.045	90.871	80.8817
5.5	108.422	79.8955	107.496	95.043	94.073	98.443	88.0033
6	102.637	76.117	101.148	91.149	90.039	92.054	82.7091
6.5	111.097	84.5416	112.772	99.55	98.358	100.757	93.0674
7	106.028	79.331	103.079	94.337	93.471	93.399	83.0957
7.5	112.41	84.2526	117.391	110.162	100.443	103.145	94.9137
8	106.113	78.2465	102.829	99.836	94.295	95.397	85.5916
8.5	115	84.5111	116.055	106.374	101.77	104.304	96.5599
9	107.307	76.1556	102.276	97.324	93.561	95.34	83.9907

Weeks	SHRSP males			SHRSP females		
Animal number	C2138	C2207	C2414	C2152	C2153	C2154
0.5	130.527	130.933	119.127	126.409	118.413	120.158
1	124.935	126.1	111.476	117.724	110.087	111.743
1.5	124.471	123.899	121.434	121.223	114.714	116.923
2	120.064	121.153	111.387	112.882	108.893	113.611
2.5	125.485	128.839	125.387	122.878	115.523	122.287
3	122.009	125.408	114.126	113.782	108.785	117.972
3.5	129.25	128.995	127.83	123.306	114.386	120.956
4	126.262	124.961	117.514	115.836	107.387	114.997
4.5	131.134	134.176	131.626	124.675	113.38	120.486
5	129.478	129.292	119.251	116.786	107.912	110.61
5.5	137.542	138.618	138.681	129.27	116.212	121.815
6	133.18	133.612	125.847	117.236	110.247	110.947
6.5	150.313	161.786	158.861	133.832	125.488	131.31
7	136.838	143.755	144.296	120.308	112.836	116.241
7.5	157.56	177.718	174.854	140.973	128.066	138.476
8	137.566	157.773	157.811	125.859	114.458	119.697
8.5	167.59	196.246	189.867	144.782	128.574	140.473
9	148.239	178.454	168.286	128.335	117.276	121.42

Weeks		WKY.SP.Gla2c males			WKY.SP.Gla2c female		
	Animal number	T560	T561	T562	T564	T566	T606
0.5		114.379	112.1	105.86	92.8543	107.423	101.13
1		108.892	104.709	102.093	86.682	102.463	93.372
1.5		112.425	108.092	102.309	89.7384	105.445	95.54
2		107.875	102.665	98.245	85.9484	98.59	89.926
2.5		115.194	108.431	101.53	96.708	107.146	97.223
3		113.018	105.204	98.103	90.4111	99.113	91.651
3.5		118.526	110.831	103.543	96.1869	107.763	93.365
4		112.787	105.137	99.429	88.6314	100.007	88.15
4.5		116.778	108.892	102.942	93.5169	107.933	91.908
5		113.526	103.883	98.694	86.7681	99.954	87.615
5.5		118.056	111.814	101.207	93.0533	110.322	95.043
6		114.997	106.081	98.383	84.9679	101.827	91.149
6.5		123.233	119.888	107.822	99.7063	115.58	99.55
7		118.556	108.392	99.872	87.5914	104.22	94.337
7.5		129.23	123.191	108.691	98.9097	114.53	110.162
8		118.98	111.004	99.795	87.563	102.993	99.836
8.5		128.202	123.722	107.229	98.9437	114.382	106.374
9		120.789	114.503	99.88	86.9147	103.31	97.324

Weeks		WKY.SP.Gla2d males			WKY.SP.Gla2d female		
	Animal number	T497	T499	T555	T556	T544	T545
0.5		108.151	102.835	110.216	91.947	86.7571	90.315
1		99.159	94.685	104.962	84.925	83.1253	84.608
1.5		97.628	97.249	100.538	87.48	91.0444	84.328
2		93.54	90.118	95.858	81.717	86.0274	80.066
2.5		100.772	98.673	99.076	90.396	93.873	87.477
3		97.921	93.23	94.573	83.506	89.8827	81.064
3.5		100.93	94.128	96.181	90.862	68.5974	101.892
4		95.316	90.242	92.315	82.305	74.4593	92.134
4.5		103.599	96.723	97.707	91.977	63.9839	97.854
5		98.175	90.955	94.165	84.718	64.873	88.751
5.5		104.5	97.876	98.211	93.437	67.7033	91.539
6		97.419	91.836	95.702	84.755	67.8129	85.965
6.5		112.432	104.374	107.996	99.905	78.951	100.856
7		98.748	96.034	99.845	87.382	68.8234	87.366
7.5		110.86	104.773	109.323	100.791	77.577	102.174
8		98.41	95.91	98.615	85.776	69.5073	87.815
8.5		111.707	104.679	112.034	97.684	76.5854	102.285
9		97.936	95.674	98.925	84.1	68.0933	89.264

APPENDIX 11.

Body weight, heart weight, left ventricle plus septum weight, heart weight-body weight ratio, and left ventricle plus septum-body weight ratio for each congenic animal.

WKY MALES

	A1283	A1284	A1287	A1529
BW (g)	446	432	414	340
HW (mg)	1.45	1.44	1.38	1.08
LV+S (mg)	1.01	0.97	0.96	0.74
HW/BW (mg/g)	3.32	3.4	3.41	3.26
LV+S/BW (mg/g)	2.31	2.29	2.37	2.24

WKY FEMALES

	A1274	A1275	A1276	A1534
BW (g)	231	222	242	240
HW (mg)	0.87	0.76	0.83	0.86
LV+S (mg)	0.63	0.53	0.6	0.58
HW/BW (mg/g)	3.92	3.57	3.56	3.72
LV+S/BW (mg/g)	2.84	2.49	2.58	2.51

SHRSP MALES

	C2138	C2207	C2414
BW (g)	274	316	236
HW (mg)	1.22	1.28	1.09
LV+S (mg)	0.91	1.04	0.82
HW/BW (mg/g)	4.6	4.17	4.61
LV+S/BW (mg/g)	3.43	3.39	3.47

SHRSP FEMALES

	C2152	C2153	C2154
BW (g)	206	204	226
HW (mg)	0.9	0.94	0.99
LV+S (mg)	0.72	0.73	0.78
HW/BW (mg/g)	4.57	4.95	4.56
LV+S/BW (mg/g)	3.65	3.84	3.59

SP.WKYGla2a MALES

	N555	N765	N863
BW (g)	316	352	342
HW (mg)	1.27	1.44	1.32
LV+S (mg)	0.93	1.16	1.03
HW/BW (mg/g)	4.14	4.19	3.96
LV+S/BW (mg/g)	3.19	3.38	3.09

SP.WKYGla2a FEMALES

	N783	N784	N946

BW (g)	214	192	206
HW (mg)	0.97	0.83	0.81
LV+S (mg)	0.74	0.68	0.56
HW/BW (mg/g)	4.73	4.54	4.11
LV+S/BW (mg/g)	3.61	3.72	2.84

SP.WKYGla2b MALES

	N811	N812	N813
BW (g)	324	234	216
HW (mg)	1.57	1.17	1.13
LV+S (mg)	1.32	0.99	0.86
HW/BW (mg/g)	4.98	5.2	5.45
LV+S/BW (mg/g)	4.19	4.4	4.15

SP.WKYGla2b FEMALES

	N816	N817	N818
BW (g)	192	202	174
HW (mg)	0.81	0.84	0.75
LV+S (mg)	0.64	0.62	0.57
HW/BW (mg/g)	4.43	4.35	4.55
LV+S/BW (mg/g)	3.49	3.21	3.45

WKY.SPGla2c MALES

	T560	T561	T562
BW (g)	334	316	308
HW (mg)	1.08	1.02	1.09
LV+S (mg)	0.78	0.74	0.79
HW/BW (mg/g)	3.32	3.32	3.65
LV+S/BW (mg/g)	2.4	2.41	2.64

WKY.SPGla2c FEMALES

	T564	T565	T566	T606
BW (g)	208	210	216	214
HW (mg)	0.78	0.77	0.85	0.78
LV+S (mg)	0.55	0.56	0.66	0.56
HW/BW (mg/g)	3.92	3.83	4.11	3.8
LV+S/BW (mg/g)	2.76	2.79	3.19	2.73

WKY.SPGla2d MALES

	T487	T497	T499	T555
BW (g)	324	344	374	368
HW (mg)	1.25	1.25	1.12	1.21
LV+S (mg)	1.03	0.95	0.82	0.9
HW/BW (mg/g)	3.96	3.73	3.07	3.37
LV+S/BW (mg/g)	3.27	2.84	2.25	2.51

WKY.SPGla2d FEMALES

	T556	T544	T545
BW (g)	234	250	248
HW (mg)	0.89	0.93	0.89
LV+S (mg)	0.66	0.72	0.61
HW/BW (mg/g)	3.96	3.86	3.72
LV+S/BW (mg/g)	2.93	2.99	2.55

APPENDIX 12.

For the analysis of radiation hybrid mapping data many different methods have been proposed, currently there are four main software packages that have been used to construct complete maps from the data: RHMAP (Boehnke *et al.* 1991; Lunetta *et al.* 1995b; Lunetta *et al.* 1996a), RHMAPPER (Stein, 1996) SAMAPPER (Stewart *et al.* 1997) and MultiMap (Matise *et al.* 1994).

RHMAP uses a combination of minimising the obligate number of breaks required to explain the observed retention patterns together with maximum-likelihood retention analysis. RHMAPPER uses a Markov model applied to genetic analysis to obtain optimal position for the loci on a linear map. SAMAPPER and MultiMap use a combination of these approaches. Each of these methods models the retention of different chromosomal fragments in the different clones comprising a radiation hybrid panel. The parameters in these models are the breakage frequencies between all pairs of markers, and the retention frequencies of different fragments (which may depend on chromosomal location), which are adjusted to maximise the likelihood of the data in the context of the current model. Also different models of fragment retention can be specified, such as equal retention, centromeric retention, and the left end-point model.

RHMAP was used to analyse our data, this package is easy accessible from the web site http://www.sph.umich.edu/group/statgen/software in both Unix

and PC based implementations, and is accompanied with a complete manual available on line. We used the RHMAP package program v 3.0 to carry out the analysis. This is a statistical package for multipoint radiation hybrid mapping analysis developed by Michael Boehnke (Boehnke *et al.* 1991). It consists of a set of three programs in Fortran 77 that provides the means for a complete statistical analysis of radiation hybrid mapping data.

A.1 MATHEMATICAL ASSUMPTIONS IN THE RHMAP SOFTWARE.

In the early papers on radiation hybrid methodology, the way to produce an estimated distance between two loci was the method of moments (Cox *et al.* 1990). This method assumed independent retention of fragments and random breakage along the chromosome, thus allowing breakage to be modelled as a Poisson process. The breakage probability θ for a given interval can be converted to an additive distance D by the formula $D= -\ln(1 - \theta)$, in analogy to Haldane's no interference mapping function (Haldane, 1919). The resulting units of distance for D are called Rays. Retention should be highest for markers close to the centromere, intermediate for markers close to the telomere, and least for mid arm markers, since retained markers must be incorporated into a hybrid rearrangement that includes a centromere and usually one telomere per arm.

To order loci along the chromosome, Cox *et al* (1990) chose the order that minimised the sum of the distance estimates between adjacent linked loci in

the map. Having chosen an order, they estimated the local support for this order by comparing likelihood for the four-locus orders in which the internal two loci are interchanged. For this purpose, they calculated likelihood for four-locus orders at the parameter estimates obtained from the various two analyses.

For the analysis of radiation hybrids there are two sets of crucial parameters that need to be estimated to determine the efficiency of a radiation hybrid panel: the probability of retention P and the breakage probability θ.

A.1.1 PROBABILITY OF RETENTION.

The probability of retention P is defined as the probability that a DNA fragment is present in a radiation hybrid clone (Cox *et al.* 1990). P is also the fraction of radiation hybrid clones that contains a specific DNA fragments as different radiation hybrid clones contain different pieces of the chromosomes. Finally, P is the average proportion of the target chromosome present in any radiation hybrid clones. P is not under experimental control but is a function of the radiation dose and the specific donor-recipient cell line used to prepare the panel. The value of P varies from 30% to 50% depending on the panel and the marker used and some studies show increased retention frequencies for markers close to the centromere (Lawrence *et al.* 1991). For any collection of marker loci, the observed retention frequencies may vary from locus to locus. These fluctuations in P may be random and not statistically significant, may appear to be random

and yet be statistically significant or may display a pattern such as higher retention values near the centromere (Cox *et al.* 1990). Although the reasons for such variation are currently unknown, it is suspected that retention of some specific DNA segments may lead to differential survival of radiation hybrid clones.

The analysis of radiation hybrid mapping data is performed under two major assumptions. Firstly, the different segments of DNA within a radiation hybrid clone obtained by X-ray irradiation are lost or retained independently of one another. However, non-random retention has been observed but the effects noted to date were small and did not appear to cause incorrect orders of loci to bias estimates of distances. Secondly, it is assumed that P is constant along the chromosome (Cox *et al.* 1990; Gorski *et al.* 1992).

A.1.2 BREAKAGE PROBABILITY AND DISTANCE CALCULATIONS.

The breakage probability or θ is defined as the probability that two marker loci are separated by one or more X-irradiation-induced breaks. The θ value represents an estimate of distance between two marker loci because as the physical distance between two marker loci increases, the probability of breakage increases. For completely linked loci $\theta = 0$, whereas for unlinked loci $\theta = 1$. The term linkage in the context of radiation hybrid data analysis is an operational term referring to physical linkage and is unrelated to meiotic recombination (Cox *et al.* 1990).

In contrast to P, θ is partially under experimental control, as it depends on the donor-recipient cell line used and on how much X-irradiation is adsorbed. The distance estimates between loci are a function of the radiation dose used for generating the radiation hybrid panel, so θ can be increased by increasing the radiation dose (Richard *et al.* 1991; Burmeister *et al.* 1991).

The breakage probability is a poor measure of distance for two reasons. Firstly, θ is a measure of probability and not an additive measure of distance. Secondly, θ distinguishes between no breaks and multiple breaks but it does not provide any information on the actual number of breaks between two marker loci because any radiation hybrid clone could have none, one, two or more breaks between two markers. The average number of breaks between two markers is a measure of distance and it is calculated assuming that the number of breaks is determined by a homogeneous Poisson process having the average w, so that:

$$1 - \theta = e^{-w} \text{ and}$$

$$w = - \ln(1 - \theta)$$

The sum of all w values over all adjacent mapped intervals is its length for an entire chromosome or a region studied, and this is interpreted as the average number of breaks for the entire segment studied.

A.1.3 LOCI ORDERING METHODS.

One of the limitations of early radiation hybrid methods was that different panels had to be prepared for each chromosome, and the mathematical

models applied were developed for single chromosome hybrid panels. The algorithms in RHMAP allow working with whole genome radiation hybrid panels. The advantage of using whole genome panels as originally proposed by Goss and Harris (Goss and Harris, 1975) is that a single panel of hybrids can be used to map all the chromosomes. In spite of the fact that multiple copies of a chromosome per clone obscures fragment retention patterns, diploid radiation hybrids provide other advantages over haploid hybrids besides ease of generation. For instances, the mapping of closely spaced loci requires fragments of small average size. Such fragments may have low retention rates in cells. Using diploid clones increases the effective retention rate per clone (Lange *et al.* 1995).

Any ordering strategy for radiation hybrid data is necessarily complex. A fundamental barrier is the shear number of orders that must be considered; for N loci, this number is either $N!/2$ or $N!$, depending on the symmetry and the model employed (Lange *et al.* 1995). This rapidly becomes impractical as N gets large; if N = 14, the number of locus orders is more than 43 billions.

It is well-established rule that two loci that are close on the same chromosome are less likely to have a break between them than the two loci that are far apart. Thus, close loci will tend to be retained or lost together, whereas distant loci will be independently retained or lost. The models used in RHMAP are based on six assumptions. First, the markers to be mapped are linearly arranged along a given chromosome. Second, each clone

contains fragments derived from c copies of this chromosome. The values c=1 and c=2 correspond to haploid and diploid hybrids, respectively. Third, it is assumed that the breaks caused by radiation along any chromosome occur according to a Poisson process. These Poisson processes are independent from chromosome to chromosome and identically distributed on homologous chromosomes. Fourth, fragments within a clone are retained and lost independently. Different fragments can be retained with different rates, but the retention processes are again independent and identically distributed from chromosome to chromosome. Fifth, breakage and retention operate independently of each other. Sixth, only the presence and not the number of markers in a clone can be detected at any locus or marker.

RHMAP has two alternative ordering methods for radiation hybrid mapping data that make use of the information on many loci simultaneously, including information on partially typed hybrids. The first of these multipoint methods is nonparametric.

Since the closer two loci are on a chromosome, the less likely it is that a break will occur between them, a reasonable ordering strategy is to minimise the number of obligate chromosomal breaks implied by the mapping data. If the number of obligate chromosome breaks per clone is summarised over all clones, then the resulting sum serves as a criterion for comparing the current order to other orders. This approach (minimum-breaks) is analogous to minimising the number of recombinants to infer order in genetic linkage mapping (Thompson, 1987). The advantage of the

minimum breaks criterion is that it depends on almost no assumptions about how breaks occur and fragments are retained (Lange *et al.* 1995).

The minimum break method is attractive because of its intuitive logic, its lack of restrictive assumptions, and its straightforward computation. However, this method provides neither estimates of distances between loci nor a comparison of relative likelihood for comparing locus orders.

Use of the second method of loci ordering is therefore required to provide estimates of the distances between adjacent loci and the relative likelihoods of the different orders under various models. For this maximum-likelihood approach RHMAP considers a variety of models for fragment retention. These models range in complexity, from assuming that all retention probabilities are equal to assuming that all retention probabilities may differ. Each maximum-likelihood model depends on the assumption of independent fragment retention and random chromosome breakage along the chromosome (Boehnke *et al.* 1991).

The minimum-breaks and the maximum-likelihood methods may be used separately as distinct approaches to identify the best locus order. Alternatively, the minimum-breaks method can provide a preliminary list of candidate orders for evaluation by the computationally more intensive maximum-likelihood method.

A.1.4 STRATEGIES TO DETERMINE THE BEST CANDIDATE ORDER USING THE MINIMUM BREAKAGE APPROACH OR THE MAXIMUM LIKELIHOOD METHOD.

Considering all the possible orders in 14 different loci is impossible for mathematical calculations using the minimum breakage approach or the more intensive maximum likelihood method. Thus, alternatives or strategies are required, and in RHMAP three are proposed to analyse the radiation hybrid mapping data.

A.1.4.1 BRANCH AND BOUND STRATEGY.

This is an approach to systematically eliminate large numbers of non-optimal solutions to a problem, without considering each solution in detail (Nijenhuis and Wilf, 1978). This is achieved by identifying early in the process a candidate solution that is optimal or nearly so and then eliminating solutions that are inferior either to the candidate solution or to a better solution encountered subsequently.

In the specific case of radiation hybrid mapping data, an order is constructed using one locus at a time, and when a partial locus order needs more breaks than the current best complete order. Then all complete orders consistent with the partial order are eliminated. A list of orders that differs from the current best by K or fewer breaks is kept. The criterion used is the minimum number of obligate breaks and this never decreases as loci are added.

To generate good initial candidate orders, branch and bound uses a "greedy" algorithm (Goodman and Hedetniemi, 1977). Begining with any of the N(N-1)/2 locus pairs, the next locus to add to the current partial locus order is determined by examining each unplaced locus and each possible position for it. The optimal position for an unplaced locus is the position that requires the smallest increase in the number of obligate chromosome breaks. The unplaced locus with the greatest difference between the mean number of breaks required by addition at non optimal positions and the number of breaks required by addition at its optimal position is then added at its optimal position; ties are broken randomly. Alternatively, the unplaced locus with the greatest difference between the number of breaks required by addition at its optimal position and the number of breaks required by addition at its next best position could be added at its optimal position. The purpose of this algorithm is to add at each stage that locus having strongest support for its optimal position.

A.1.4.2 STEPWISE LOCUS ORDERING.

The branch and bound strategy allows elimination of several orders. If the number of loci is more than 14 this strategy is impractical because the number of orders evaluated may scale exponentially. The alternative is to build orders, one locus at a time but to keep under consideration only those partial orders that are within K breaks of the current best partial order. When a partial order of the same length as the current best partial order is eliminated from consideration, all complete orders descended from it are

also eliminated. This approach considers many fewer partial orders (compared with branch and bound) at some risk of missing the overall best order. Taking larger values of the constant K increases the chance of finding the best order but also increases the computational work. The result of stepwise ordering will be a list of orders, which should include the best order or orders. Choosing at each step to add that locus whose position is most strongly supported by the data, and/or beginning with a framework map of well-placed loci, increases the probability of finding the best order(s).

A.1.4.3 SIMULATED ANNEALING.

This approach is motivated by the analogy of crystal formation in a cooling liquid. When cooled slowly, the molecules of a liquid settle into the minimum energy state for that system. If cooling is fast, the minimum energy state may not be reached; instead, the system ends up in a polycrystalline or amorphous state of somewhat greater energy (Kirkpatrick *et al.* 1983; Press *et al.* 1989). To make this simulation, the N!/2 locus orders are identified with the states of a non-stationary Markov Chain (Karlin and Taylor, 1975). The possible transitions for the Markov chain are block inversions of the current locus order. For example, in the state corresponding to locus order 1-2-3-4-5-6-7-8-9-10, the block 5-6-7-8 can be inverted to yield the new order 1-2-3-4-8-7-6-5-9-10.

The essence of simulated annealing is that, early on in the analysis, steps leading to an increase in energy (number of obligate breaks) are often taken. This protects against prematurely being trapped in a local minimum. Later steps converge to the presumable global minimum.

BIBLIOGRAPHY

Abel, K.J., Boehnke, M., Prahalad, M., Ho, P., Flejter, W.L., Watkins, M., VanderStoep, J., Chandrasekharappa, S.C., Collins, F.S. and Glover, T.W. (1993) A radiation hybrid map of the BRCA1 region of chromosome 17q12-q21. *Genomics.* **17**, 632-641.

Abriel, H., Loffing, J., Rebhun, J.F., Pratt, J.H., Schild, L., Horisberger, J.D., Rotin, D. and Staub, O. (1999) Defective regulation of the epithelial Na+ channel by Nedd4 in Liddle's syndrome. *J Clin Invest* **103**, 667-673.

Adams, R.J., Schwartz, A., Grupp, G., Grupp, I., Lee, S.W., Wallick, E.T., Powell, T., Twist, V.W. and Gathiram, P. (1982) High-affinity ouabain binding site and low-dose positive inotropic effect in rat myocardium. *Nature* **296**, 167-169.

Aitman, T.J., Glazier, A.M., Wallace, C.A., Cooper, L.D., Norsworthy, P.J., Wahid, F.N., Al-Majali, K.M., Trembling, P.M., Mann, C.J., Shoulders, C.C., Graf, D., St.Lezin, E., Kurtz, T.W., Kren, V., Pravenec, M., Ibrahimi, A., Abumrad, N.A., Stanton, L.W. and Scott, J. (1999) Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. *Nat Genet* **21**, 76-83.

Aitman, T.J., Gotoda, T., Evans, A.L., Imrie, H., Heath, K.E., Trembling, P.M., Truman, H., Wallace, C.A., Rahman, A., Dore, C., Flint, J., Kren, V., Zidek, V., Kurtz, T.W., Pravenec, M. and Scott, J. (1997) Quantitative trait loci for cellular defects in glucose and fatty acid metabolism in hypertensive rats. *Nat Genet* **16**, 197-201.

Al-Majali, K.M., Glazier, A.M., Norsworthy, P.J., Wahid, F.N., Cooper, L.D., Wallace, C.A., Scott, J., Lausen, B. and Aitman, T.J. (1999) A high-resolution radiation hybrid map of the proximal region of rat chromosome 4. *Mamm Genome* **10**, 471-476.

Alam, K.Y., Wang, Y., Dene, H. and Rapp, J.P. (1993) Renin gene nucleotide sequence of coding and regulatory regions in Dahl rats. *Clin Exp Hypertens* **15**, 599-614.

Alam, S., Liyou, N., Davis, D., Tresillian, M. and Johnson, A.G. (2000) The 460Trp polymorphism of the human alpha-adducin gene is not associated with isolated systolic hypertension in elderly Australian Caucasians. *J Hum Hypertens* **14**, 199-203.

Annest, J.L., Sing, C.F., Biron, P. and Mongeau, J.G. (1979) Familial aggregation of blood pressure and weight in adoptive families. I. Comparisons of blood pressure and weight statistics among families with

adopted, natural, or both natural and adopted children. *Am J Epidemiol* **110**, 479-491.

Argentin, S., Nemer, M., Drouin, J., Scott, G.K., Kennedy, B.P. and Davies, P.L. (1985) The gene for rat atrial natriuretic factor. *J Biol Chem* **260**, 4568-4571.

Arriza, J.L., Weinberger, C., Cerelli, G., Glaser, T.M., Handelin, B.L., Housman, D.E. and Evans, R.M. (1987) Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science* **237**, 268-275.

Atwood, L.D., Kammerer, C.M., Samollow, P.B., Hixson, J.E., Shade, R.E. and MacCluer, J.W. (1997) Linkage of essential hypertension to the angiotensinogen locus in Mexican Americans. *Hypertension.* **30**, 326-330.

Bahring, S., Nagai, T., Toka, H.R., Nitz, I., Toka, O., Aydin, A., Muhl, A., Wienker, T.F., Schuster, H. and Luft, F.C. (1997) Deletion at 12p in a Japanese child with brachydactyly overlaps the assigned locus of brachydactyly with hypertension in a Turkish family. *Am J Hum Genet* **60**, 732-735.

Baima, J., Nicolaou, M., Schwartz, F., DeStefano, A.L., Manolis, A., Gavras, I., Laffer, C., Elijovich, F., Farrer, L., Baldwin, C.T. and Gavras, H. (1999) Evidence for linkage between essential hypertension and a putative locus on human chromosome 17. *Hypertension.* **34**, 4-7.

Barbry, P. (1999) The amiloride-sensitive epithelial sodium channel: structure and function of a key molecule for salt homeostasis. In: Dominiczak, A.F., Connell, J.M. and Soubrier, F., (Eds.) *Molecular genetics of hypertension*, pp. 149-174. Oxford: BIOS Scientific Publishers Ltd.

Barlassina, C., Norton, G.R., Samani, N.J., Woodwiss, A.J., Candy, G.C., Radevski, I., Citterio, L., Bianchi, G. and Cusi, D. (2000) Alpha-adducin polymorphism in hypertensives of South African ancestry. *Am J Hypertens* **13**, 719-723.

Barley, J., Carter, N.D., Cruickshank, J.K., Jeffery, S., Smith, A., Charlett, A. and Webb, D.J. (1991) Renin and atrial natriuretic peptide restriction fragment length polymorphisms: association with ethnicity and blood pressure. *J Hypertens* **9**, 993-996.

Bazil, M.K., Krulan, C. and Webb, R.L. (1993) Telemetric monitoring of cardiovascular parameters in conscious spontaneously hypertensive rats. *J Cardiovasc Pharmacol* **22**, 897-905.

Beckman, J.S. and Weber, J.L. (1992) Survey of human and rat microsatellites. *Genomics.* **12**, 627-631.

Behringer, R. (1998) Supersonic congenics? *Nat Genet* **18**, 108

Beige, J., Hohenbleicher, H., Distler, A. and Sharma, A.M. (1999) G-Protein beta3 subunit C825T variant and ambulatory blood pressure in essential hypertension. *Hypertension.* **33**, 1049-1051.

Ben-Ishay, D., Saliternik, R. and Welner, A. (1972) Separation of two strains of rats with inbred dissimilar sensitivity to Doca-salt hypertension. *Experientia* **28**, 1321-1322.

Bender, K., Adams, M., Baverstock, P.R., den Bieman, M., Bissbort, S., Brdicka, R., Butcher, G.W., Cramer, D.V., von Deimling, O. and Festing, M.F. (1984) Biochemical markers in inbred strains of the rat (Rattus norvegicus). *Immunogenetics* **19**, 257-266.

Bettinelli, A., Bianchetti, M.G., Girardin, E., Caringella, A., Cecconi, M., Appiani, A.C., Pavanello, L., Gastaldi, R., Isimbaldi, C. and Lama, G. (1992) Use of calcium excretion values to distinguish two forms of primary renal tubular hypokalemic alkalosis: Bartter and Gitelman syndromes. *J Pediatr* **120**, 38-43.

Bianchi, G., Fox, U., Di Francesco, G.F., Giovanetti, A.M. and Pagetti, D. (1974) Blood pressure changes produced by kidney cross-transplantation between spontaneously hypertensive rats and normotensive rats. *Clin Sci Mol Med* **47**, 435-448.

Bianchi, G., Tripodi, G., Casari, G., Salardi, S., Barber, B.R., Garcia, R., Leoni, P., Torielli, L., Cusi, D. and Ferrandi, M. (1994) Two point mutations within the adducin genes are involved in blood pressure variation. *Proc Natl Acad Sci U S A* **91**, 3999-4003.

Bihoreau, M.T., Gauguier, D., Kato, N., Hyne, G., Lindpaintner, K., Rapp, J.P., James, M.R. and Lathrop, G.M. (1997) A linkage map of the rat genome derived from three F2 crosses. *Genome Res* **7**, 434-440.

Biron, P., Mongeau, J.G. and Bertrand, D. (1976) Familial aggregation of blood pressure in 558 adopted children. *Can Med Assoc J* **115**, 773-774.

Blanco, G. and Mercer, R.W. (1998) Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *Am J Physiol* **275**, F633-F650

Blaustein, M.P. and Hamlyn, J.M. (1984) Sodium transport inhibition, cell calcium, and hypertension. The natriuretic hormone/Na+-Ca2+ exchange/hypertension hypothesis. *Am J Med* **77**, 45-59.

Boehnke, M., Lange, K. and Cox, D.R. (1991) Statistical methods for multipoint radiation hybrid mapping. *Am J Hum Genet* **49**, 1174-1188.

Bongiovanni, A.M., Eberlein, W.R., Goldman, A.S. and New, M. (1967) Disorders of adrenal steroid biogenesis. *Recent Prog Horm Res* **23**, 375-449.

Bonnardeaux, A., Davies, E., Jeunemaitre, X., Fery, I., Charru, A., Clauser, E., Tiret, L., Cambien, F., Corvol, P. and Soubrier, F. (1994) Angiotensin II type 1 receptor gene polymorphisms in human essential hypertension. *Hypertension.* **24**, 63-69.

Bonnardeaux, A., Nadaud, S., Charru, A., Jeunemaitre, X., Corvol, P. and Soubrier, F. (1995) Lack of evidence for linkage of the endothelial cell nitric oxide synthase gene to essential hypertension. *Circulation.* **91**, 96-102.

Borg, E. and Viberg, A. (1980) Role of heating in non-invasive blood pressure measurements in rats. *Acta Physiol Scand* **108**, 73-75.

Botero-Velez, M., Curtis, J.J. and Warnock, D.G. (1994) Brief report: Liddle's syndrome revisited--a disorder of sodium reabsorption in the distal tubule. *N Engl J Med* **330**, 178-181.

Brand, E., Chatelain, N., Keavney, B., Caulfield, M., Citterio, L., Connell, J., Grobbee, D., Schmidt, S., Schunkert, H., Schuster, H., Sharma, A.M. and Soubrier, F. (1998) Evaluation of the angiotensinogen locus in human essential hypertension: a European study. *Hypertension.* **31**, 725-729.

Brand, E., Herrmann, S.M., Nicaud, V., Ruidavets, J.B., Evans, A., Arveiler, D., Luc, G., Plouin, P.F., Tiret, L. and Cambien, F. (1999) The 825C/T polymorphism of the G-protein subunit beta3 is not related to hypertension. *Hypertension.* **33**, 1175-1178.

Brockway, B.P. and Hassler, C.R. (1993) Application of radiotelemetry to cardiovascular measurements in pharmacology and toxicology. In: Salem, H. and Baskin, S.I., (Eds.) *New technologies and concepts for reducing drug toxicities.*, pp. 109-131. Ann Arbor: CRC Press.

Brockway, B.P., Mills, P.A. and Azar, S.H. (1991) A new method for continuous chronic measurement and recording of blood pressure, heart rate and activity in the rat via radio-telemetry. *Clin Exp Hypertens* **13**, 885-895.

Broeckel, U., Shiozawa, M., Kissebah, A.H., Provoost, A.P. and Jacob, H.J. (1998) Susceptibility genes for end-organ damage. New strategies to understand diabetic and hypertensive nephropathy. *Nephrol Dial Transplant* **13**, 840-842.

Brown, D.M., Matise, T.C., Koike, G., Simon, J.S., Winer, E.S., Zangen, S., McLaughlin, M.G., Shiozawa, M., Atkinson, O.S., Hudson, J.R.J., Chakravarti, A., Lander, E.S. and Jacob, H.J. (1998) An integrated genetic linkage map of the laboratory rat. *Mamm Genome* **9**, 521-530.

Brown, D.M., Provoost, A.P., Daly, M., Lander, E.S. and Jacob, H.J. (1996) Renal disease suceptibility and hypertension are under independent genetic control in the fawn-hooded rat. *Nat Genet* **12**, 44-51.

Bruner, C.A., Myers, J.H., Sing, C.F., Jokelainen, P.T. and Webb, R.C. (1986) Genetic association of hypertension and vascular changes in stroke-prone spontaneously hypertensive rats. *Hypertension.* **8**, 904-910.

Bruner, C.A., Myers, J.H., Sing, C.F., Jokelainen, P.T. and Webb, R.C. (1986) Genetic basis for altered vascular responses to ouabain and potassium-free solution in hypertension. *Am J Physiol* **251**, H1276-H1282

Bunag, R.D. (1983) Facts and fallacies about measuring blood pressure in rats. *Clin Exp Hypertens* **5**, 1659-1681.

Burmeister, M., Kim, S., Price, E.R., de Lange, T., Tantravahi, U., Myers, R.M. and Cox, D.R. (1991) A map of the distal region of the long arm of human chromosome 21 constructed by radiation hybrid mapping and pulsed-field gel electrophoresis. *Genomics.* **9**, 19-30.

Byrom, F.B. and Wilson, C.A. (1938) A plethysmographic method for measuring blood pressure in the rat. *J Physiol* **93**, 301-304.

Cai, L., Schalkwyk, L.C., Schoeberlein-Stehli, A., Zee, R.Y., Smith, A., Haaf, T., Georges, M., Lehrach, H. and Lindpaintner, K. (1997) Construction and characterization of a 10-genome equivalent yeast artificial chromosome library for the laboratory rat, Rattus norvegicus. *Genomics.* **39**, 385-392.

Canessa, C.M., Schild, L., Buell, G., Thorens, B., Gautschi, I., Horisberger, J.D. and Rossier, B.C. (1994) Amiloride-sensitive epithelial Na+ channel is made of three homologous subunits. *Nature* **367**, 463-467.

Casto, R. and Printz, M.P. (1988) Genetic transmission of hyper-responsivity in crosses between spontaneously hypertensive and Wistar-Kyoto rats. *J Hypertens Suppl* **6**, S52-S54

Caulfield, M., Lavender, P., Farrall, M., Munroe, P., Lawson, M., Turner, P. and Clark, A.J. (1994) Linkage of the angiotensinogen gene to essential hypertension. *N Engl J Med* **330**, 1629-1633.

Caulfield, M., Lavender, P., Newell-Price, J., Farrall, M., Kamdar, S., Daniel, H., Lawson, M., De Freitas, P., Fogarty, P. and Clark, A.J. (1995) Linkage of the angiotensinogen gene locus to human essential hypertension in African Caribbeans. *J Clin Invest* **96**, 687-692.

Chang, S.S., Grunder, S., Hanukoglu, A., Rosler, A., Mathew, P.M., Hanukoglu, I., Schild, L., Lu, Y., Shimkets, R.A., Nelson-Williams, C., Rossier, B.C. and Lifton, R.P. (1996) Mutations in subunits of the epithelial sodium channel cause salt wasting with hyperkalaemic acidosis, pseudohypoaldosteronism type 1. *Nat Genet* **12**, 248-253.

Chua, S.C., Szabo, P., Vitek, A., Grzeschik, K.H., John, M. and White, P.C. (1987) Cloning of cDNA encoding steroid 11 beta-hydroxylase (P450c11). *Proc Natl Acad Sci U S A* **84**, 7193-7197.

Cicila, G.T., Choi, C., Dene, H., Lee, S.J. and Rapp, J.P. (1999) Two blood pressure/cardiac mass quantitative trait loci on chromosome 3 in dahl rats. *Mamm Genome* **10**, 112-116.

Cicila, G.T., Dukhanina, O.I., Kurtz, T.W., Walder, R., Garrett, M.R., Dene, H. and Rapp, J.P. (1997) Blood pressure and survival of a chromosome 7 congenic strain bred from Dahl rats. *Mamm Genome* **8**, 896-902.

Cicila, G.T., Rapp, J.P., Wang, J.M., St.Lezin, E., Ng, S.C. and Kurtz, T.W. (1993) Linkage of 11 beta-hydroxylase mutations with altered steroid biosynthesis and blood pressure in the Dahl rat. *Nat Genet* **3**, 346-353.

Clark, J.S., Jeffs, B., Davidson, A.O., Lee, W.K., Anderson, N.H., Bihoreau, M.T., Brosnan, M.J., Devlin, A.M., Kelman, A.W., Lindpaintner, K. and Dominiczak, A.F. (1996) Quantitative trait loci in genetically hypertensive rats. Possible sex specificity. *Hypertension.* **28**, 898-906.

Colhoun, H. (1999) Confirmation needed for genes for hypertension. *Lancet* **353**, 1200-1201.

Collison, M., Glazier, A.M., Graham, D., Morton, J.J., Dominiczak, M.H., Aitman, T.J., Connel, J., Gould, G.W. and Dominiczak, A.F. (2000) *Cd36* molecular mechanisms of insulin resistance in the stroke prone spontaneously hypertensive rat. *Diabetes* -In press

Conrad, C.H., Brooks, W.W., Robinson, K.G. and Bing, O.H. (1991) Impaired myocardial function in spontaneously hypertensive rats with heart failure. *Am J Physiol* **260**, H136-H145

Corvol, P. and Jeunemaitre, X. (1997) Molecular genetics of human hypertension: role of angiotensinogen. *Endocr Rev* **18**, 662-677.

Corvol, P., Persu, A., Gimenez-Roqueplo, A.P. and Jeunemaitre, X. (1999) Seven lessons from two candidate genes in human essential hypertension: angiotensinogen and epithelial sodium channel. *Hypertension.* **33**, 1324-1331.

Cox, D.R., Burmeister, M., Price, E.R., Kim, S. and Myers, R.M. (1990) Radiation hybrid mapping: a somatic cell genetic method for constructing high-resolution maps of mammalian chromosomes. *Science* **250**, 245-250.

Crambert, G., Hasler, U., Beggah, A.T., Yu, C., Modyanov, N.N., Horisberger, J.D., Lelievre, L. and Geering, K. (2000) Transport and pharmacological properties of nine different human Na, K-ATPase isozymes. *J Biol Chem* **275**, 1976-1986.

Cusi, D., Barlassina, C., Azzani, T., Casari, G., Citterio, L., Devoto, M., Glorioso, N., Lanzani, C., Manunta, P., Righetti, M., Rivera, R., Stella, P., Troffa, C., Zagato, L. and Bianchi, G. (1997) Polymorphisms of alpha-adducin and salt sensitivity in patients with essential hypertension. *Lancet* **349**, 1353-1357.

Dahl, L.K., Heine, M. and Tassinari, L. (1962) Role of genetic factors in susceptibility to experimental hypertension due to chronic excess of salt ingestion. *Nature* **194**, 480-482.

Darvasi, A. (1998) Experimental strategies for the genetic dissection of complex traits in animal models. *Nat Genet* **18**, 19-24.

Darvasi, A., Weinreb, A., Minke, V., Weller, J.I. and Soller, M. (1993) Detecting marker-QTL linkage and estimating QTL gene effect and map location using a saturated genetic map. *Genetics.* **134**, 943-951.

Dave-Sharma, S., Wilson, R.C., Harbison, M.D., Newfield, R., Azar, M.R., Krozowski, Z.S., Funder, J.W., Shackleton, C.H., Bradlow, H.L., Wei, J.Q., Hertecant, J., Moran, A., Neiberger, R.E., Balfe, J.W., Fattah, A., Daneman, D., Akkurt, H.I., De Santis, C. and New, M.I. (1998) Examination of genotype and phenotype relationships in 14 patients with apparent mineralocorticoid excess. *J Clin Endocrinol Metab* **83**, 2244-2254.

Davidson, A.O., Schork, N.J., Jaques, B.C., Kelman, A.W., Sutcliffe, R.G., Reid, J.L. and Dominiczak, A. (1995) Blood pressure in genetically hypertensive rats. Influence of the Y chromosome. *Hypertension.* **26**, 452-459.

Deboo, G.J. and Fryer, T.B. (1965) Miniature biopotential telemetry system. *Am J Med Elect* **4**, 138-145.

Deng, A.Y., Dene, H. and Rapp, J.P. (1994) Mapping of a quantitative trait loci for blood pressure on rat chromosome 2. *J Clin Invest* **94**, 431-436.

Deng, A.Y., Dene, H. and Rapp, J.P. (1997) Congenic strains for the blood pressure quantitative trait locus on rat chromosome 2. *Hypertension.* **30**, 199-202.

Deng, A.Y. and Rapp, J.P. (1995) Locus for the inducible, but not a constitutive, nitric oxide synthase cosegregates with blood pressure in the Dahl salt-sensitive rat. *J Clin Invest* **95**, 2170-2177.

Deng, A.Y., Rapp, J.P., Kato, H. and Bihoreau, M.T. (1995) Linkage mapping of the neuronal nitric oxide synthase gene (Nos1) to rat chromosome 12. *Mamm Genome* **6**, 824

Deng, Y. and Rapp, J.P. (1992) Cosegregation of blood pressure with angiotensin converting enzyme and atrial natriuretic peptide receptor genes using Dahl salt-sensitive rats. *Nat Genet* **1**, 267-272.

Dietrich, W.F., Miller, J., Steen, R., Merchant, M.A., Damron-Boles, D., Husain, Z., Dredge, R., Daly, M.J., Ingalls, K.A. and O'Connor, T.J. (1996) A comprehensive genetic map of the mouse genome. *Nature* **380**, 149-152.

Dluhy, R.G. and Lifton, R.P. (1995) Glucocorticoid-remediable aldosteronism (GRA): diagnosis, variability of phenotype and regulation of potassium homeostasis. *Steroids* **60**, 48-51.

Dominiczak, A.F., Clark, J.S., Jeffs, B., Anderson, N.H., Negrin, C.D., Lee, W.K. and Brosnan, M.J. (1998) Genetics of experimental hypertension. *J Hypertens* **16**, 1859-1869.

Dominiczak, A.F., Jeffs, B. and Connell, J.M. (1998) New genetic concepts in hypertensive cardiovascular disease. *Curr Opin Cardiol* **13**, 304-311.

Dominiczak, A.F., Lazar, D.F., Das, A.K. and Bohr, D.F. (1991) Lipid bilayer in genetic hypertension. *Hypertension.* **18**, 748-757.

Dominiczak, A.F. and Lindpaintner, K. (1994) Genetics of hypertension: a current appraisal. *News in Physiological Science* 246-251.

Dominiczak, A.F., Negrin, D.C., Clark, J.S., Brosnan, M.J., McBride, M.W. and Alexander, M.Y. (2000) Genes and hypertension: from gene mapping

in experimental models to vascular gene transfer strategies. *Hypertension.* **35**, 164-172.

Dubay, C., Vincent, M., Samani, N.J., Hilbert, P., Kaiser, M.A., Beressi, J.P., Kotelevtsev, Y., Beckmann, J.S., Soubrier, F. and Sassard, J. (1993) Genetic determinants of diastolic and pulse pressure map to different loci in Lyon hypertensive rats. *Nat Genet* **3**, 354-357.

Dukhanina, O.I., Dene, H., Deng, A.Y., Choi, C.R., Hoebee, B. and Rapp, J.P. (1997) Linkage map and congenic strains to localize blood pressure QTL on rat chromosome 10. *Mamm Genome* **8**, 229-235.

Dupont, J., Dupont, J.C., Froment, A., Milon, H. and Vincent, M. (1973) Selection of three strains of rats with spontaneously different levels of blood pressure. *Biomedicine* **19**, 36-41.

Duyk, G.M., Kim, S.W., Myers, R.M. and Cox, D.R. (1990) Exon trapping: a genetic screen to identify candidate transcribed sequences in cloned mammalian genomic DNA. *Proc Natl Acad Sci U S A* **87**, 8995-8999.

Ewing, B. and Green, P. (2000) Analysis of expressed sequence tags indicates 35,000 human genes. *Nat Genet* **25**, 232-234.

Falconer, D.S. (1989) *Introduction to quantitative trait genetics*, Essex U.K.: Longman, Scientific and Technical.

Fasola, A.F., Martz, B.L. and Helmer, O.M. (1966) Renin activity during supine exercise in normotensives and hypertensives. *J Appl Physiol* **21**, 1709-1712.

Feinleib, M., Garrison, R.J., Fabsitz, R., Christian, J.C., Hrubec, Z., Borhani, N.O., Kannel, W.B., Rosenman, R., Schwartz, J.T. and Wagner, J.O. (1977) The NHLBI twin study of cardiovascular disease risk factors: methodology and summary of results. *Am J Epidemiol* **106**, 284-285.

Ferrari, P., Ferrandi, M., Torielli, L., Canessa, M. and Bianchi, G. (1987) Relationship between erythrocyte volume and sodium transport in the Milan hypertensive rat and age-dependent changes. *J Hypertens* **5**, 199-206.

Fornage, M., Amos, C.I., Kardia, S., Sing, C.F., Turner, S.T. and Boerwinkle, E. (1998) Variation in the region of the angiotensin-converting enzyme gene influences interindividual differences in blood pressure levels in young white males. *Circulation.* **97** , 1773-1779.

Franklin, D., Watson, N.M. and Van Clitters, R.S. (1964) Blood velocity telemetered from untethered animals. *Nature* **203**, 528-532.

Frantz, S.A., Kaiser, M., Gardiner, S.M., Gauguier, D., Vincent, M., Thompson, J.R., Bennett, T. and Samani, N.J. (1998) Successful isolation of a rat chromosome 1 blood pressure quantitative trait locus in reciprocal congenic strains. *Hypertension.* **32**, 639-646.

Frazer, K.A., Boehnke, M., Budarf, M.L., Wolff, R.K., Emanuel, B.S., Myers, R.M. and Cox, D.R. (1992) A radiation hybrid map of the region on human chromosome 22 containing the neurofibromatosis type 2 locus. *Genomics.* **14**, 574-584.

Frohlich, E.D. (1986) Is the spontaneously hypertensive rat a model for human hypertension? *J Hypertens Suppl* **4**, S15-S19

Frossard, P.M., Obineche, E.N., Elshahat, Y.I. and Lestringant, G.G. (1997) Deletion polymorphism in the angiotensin-converting enzyme gene is not associated with hypertension in a Gulf Arab population. *Clin Genet* **51**, 211-213.

Fujisawa, T., Ikegami, H., Yamato, E., Hamada, Y., Kamide, K., Rakugi, H., Higaki, J., Murakami, H., Shimamoto, K. and Ogihara, T. (1997) Trp64Arg mutation of beta3-adrenergic receptor in essential hypertension: insulin resistance and the adrenergic system. *Am J Hypertens* **10**, 101-105.

Furspan, P.B., Jokelainen, P.T., Sing, C.F. and Bohr, D.F. (1987) Genetic relationship between a lymphocyte membrane abnormality and blood pressure in spontaneously hypertensive stroke prone and Wistar-Kyoto rats. *J Hypertens* **5**, 293-297.

Ganguly, A., Weinberger, M.H., Guthrie, G.P. and Fineberg, N.S. (1984) Adrenal steroid responses to ACTH in glucocorticoid-suppressible aldosteronism. *Hypertension.* **6**, 563-567.

Garrett, M.R., Dene, H., Walder, R., Zhang, Q.Y., Cicila, G.T., Assadnia, S., Deng, A.Y. and Rapp, J.P. (1998) Genome scan and congenic strains for blood pressure QTL using dahl salt- sensitive rats. *Genome Res* **8**, 711-723.

Gauguier, D., Kaisaki, P., Rouard, M., Wallis, R.H., Browne, J., Rapp, J. and Bihoreau, M.T. (1999) A gene map of the rat derived from linkage analysis and related regions in mouse and human genomics. *Mamm Genome* **10**, 675-686.

Geering, K. (1997) Na,K-ATPase. *Curr Opin Nephrol Hypertens* **6**, 434-439.

Geller, D.S., Rodriguez-Soriano, J., Vallo, B.A., Schifter, S., Bayer, M., Chang, S.S. and Lifton, R.P. (1998) Mutations in the mineralocorticoid

receptor gene cause autosomal dominant pseudohypoaldosteronism type I. *Nat Genet* **19**, 279-281.

Gitelman, H.J., Graham, J.B. and Welt, L.G. (1966) A new familial disorder characterized by hypokalemia and hypomagnesemia. *Trans Assoc Am Physicians* **79**, 221-235.

Goldblatt, H., Lynch, J. and Hanzal, R.F. (1934) The production of persistent elevation of systolic blood pressure by means of renal ischemia. *Journal of Experimental Medicine* **59**, 347-379.

Goodman, E. and Hedetniemi, T. (1977) *Introduction to the design and analysis of algorithms*, New York: McGraw-Hill.

Gordon, H.W. (1994) Hypertensive Vascular disease. In: Isselbacher, K.J., Braunwald, E., Wilson, J.D., Martin, J.B., Faucy, A.S. and Kasper, D.L., (Eds.) *Harrison's Principles of Internal Medicine*, 13 edn. pp. 1116-1131.

Gorski, J.L., Boehnke, M., Reyner, E.L. and Burright, E.N. (1992) A radiation hybrid map of the proximal short arm of the human X chromosome spanning a incontinentia pigmenti1 (IP 1) translocation breakpoints. *Genomics.* **14**, 657-665.

Goss, S.J. and Harris, H. (1975) New method for mapping genes in human chromosomes. *Nature* **255**, 680-684.

Goss, S.J. and Harris, H. (1977) Gene transfer by means of cell fusion. II. The mapping of 8 loci on human chromosome 1 by statistical analysis of gene assortment in somatic cell hybrids. *J.Cell Sci.* **25:39-57**, 39-57.

Gotoda, T., Lizuka, Y., Kato, N., Osuga, J., Bihoreau, M.T., Murakami, T., Yamori, Y., Shimano, H., Ishibashi, S. and Yamada, N. (1999) Absence of Cd36 mutation in the original spontaneously hypertensive rats with insulin resistance. *Nat Genet* **22**, 226-228.

Gu, L., Dene, H., Deng, A.Y., Hoebee, B., Bihoreau, M.T., James, M. and Rapp, J.P. (1996) Genetic mapping of two blood pressure quantitative trait loci on rat chromosome 1. *J Clin Invest* **97**, 777-788.

Guiol, C., Ledoussal, C. and Surge, J.M. (1992) A radiotelemetry system for chronic measurement of blood pressure and heart rate in the unrestrained rat validation of the method. *J Pharmacol Toxicol Methods* **28**, 99-105.

Haldane, J.B.S. (1919) The combination of linkage values, and the calculation of distances between the loci of linked factor. *J.Genet.* **8**, 299-309.

Hall, C.E. and Gomez-Sanchez, C.E. (1986) Hypertensive potency of 18-oxocortisol in the rat. *Hypertension.* **8**, 317-322.

Hamet, P. (1996) Environmentally-regulated genes of hypertension. *Clin Exp Hypertens* **18**, 267-278.

Hamet, P., Kaiser, M.A., Sun, Y.L., Page, V., Vincent, M., Kren, V., Pravenec, M., Kunes, J., Tremblay, J. and Samani, N.J. (1996) HSP27 locus cosegregate with left ventricular mass independently of blood pressure. *Hypertension.* **28**, 1112-1117.

Hamet, P., Kong, D., Pravenec, M., Kunes, J., Kren, V., Klir, P., Sun, Y.L. and Tremblay, J. (1992) Restriction fragment length polymorphism of hsp70 gene, localized in the RT1 complex, is associated with hypertension in spontaneously hypertensive rats. *Hypertension.* **19**, 611-614.

Hamet, P., Pausova, Z., Adarichev, V., Adaricheva, K. and Tremblay, J. (1998) Hypertension: genes and environment. *J Hypertens* **16**, 397-418.

Hansson, J.H., Nelson-Williams, C., Suzuki, H., Schild, L., Shimkets, R., Lu, Y., Canessa, C., Iwasaki, T., Rossier, B. and Lifton, R.P. (1995) Hypertension caused by a truncated epithelial sodium channel gamma subunit: genetic heterogeneity of Liddle syndrome. *Nat Genet* **11**, 76-82.

Harrap, S.B. and Doyle, A.E. (1988) Genetic co-segregation of renal haemodynamics and blood pressure in the spontaneously hypertensive rat. *Clin Sci* **74**, 63-69.

Harris, E.L., Dene, H. and Rapp, J.P. (1993) SA gene and blood pressure cosegregation using Dahl salt-sensitive rats. *Am J Hypertens* **6**, 330-334.

Harris, E.L., Grigor, M.R. and Thompson, C.M. (1998) Cosegregation of the Tnfalpha locus with cardiovascular phenotypes in the F2 generation of a New Zealand genetically hypertensive and Brown Norway cross. *Clin Exp Pharmacol Physiol* **25**, 204-207.

Harris, E.L., Phelan, E.L., Thompson, C.M., Millar, J.A. and Grigor, M.R. (1995) Heart mass and blood pressure have separate genetic determinants in the New Zealand genetically hypertensive (GH) rat. *J Hypertens* **13**, 397-404.

Havlik, R.J., Garrison, R.J., Feinleib, M., Kannel, W.B., Castelli, W.P. and McNamara, P.M. (1979) Blood pressure aggregation in families. *Am J Epidemiol* **110**, 304-312.

Hedrich, H.J., Fangmann, J., Reetz, I.C., Kloting, I., Kornerup, H.A. and Wonigeit, K. (1990) Genetic characterization of different diabetes-prone

and -resistant BB-rat strains with special reference to RT6. *Transplant Proc* **22**, 2570-2571.

Hegele, R.A., Harris, S.B., Hanley, A.J., Cao, H. and Zinman, B. (1998) G protein beta3 subunit gene variant and blood pressure variation in Canadian Oji-Cree. *Hypertension.* **32**, 688-692.

Heiberg, A., Magnus, P., Berg, K. and Nance, W.E. (1981) Blood pressure in Norwegian twins. *Prog Clin Biol Res* **69 Pt C**, 163-168.

Heller, J., Hellerova, S., Dobesova, Z., Kunes, J. and Zicha, J. (1993) The Prague Hypertensive Rat: a new model of genetic hypertension. *Clin Exp Hypertens* **15**, 807-818.

Herrera, V.L. and Ruiz-Opazo, N. (1990) Alteration of alpha 1 Na+,K(+)-ATPase 86Rb+ influx by a single amino acid substitution. *Science* **249**, 1023-1026.

Herrera, V.L., Xie, H.X., Lopez, L.V., Schork, N.J. and Ruiz-Opazo, N. (1998) The alpha1 Na,K-ATPase gene is a susceptibility hypertension gene in the Dahl salt-sensitiveHSD rat. *J Clin Invest* **102**, 1102-1111.

Hilbert, P., Lindpaintner, K., Beckmann, J.S., Serikawa, T., Soubrier, F., Dubay, C., Cartwright, P., De Gouyon, B., Julier, C. and Takahasi, S. (1991) Chromosomal mapping of two genetic loci associated with blood-pressure regulation in hereditary hypertensive rats. *Nature* **353**, 521-529.

Hofstra, R.M., Landsvater, R.M., Ceccherini, I., Stulp, R.P., Stelwagen, T., Luo, Y., Pasini, B., Hoppener, J.W., van Amstel, H.K. and Romeo, G. (1994) A mutation in the RET proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. *Nature* **367**, 375-376.

Hsueh, W.C., Mitchell, B.D., Schneider, J.L., Wagner, M.J., Bell, C.J., Nanthakumar, E. and Shuldiner, A.R. (2000) QTL influencing blood pressure maps to the region of PPH1 on chromosome 2q31-34 in Old Order Amish. *Circulation.* **101**, 2810-2816.

Hubner, N., Lee, Y.A., Lindpaintner, K., Ganten, D. and Kreutz, R. (1999) Congenic substitution mapping excludes Sa as a candidate gene locus for a blood pressure quantitative trait locus on rat chromosome 1. *Hypertension.* **34**, 643-648.

Hughes, C.A. and Bennett, V. (1995) Adducin: a physical model with implications for function in assembly of spectrin-actin complexes. *J Biol Chem* **270**, 18990-18996.

Innes, B.A., McLaughlin, M.G., Kapuscinski, M.K., Jacob, H.J. and Harrap, S.B. (1998) Independent genetic susceptibility to cardiac hypertrophy in inherited hypertension. *Hypertension.* **31**, 741-746.

Inoue, I., Nakajima, T., Williams, C.S., Quackenbush, J., Puryear, R., Powers, M., Cheng, T., Ludwig, E.H., Sharma, A.M., Hata, A., Jeunemaitre, X. and Lalouel, J.M. (1997) A nucleotide substitution in the promoter of human angiotensinogen is associated with essential hypertension and affects basal transcription in vitro. *J Clin Invest* **99**, 1786-1797.

Iselius, L., Morton, N.E. and Rao, D.C. (1983) Family resemblance for blood pressure. *Hum Hered* **33**, 277-286.

Ishikawa, K., Katsuya, T., Sato, N., Nakata, Y., Takami, S., Takiuchi, S., Fu, Y., Higaki, J. and Ogihara, T. (1998) No association between alpha-adducin 460 polymorphism and essential hypertension in a Japanese population. *Am J Hypertens* **11**, 502-506.

Iwai, N. and Inagami, T. (1991) Isolation of preferentially expressed genes in the kidneys of hypertensive rats. *Hypertension.* **17**, 161-169.

Iwai, N. and Inagami, T. (1992) Identification of a candidate gene responsible for the high blood pressure of spontaneously hypertensive rats. *J Hypertens* **10**, 1155-1157.

Iwai, N., Kinoshita, M. and Shimoike, H. (1999) Chromosomal mapping of quantitative trait loci that influence renal hemodynamic functions. *Circulation.* **100**, 1923-1929.

Iwai, N., Kurtz, T.W. and Inagami, T. (1992) Further evidence of the SA gene as a candidate gene contributing to the hypertension in spontaneously hypertensive rat. *Biochem Biophys Res Commun* **188**, 64-69.

Iwai, N., Tsujita, Y. and Kinoshita, M. (1998) Isolation of a chromosome 1 region that contributes to high blood pressure and salt sensitivity. *Hypertension.* **32**, 636-638.

Jacob, H.J. (1999) Functional genomics and rat models. *Genome Res* **9**, 1013-1016.

Jacob, H.J., Brown, D.M., Bunker, R.K., Daly, M.J., Dzau, V.J., Goodman, A., Koike, G., Kren, V., Kurtz, T. and Lernmark, A. (1995) A genetic linkage map of the laboratory rat, Rattus norvegicus. *Nat Genet* **9**, 63-69.

Jacob, H.J., Brown, D.M., Bunker, R.K., Daly, M.J., Dzau, V.J., Goodman, A., Koike, G., Kren, V., Kurtz, T.W., Lernmark, A., Levan, G., Mao, Y., Pettersson, A., Pravenec, M., Simon, J.S., Szpirer, C., Szpirer, J., Trolliet,

M.R., Winer, E.S. and Lander, E.S. (1995) A genetic linkage map of the laboratory rat, Rattus norvegicus. *Genetics.* **9**, 63-69.

Jacob, H.J., Lindpaintner, K., Lincoln, S.E., Kusumi, K., Bunker, R.K., Mao, Y.P., Ganten, D., Dzau, V.J. and Lander, E.S. (1991) Genetic mapping of a gene causing hypertension in the stroke-prone spontaneously hypertensive rat. *Cell.* **67**, 213-224.

James, M.R. and Lindpaintner, K. (1997) Why map the rat? *Trends Genet.* **13**, 171-173.

Jamieson, A., Slutsker, L., Inglis, G.C., Fraser, R., White, P.C. and Connell, J.M. (1995) Glucocorticoid-suppressible hyperaldosteronism: effects of crossover site and parental origin of chimaeric gene on phenotypic expression. *Clin Sci* **88**, 563-570.

Jeffreys, A.J. (1979) DNA sequence variants in the G gamma-, A gamma-, delta- and beta-globin genes of man. *Cell.* **18**, 1-10.

Jeffreys, A.J., Craig, I.W. and Francke, U. (1979) Localisation of the G gamma-, A gamma-, delta- and beta-globin genes on the short arm of human chromosome 11. *Nature* **281**, 606-608.

Jeffreys, A.J., Wilson, V. and Thein, S.L. (1985) Hypervariable 'minisatellite' regions in human DNA. *Nature* **314**, 67-73.

Jeffs, B., Clark, J.S., Anderson, N., Gratton, J., Brosnan, M.J., Gauguier, D., Reid, J.L., Macrae, I.M. and Dominiczak, A. (1997) Sensitivity to cerebral ischemic insult in a rat model of stroke is determined by a single genetic locus. *Nat Genet* **16**, 364-367.

Jeffs, B., Negrin, C.D., Graham, D., Clark, J.S., Anderson, N., Gauguier, D. and Dominiczak, A.F. (2000) Applicability of a "speed" congenic strategy to dissect blood pressure quantitative trait loci on rat chromosome 2. *Hypertension.* **35**, 179-187.

Jeunemaitre, X., Charru, A., Chatellier, G., Dumont, C., Sassano, P., Soubrier, F., Menard, J. and Corvol, P. (1993) M235T variant of the human angiotensinogen gene in unselected hypertensive patients. *J Hypertens Suppl* **11 Suppl 5**, S80-S81

Jeunemaitre, X., Inoue, I., Williams, C., Charru, A., Tichet, J., Powers, M., Sharma, A.M., Gimenez-Roqueplo, A.P., Hata, A., Corvol, P. and Lalouel, J.M. (1997) Haplotypes of angiotensinogen in essential hypertension. *Am J Hum Genet* **60**, 1448-1460.

Jeunemaitre, X., Lifton, R.P., Hunt, S.C., Williams, R.R. and Lalouel, J.M. (1992) Absence of linkage between the angiotensin converting enzyme locus and human essential hypertension. *Nat Genet* **1**, 72-75.

Jeunemaitre, X., Rigat, B., Charru, A., Houot, A.M., Soubrier, F. and Corvol, P. (1992) Sib pair linkage analysis of renin gene haplotypes in human essential hypertension. *Hum Genet* **88**, 301-306.

Jeunemaitre, X., Soubrier, F., Kotelevtsev, Y.V., Lifton, R.P., Williams, C.S., Charru, A., Hunt, S.C., Hopkins, P.N., Williams, R.R. and Lalouel, J.M. (1992) Molecular basis of human hypertension: role of angiotensinogen. *Cell.* **71**, 169-180.

Jiang, J., Stec, D.E., Drummond, H., Simon, J.S., Koike, G., Jacob, H.J. and Roman, R.J. (1997) Transfer of a salt-resistant renin allele raises blood pressure in Dahl salt-sensitive rats. *Hypertension.* **29**, 619-627.

Judy, W.V. and Farrell, S.K. (1979) Arterial baroreceptor reflex control of sympathetic nerve activity in the spontaneously hypertensive rat. *Hypertension.* **1**, 605-614.

Judy, W.V., Watanabe, A.M., Murphy, W.R., Aprison, B.S. and Yu, P.L. (1979) Sympathetic nerve activity and blood pressure in normotensive backcross rats genetically related to the spontaneously hypertensive rat. *Hypertension.* **1**, 598-604.

Julier, C., Delepine, M., Keavney, B., Terwilliger, J., Davis, S., Weeks, D.E., Bui, T., Jeunemaitre, X., Velho, G., Froguel, P., Ratcliffe, P., Corvol, P., Soubrier, F. and Lathrop, G.M. (1997) Genetic susceptibility for human familial essential hypertension in a region of homology with blood pressure linkage on rat chromosome 10. *Hum Mol Genet* **6**, 2077-2085.

Kagimoto, M., Winter, J.S., Kagimoto, K., Simpson, E.R. and Waterman, M.R. (1988) Structural characterization of normal and mutant human steroid 17 alpha-hydroxylase genes: molecular basis of one example of combined 17 alpha-hydroxylase/17,20 lyase deficiency. *Mol Endocrinol* **2**, 564-570.

Karet, F.E. and Lifton, R.P. (1997) Mutations contributing to human blood pressure variation. *Recent Prog Horm Res* **52**, 263-276.

Karlin, S. and Taylor, H.M. (1975) *A first course in stochastic processes*, 2nd edn. New York: Academic Press.

Kato, N., Hyne, G., Bihoreau, M.T., Gauguier, D., Lathrop, G.M. and Rapp, J.P. (1999) Complete genome searches for quantitative trait loci controlling

blood pressure and related traits in four segregating populations derived from Dahl hypertensive rats. *Mamm Genome* **10**, 259-265.

Kato, N., Sugiyama, T., Morita, H., Kurihara, H., Yamori, Y. and Yazaki, Y. (1998) G protein beta3 subunit variant and essential hypertension in Japanese. *Hypertension.* **32**, 935-938.

Kato, N., Sugiyama, T., Nabika, T., Morita, H., Kurihara, H., Yazaki, Y. and Yamori, Y. (1998) Lack of association between the alpha-adducin locus and essential hypertension in the Japanese population. *Hypertension.* **31**, 730-733.

Katsuya, T., Higaki, J., Miki, T., Kohara, K., Yagisawa, H., Tanase, H., Mikami, H., Serikawa, T., Nojima, H. and Ogihara, T. (1992) Hypotensive effect associated with a phospholipase C-delta 1 gene mutation in the spontaneously hypertensive rat. *Biochem Biophys Res Commun* **187**, 1359-1366.

Katsuya, T., Higaki, J., Zhao, Y., Miki, T., Mikami, H., Serikawa, T. and Ogihara, T. (1993) A neuropeptide Y locus on chromosome 4 cosegregates with blood pressure in the spontaneously hypertensive rat. *Biochem Biophys Res Commun* **192**, 261-267.

Khoury, M.J. and Wagener, D.K. (1995) Epidemiological evaluation of the use of genetics to improve the predictive value of disease risk factors. *Am J Hum Genet* **56**, 835-844.

Kim, H.S., Krege, J.H., Kluckman, K.D., Hagaman, J.R., Hodgin, J.B., Best, C.F., Jennette, J.C., Coffman, T.M., Maeda, N. and Smithies, O. (1995) Genetic control of blood pressure and the angiotensinogen locus. *Proc Natl Acad Sci U S A* **92**, 2735-2739.

Kimura, S., Mullins, J.J., Bunnemann, B., Metzger, R., Hilgenfeldt, U., Zimmermann, F., Jacob, H., Fuxe, K., Ganten, D. and Kaling, M. (1992) High blood pressure in transgenic mice carrying the rat angiotensinogen gene. *EMBO J* **11**, 821-827.

Kirkpatrick, S., Gelatt, C.D. and Vecchi, M.P. (1983) Optimization by stimulated annealing. *Science* **220**, 671-680.

Kitanaka, S., Katsumata, N., Tanae, A., Hibi, I., Takeyama, K., Fuse, H., Kato, S. and Tanaka, T. (1997) A new compound heterozygous mutation in the 11 beta-hydroxysteroid dehydrogenase type 2 gene in a case of apparent mineralocorticoid excess. *J Clin Endocrinol Metab* **82**, 4054-4058.

Koike, G. and Jacob, H.J. (1998) Hypertension. In: Jameson, J.L., (Ed.) *Principles of molecular medicine*, pp. 145-155. Human Press Inc.

Kosambi, D.D. (1943) The estimation of map units from recombination fraction. *Annals of Eugenics* **12**, 172-175.

Kren, V., Pravenec, M., Lu, S., Krenova, D., Wang, J.M., Wang, N., Merriouns, T., Wong, A., St.Lezin, E., Lau, D., Szpirer, C., Szpirer, J. and Kurtz, T.W. (1997) Genetic isolation of a region of chromosome 8 that exerts major effects on blood pressure and cardiac mass in the spontaneously hypertensive rat. *J Clin Invest* **99**, 577-581.

Kreutz, R., Hubner, N., James, M.R., Bihoreau, M.T., Gauguier, D., Lathrop, G.M., Ganten, D. and Lindpaintner, K. (1995) Dissection of a quantitative trait locus for genetic hypertension on rat chromosome 10. *Proc Natl Acad Sci U S A* **92**, 8778-8782.

Krushkal, J., Ferrell, R., Mockrin, S.C., Turner, S.T., Sing, C.F. and Boerwinkle, E. (1999) Genome-wide linkage analyses of systolic blood pressure using highly discordant siblings. *Circulation.* **99**, 1407-1410.

Kuijpers, M.H. and Gruys, E. (1984) Spontaneous hypertension and hypertensive renal disease in the fawn-hooded rat. *Br J Exp Pathol* **65**, 181-190.

Kunz, R., Kreutz, R., Beige, J., Distler, A. and Sharma, A.M. (1997) Association between the angiotensinogen 235T-variant and essential hypertension in whites: a systematic review and methodological appraisal. *Hypertension.* **30**, 1331-1337.

Kurtz, T.W., Montano, M., Chan, L. and Kabra, P. (1989) Molecular evidence of genetic heterogeneity in Wistar-Kyoto rats: implications for research with the spontaneously hypertensive rat. *Hypertension.* **13**, 188-192.

Kurtz, T.W. and Morris, R.C.J. (1987) Biological variability in Wistar-Kyoto rats. Implications for research with the spontaneously hypertensive rat. *Hypertension.* **10**, 127-131.

Kurtz, T.W., Simonet, L., Kabra, P.M., Wolfe, S., Chan, L. and Hjelle, B.L. (1990) Cosegregation of the renin allele of the spontaneously hypertensive rat with an increase in blood pressure. *J Clin Invest* **85**, 1328-1332.

Kurtz, T.W., St.Lezin, E.M. and Pravenec, M. (1994) Development of hypertensive strains. In: Swales, J.D., (Ed.) *Text book of hypertension.*, pp. 441-447. Oxford: Blackwell.

Kvetnansky, R., Sun, C.L., Lake, C.R., Thoa, N., Torda, T. and Kopin, I.J. (1978) Effect of handling and forced immobilization on rat plasma levels of

epinephrine, norepinephrine, and dopamine-beta-hydroxylase. *Endocrinology* **103**, 1868-1874.

Laird, P.W., Zijderveld, A., Linders, K., Rudnicki, M.A., Jaenisch, R. and Berns, A. (1991) Simplified mammalian DNA isolation procedure. *Nucleic Acids Res* **19**, 4293

Lamprecht, F., Williams, R.B. and Kopin, I.J. (1973) Serum dopamine-beta-hydroxylase during development of immobilization-induced hypertension. *Endocrinology* **92**, 953-956.

Lande, R. and Thompson, R. (1990) Efficiency of marker-assisted selection in the improvement of quantitative traits. *Genetics.* **124**, 743-756.

Lander, E.S. and Botstein, D. (1986) Strategies for studying heterogeneous genetic traits in humans by using a linkage map of restriction fragment length polymorphisms. *Proc Natl Acad Sci U S A* **83**, 7353-7357.

Lander, E.S. and Botstein, D. (1989) Mapping mendelian factors underlying quantitative traits using RFLP linkage maps. *Genetics.* **121**, 185-199.

Lander, E.S., Green, P., Abrahamson, J., Barlow, A., Daly, M.J., Lincoln, S.E. and Newburg, L. (1987) Mapmaker: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics.* **1**, 174-181.

Lander, E.S. and Kruglyak, L. (1995) Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet* **11**, 241-247.

Lander, E.S. and Schork, N.J. (1994) Genetic dissection of complex traits. *Science* **265**, 2037-2048.

Lange, K., Boehnke, M., Cox, D.R. and Lunetta, K.L. (1995) Statistical methods for polyploid radiation hybrid mapping. *Genome Res* **5**, 136-150.

Latif, F., Tory, K., Gnarra, J., Yao, M., Duh, F.M., Orcutt, M.L., Stackhouse, T., Kuzmin, I., Modi, W. and Geil, L. (1993) Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* **260**, 1317-1320.

Lawrence, S., Morton, N.E. and Cox, D.R. (1991) Radiation hybrid mapping. *Proc Natl Acad Sci U S A* **88**, 7477-7480.

Lesage, S., Velho, G., Vionnet, N., Chatelain, N., Demenais, F., Passa, P., Soubrier, F. and Froguel, P. (1997) Genetic studies of the renin-angiotensin system in arterial hypertension associated with non-insulin-dependent diabetes mellitus. *J Hypertens* **15**, 601-606.

Levan, G. (1974) Nomenclature for G-bands in rat chromosomes. *Hereditas* **77**, 37-52.

Levine, R.S., Hennekens, C.H., Perry, A., Cassady, J., Gelband, H. and Jesse, M.J. (1982) Genetic variance of blood pressure levels in infant twins. *Am J Epidemiol* **116**, 759-764.

Li, A., Li, K.X., Marui, S., Krozowski, Z.S., Batista, M.C., Whorwood, C.B., Arnhold, I.J., Shackleton, C.H., Mendonca, B.B. and Stewart, P.M. (1997) Apparent mineralocorticoid excess in a Brazilian kindred: hypertension in the heterozygote state. *J Hypertens* **15**, 1397-1402.

Liang, F., Holt, I., Pertea, G., Karamycheva, S., Salzberg, S.L. and Quackenbush, J. (2000) Gene index analysis of the human genome estimates approximately 120, 000 genes. *Nat Genet* **25**, 239-240.

Liddle, G.W., Bledsoe, T. and Coppage, W.S.J. (1974) Hypertension reviews. *J Tenn Med Assoc* **67**, 669

Lifton, R.P. (1995) Genetic determinants of human hypertension. *Proc Natl Acad Sci U S A* **92**, 8545-8551.

Lifton, R.P., Dluhy, R.G., Powers, M., Rich, G.M., Cook, S., Ulick, S. and Lalouel, J.M. (1992) A chimaeric 11 beta-hydroxylase/aldosterone synthase gene causes glucocorticoid-remediable aldosteronism and human hypertension. *Nature* **355**, 262-265.

Lifton, R.P., Dluhy, R.G., Powers, M., Rich, G.M., Gutkin, M., Fallo, F., Gill, J.R.J., Feld, L., Ganguly, A. and Laidlaw, J.C. (1992) Hereditary hypertension caused by chimaeric gene duplications and ectopic expression of aldosterone synthase. *Nat Genet* **2**, 66-74.

Lifton, R.P., Dluhy, R.G., Powers, M., Ulick, S. and Lalouel, J.M. (1992) The molecular basis of glucocorticoid-remediable aldosteronism, a Mendelian cause of human hypertension. *Trans Assoc Am Physicians* **105**, 64-71.

Lifton, R.P., Hunt, S.C., Williams, R.R., Pouyssegur, J. and Lalouel, J.M. (1991) Exclusion of the Na(+)-H+ antiporter as a candidate gene in human essential hypertension. *Hypertension.* **17**, 8-14.

Lifton, R.P. and Jeunemaitre, X. (1993) Finding genes that cause human hypertension. *J Hypertens* **11**, 231-236.

Lin, D., Harikrishna, J.A., Moore, C.C., Jones, K.L. and Miller, W.L. (1991) Missense mutation serine106 proline causes 17 alpha-hydroxylase deficiency. *J Biol Chem* **266**, 15992-15998.

Lindpaintner, K. (1992) Genetic linkage analysis in hypertension: principles and practice. *J Hypertens* **10**, 121-124.

Lindpaintner, K., Hilbert, P., Ganten, D., Nadal-Ginard, B., Inagami, T. and Iwai, N. (1993) Molecular genetics of the S_A -gene: cosegregation with hypertension and mapping to rat cromosome 1. *J Hypertens* **11**, 19-23.

Lindpaintner, K., Takahashi, S. and Ganten, D. (1990) Structural alterations of the renin gene in stroke-prone spontaneously hypertensive rats: examination of genotype-phenotype correlations. *J Hypertens* **8**, 763-773.

Lindsey, J.R. (1979) Historical Foundations. In: Barker, H.J. and Lindsey, J.R., (Eds.) *The laboratory rat. Biology and diseases*, pp. 1-36. New York: Academic.

Lodwick, D., Zagato, L., Kaiser, M.A., Torielli, L., Casari, G., Bianchi, G. and Samani, N.J. (1998) Genetic analysis of the SA and Na+/K+-ATPase alpha1 genes in the Milan hypertensive rat. *J Hypertens* **16**, 139-144.

Longini, I.M.J., Higgins, M.W., Hinton, P.C., Moll, P.P. and Keller, J.B. (1984) Environmental and genetic sources of familial aggregation of blood pressure in Tecumseh, Michigan. *Am J Epidemiol* **120**, 131-144.

Lovenberg, W. (1987) Animal models for hypertension research. *Prog Clin Biol Res* **229**, 225-240.

Lovett, M. (1994) Fishing for complements: finding genes by direct selection. *Trends Genet* **10**, 352-357.

Lunetta, K.L., Boehnke, M., Lange, K. and Cox, D.R. (1995b) Experimental design and error detection for polyploid radiation hybrid mapping. *Genome Res* **5**, 151-163.

Lunetta, K.L., Boehnke, M., Lange, K. and Cox, D.R. (1996a) Selected locus and multiple panel models for radiation hybrid mapping. *Am J Hum Genet* **59**, 717-725.

Maguchi, M., Kohara, K., Okura, T., Li, S., Takezaki, M., Nishida, W. and Hiwada, K. (1996) Angiotensin-converting enzyme gene polymorphism in

essential hypertensive patients in Japanese population. *Angiology* **47**, 643-648.

Maixent, J.M., Charlemagne, D., de la Chapelle, B. and Lelievre, L.G. (1987) Two Na,K-ATPase isoenzymes in canine cardiac myocytes. Molecular basis of inotropic and toxic effects of digitalis. *J Biol Chem* **262**, 6842-6848.

Mangin, B., Goffinet, B. and Rebai, A. (1994) Constructing confidence intervals for QTL location. *Genetics.* **138**, 1301-1308.

Mansfield, T.A., Simon, D.B., Farfel, Z., Bia, M., Tucci, J.R., Lebel, M., Gutkin, M., Vialettes, B., Christofilis, M.A., Kauppinen-Makelin, R., Mayan, H., Risch, N. and Lifton, R.P. (1997) Multilocus linkage of familial hyperkalaemia and hypertension, pseudohypoaldosteronism type II, to chromosomes 1q31-42 and 17p11-q21. *Nat Genet* **16**, 202-205.

Manunta, P., Burnier, M., D'Amico, M., Buzzi, L., Maillard, M., Barlassina, C., Lanella, G., Cusi, D. and Bianchi, G. (1999) Adducin polymorphism affects renal proximal tubule reabsorption in hypertension. *Hypertension.* **33**, 694-697.

Markel, A.L. (1985) Experimental models of inherited arterial hypertension conditioned by stress. *Izvestia Acad Nauk SSR Seria Biol* **3**, 466-469.

Markel, P., Shu, P., Ebeling, C., Carlson, G.A., Nagle, D.L., Smutko, J.S. and Moore, K.J. (1997) Theoretical and empirical issues for marker-assisted breeding of congenic mouse strains. *Nat Genet* **17**, 280-284.

Markel, P.D., Bennett, B., Beeson, M., Gordon, L. and Johnson, T.E. (1997) Confirmation of quantitative trait loci for ethanol sensitivity in long- sleep and short-sleep mice. *Genome Res* **7**, 92-99.

Materson, B.J., Reda, D.J., Cushman, W.C., Massie, B.M., Freis, E.D., Kochar, M.S., Hamburger, R.J., Fye, C., Lakshman, R. and Gottdiener, J. (1993) Single-drug therapy for hypertension in men. A comparison of six antihypertensive agents with placebo. The Department of Veterans Affairs Cooperative Study Group on Antihypertensive Agents. *N Engl J Med* **328**, 914-921.

Matise, T.C., Perlin, M. and Chakravarti, A. (1994) Automated construction of genetic linkage maps using an expert system (MultiMap): A human genome linkage map. *Nat Genet* **6**, 384-390.

Matsukawa, N., Nonaka, Y., Higaki, J., Nagano, M., Mikami, H., Ogihara, T. and Okamoto, M. (1993) Dahl's salt-resistant normotensive rat has

mutations in cytochrome P450(11 beta), but the salt-sensitive hypertensive rat does not. *J Biol Chem* **268**, 9117-9121.

Matsumoto, C., Nara, Y., Ikeda, K., Nabika, T., Sawamura, M. and Yamori, Y. (1995) A new locus on chromosome 3 strongly linked with salt-sensitive high blood pressure in female F2 from SHRSP and WKY rats. *Clin Exp Pharmacol Physiol Suppl* **1**, S2-S3

Matsumoto, C., Nara, Y., Ikeda, K., Tamada, T., Mashimo, T., Nabika, T., Sawamura, M. and Yamori, Y. (1996) Cosegregation of the new region on chromosome 3 with salt-induced hypertension in female F2 progeny from stroke-prone spontaneously hypertensive and Wistar-Kyoto rats. *Clin Exp Pharmacol Physiol* **23**, 1028-1034.

Matsumoto, K., Yamada, T., Natori, T., Ikeda, K., Yamada, J. and Yamori, Y. (1991) Genetic variability in SHR (SHRSR), SHRSP and WKY strains. *Clin Exp Hypertens* **13**, 925-938.

McIlhany, M.L., Shaffer, J.W. and Hines, E.A.J. (1975) The heritability of blood pressure: an investigation of 200 pairs of twins using the cold pressor test. *Johns Hopkins Med J* **136**, 57-64.

McKusick, V.A. (1975) The growth and development of human genetics as a clinical discipline. *Am J Hum Genet* **27**, 261-273.

Mitchell, B.D., Ghosh, S., Schneider, J.L., Birznieks, G. and Blangero, J. (1997) Power of variance component linkage analysis to detect epistasis. *Genet Epidemiol* **14**, 1017-1022.

Miura, K., Yasuda, K., Yanase, T., Yamakita, N., Sasano, H., Nawata, H., Inoue, M., Fukaya, T. and Shizuta, Y. (1996) Mutation of cytochrome P-45017 alpha gene (CYP17) in a Japanese patient previously reported as having glucocorticoid-responsive hyperaldosteronism: with a review of Japanese patients with mutations of CYP17. *J Clin Endocrinol Metab* **81**, 3797-3801.

Moley, J.F., Brother, M.B., Fong, C.T., White, P.S., Baylin, S.B., Nelkin, B., Wells, S.A. and Brodeur, G.M. (1992) Consistent association of 1p loss of heterozygosity with pheochromocytomas from patients with multiple endocrine neoplasia type 2 syndromes. *Cancer Res* **52**, 770-774.

Moll, P.P., Harburg, E., Burns, T.L., Schork, M.A. and Ozgoren, F. (1983) Heredity, stress and blood pressure, a family set approach: the Detroit Project revisited. *J Chronic Dis* **36**, 317-328.

Monder, C., Stewart, P.M., Lakshmi, V., Valentino, R., Burt, D. and Edwards, C.R. (1989) Licorice inhibits corticosteroid 11 beta-

dehydrogenase of rat kidney and liver: in vivo and in vitro studies. *Endocrinology* **125**, 1046-1053.

Mongeau, J.G. (1989) Heredity and blood pressure. *Semin Nephrol* **9**, 208-216.

Morel, L., Yu, Y., Blenman, K.R., Caldwell, R.A. and Wakeland, E.K. (1996) Production of congenic mouse strains carrying genomic intervals containing SLE-susceptibility genes derived from the SLE-prone NZM2410 strain. *Mamm Genome* **7**, 335-339.

Mornet, E., Dupont, J., Vitek, A. and White, P.C. (1989) Characterization of two genes encoding human steroid 11 beta-hydroxylase (P-450(11) beta). *J Biol Chem* **264**, 20961-20967.

Morris, B.J., Zee, R.Y. and Schrader, A.P. (1994) Different frequencies of angiotensin-converting enzyme genotypes in older hypertensive individuals. *J Clin Invest* **94**, 1085-1089.

Morris, B.J., Zee, R.Y., Ying, L.H. and Griffiths, L.R. (1993) Independent, marked associations of alleles of the insulin receptor and dipeptidyl carboxypeptidase-I genes with essential hypertension. *Clin Sci (Colch)* **85**, 189-195.

Morton, N.E., Gulbrandsen, C.L., Rao, D.C., Rhoads, G.G. and Kagan, A. (1980) Determinants of blood pressure in Japanese-American Families. *Hum Genet* **53**, 261-266.

Mulligan, L.M., Kwok, J.B., Healey, C.S., Elsdon, M.J., Eng, C., Gardner, E., Love, D.R., Mole, S.E., Moore, J.K. and Papi, L. (1993) Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature* **363**, 458-460.

Mulvany, M.J. (1986) Role of vascular structure in blood pressure development of the spontaneously hypertensive rat. *J Hypertens Suppl* **4**, S61-S63

Mune, T., Rogerson, F.M., Nikkila, H., Agarwal, A.K. and White, P.C. (1995) Human hypertension caused by mutations in the kidney isozyme of 11 beta-hydroxysteroid dehydrogenase. *Nat Genet* **10**, 394-399.

Nabika, T., Bonnardeaux, A., James, M., Julier, C., Jeunemaitre, X., Corvol, P., Lathrop, M. and Soubrier, F. (1995) Evaluation of the SA locus in human hypertension. *Hypertension.* **25**, 6-13.

Naftilan, A.J., Williams, R., Burt, D., Paul, M., Pratt, R.E., Hobart, P., Chirgwin, J. and Dzau, V.J. (1989) A lack of genetic linkage of renin gene

restriction fragment length polymorphisms with human hypertension. *Hypertension.* **14**, 614-618.

Nara, Y., Nabika, T., Ikeda, K., Sawamura, M., Mano, M., Endo, J. and Yamori, Y. (1993) Basal high blood pressure cosegregates with the loci on chromosome 1 in the F2 generation from crosses between normotensive Wistar Kyoto rats and stroke-prone spontaneously hypertensive rats. *Biochem Biophys Res Commun* **194**, 1344-1351.

Nijenhuis, A. and Wilf, H.S. (1978) *Combinatorial algorithm*, 2nd edn. New York: Academic Press.

Niu, T., Xu, X., Rogus, J., Zhou, Y., Chen, C., Yang, J., Fang, Z., Schmitz, C., Zhao, J., Rao, V.S. and Lindpaintner, K. (1998) Angiotensinogen gene and hypertension in Chinese. *J Clin Invest* **101**, 188-194.

O'Brien, S.J., Menotti-Raymond, M., Murphy, W.J., Nash, W.G., Wienberg, J., Stanyon, R., Copeland, N.G., Jenkins, N.A., Womack, J.E. and Marshall, G.J. (1999) The promise of comparative genomics in mammals. *Science* **286**, 458-462.

O'Connor, D.T., Kailasam, M.T. and Parmer, R.J. (1996) Complex trait genetics: new methods yield a result for essential hypertension. *J Clin Invest* **97**, 1997-1998.

Oberfield, S.E., Levine, L.S., Stoner, E., Chow, D., Rauh, W., Greig, F., Lee, S.M., Lightner, E., Witte, M. and New, M.I. (1981) Adrenal glomerulosa function in patients with dexamethasone-suppressible hyperaldosteronism. *J Clin Endocrinol Metab* **53**, 158-164.

Okamoto, K. and Aoki, K. (1963) Development of a strain of spontaneously hypertensive rat. *Japanese Circulation Journal* **27**, 282-293.

Okamoto, K., Yamori, Y. and Nagaoka, A. (1974) Establishment of the stroke-prone spontaneously hypertensive rat (SHR). *Circulation Research* **34**, I-143-I-153

Pascoe, L., Curnow, K.M., Slutsker, L., Connell, J.M., Speiser, P.W., New, M.I. and White, P.C. (1992) Glucocorticoid-suppressible hyperaldosteronism results from hybrid genes created by unequal crossovers between CYP11B1 and CYP11B2. *Proc Natl Acad Sci U S A* **89**, 8327-8331.

Paterson, A.H., DeVerna, J.W., Lanini, B. and Tanksley, S.D. (1990) Fine mapping of quantitative trait loci using selected overlapping recombinant chromosomes, in an interspecies cross of tomato. *Genetics.* **124**, 735-742.

Paterson, A.H., Lander, E.S., Hewitt, J.D., Peterson, S., Lincoln, S.E. and Tanksley, S.D. (1988) Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. *Nature* **335**, 721-726.

Peng, L., Martin-Vasallo, P. and Sweadner, K.J. (1997) Isoforms of Na,K-ATPase alpha and beta subunits in the rat cerebellum and in granule cell cultures. *J Neurosci* **17**, 3488-3502.

Perera, G.A., Gearing, F.R. and Schweitzer, M.D. (1972) A family study of primary hypertension: final report. *J Chronic Dis* **25**, 127-132.

Pickering, G. (1967) The inheritance of arterial pressure. In: Stamler, J., Stamler, R. and Pullman T.N., (Eds.) *The epidemiology of hypertension*, pp. 18-27. New York: Grune & Stratton.

Pinkel, D., Landegent, J., Collins, C., Fuscoe, J., Segraves, R., Lucas, J. and Gray, J. (1988) Fluorescence in situ hybridization with human chromosome-specific libraries: detection of trisomy 21 and translocations of chromosome 4. *Proc Natl Acad Sci U S A* **85**, 9138-9142.

Pinto, Y.M., Paul, M. and Ganten, D. (1998) Lessons from rat models of hypertension: from Goldblatt to genetic engineering. *Cardiovasc Res* **39**, 77-88.

Platt, R. (1967) The influence of heredity. In: Stamler, J., Stamler, R. and Pullman T.N., (Eds.) *The epidemiology of hypertension*, pp. 9-17. New York: Grune & Stratton.

Pontecorvo, G. (1975) Production of mammalian somatic cell hybrids by means of polyethylene glycol treatment. *Somatic.Cell Genet.* **1**, 397-400.

Pravenec, M., Gauguier, D., Schott, J.J., Buard, J., Kren, V., Bila, V., Szpirer, C., Szpirer, J., Wang, J.M. and Huang, H. (1995) Mapping of quantitative trait loci for blood pressure and cardiac mass in the rat by genome scanning of recombinant inbred strains. *J Clin Invest* **96**, 1973-1978.

Pravenec, M., Kren, V., Kunes, J., Scicli, A.G., Carretero, O.A., Simonet, L. and Kurtz, T.W. (1991) Cosegregation of blood pressure with a kallikrein gene family polymorphism. *Hypertension.* **17**, 242-246.

Pravenec, M., Krenova, D., Kren, V., Zidek, V., Simakova, M., Musilova, A., Bottger, A., van Zutphen, B.F., St Lezin, E. and Kurtz, T.W. (1999) Congenic strains for genetic analysis of hypertension and dyslipidemia in the spontaneously hypertensive rat. *Transplant Proc* **31**, 1555-1556.

Press, W.H., Flannery, B.P., Teukolsky, S.A. and Vetterling, W.T. (1989) *Numerical recipes: the art of scientific computing (fortran version)*, Cambridge: Cambridge University press.

Ranade, K., Hsuing, A.C., Wu, K.D., Chang, M.S., Chen, Y.T., Hebert, J., Chen, Y.I., Olshen, R., Curb, D., Dzau, V., Botstein, D., Cox, D. and Risch, N. (2000) Lack of evidence for an association between alpha-adducin and blood pressure regulation in Asian populations. *Am J Hypertens* **13**, 704-709.

Rapp, J.P. (1982) A genetic locus (Hyp-2) controlling vascular smooth muscle response in spontaneously hypertensive rats. *Hypertension.* **4**, 459-467.

Rapp, J.P. (1983) A paradigm for identification of primary genetic causes of hypertension in rats. *Hypertension.* **1**, I-198-I-203

Rapp, J.P. (1983) Genetics of experimental and human hypertension. In: Genest, J., Kuchel.O. and Hamet, P., (Eds.) *Hypertension*, pp. 582-598. New York: McGraw-Hill.

Rapp, J.P. (1991) Dissecting the primary cuses of genetic hypertension in rats. *Hypertension.* **18**, I-18-I-28

Rapp, J.P. (2000) Genetic analysis of inherited hypertension in the rat. *Physiol Rev* **80**, 135-172.

Rapp, J.P. and Dahl, L.K. (1976) Mutant forms of cytochrome P-450 controlling both 18- and 11beta-steroid hydroxylation in the rat. *Biochemistry* **15**, 1235-1242.

Rapp, J.P. and Deng, A.Y. (1995) Detection and positional cloning of blood pressure quantitative trait loci: is it possible? Identifying the genes for genetic hypertension. *Hypertension.* **25**, 1121-1128.

Rapp, J.P., Garrett, M.R., Dene, H., Meng, H., Hoebee, B. and Lathrop, G.M. (1998a) Linkage analysis and construction of a congenic strain for a blood pressure QTL on rat chromosome 9. *Genomics.* **51**, 191-196.

Rapp, J.P., Garrett, M.R. and Deng, A.Y. (1998b) Construction of a double congenic strain to prove an epistatic interaction on blood pressure between rat chromosomes 2 and 10. *J Clin Invest* **101**, 1591-1595.

Rapp, J.P., Wang, S.M. and Dene, H. (1989) A genetic polymorphism in the renin gene of Dahl rats cosegregates with blood pressure. *Science* **243**, 542-544.

Richard, C.W., Boehnke, M., Berg, D.J., Lichy, J.H., Meeker, T.C., Hauser, E., Myers, R.M. and Cox, D.R. (1993) A radiation hybrid map of the distal short arm of human chromosome 11, containing the Beckwith-Wiedemann and associated embryonal tumor disease loci. *Am J Hum Genet* **52**, 915-921.

Richard, C.W., Withers, D.A., Meeker, T.C., Maurer, S., Evans, G.A., Myers, R.M. and Cox, D.R. (1991) A radiation hybrid map of the proximal long arm of human chromosome 11 containing the multiple endocrine neoplasia type 1 (MEN-1) and bcl-1 disease loci. *Am J Hum Genet* **49**, 1189-1196.

Risch, N. (1990) Genetic linkage and complex diseases, with special reference to psychiatric disorders. *Genet Epidemiol* **7**, 3-16.

Risch, N. and Merikangas, K. (1996) The future of genetic studies of complex human diseases. *Science* **273**, 1516-1517.

Robinson, M., Grim, C.E., ilson, T.W., Nicholson, G.D., ell, G.C. and ienne, C.M. (1996) Nuclear and mitochondrial DNA are associated with 24 hours blood pressure in Afro-Caribbeans. *Am J Hypertens* **9**, 30A

Roest, C.H., Jaillon, O., Bernot, A., Dasilva, C., Bouneau, L., Fischer, C., Fizames, C., Wincker, P., Brottier, P., Quetier, F., Saurin, W. and Weissenbach, J. (2000) Estimate of human gene number provided by genome-wide analysis using Tetraodon nigroviridis DNA sequence. *Nat Genet* **25**, 235-238.

Rose, R.J., Miller, J.Z., Grim, C.E. and Christian, J.C. (1979) Aggregation of blood pressure in the families of identical twins. *Am J Epidemiol* **109**, 503-511.

Rubattu, S., Volpe, M., Kreutz, R., Ganten, U., Ganten, D. and Lindpaintner, K. (1996) Chromosomal mapping of quantitative trait loci contributing to stroke in a rat model of complex human disease. *Nat Genet* **13**, 429-434.

Rubenson, D., Griffin, J.C., Ford, A., Claude, J., Reitz, B., Knutti, J., Billingham, M. and Harrison, D.C. (1984) Telemetry of electrophysiologic variables from conscious dogs: system design, validation, and serial studies. *Am Heart J* **107**, 90-96.

Ruiz-Opazo, N., Barany, F., Hirayama, K. and Herrera, V.L. (1994) Confirmation of mutant alpha 1 Na,K-ATPase gene and transcript in Dahl salt-sensitive/JR rats. *Hypertension.* **24**, 260-270.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**, 1350-1354.

Salardi, S., Saccardo, B., Borsani, G., Modica, R., Ferrandi, M., Tripodi, M.G., Soria, M., Ferrari, P., Baralle, F.E. and Sidoli, A. (1989) Erythrocyte adducin differential properties in the normotensive and hypertensive rats of the Milan strain. Characterization of spleen adducin m-RNA. *Am J Hypertens* **2**, 229-237.

Samani, N.J., Gauguier, D., Vincent, M., Kaiser, M.A., Bihoreau, M.T., Lodwick, D., Wallis, R., Parent, V., Kimber, P., Rattray, F., Thompson, J.R., Sassard, J. and Lathrop, M. (1996) Analysis of quantitative trait loci for blood pressure on rat chromosomes 2 and 13. Age-related differences in effect. *Hypertension.* **28**, 1118-1122.

Samani, N.J. and Lodwick, D. (1993) Elucidating the genetic basis of spontaneous hypertension: a perspective. *J Hum Hypertens* **7**, 167-171.

Samani, N.J., Lodwick, D., Vincent, M., Dubay, C., Kaiser, M.A., Kelly, M.P., Lo, M., Harris, J., Sassard, J. and Lathrop, M. (1993) A gene differentially expressed in the kidney of the spontaneously hypertensive rat cosegregates with increased blood pressure. *J Clin Invest* **92**, 1099-1103.

Samani, N.J., Swales, J.D., Jeffreys, A.J., Morton, D.B., Naftilan, A.J., Lindpaintner, K., Ganten, D. and Brammar, W.J. (1989) DNA fingerprinting of spontaneously hypertensive and Wistar-Kyoto rats: implications for hypertension research. *J Hypertens* **7**, 809-816.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74**, 5463-5467.

Sanger, F., Nicklen, S. and Coulson, A.R. (1992) DNA sequencing with chain-terminating inhibitors. 1977. *Biotechnology* **24**, 104-108.

Saunders, E., Weir, M.R., Kong, B.W., Hollifield, J., Gray, J., Vertes, V., Sowers, J.R., Zemel, M.B., Curry, C. and Schoenberger, J. (1990) A comparison of the efficacy and safety of a beta-blocker, a calcium channel blocker, and a converting enzyme inhibitor in hypertensive blacks. *Arch Intern Med* **150**, 1707-1713.

Schambelan, M., Sebastian, A. and Rector, F.C.J. (1981) Mineralocorticoid-resistant renal hyperkalemia without salt wasting (type II pseudohypoaldosteronism): role of increased renal chloride reabsorption. *Kidney Int* **19**, 716-727.

Schild, L., Canessa, C.M., Shimkets, R.A., Gautschi, I., Lifton, R.P. and Rossier, B.C. (1995) A mutation in the epithelial sodium channel causing Liddle disease increases channel activity in the Xenopus laevis oocyte expression system. *Proc Natl Acad Sci U S A* **92**, 5699-5703.

Schild, L., Lu, Y., Gautschi, I., Schneeberger, E., Lifton, R.P. and Rossier, B.C. (1996) Identification of a PY motif in the epithelial Na channel subunits as a target sequence for mutations causing channel activation found in Liddle syndrome. *EMBO J* **15**, 2381-2387.

Schmidt, S., Sharma, A.M., Zilch, O., Beige, J., Walla-Friedel, M., Ganten, D., Distler, A. and Ritz, E. (1995) Association of M235T variant of the angiotensinogen gene with familial hypertension of early onset. *Nephrol Dial Transplant* **10**, 1145-1148.

Schmidt, S., van, H., I, Grobbee, D.E., Ganten, D. and Ritz, E. (1993) Polymorphism of the angiotensin I converting enzyme gene is apparently not related to high blood pressure: Dutch Hypertension and Offspring Study. *J Hypertens* **11**, 345-348.

Schork, N.J. (1997) Genetically complex cardiovascular traits. Origins, problems, and potential solutions. *Hypertension.* **29**, 145-149.

Schork, N.J., Krieger, J.E., Trolliet, M.R., Franchini, K.G., Koike, G., Krieger, E.M., Lander, E.S., Dzau, V.J. and Jacob, H.J. (1995) A biometrical genome search in rats reveals the multigenic basis of blood pressure variation. *Genome Res* **5**, 164-172.

Schork, N.J., Nath, S.P., Lindpaintner, K. and Jacob, H.J. (1996) Extension to quantitative trait locus mapping in experimental organisms. *Hypertension.* **28**, 1104-1111.

Schunkert, H., Hense, H.W., Doring, A., Riegger, G.A. and Siffert, W. (1998) Association between a polymorphism in the G protein beta3 subunit gene and lower renin and elevated diastolic blood pressure levels. *Hypertension.* **32**, 510-513.

Schuster, H., Wienker, T.E., Bahring, S., Bilginturan, N., Toka, H.R., Neitzel, H., Jeschke, E., Toka, O., Gilbert, D., Lowe, A., Ott, J., Haller, H. and Luft, F.C. (1996) Severe autosomal dominant hypertension and brachydactyly in a unique Turkish kindred maps to human chromosome 12. *Nat Genet* **13**, 98-100.

Sebkhi, A., Zhao, L., Lu, L., Haley, C.S., Nunez, D.J. and Wilkins, M.R. (1999) Genetic determination of cardiac mass in normotensive rats: results from an F344xWKY cross. *Hypertension.* **33**, 949-953.

Serikawa, T., Kuramoto, T., Hilbert, P., Mori, M., Yamada, J., Dubay, C.J., Lindpaintner, K., Ganten, D., Guenet, J.L., Lathrop, G.M. and Beckmann, J.S. (1992) Rat gene mapping using PCR-analyzed microsatellites. *Genetics.* **131**, 701-721.

Sessler, F.M., Jokelainen, P.T., Sing, C.F., Strack, A.M. and Malvin, R.L. (1986) Renin heterogeneity in stroke-prone hypertensive and normotensive rats. *Am J Physiol* **251**, E367-E372

Seyde, W.C., McGowan, L., Lund, N., Duling, B. and Longnecker, D.E. (1985) Effects of anesthetics on regional hemodynamics in normovolemic and hemorrhaged rats. *Am J Physiol* **249**, H164-H173

Shibota, M., Nagaoka, A., Shino, A. and Iwatsuka, H. (1976) Proceedings: Cerebral vascular permeability in stroke-prone SHR. *Jpn Heart J* **17**, 371-372.

Shimkets, R.A. and Lifton, R.P. (1996) Recent advances in the molecular genetics of hypertension. *Curr Opin Nephrol Hypertens* **5**, 162-165.

Shimkets, R.A., Warnock, D.G., Bositis, C.M., Nelson-Williams, C., Hansson, J.H., Schambelan, M., Gill, J.R.J., Ulick, S., Milora, R.V. and Findling, J.W. (1994) Liddle's syndrome: heritable human hypertension caused by mutations in the beta subunit of the epithelial sodium channel. *Cell.* **79**, 407-414.

Siffert, W., Rosskopf, D., Siffert, G., Busch, S., Moritz, A., Erbel, R., Sharma, A.M., Ritz, E., Wichmann, H.E., Jakobs, K.H. and Horsthemke, B. (1998) Association of a human G-protein beta3 subunit variant with hypertension. *Nat Genet* **18**, 45-48.

Silver, L.M. (1995) *Mouse Genetics*, New York, N.Y.: Oxford University Press, Inc.

Simon, D.B., Bindra, R.S., Mansfield, T.A., Nelson-Williams, C., Mendonca, E., Stone, R., Schurman, S., Nayir, A., Alpay, H., Bakkaloglu, A., Rodriguez-Soriano, J., Morales, J.M., Sanjad, S.A., Taylor, C.M., Pilz, D., Brem, A., Trachtman, H., Griswold, W., Richard, G.A., John, E. and Lifton, R.P. (1997) Mutations in the chloride channel gene, CLCNKB, cause Bartter's syndrome type III. *Nat Genet* **17**, 171-178.

Simon, D.B., Karet, F.E., Hamdan, J.M., DiPietro, A., Sanjad, S.A. and Lifton, R.P. (1996) Bartter's syndrome, hypokalaemic alkalosis with

hypercalciuria, is caused by mutations in the Na-K-2Cl cotransporter NKCC2. *Nat Genet* **13**, 183-188.

Simon, D.B., Karet, F.E., Rodriguez-Soriano, J., Hamdan, J.H., DiPietro, A., Trachtman, H., Sanjad, S.A. and Lifton, R.P. (1996) Genetic heterogeneity of Bartter's syndrome revealed by mutations in the K+ channel, ROMK. *Nat Genet* **14**, 152-156.

Simon, D.B. and Lifton, R.P. (1998) Ion transporter mutations in Gitelman's and Bartter's syndromes. *Curr Opin Nephrol Hypertens* **7**, 43-47.

Simon, D.B. and Lifton, R.P. (1998) Mutations in Na(K)Cl transporters in Gitelman's and Bartter's syndromes. *Curr Opin Cell Biol* **10**, 450-454.

Simon, D.B., Nelson-Williams, C., Bia, M.J., Ellison, D., Karet, F.E., Molina, A.M., Vaara, I., Iwata, F., Cushner, H.M., Koolen, M., Gainza, F.J., Gitleman, H.J. and Lifton, R.P. (1996) Gitelman's variant of Bartter's syndrome, inherited hypokalaemic alkalosis, is caused by mutations in the thiazide-sensitive Na-Cl cotransporter. *Nat Genet* **12**, 24-30.

Smirk, F.H. and Haller, W.H. (1958) Inherited hypertension in rats. *Nature* **182**, 727-728.

Smith, T.L. and Hutchins, P.M. (1980) Anesthetic effects on hemodynamics of spontaneously hypertensive and Wistar-Kyoto rats. *Am J Physiol* **238**, H539-H544

Smithies, O. and Kim, H.S. (1994) Targeted gene duplication and disruption for analyzing quantitative genetic traits in mice. *Proc Natl Acad Sci U S A* **91**, 3612-3615.

Snell, G.D. (1948) Methods for the study of histocompatibility genes. *J Genet* **49**, 87-108.

Snyder, P.M., Price, M.P., McDonald, F.J., Adams, C.M., Volk, K.A., Zeiher, B.G., Stokes, J.B. and Welsh, M.J. (1995) Mechanism by which Liddle's syndrome mutations increase activity of a human epithelial Na+ channel. *Cell.* **83**, 969-978.

Soller, M. and Brody, T.S. (1976) On the power of experimental designs for the detection of linkage between marker loci and quantitative trait loci in crosses between inbred lines. *Theoretical and Applied Genetics* **47**, 35-39.

St.Lezin, E., Liu, W., Wang, J.M., Wang, N., Kren, V., Krenova, D., Musilova, A., Zdobinska, M., Zidek, V., Lau, D. and Pravenec, M. (1997)

Genetic isolation of a chromosome 1 region affecting blood pressure in the spontaneously hypertensive rat. *Hypertension.* **30**, 854-859.

St.Lezin, E., Liu, W., Wang, J.M., Yang, Y., Qi, N., Kren, V., Zidek, V., Kurtz, T.W. and Pravenec, M. (2000) Genetic analysis of rat chromosome 1 and the Sa gene in spontaneous hypertension. *Hypertension.* **35**, 225-230.

St.Lezin, E., Liu, W., Wang, N., Wang, J.M., Kren, V., Zidek, V., Zdobinska, M., Krenova, D., Bottger, A., van Zutphen, B.F. and Pravenec, M. (1998) Effect of renin gene transfer on blood pressure in the spontaneously hypertensive rat. *Hypertension.* **31**, 373-377.

St.Lezin, E., Pravenec, M. and Kurtz, T.W. (1993) New genetic models for hypertension research. *Trends Cardiovasc Med* **3**, 119-123.

St.Lezin, E., Zhang, L., Yang, Y., Wang, J.M., Wang, N., Qi, N., Steadman, J.S., Liu, W., Kren, V., Zidek, V., Krenova, D., Churchill, P.C., Churchill, M.C. and Pravenec, M. (1999) Effect of chromosome 19 transfer on blood pressure in the spontaneously hypertensive rat. *Hypertension.* **33**, 256-260.

St.Lezin, E.M., Pravenec, M., Wong, A.L., Liu, W., Wang, N., Lu, S., Jacob, H.J., Roman, R.J., Stec, D.E., Wang, J.M., Reid, I.A. and Kurtz, T.W. (1996) Effects of renin gene transfer on blood pressure and renin gene expression in a congenic strain of Dahl salt-resistant rats. *J Clin Invest* **97**, 522-527.

Staessen, J.A., Kuznetsova, T., Wang, J.G., Emelianov, D., Vlietinck, R. and Fagard, R. (1999) M235T angiotensinogen gene polymorphism and cardiovascular renal risk. *J Hypertens* **17**, 9-17.

Stanton, H.G. (1971) Experimental hypertension. In: Schwartz, A., (Ed.) *Methods in pharmacology.*, pp. 125-150. New York: Meredith Corporation.

Staub, O., Dho, S., Henry, P., Correa, J., Ishikawa, T., McGlade, J. and Rotin, D. (1996) WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na+ channel deleted in Liddle's syndrome. *EMBO J* **15**, 2371-2380.

Steen, R.G.K.-B.A.E., Glenn, C., Gullings-Handley, J., Van Etten, W., Atkinson, O.S., Appel, D., Twigger, S., Muir, M., Mull, T., Granados, M., Kissebah, M., Russo, K., Crane, R., Popp, M., Peden, M., Matise, T.C., Brown, D.M., Lu, J., Kingsmore, S., Tonellato, P.J., Rozen, S., Slonin, D., Young, P. and Jacob, H.J. (1999) A high-density integrated genetic linkage and radiation hybrid map of the laboratory rat. *Genome Res* **6**, 1-8.

Stein, L. http://www-genome.wi.mit.edu/ftp/pub/software/rhmapper. RHMAPPER, installation and user's guide. 1996
Internet Web address

Stephanova, E., Tissir, F., Dusetti, N., Iovanna, J., Szpirer, J. and Szpirer, C. (1996) The rat genes encoding the pancreatitis-associated proteins I, II and III (Pap1, Pap2, Pap3), and the lithostathin/pancreatic stone protein/regeneration protein (Reg) colocalize at 4q33-->q34. *Cytogenet Cell Genet* **72**, 83-85.

Stevens, P.A. and Brown, M.J. (1995) Genetic variability of the ET-1 and the ETA receptor genes in essential hypertension. *J Cardiovasc Pharmacol* **26 Suppl 3**, S9-12.

Stewart, E.A., McKusick, K.B., Aggarwal, A., Bajorek, E., Brady, S., Chu, A., Fang, N., Hadley, D., Harris, M., Hussain, S., Lee, R., Maratukulam, A., O'Connor, K., Perkins, S., Piercy, M., Qin, F., Reif, T., Sanders, C., She, X., Sun, W.L., Tabar, P., Voyticky, S., Cowles, S., Fan, J.B. and Cox, D.R. (1997) An STS-based radiation hybrid map of the human genome. *Genome Res* **7**, 422-433.

Stewart, P.M., Krozowski, Z.S., Gupta, A., Milford, D.V., Howie, A.J., Sheppard, M.C. and Whorwood, C.B. (1996) Hypertension in the syndrome of apparent mineralocorticoid excess due to mutation of the 11 beta-hydroxysteroid dehydrogenase type 2 gene. *Lancet* **347**, 88-91.

Stewart, P.M., Wallace, A.M., Valentino, R., Burt, D., Shackleton, C.H. and Edwards, C.R. (1987) Mineralocorticoid activity of liquorice: 11-beta-hydroxysteroid dehydrogenase deficiency comes of age. *Lancet* **2**, 821-824.

Summers, K.M., West, J.A., Huggard, P.R. and West, M.J. (1993) Angiotensin-converting enzyme and regulation of blood pressure in a large Australian family. *Clin Exp Pharmacol Physiol* **20**, 320-323.

Sutherland, D.J., Ruse, J.L. and Laidlaw, J.C. (1966) Hypertension, increased aldosterone secretion and low plasma renin activity relieved by dexamethasone. *Can Med Assoc J* **95** , 1109-1119.

Svetkey, L.P., Chen, Y.T., McKeown, S.P., Preis, L. and Wilson, A.F. (1997) Preliminary evidence of linkage of salt sensitivity in black Americans at the beta 2-adrenergic receptor locus. *Hypertension.* **29**, 918-922.

Svetkey, L.P., Timmons, P.Z., Emovon, O., Anderson, N.B., Preis, L. and Chen, Y.T. (1996) Association of hypertension with beta2- and alpha2c10-adrenergic receptor genotype. *Hypertension.* **27**, 1210-1215.

Tanase, H., Suzuki, Y., Ooshima, A., Yamori, Y. and Okamoto, K. (1970) Genetic analysis of blood pressure in spontaneously hypertensive rats. *Jpn Circ J* **34**, 1197-1212.

Thompson, E.A. (1987) Crossover counts and likelihood in multipoint linkage analysis. *IMA J Math Appl Med Biol* **4**, 93-108.

Tigerstedt, R. and Bergman, P.G. (1898) Niere und kreisbuf. *Scand Arch Physiol* **8**, 223-279.

Toka, H.R., Bahring, S., Chitayat, D., Melby, J.C., Whitehead, R., Jeschke, E., Wienker, T.F., Toka, O., Schuster, H. and Luft, F.C. (1998) Families with autosomal dominant brachydactyly type E, short stature, and severe hypertension. *Ann Intern Med* **129**, 204-208.

Turner, M.T., Johnson, M.L. and Ely, D.L. (1991) Separate sex-influenced and genetic components in spontaneously hypertensive rat hypertension. *Hypertension.* **17**, 1097-1103.

Ulick, S., Levine, L.S., Gunczler, P., Zanconato, G., Ramirez, L.C., Rauh, W., Rosler, A., Bradlow, H.L. and New, M.I. (1979) A syndrome of apparent mineralocorticoid excess associated with defects in the peripheral metabolism of cortisol. *J Clin Endocrinol Metab* **49**, 757-764.

Ulick, S., Tedde, R. and Mantero, F. (1990) Pathogenesis of the type 2 variant of the syndrome of apparent mineralocorticoid excess. *J Clin Endocrinol Metab* **70**, 200-206.

Van Ooijen, J.W. (1992) Accuracy of mapping quantitative trait loci in autogamus species. *Theoretical and Applied Genetics* **84**, 803-811.

Vincent, M., Cartier, R., Privat, P., Benzoni, D., Samani, N.J. and Sassard, J. (1996) Major cardiovascular risk factors in Lyon hypertensive rats. A correlation analysis in a segregating population. *J Hypertens* **14**, 469-474.

Wakeland, E., Morel, L., Achey, K., Yui, M. and Longmate, J. (1997) Speed congenics: a classic technique in the fast lane (relatively speaking). *Immunol.Today* **18**, 472-477.

Walker, B.R., Stewart, P.M., Shackleton, C.H., Padfield, P.L. and Edwards, C.R. (1993) Deficient inactivation of cortisol by 11 beta-hydroxysteroid dehydrogenase in essential hypertension. *Clin Endocrinol* **39**, 221-227.

Walker, W.G., Whelton, P.K., Saito, H., Russell, R.P. and Hermann, J. (1979) Relation between blood pressure and renin, renin substrate, angiotensin II, aldosterone and urinary sodium and potassium in 574 ambulatory subjects. *Hypertension.* **1**, 287-291.

Walter, M.A. and Goodfellow, P.N. (1993) Radiation hybrids: irradiation and fusion gene transfer. *Trends Genet.* **9**, 352-356.

Walter, M.A. and Goodfellow, P.N. (1993) Radiation hybrids: irradiation and fusion gene transfer. *Elsevier Science Publishers Ltd UK* **9**, 352-356.

Ward, R. (1990) Familial agregation and genetic epidemiology of blood pressure. In: Laragh, J.H. and Brenner, B.M., (Eds.) *Hypertension: Pathology, Diagnosis and Management*, pp. 811-1000. New York: Raven Press.

Warrington, J.A., Bailey, S.K., Armstrong, E., Aprelikova, O., Alitalo, K., Dolganov, G.M., Wilcox, A.S., Sikela, J.M., Wolfe, S.F. and Lovett, M. (1992) A radiation hybrid map of 18 growth factor, growth factor receptor, hormone receptor, or neurotransmitter receptor genes on the distal region of the long arm of chromosome 5. *Genomics.* **13**, 803-808.

Warrington, J.A., Hall, L.V., Hinton, L.M., Miller, J.N., Wasmuth, J.J. and Lovett, M. (1991) Radiation hybrid map of 13 loci on the long arm of chromosome 5. *Genomics.* **11**, 701-708.

Watanabe, T.K., Bihoreau, M.T., McCarthy, L.C., Kiguwa, S.L., Hishigaki, H., Tsuji, A., Browne, J., Yamasaki, Y., Mizoguchi-Miyakita, A., Oga, K., Ono, T., Okuno, S., Kanemoto, N., Takahashi, E., Tomita, K., Hayashi, H., Adachi, M., Webber, C., Davis, M., Kiel, S., Knights, C., Smith, A., Critcher, R., Miller, J. and James, M.R. (1999) A radiation hybrid map of the rat genome containing 5,255 markers. *Nat Genet* **22**, 27-36.

Weinberger, M.H., Miller, J.Z., Luft, F.C., Fineberg, N.S., Grim, C.E. and Christian, J.C. (1981) Genetic aspects of the blood pressure control system. *Clin Exp Hypertens* **3**, 569-581.

White, P.C., Dupont, J., New, M.I., Leiberman, E., Hochberg, Z. and Rosler, A. (1991) A mutation in CYP11B1 (Arg-448-His) associated with steroid 11 beta-hydroxylase deficiency in Jews of Moroccan origin. *J Clin Invest* **87**, 1664-1667.

Williams, G.H., Hollenberg, N.K., Hopkins, P.M. and Jeunemaitre, X. (1996) The role of intermediate phenotypes in essential hypertension: non-modulation as a model. *Endocr Res* **22**, 675-680.

Williams, J.R., Harrison, T.R. and Grollman, A. (1939) A simple method for determining the systolic blood pressure of the unanesthetized rat. *J Clin Invest* **18**, 373-376.

Wilson, R.C., Dave-Sharma, S., Wei, J.Q., Obeyesekere, V.R., Li, K., Ferrari, P., Krozowski, Z.S., Shackleton, C.H., Bradlow, L., Wiens, T. and

New, M.I. (1998) A genetic defect resulting in mild low-renin hypertension. *Proc Natl Acad Sci U S A* **95**, 10200-10205.

Wilson, R.C., Harbison, M.D., Krozowski, Z.S., Funder, J.W., Shackleton, C.H., Hanauske-Abel, H.M., Wei, J.Q., Hertecant, J., Moran, A. and Neiberger, R.E. (1995) Several homozygous mutations in the gene for 11 beta-hydroxysteroid dehydrogenase type 2 in patients with apparent mineralocorticoid excess. *J Clin Endocrinol Metab* **80**, 3145-3150.

Wilson, R.K., Chen, C., Avdalovic, N., Burns, J. and Hood, L. (1990) Development of an automated procedure for fluorescent DNA sequencing. *Genomics.* **6**, 626-634.

Wu, D.A., Bu, X., Warden, C.H., Shen, D.D., Jeng, C.Y., Sheu, W.H., Fuh, M.M., Katsuya, T., Dzau, V.J., Reaven, G.M., Lusis, A.J., Rotter, J.I. and Chen, Y.D. (1996) Quantitative trait locus mapping of human blood pressure to a genetic region at or near the lipoprotein lipase gene locus on chromosome 8p22. *J Clin Invest* **97**, 2111-2118.

Xu, X., Rogus, J.J., Terwedow, H.A., Yang, J., Wang, Z., Chen, C., Niu, T., Wang, B., Xu, H., Weiss, S., Schork, N.J. and Fang, Z. (1999) An extreme-sib-pair genome scan for genes regulating blood pressure. *Am J Hum Genet* **64**, 1694-1701.

Yagil, C., Sapojnikov, M., Kreutz, R., Katni, G., Lindpaintner, K., Ganten, D. and Yagil, Y. (1998) Salt susceptibility maps to chromosomes 1 and 17 with sex specificity in the Sabra rat model of hypertension. *Hypertension.* **31**, 119-124.

Yagil, C., Sapojnikov, M., Kreutz, R., Zurcher, H., Ganten, D. and Yagil, Y. (1999) Role of chromosome X in the Sabra rat model of salt-sensitive hypertension. *Hypertension.* **33**, 261-265.

Yamada, J., Kuramoto, T. and Serikawa, T. (1994) A rat genetic linkage map and comparative maps for mouse or human homologous rat genes. *Mamm Genome* **5**, 63-83.

Yamori, Y., Horie, R., Handa, H., Sato, M. and Fukase, M. (1976) Pathogenetic similarity of strokes in stroke-prone spontaneously hypertensive rats and humans. *Stroke* **7**, 46-53.

Yamori, Y., Mori, C., Nishio, T., Ooshima, A., Horie, R., Ohtaka, M., Soeda, T., Saito, M., Abe, K., Nara, Y., Nakao, Y. and Kihara, M. (1979) Cardiac hypertrophy in early hypertension. *Am J Cardiol* **44**, 964-969.

Yamori, Y., Nara, Y., Kihara, M., Horie, R. and Ooshima, A. (1981) Sodium and other dietary factors in experimental and human hypertension:

the Japanese experience. In: Laragh, J.H., Buhler, F.R. and Seldin, D.W., (Eds.) *Frontiers in hypertension research*, pp. 46-48. New York: Springer-Verlag.

Yamori, Y., Ooshima, A. and Okamoto, K. (1972) Genetic factors involved in spontaneous hypertension in rats an analysis of F 2 segregate generation. *Jpn Circ J* **36**, 561-568.

Yanase, T., Kagimoto, M., Suzuki, S., Hashiba, K., Simpson, E.R. and Waterman, M.R. (1989) Deletion of a phenylalanine in the N-terminal region of human cytochrome P-450(17 alpha) results in partial combined 17 alpha-hydroxylase/17,20-lyase deficiency [published erratum appears in J Biol Chem 1989 Dec 15;264(35):21433]. *J Biol Chem* **264**, 18076-18082.

Yui, M.A., Muralidharan, K., Moreno-Altamirano, B., Perrin, G., Chestnut, K. and Wakeland, E.K. (1996) Production of congenic mouse strains carrying NOD-derived diabetogenic genetic intervals: an approach for the genetic dissection of complex traits. *Mamm Genome* **7**, 331-334.

Zee, R.Y., Stephen, A.L., Iwai, N. and Morris, B.J. (1997) Association analyses of S(A) gene variant in essential hypertensives. *Am J Hypertens* **10**, 235-242.

Zee, R.Y., Ying, L.H., Morris, B.J. and Griffiths, L.R. (1991) Association and linkage analyses of restriction fragment length polymorphisms for the human renin and antithrombin III genes in essential hypertension. *J Hypertens* **9**, 825-830.

Zhang, L., Summers, K.M. and West, M.J. (1996) Angiotensin I converting enzyme gene cosegregates with blood pressure and heart weight in F2 progeny derived from spontaneously hypertensive and normotensive Wistar-Kyoto rats. *Clin Exp Hypertens* **18**, 753-771.

Zhang, L., Summers, K.M. and West, M.J. (1996) Cosegregation of genes on chromosome 5 with heart weight and blood pressure in genetic hypertension. *Clin Exp Hypertens* **18**, 1073-1087.

Zhang, Q.Y., Dene, H., Deng, A.Y., Garrett, M.R., Jacob, H.J. and Rapp, J.P. (1997) Interval mapping and congenic strains for a blood pressure QTL on rat chromosome 13. *Mamm Genome* **8**, 636-641.

Zinner, S.H., Levy, P.S. and Kass, E.H. (1971) Familial aggregation of blood pressure in childhood. *N Engl J Med* **284**, 401-404.

PRESENTATIONS

Negrin, C.D., Graham, D., Jeffs, B., Clark, J.S., Beatty, E., Gauguier, D., Dominiczak, A.F. (1999) Confirmation of blood pressure loci on rat chromosome 2 and Y: a speed congenic strategy. British Society for Cardiovascular Research, University of Glasgow, Scotland. (Poster).

Negrin, C.D., Graham, D, Jeffs, B., Clark, J.S., McBride, M.W., Anderson, N.H., Gauguier, D., Dominiczak, A.F. (1999) Two blood pressure quantitative trait loci on rat chromosome 2: a speed congenic strategy. *J Hypertens* **13**, 4-5. (Oral).

Negrin, C.D., Graham, D, McBride, M.W., Clark, J.S., Anderson, N.H., Carswell, H.V.O., Carr, F.J., Macrae, I.M., Dominiczak, A.F. (2000) Investigation of sensitivity to cerebral ischemia and blood pressure regulation in Y chromosome consomic strains. *J Hypertens* **18 (Suppl. 2)**, S116. (Oral).

Negrin, C.D., Graham, D, McBride, M.W., Clark, J.S., Anderson, N.H., Carswell, H.V.O., Carr, F.J., Macrae, I.M., Dominiczak, A.F. (2000) Y chromosome consomic strains to study blood pressure regulation and vulnerability to cerebral ischemia. *J Hypertens* **18 (Suppl. 4)**, S2. (Oral).

PUBLICATIONS

Brosnan, M.J., Clark, J.S., Jeffs, B., **Negrin, C.D.**, Van Vooren, P., Arribas, S.M., Carswell, H., Aitman, T.J., Szpirer, C., Macrae, I.M. and Dominiczak, A.F. (1999) Genes encoding atrial and brain natriuretic

peptides as candidates for sensitivity to brain ischemia in stroke-prone hypertensive rats. *Hypertension.* **33**, 290-297.

Dominiczak, A.F., Clark, J.S., Jeffs, B., Anderson, N.H., **Negrin, C.D.**, Lee, W.K. and Brosnan, M.J. (1998) Genetics of experimental hypertension. *J Hypertens* **16**, 1859-1869.

Dominiczak, A.F., **Negrin, D.C.**, Clark, J.S., Brosnan, M.J., McBride, M.W. and Alexander, M.Y. (2000) Genes and hypertension: from gene mapping in experimental models to vascular gene transfer strategies. *Hypertension.* **35**, 164-172.

Jeffs, B., **Negrin, C.D.**, Graham, D., Clark, J.S., Anderson, N., Gauguier, D. and Dominiczak, A.F. (2000) Applicability of a "speed" congenic strategy to dissect blood pressure quantitative trait loci on rat chromosome 2. *Hypertension.* **35**, 179-187.