

Characterisation of Rat Lines Transgenic for the Mouse *Ren-2d* cDNA

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Abstract

The rat strain TGR(mRen-2)27 is a monogenic model of hypertension expressing the mouse *Ren-2* gene in a number of tissues. One of the main features of this model is that it exhibits elevated plasma prorenin levels which has been implicated in the phenotype of hypertension. To investigate the role elevated plasma prorenin plays in the hypertensive TGR(mRen-2)27 rat prorenin was expressed in a non-renal tissue distant from the renal juxtaglomerular apparatus which is the only known site of conversion of prorenin to active renin in rat. The transgene design consisted of the promoter elements of the human α 1 anti-trypsin gene, to direct expression of the transgene to the liver, fused to the mouse *Ren-2^d* cDNA.

This transgene was introduced into the rat genome by microinjection. Lines were established in both Sprague-Dawley outbred and Fischer/344 inbred rat strains and examined in detail. Expression analysis of the transgene in the lines TG2, TG3, TG7 and TG12 showed detectable expression was limited to the liver, and that there was an elevation of plasma prorenin with no increase in plasma active renin.

The percentage of plasma active renin that was transgene derived was greater than 80% and all lines studied showed pronounced left ventricular cardiac hypertrophy at an early age, which in the case of Fischer lines TG7 and TG12, was quantified by echocardiography. At six weeks of age lines TG7 and TG12 showed left ventricular mass indices (LVMI) of 3.5 as opposed to controls showing a LVMI of 1.6 at the same age. The results demonstrate that in the TGR(α 1AT/*Ren-2*) rat extra-renal expression of *Ren-2* prorenin, directly or indirectly, results in hypertension with extreme cardiac hypertrophy. This model also further refines our understanding of the basis of hypertension in the classic TGR(mRen-2)-27 rat.

Dedication

For my parents and Gemma

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Abbreviations

| | |
|-------------------------------|---|
| α1AT | alpha 1 anti-trypsin |
| AI/AII | angiotensin I/II |
| ACE | angiotensin-converting enzyme |
| ACTH | adrenocorticotropin |
| Ala | alanine |
| Ao | angiotensinogen |
| ARAS | athermatous renal artery stenosis |
| Arg | arginine |
| BP | blood pressure |
| BSA | bovine serum albumin |
| cAMP | cyclic adenosine monophosphate |
| cDNA | complementary deoxyribonucleic acid |
| CHD | coronary heart disease |
| CVD | coardiovascular disease |
| Cys | cysteine |
| DBP | diastolic blood pressure |
| DEPC | diethyl pyrocarbonate |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| DOC | deoxycorticosterone |
| EDD | end diastolic diameter |
| EDTA | ethylenediaminetetraacetic acid |
| ES | embryonic stem cell |
| F | Fischer/F344 |
| FSH | follicle stimulating hormone |
| GRA | glucocorticoid remediable hyperaldosteronism |
| hCG | human chorionic gonadotrophin |
| IAP | intracisternal A particle |
| IVS | interventricular septum |
| JGA | juxtaglomerular apparatus |
| JNC V | Joint National Committee for Detection, Evaluation and Treatment of High Blood Pressure |
| kb | kilobase |
| kDa | kiloDalton |
| kcat | maximal catalytic rate |
| km | Michaelis-Menton constant |

| | |
|--------------|---|
| LCR | locus control region |
| LH | luteinising hormone |
| Lys | lysine |
| MAR | matrix attachment region |
| Met | methionine |
| mmHg | millimeters of mercury |
| mRNA | messenger ribonucleic acid |
| MT | metallothionein |
| MW | molecular weight |
| PBS | phosphate buffered solution |
| PCR | polymerase chain reaction |
| PMSG | pregnant mare serum gonadotrophin |
| PNMT | phenylethanolamine N-methyl transferase |
| PWT | peripheral wall thickness |
| RAS | renin-angiotensin system |
| RFLP | restriction fragment length polymorphism |
| SBP | systolic blood pressure |
| SD | Sprague Dawley |
| SDS | sodium dodecyl sulphate |
| SHR | spontaneously hypertensive rat |
| SHRSP | stroke prone spontaneously hypertensive rat |
| SMG | submaxillary gland |
| SSC | standard sodium citrate |
| SSR | simple sequence repeats |
| TAE | tris-acetate/EDTA buffer |
| TBE | tris-borate/EDTA buffer |
| TEMED | N, N, N', N'-tetramethylenediamine |
| Tris | N-tris [hydroxymethyl] aminomethane |
| VNTR | variable number of tandem repeats |
| WKY | Wistar Kyoto rat |

| | | |
|---|--------|-----------------------|
| k | kilo- | (x10 ³) |
| c | centi- | (x10 ⁻²) |
| m | milli- | (x10 ⁻³) |
| μ | micro- | (x10 ⁻⁶) |
| n | nano- | (x10 ⁻⁹) |
| p | pico- | (x10 ⁻¹²) |

CHAPTER 1

Introduction

1.1 Classification of Hypertension

As many as 50 million Americans have elevated blood pressure or are taking antihypertensive medication. This estimate is taken from the random sample of American adults in the National Health and Nutrition Examination Survey III. The prevalence of high blood pressure increases with age, is far greater for blacks than whites, and in both races is greater in less educated than more educated people. It is especially prevalent and devastating in lower socioeconomic groups. In young adulthood and early middle age, high blood pressure is more prevalent in men than for women, thereafter, the reverse is true (Rocella et al. 1989). Nonfatal and fatal cardiovascular diseases (CVDs) - including coronary heart disease (CHD) and stroke - and renal disease all increase in mortality risk progressively with higher levels of both systolic and diastolic blood pressures (Anderson et al. 1991).

Individuals are classified as being hypertensive or normotensive by their blood pressure levels. These categories aid in prognosis, decisions of therapy, and concentrate efforts on individuals who are most likely to benefit from treatment. The US Joint National Committee for Detection, Evaluation and Treatment of High Blood Pressure (JNC V), (JNC V 1993) defined the optimal blood pressure as a systolic blood pressure less than 120 mm Hg and a diastolic blood pressure less than 80 mm Hg. The high normal range of blood pressure in an individual is a systolic blood pressure (SBP) reading between 130 and 139 mm Hg or a diastolic blood pressure (DBP) reading between 85 and 89 mmHg. High systolic (≥ 140 mm Hg) or diastolic (≥ 90 mm Hg) pressure, when confirmed on two or more visits, is diagnosed as hypertension. Hypertension is further categorised into four stages corresponding to the patient's level of systolic and diastolic blood pressure. The most common form of hypertension is stage 1 (80%) with stage four the least common (3%) (See table 1.1).

Despite the practicalities of this system it is necessary to be aware that the cut-off points on this scale represent an operational guideline and not a biological distinction between hypertension and normotension. Population characteristics such as age, gender and race all influence the prevalence of hypertension (Hollenberg 1992).

Age has the greatest impact, using the JNC V criteria, with 65% of those aged ≥ 80 years displaying varying degrees of hypertension. Gender plays a less significant role than other demographic factors in the epidemiology of hypertension. An example of one of the outcomes of hypertension in the different sexes is that coronary artery disease occurs less frequently in women than men until 6-10 years post-menopause. After the menopause, considerations for anti-hypertensive therapy selection in women become identical to those in men. In the case of race, recent studies support the view that blacks have higher blood pressure than whites at all ages (Manatunga et al. 1993). There is evidence for structural changes in the resistance vessels of the peripheral vasculature and the kidney, which are apparent in young blacks, both may possibly be related to the development of hypertension.

Table 1.1

Classification of Blood Pressure for Adults Aged 18 Years and Older*

| Category | Systolic, mmHg | Diastolic, mmHg |
|----------------------------|----------------|-----------------|
| Normal | <130 | <85 |
| High Normal [†] | 130-139 | 85-89 |
| Hypertension ^{††} | | |
| Stage 1 | 140-159 | 90-99 |
| Stage 2 | 160-179 | 100-119 |
| Stage 3 | 180-209 | 110-119 |
| Stage 4 | ≥ 210 | ≥ 120 |

*Adapted from JNC V (JNC V 1993)

[†]Optimal blood pressure with respect to cardiovascular risk is less than 120 mmHg systolic and less than 80 mmHg diastolic. However, unusually low readings are evaluated for clinical significance.

^{††}Based on the average of two or more readings taken at each of two or more visits after an initial screening.

1.1.1 Essential Hypertension:

No single factor has been found to be responsible for the onset of essential or idiopathic hypertension (Ayman 1934; Kurtz et al. 1993). Early stage essential hypertension may be due to a small increase in cardiac output, possibly caused by sympathetic overactivity. Later in the disease the cardiac output is normal but

peripheral resistance is increased. In essential hypertension the baroreceptor reflexes operate at a higher pressure. Increased blood pressure should stimulate a bradycardia through the baroreceptor mechanism of the carotid sinus, but this does not occur in essential hypertension.

There is a strong genetic tendency to develop essential hypertension, which can be exacerbated by factors such as obesity, stress, and excessive salt intake. It has been postulated that a defect in renal salt management may underlie many cases of essential hypertension. Minor disturbances in renal function, below the level of detection could lead to a gradual accumulation of salt and water in the body, resulting in progressive increase in arterial pressure.

In benign essential hypertension arteriosclerosis of the major renal arteries, and changes in the intrarenal vasculature, occurs (Brown et al. 1974). The small vessels and arterioles exhibit myointimal thickening and the vessel wall becomes hyalinized. Concentric reduplication of the internal elastic lamina and endothelial proliferation produce an "onion skin" appearance (Hsu et al. 1980).

In only 10% of hypertension cases is the cause of the hypertension known, these are termed secondary hypertension. These causes can be divided into renal, endocrine, cardiovascular, pregnancy and drug related.

1. *Renal causes.* Renal diseases are the most common causes of secondary hypertension (80% of cases) (Conn et al. 1972), see table 1.2.

Arthermatous renal artery stenosis (ARAS):

Patients usually present with atheroma elsewhere before developing atheroma of the renal arteries and also should be considered in patients with histories of myocardial ischaemia or stroke. Stenosis usually affects the proximal portion of the renal arteries and in nearly 30% of the cases is predominantly ostial (Sos et al. 1983). In ARAS stenosis progression is common. Quality of blood pressure control does not seem to influence progression and between 8%-17% of stenoses progress to occlusion in 3-4 years (Schreiber et al. 1984)

Table 1.2

| Renovascular | Renal Parenchymal |
|-------------------------------|--|
| Atherosclerosis | Glomerulonephritis |
| Fibromuscular dysplasia (FMD) | Pyelonephritis |
| Takayasu's arteritis | Nephrocalcinosis |
| Middle aortic syndrome | Neoplasms |
| Neurofibromatosis | Glomerulosclerosis |
| Dissecting aneurysm aorta | Radiation nephritis |
| Renal artery aneurysm | Obstructive uropathies and hydronephritis |
| Renal arteriovenous fistula | Renin-secreting tumours (eg. Wilms tumour) |
| Radiation arteritis | Renal Trauma |
| Renal artery embolus | |
| Renal transplantation | |
| Extrinsic obstruction | |

(Adapted from the Essential Hypertension ed Laragh and Blumefield)

2. Endocrine causes.

Conn's Syndrome: True Conn's syndrome, as the classic form of mineralocorticoid excess with hypertension, is due to an aldosterone-secreting adenoma of the adrenal cortex. The disease is more often found in women than in men. Several patients have been described with both acromegaly and Conn's syndrome. The hypertension is accompanied by hypokalaemia, contraction of body potassium content and expansion of body sodium content.

Acromegaly: Acromegaly is the result of excessive production of growth hormone in an adult subject. The usual cause of acromegaly is a tumour of the anterior pituitary gland producing an excess of growth hormone. Due to varied definitions of 'hypertension' and of methods of blood pressure measurement, accurate estimates of the prevalence of hypertension in acromegaly are difficult. Despite this, many series report high blood pressure in between 18-45% of cases, with the greatest frequency seen in older patients and women (Fraser et al. 1989).

Cushing's Syndrome: Cushing's syndrome results from a prolonged excess of glucocorticoids, notably cortisol. Hypertension, occurring in about 75-85% of patients is a frequent accompaniment of this uncommon disease (Padfield et al. 1993). Cushing's syndrome is caused by an abnormality of the anterior pituitary gland. Such primary pituitary disease or dysfunction of the hypothalamic-pituitary axis accounts for 70% of all cases.

Phaeochromocytoma: Phaeochromocytomas are tumours of the chromaffin cells. Despite the majority of these tumours lying within the adrenal medulla, they can also be widely distributed at other sites where chromaffin cells occur, such as in apposition to sympathetic nerve plexuses in the neck, chest, urinary bladder, rectum, testes or ovaries. These tumours can secrete excessive quantities of noradrenalin and adrenalin, together with the catecholamine precursors dopamine and DOPA. Noradrenalin is the hormone predominantly released, and seems to be ubiquitous in non-familial phaeochromocytomas. Noradrenalin causes peripheral vasoconstriction, elevation of both systolic and diastolic pressures and reflex bradycardia; adrenalin raises systolic pressure, lowers the diastolic pressure and increases heart rate.

3. *Cardiovascular causes.*

Coarctation of the aorta: Coarctation of the aorta results from an abnormality in development of the aortic arch system, such that there persists a structural narrowing of the aorta usually in its descending intrathoracic course. An extensive collateral arterial system develops to circumvent the coarctation and thus supply adequate blood to the lower part of the body. Hypertension, readily detected in the upper limbs, is indicative of coarctation (Rocchini et al. 1976).

4. *Pregnancy.*

Hypertension in the early stages of pregnancy is usually essential hypertension. However, the definition of hypertension during pregnancy is arbitrarily taken as systolic BP in excess of 140 mmHg and/or a fourth phase diastolic over 90 mmHg (JNC V 1993)(table 1.1). Pre-eclampsia or toxæmia of pregnancy is diagnosed when hypertension develops in the last three months and is associated with oedema and proteinuria.

5. *Drugs.*

Oestrogen-containing contraceptives, other steroids, liquorice and vasopressin may all cause hypertension. Oestrogens cause a rise in the level of plasma angiotensinogen concentration. Due to the absence of a compensatory fall in plasma concentration of renin, plasma levels of angiotensin II rise, contributing to blood pressure elevation (JNC V 1993).

1.1.2 Malignant Hypertension:

Malignant hypertension is a rare disorder with a high but declining mortality and morbidity from renal cerebral and cardiac complications. It is a disorder that presents with a rapidly and deteriorating renal function. Of the patients with essential hypertension only 1-2% are thought to develop malignant phase hypertension,

precipitated by a sudden rise in blood pressure (Houston 1989). Clinical features of malignant hypertension include visual blurring, dizziness, headaches, fatigue and weight loss. However, many patients remain asymptomatic (Kincaid-Smith 1982)

The early reports of malignant hypertension described the condition without treatments (Keith et al. 1928). Macroscopic changes were not seen in the kidney with the main renal findings being at the microscopic level, with diffuse changes affecting smaller arteries and arterioles, but not larger arteries. Specifically, intimal hyperplasia with deposits of lipid, fatty acids and calcium resulted in significant luminal narrowing (Keith et al. 1928). A further series of cases described a degree of glomerular capillary tuft collapse, capillary thrombosis, glomerulosclerosis and evidence of crescent formation in addition to the arteriolar changes previously reported. "Onion skinning" intimal proliferation, with concentric lamellae of basement membrane and granulofilamentous material and a thin layer of hypertrophic medial cells stretched around the circumference, was shown by light microscope studies of affected kidneys to be caused by smooth muscle cells (Hsu et al. 1980; Dunn 1982). Electron microscopy showed that fibrinoid necrosis consisted of three components, fibrin, fibrinoid and cellular necrosis. Arterial luminal thromboses consisted of both fibrin and platelets (Dunn 1982). As a result of natriuresis and diuresis the renal renin-angiotensin system is activated. This may cause a cycle of increasing blood pressure and continuous renal damage.

1.1.3 Treatment of Essential and Malignant Hypertension:

General Measures:

Weight reduction is one of the most common 'life style' alterations made in clinically hypertensive patients (Birkenhager et al. 1984; Ward 1990; Hollenberg 1992). This leads to a fall in blood pressure and a reduction of the artefactually increased cuff measurements. Weight reduction in patients 10%, or greater, above their ideal weight enhances the blood-pressure lowering effects of concurrent antihypertensive agents. Reducing the intake in a heavy drinker leads to a small reduction in blood pressure. Salt restriction requires reduction of dietary salt. This often takes the form of no added salt at the table. Regular exercise in conjunction with a reduction in stress can lead to a reduction in blood pressure.

Drug Treatment:

Vasodilators:

Angiotensin-converting enzyme (ACE) inhibitors, such as Captopril and Enalapril, block the conversion of angiotensin I to angiotensin II, promote vasodilation and decrease aldosterone levels. Angiotensin II is a potent vasoconstrictor. ACE inhibitors also block the degradation of bradykinin, a vasodilator, which results in increased levels of plasma bradykinin. Calcium antagonists such as Nifedipine or Verapamil block inward movement of calcium ions across cell membranes and cause smooth-muscle relaxation reducing blood pressure primarily by arteriolar dilatation, but also by negative inotropy (force of cardiac contraction) (Opie 1990). Alpha-1 adrenergic antagonists such as Prazosin and Indoramin are post-synaptic alpha-blockers causing vasodilation and are very effective hypotensive drugs.

Diuretics:

Loop diuretics (e.g., Frusemide) and thiazide diuretics (e.g., Cyclopentiazide) are equally effective at lowering blood pressure and work by decreasing plasma volume and extracellular fluid volume followed by decreasing total peripheral resistance and normalisation of cardiac output. Thiazides are often preferred as the duration of action is longer and the diuresis is less severe. Loop diuretics are restricted to those with cardiac or renal impairment for whom an additional diuretic effect is required.

Beta-adrenergic antagonists:

Beta-adrenergic antagonists decrease cardiac output and increase total peripheral resistance. According to the JNC V report, beta-adrenergic antagonists should be used as the optimal first steps of pharmacological intervention in hypertension. This view is disputed by Weber who draws attention to the limitations of the study (SHEP) from which these conclusions were made and suggests ACE inhibitors may be as effective (Weber et al. 1990).

1.2 Genetics of Hypertension

Genetic analysis of essential hypertension is hampered by the fact that the disease is polygenic and heterogeneous in nature (Ward 1990). Essential hypertension is likely to involve epistatic and ecogenetic interactions (Lindpaintner 1993). Population studies (Miall et al. 1963) have shown that between 12-35% of blood pressure variation can be

attributed to genetic causes. Studying the genetics of hypertension has taken the form of sib-pair analysis, kindreds with a high incidence of the disease, and animal models of genetic hypertension. The results are applied to humans to identify genes that have an influence on the clinical features of this polygenic disease.

The study performed by Zinner *et al* (Zinner *et al.* 1971), on children aged 2-14 years from 192 families, did demonstrate familial aggregation. By studying the distribution of mean family scores it was shown that they differed significantly from the expected normal distribution. A weak correlation between infant blood pressure and maternal blood pressure was found at birth, but a significant relationship with paternal blood pressure was not found until one month old (Zinner *et al.* 1985). At birth, infant blood pressure could be affected by the intra-uterine environment which in turn may be influenced by both maternal genes and environment.

Biochemical and physiological abnormalities have been shown in hypertensive families and animals, but results are difficult to interpret whether a particular abnormality is a cause or consequence of the disease. Despite no single biochemical defect being shown to be causative for essential hypertension, these studies provide indications as to the genes responsible for blood pressure regulation (Padfield *et al.* 1993).

Restriction fragment length polymorphism (RFLP) analysis takes advantage of variation in restriction sites present in the human genome to map polymorphisms that segregate with the desired trait (see figure 1.1). Polymerase chain reaction (PCR) is now used in a similar fashion to amplify regions of the genome which varies from individual to individual. These approaches have proved successful for diseases having Mendelian inheritance (Cawthon *et al.* 1990). For polygenic traits it may not be possible to study a homogeneous population as different families segregate from different loci. A quantitative trait that is a continuous variable, e.g. blood pressure, is often best analysed as a quantitative phenotype, which avoids making strong assumptions about the form of its association with the disease of interest.

Liddle's Syndrome (pseudaldosteronism) has long been recognised as having an autosomal dominant mode of inheritance with constitutive activation of the Na^+H^+ exchange channel in the distal tubule (Botero-Velez *et al.* 1994). Recently polymorphisms for the gene coding for the β subunit of the epithelial sodium channel have been found to be linked with hypertension in five kindreds with the syndrome (Shimkets *et al.* 1994). It is therefore quite possible that other single gene defects may be identified causing hypertension in certain kindred (Kurtz 1994).

Glucocorticoid remediable hyperaldosteronism (GRA) does not reliably exhibit clinical features or blood chemistry that would have allowed clear distinction from

essential hypertension after standard investigations. The diagnosis of GRA requires demonstrating elevated urinary excretion of steroid metabolites 18-hydroxycortisol and 18-oxocortisol. Lifton *et al* identified linkage of GRA with an RFLP occurring at a fusion event between the ACTH responsive regulatory sequences of the 11 β -hydroxylase gene and the coding sequences of the aldosterone synthase gene. This resulted in ectopic expression of aldosterone synthase in the adrenal zona fasciculata in response to stimulation by ACTH rather than angiotensin II (Ang II) or potassium (Lifton *et al.* 1992).

One of the most intensely studied blood pressure regulatory systems is the renin-angiotensin-system. There is a *Bgl II* RFLP in the rat renin gene as a result of a variable number of tandem repeats (VNTR) of a 38bp sequence present in the first intron. Several renin alleles have been identified in hypertensive and normotensive strains of rat.

Using established rat strains Kurtz *et al* (1990) identified one or two SHR renin alleles in a SHR/Lewis cross resulting in higher blood pressure in the animals which inherited the SHR allele (Kurtz *et al.* 1990). Both SHR and inbred Dahl salt-hypertension resistant rats (SR/Jr) were found to carry the same renin allele based on studies of the first intron VNTR and a *Hind III* site in the fifth intron which distinguishes the allele. This meant that in an F₂ cross of the inbred salt-hypertension sensitive Dahl rat (SS/Jr) and SHR, the SHR contributed the r allele which lowered blood pressure. Therefore in the F₂ progeny homozygous for the s allele derived from SS/Jr showed higher blood pressure was seen and rats homozygous for the r allele derived from SHR showed lower blood pressure (Rapp *et al.* 1994).

Two groups have shown linkage of loci with basal blood pressure variance in response to sodium loading in TG2 SHRSP/WKY crosses. Jacob *et al* (1991) developed a series of microsatellite markers for the rat based on simple sequence repeats (SSR) (Jacob *et al.* 1991). Hilbert *et al* (1991) used a combination of minisatellite probes, microsatellite markers and an RFLP previously identified in the SHRSP renin gene (Hilbert *et al.* 1991). In common to both studies, a locus on chromosome 10 named Bp1 or BP/SP-1 respectively, showed significant linkage disequilibrium with sodium loaded blood pressure. The locus was identified by both groups to be in the region of the angiotensin converting enzyme gene. The sequences for the respective ACE cDNAs, as obtained by screening cDNA libraries from SHRSP and WKY, showed that they differed by 5 nucleotides in the coding regions, only one of which resulted in an amino acid substitution (Lys₂₀₇->Arg₂₀₇) in SHR-SP.

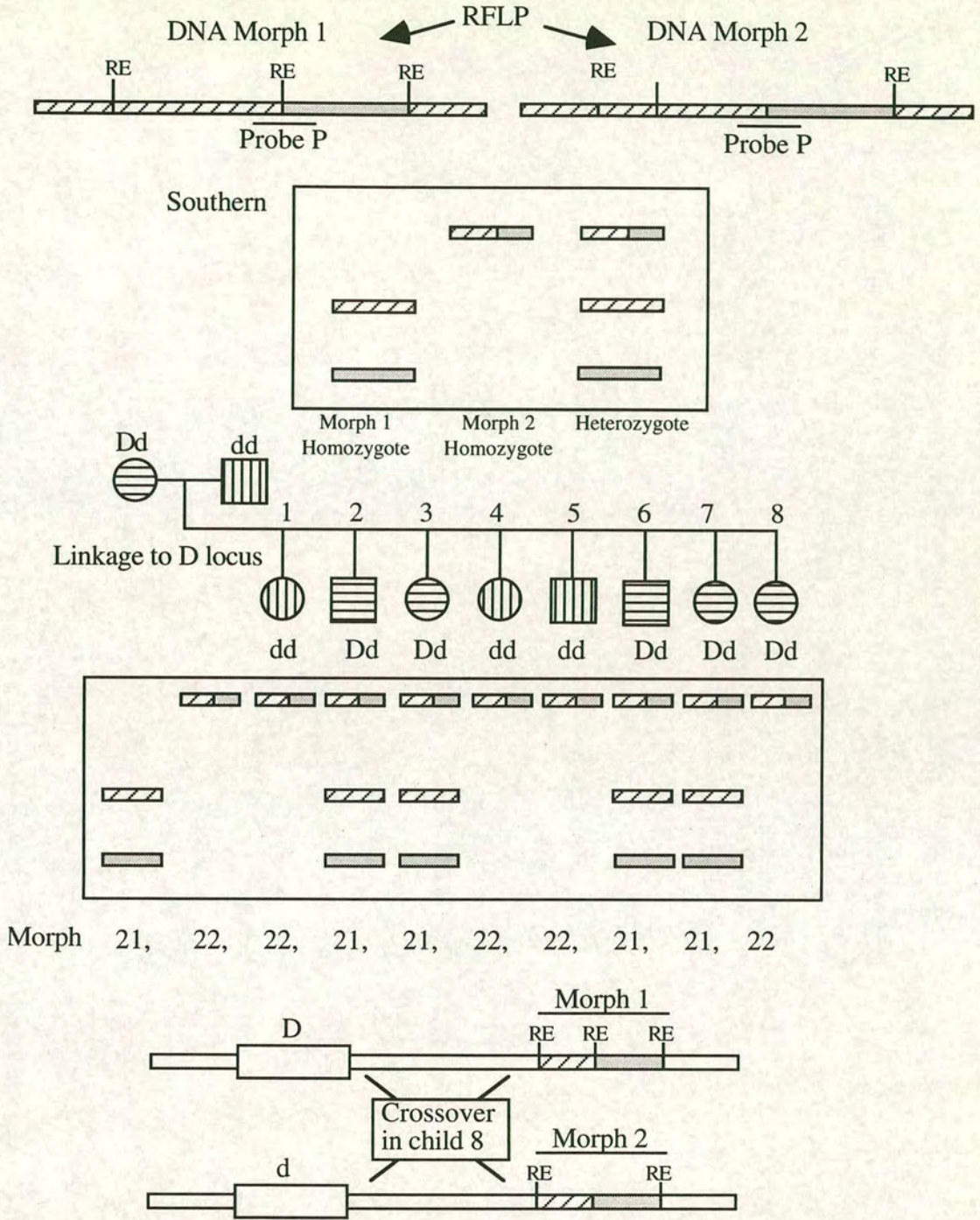


Figure 1.1

The detection and inheritance of a restriction fragment length polymorphism (RFLP). A probe P detects two DNA polymorphisms when the DNA is cut by a certain restriction enzyme (RE). The pedigree of the dominant disease phenotype D shows linkage of the D locus to the RFLP locus: only child 8 is a recombinant.

Enzymatic analysis revealed similar K_m and V_{max} for ACE extracted from lung tissue from both strains (Koike et al. 1994). However, another study failed to identify linkage between ACE alleles and hypertension in an F2 (SHR x WKY) population using CA repeat elements to differentiate ACE alleles, but found linkage in an F2 (Dahl SS/Jr x MNS) population (Deng et al. 1992).

Phenylethanolamine N-methyl transferase (PNMT), an enzyme involved in catecholamine synthesis, has been found to be elevated in the brains of SHR-SP. The gene for the enzyme has been mapped to the region 17q22-17q24 in humans which is within the conserved linkage group syntenic to the region in the rat where the locus BP/SP-1 (or Bp1) was found (Bell 1992). The PNMT gene has now been located to Chromosome 10 in the rat, but no sequence differences were identified between the cloned genes obtained from SHRSP and WKY genomic DNA libraries, including 1.0 kb of 5' sequence containing known regulatory regions and all exons (Koike et al. 1994).

In addition to the RAS, other regulatory systems potentially involved in blood pressure homeostasis have been implicated. The kallikrein gene family or closely linked genes has been marked with an RFLP using a pancreatic kallikrein cDNA probe which cross hybridised with a number of closely related kallikrein genes. This demonstrated that inheritance of the SHR allele in recombinant inbred strains derived from SHR and BN progenitors, is associated with significantly greater median systolic, diastolic and mean blood pressures (Pravenec et al. 1991).

The results of linkage between candidate genes and hypertension in animal models of genetic hypertension can not be directly translated into the human situation. Association studies in humans look for a difference in the observed frequency of an allele at a marker locus comparing affected cases to unaffected, unrelated controls and for evidence of the loss of expected random segregation of alleles in a population. An allele is said to exhibit linkage disequilibrium with a trait if it occurs at a significantly higher frequency in the affected group compared with the unaffected control group (Griffiths et al. 1993). Despite linkage of an allele, however, causality is not implied. One allele may lie close to another which is responsible for the trait and be in linkage disequilibrium with it.

Linkage studies test for a departure in the random segregation of both traits and genetic markers in families and look for evidence of excess sharing of a particular allele in affected relatives that is not consistent with random Mendelian segregation (Lander et al. 1994). Sibling or relative pairs may be examined, but analysis can be complicated by incomplete or delayed penetrance of the phenotype. Both these methods are very

dependent on a low likelihood of recombination events (<1%) occurring between the marker and the trait.

In the case of polygenic disease, the relatively small contribution to the overall phenotype made by a single locus may make its effects too small to detect by allele sharing methods (Soubrier 1993). A different approach is to search directly for mutations in candidate genes. Sequence variations do not always alter function as they may not alter the amino acid sequence or they may occur in non-coding regions, but a mutation can be followed through a pedigree and its relationship with a phenotype be ascertained (Lifton et al. 1992; Lifton et al. 1993).

A sib-pair linkage study using a dinucleotide repeat sequence 3' of the angiotensinogen gene, looked at hypertensive siblings from Salt Lake City and Paris. The study found a significant excess of shared alleles in male Parisian hypertensive siblings, but greater statistical significance was achieved when severe hypertensives from both populations were analysed together (Jeunemaitre et al. 1992). Fifteen angiotensinogen variants were identified and sequenced, five with 5' nucleotide substitutions and ten with silent or missense variants. Only variants with the resulting amino acid substitutions M235T and T174M showed linkage disequilibrium, both elevated blood pressure and increased plasma angiotensinogen levels were found in heterozygote and homozygote M235T carriers (Jeunemaitre et al. 1992). Substrate level may well be important as the K_m for the enzymatic cleavage of angiotensinogen by renin is close to the plasma level. Two groups have reported linkage of the angiotensinogen locus with pre-eclampsia, using the 3' dinucleotide repeat sequence in a linkage study and the M235T variant in an association study (Arngrimsson et al. 1993; Ward et al. 1993).

Two studies have shown linkage of hypertension, but not obesity, with the insulin receptor (R1-) allele based on an *Rsa* I RFLP occurring from the insertion of a CA repeat sequence in intron 9 (Ying et al. 1991). This result is of interest due to the association of insulin resistance with essential hypertension. Conflicting results were obtained using a microsatellite polymorphism located in intron 2, where derived allele frequencies showed similar distributions between hypertensive and normotensive subjects suggesting that the causative polymorphism may lie closer to intron 9 than to intron 2 (Zee et al. 1993). An association study has recently described a marker in close proximity to the α -adducin locus and hypertension (Casari et al. 1995). This membrane skeleton protein was previously implicated as a potential factor in the genetic hypertension of the MHS rat by altering ion transport (Bianchi et al. 1984).

These results support the view that variants of 'candidate' genes may contribute to the heritable factors of an individual's variation in blood pressure and affect their

subsequent response to the environment (Harrap 1994). The important contribution to blood pressure homeostasis of the RAS and the finding of genetic polymorphisms occurring in components of this system in both rat models and in humans, has led directly to the study of the contribution of individual genes, in particular by alterations in the regulation of their expression and function. New methods, in particular transgenesis, have been developed which may help identify how hypertension might result from altered gene regulation and function (Ganten et al. 1991).

The Renin Angiotensin System

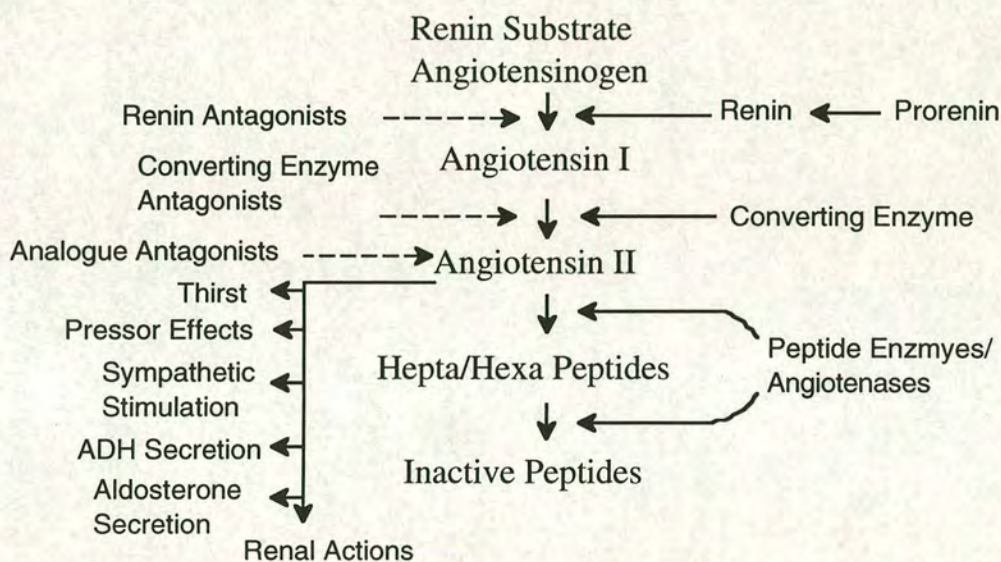


Figure 1.2 The renin-angiotensin system, biochemical pathway and physiological consequences.

1.3 Prorenin

In 1971 Lumbers (Lumbers 1971) discovered that an inactive form of human renin could be activated by partial hydrolysis which led to the postulated existence of prorenin, a renin precursor. It was only in 1981 that direct evidence was reported for the fact the synthesis of renin involved a "pro" form (Morris et al. 1981). Any physiological role for prorenin remains to be shown except from its part in the production of active renin. Production of the deca-peptide angiotensin I (AI) by the

action of renin on liver derived angiotensinogen is the rate limiting step of the renin-angiotensinogen system (RAS). The biologically active peptide produced by this cascade is angiotensin II, an octapeptide that is known to play a pivotal role in blood pressure regulation and sodium-potassium homeostasis (Peach 1977; Vecsei et al. 1978).

The primary translation product of the renin mRNA is preprorenin (Morris et al. 1983). The 2kDa signal sequence directs preprorenin to the lumen of the rough endoplasmic reticulum for processing to prorenin. In the case of the mouse *Ren2* renin gene the preprorenin is 45kDa which is then converted to a 43kDa prorenin. In contrast to other renin genes the product of the mouse *Ren-2* gene generates a prorenin that is not glycosylated (Paul et al. 1988).

It is thought that these sugar moieties may have some effect on conversion of prorenin to renin but are not essential. Baxter *et al* (Baxter et al. 1989) transfected AtT-20 cells with a human preprorenin expression vector in which the glycosylation sites had been mutated and found that the cells actually processed more prorenin to renin than normally glycosylated preprorenin. This suggests that these sugar moieties may regulate or retard processing of prorenin to renin.

Aspartyl proteases have similar three-dimensional structures and the structures of pepsinogen, *Rhizopus* pepsin, and penicillopepsin have been reported as has that of recombinant human renin (Sielecki et al. 1989). The structural cores and active sites of renin are highly conserved with other aspartyl proteases. Despite the similarities there are variations in the surface residues that are critical for the differences in substrate specificity. These include the "flap" areas; the carboxy terminal domains that may affect the pH profile, allowing renin to have activity at a relatively high pH of 5.5-7.5; and the carbohydrate moieties at the glycosylation sites, which may affect intracellular transit time (Paul et al. 1988). Protein modelling has shown that the pro-segment of prorenin can fit into the substrate binding cleft of the renin molecule and that more than three ionic interactions between the pro-segment and renin contribute to keeping the prorenin structure in an inactive form (Shiratori et al. 1990).

Secreted prorenin is the product of the constitutive pathway (Pratt et al. 1987). The regulation of this pathway is minimal with pro hormone being delivered to the cell surface at a constant rate (Kelly 1986). The primary product of the regulated secretory pathway is active renin (Pratt et al. 1987). This pathway accumulates secreted proteins, releasing them in response to physiological stimuli. This response is mediated through the dense secretory granules of the juxtaglomerular cells which also serve as the major site of prorenin conversion to renin (Taugner et al. 1986). Certain strains of mouse which carry a gene duplication for renin have expression in the

submaxillary gland and this also contributes significantly to the level of circulating renin (Mullins et al. 1989).

Activation of prorenin occurs by proteolytic cleavage of the 43 amino acid pro-segment after the dibasic Lys Arg residues in all species (Catanzaro et al. 1983). The first amino acid of renin is a leucine. The "maturase" has not yet been conclusively defined although any candidate enzyme would have to be able to activate prorenin, correctly cleave the pro-segment, lack any renin degradation activity and colocalise within the renin secretory granules. In the human kidney cathepsin B has been proposed as the renin maturase (Wang et al. 1991). Cathepsin B activates crude

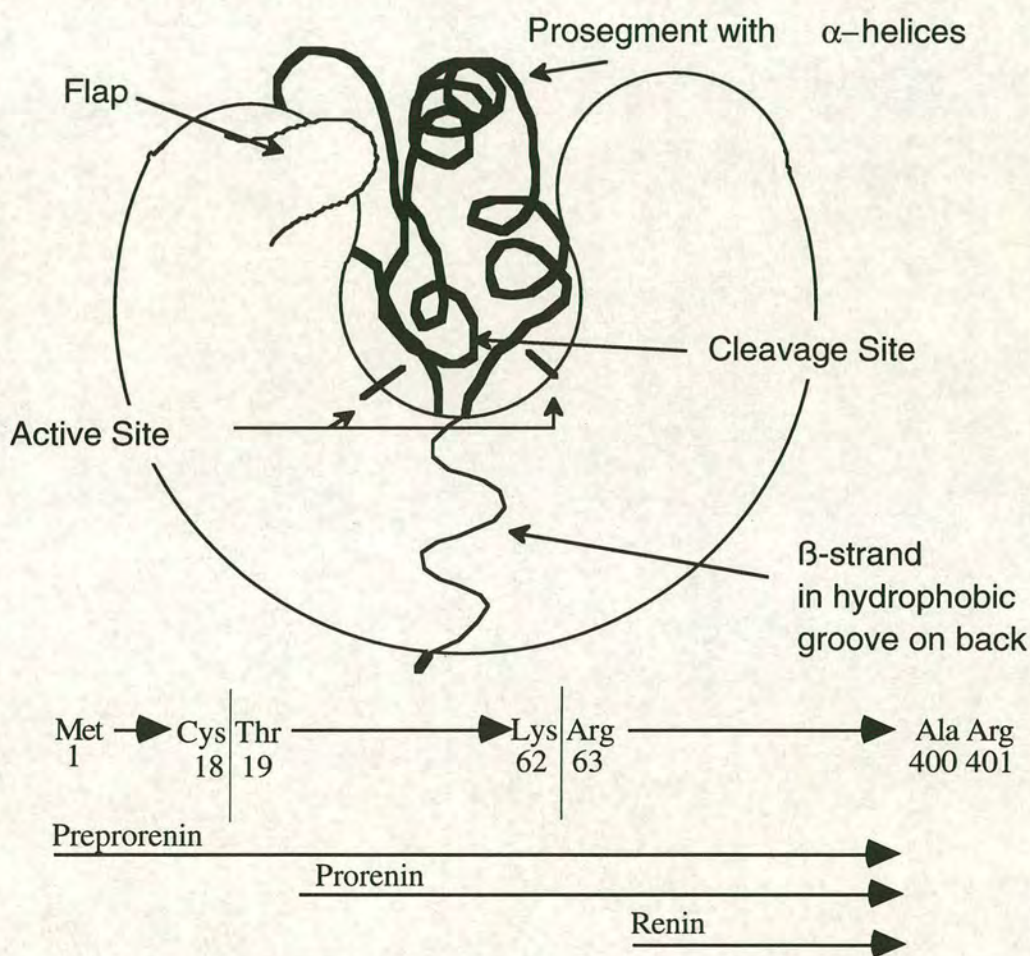


Figure 1.3 Schematic representation of the structure of human prorenin, deduced from the known structure of pepsinogen and human renin (Sielecki et al. 1989; Shiratori et al. 1990) and the peptide sequences involved in processing of preprorenin to active renin (Hsueh et al. 1991).

preparations of human prorenin and decreases its molecular weight (Takahashi et al. 1992). It has also been shown to correctly cleave the pro-segment, does not degrade renin, and colocalises with the secretory granules (Taugner et al. 1986).

Prorenin can be reversibly activated by acidification or cryoactivation (Leckie et al. 1980). These result in a conformational change where the pro-segment is removed from its normal inhibitory position. It is possible that there is local activation of this sort in a given organ where the local prorenin level could be much higher than in the circulation.

Prorenin is an unusual zymogen as it is present at a much higher concentration than renin in the circulation of humans. Possibly as much as 90% of total renin (the sum of active and inactive renin) in humans is prorenin (Sealey et al. 1980). It is possible that prorenin is present in plasma at such high concentrations to act as a pool of potential renin, supplementing renin release from the kidneys.

However, if plasma prorenin is not a pool of potential renin then there are two obvious possibilities: 1) prorenin is a functionless protein or, 2) it has a function of its own that does not require cleavage of the pro-segment. Osmond, Sealey & Mackenzie (1991) (Osmond et al. 1991) put forward six possible functions of prorenin.

1) Prorenin could be a source of "quick renin" so that it would be rapidly converted to renin in the circulation, only if there is some way of activating it endogenously.

2) Prorenin could play a role in circulation that is not mediated by renin but within the bounds of the known physiological effects of the renin angiotensin system.

3) Prorenin could have a direct effect, unrelated to the reported actions of renin angiotensin.

4) Prorenin may be a vehicle for transporting renin in an inactive form to a given target organ where it is either activated to renin or acts directly with a related function to the known effects of renin angiotensin.

5) Prorenin may be a vehicle for transporting renin in an inactive form to a given target organ where its effect is outwith the known effects of renin angiotensin.

6) Prorenin in the circulation may merely be overflow from renal and (or) extrarenal sources.

The clinical implications of plasma prorenin levels are apparent in a number of conditions. For the most part, in normal individuals prorenin increases with chronic

volume depletion, during the luteal phases of the menstrual cycle and pregnancy (Sealey et al. 1986; Troffa et al. 1991).

Patients with diabetes mellitus (Types I & II) often exhibit elevated plasma prorenin levels, and these have been implicated as a potential marker of nephropathy. It also has been found that elevated prorenin levels are an indicator of vascular complications in Type I diabetes mellitus (Luetscher et al. 1989).

The rise in circulating prorenin seen in diabetes has been hypothesised to be the result of impaired conversion of prorenin to renin in the kidney (deLeiva et al. 1976). This would suggest that either a diabetic kidney has defective storage granules or impaired proteolytic processing. In support of this is the report that in the streptozotocin diabetic rat there is defective prokallikrein-to-kallikrein conversion and decreased levels of cathepsin B.

1.4 Renin

Renin is a single chain glycosylated carboxypeptidase of MW 41kD. It cleaves angiotensinogen, with a degree of specificity which is unlike other members of the aspartyl protease family, with an optimal function at pH 5.5 - 6.0 (Baxter et al. 1991; Campbell et al. 1991). The active site of renin lies in the cleft between the two lobes of the protein.

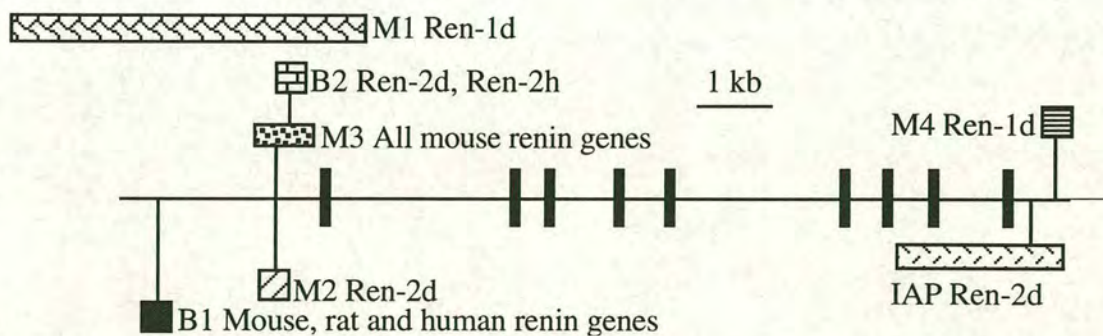


Figure 1.4

Basic structure of murine genes. The nine exons (solid boxes) and eight introns (solid line between exons) are shown. IAP, intracisternal A particle (Sigmund et al. 1991).

Both human and rat genomes contain a single copy of the renin gene on chromosome 1 and chromosome 13 respectively (Sigmund et al. 1991). In the mouse the renin structural genes also reside on chromosome 1 and are tightly linked to the *Rnr* locus. These are expressed highly in the kidney, but in mice high expression is also found in the submaxillary gland (SMG). Strains of mouse such as C57BL/6 and Balb/c have one renin gene (*Ren-1c*), but certain strains such as DBA and Swiss have two (*Ren-1d* and *Ren-2d*) (Field et al. 1984). *Ren-2d* is thought to have arisen as a result of a gene duplication event 2.5 - 5.5 million years ago (Dickinson et al. 1984; Holm et al. 1984) and it encodes a thermolabile, non-glycosylated isozyme, present at high levels in the granular convoluted tubules of the SMG. In two renin gene mice *Ren-1d* is expressed mainly in the JGA cells of the kidney at an approximately equivalent level to *Ren-2d* expression in the SMG on a per cell basis. Field and Gross (1985) demonstrated that all three mouse genes were expressed approximately equivalently in the kidney (Field et al. 1985).

Table 1.3

| Tissue | Cell Type | Genes |
|---------------------|------------------------------------|---|
| Kidney | Juxtaglomerular | <i>Ren 1c</i> = <i>Ren-1d</i> = <i>Ren-2d</i> |
| Submandibular Gland | Glomerular Convoluted Tubules | <i>Ren-2d</i> » <i>Ren 1c</i> » <i>Ren-1d</i> |
| Foetal Adrenal | Developing Cortex | <i>Ren-2d</i> = <i>Ren-1d</i> = <i>Ren 1c</i> |
| Adult Adrenal | X-zone, Zona Fasciculata | <i>Ren-2d</i> = <i>Ren-1d</i> » <i>Ren 1c</i> |
| Testes | Interstitial Leydig Cells | <i>Ren-1d</i> » <i>Ren-2d</i> = <i>Ren 1c</i> |
| Ovary | Theca, Corpus Luteum | <i>Ren-2d</i> = <i>Ren-1d</i> , <i>Ren 1c</i> |
| Anterior Prostate | Glandular Epithelium | <i>Ren 1c</i> » <i>Ren-1d</i> = <i>Ren-2d</i> |
| Foetal Subcutaneous | Specific population of Fibroblasts | <i>Ren 1c</i> = <i>Ren-1d</i> » <i>Ren-2d</i> |

Adapted from (Sigmund et al. 1991)

All mouse renin genes consist of 9 exons and 8 introns with a high degree of homology between cDNA sequences, 97% between *Ren-1c* and *Ren-2d* and 99% between *Ren-1c* and *Ren-1d* (Sigmund et al. 1991). Both types of mouse strain are inducible with androgen showing higher levels of expression particularly in male mouse SMG, but also in female rat and mouse liver.

Two highly repetitive elements have been mapped within the renin locus: a B2 repetitive element is unique to the 5' region of the *Ren-2d* gene and a B1 element is

present within the 5' flanking region of all three mouse genes. An 0.5kb insertion flanked by five nucleotide direct repeats, designated M3 (Mouse insertion 3), reside within 80bp of the transcription start site of all three mouse genes. A partial intracisternal A particle (IAP) genome lies 1kb 3' of exon 9 in *Ren-2d* but is not present in either *Ren-1* allele. The largest insertion is M1, a 7.2kb sequence flanked by short direct repeats found between *Ren-2* and *Ren-1*.

Significant homology exists between the mouse and rat renin, approximately 88% and between the mouse and human renin genes, approximately 78%. At the amino acid level, all three mouse renin genes exhibit ~97% homology. The most significant differences are between the Ren-1 and Ren-2 proteins at three asparagine-linked glycosylation sites, which may account for the differences in glycosylation and thermostability.

1.5 Angiotensinogen

Angiotensinogen is a glycoprotein identified in the α -2 globulin fraction of plasma. It is the only known substrate for renin and is the precursor of all the angiotensin peptides (Lynch et al. 1991). Tissue specificity and hormonal responsiveness are controlled by cis-acting elements in the 5' flanking region of the gene. Despite different sizes of mRNA being produced by multiple polyadenylation sites it does not result in altered substrate specificity. It appears that hormonal stimuli and ontogeny affect the choice of polyadenylation site (Ohkubo et al. 1986). Angiotensinogen is post-translationally modified by glycosylation resulting in AOs with different isoelectric points and molecular weights. These AO isoforms are all equally effective renin substrates (Ohkubo et al. 1986). The functional significance of AO isoforms remains unclear, but one high molecular weight molecule has been postulated to associate with hypertension in pregnant women (Lynch et al. 1991; Ward et al. 1993), and linkage studies demonstrated a correlation between high blood pressure and AO isoforms (Jeunemaitre et al. 1992).

In adult mammals the greatest source of AO is the liver, where it is synthesised and released constitutively (Lynch et al. 1991). Angiotensinogen has also been shown to be expressed in brown and white fat, brain, kidney, adrenal, ovary, testes, vascular wall, and heart (Campbell et al. 1984; Stornetta et al. 1988; Ingelfinger et al. 1990). The expression of the AO gene seems to be regulated by a number of physiological and pathological conditions. Angiotensinogen mRNA increases with salt depletion, estradiol, androgens, mineralocorticoids, thyroxine and AII and decreases in response to insulin administration (Ellison et al. 1989). Localisation and regulation of AO in the

kidney proximal tubular cells suggests that Ao could be released either into the lumen or into the renal interstitium, providing a source of substrate for the intrarenal generation of angiotensins (Hunt et al. 1992).

1.6 Angiotensin-Converting Enzyme

Angiotensin-converting enzyme is a zinc-containing peptidyl-dipeptidase that hydrolyzes carboxyl terminal dipeptides from small peptides (Bernstein et al. 1989). Angiotensin-converting enzyme (ACE) cleaves the decapeptide AI to the biologically active octapeptide AII (Beldent et al. 1993). However, ACE is also capable of hydrolysing other substrates, including kinins and is the same enzyme as kininase II which degrades bradykinin. Therefore, ACE has a dual function in generating a vasoconstrictor and inactivating a vasodilator. The entire ACE protein is encoded by a single gene consisting of 26 exons (Bernstein et al. 1988). Differential splicing results in 2 mRNA species, which are 4.9 and 4.1kb long and are expressed in the lung and the testes respectively. The C-terminus contains hydrophobic amino acids which are responsible for the ACE anchorage to the membrane of several cell types, including vascular endothelium. The soluble form of ACE found in plasma and seminal fluid is thought to be derived from the membrane bound enzyme after cleavage of its extracellular domain from the cell surface.

Angiotensin-converting enzyme has been detected in almost all vascular endothelium tested (Caldwell et al. 1976). It is also present in the brush border of the proximal tubule, small intestine and choroid plexus (Bruneval et al. 1986). These sites suggest a role for ACE in water and solute adsorption and possibly degradation of small peptides. The location of ACE on the endothelial surface makes it strategically positioned for the generation of AII and the regulation of systemic vascular tone. In the kidney, ACE is located in the endothelium of arterioles, proximal tubules and glomerular capillaries.

The gene expression of ACE is enhanced by cAMP, glucocorticoids and thyroxine and may be down regulated by AII. Genetic studies in humans indicate that ACE variants are involved in cardiovascular pathology. A deletion polymorphism in the ACE gene may increase the risk of myocardial infarction (Cambien et al. 1992).

1.7 The Angiotensin II Receptors

The angiotensin II receptors are distinguished by their physiological role and mode of inhibition. AT₁ is inhibited by Losartan, DTT and mediates the pressor effects

of AII (Bumpus et al. 1991). AT₂ is inhibited by the non-peptide PD12377 and its structural analogues. The physiological role for AT₂ has yet to be defined. The most common source for membranes for AII binding was the adrenal cortex, recognised as containing a mixture of the receptor types (Chiu et al. 1989; Whitebread et al. 1989). Autoradiography studies of the adrenal gland demonstrated the heterogeneity of AT receptors in the cortex and a relatively pure population of AT₂ sites in the rat adrenal medulla (Chiu et al. 1989). In the rat kidney only the AT₁ receptor is reported (Chang et al. 1991; Edwards et al. 1992). In contrast, the human kidney shows a unique distribution in which AT₂ sites are present on the large preglomerular vessels of the renal cortex (Grone et al. 1992).

The type 1 receptor (AT₁) belongs to the class of G-protein-coupled seven transmembrane receptors (Bunkenburg et al. 1991; Iwai et al. 1991). The receptor protein is 359 amino acids long. The native protein is probably subject to post-translational modification, with multiple potential N-glycosylation sites found in the extracellular domains (Sasaki et al. 1991; Iwai et al. 1992). In the rat two different receptor subtypes, type 1 α and 1B, have been identified. Sequence comparison between these two subtypes shows a high degree of homology (94%) at the protein level. Most changes are highly conservative particularly in the transmembrane regions.

By interacting with specific receptors AII elicits multiple cellular responses. The coupling of the receptors involves a number of second-messenger systems. Angiotensin II stimulates phospholipase C (through a G-protein), the production of inositol 1, 4, 5-trisphosphate, diacylglycerol and the mobilisation of intracellular Ca²⁺ (Griendling et al. 1989; Griendling et al. 1989). Angiotensin II also stimulates phospholipase A₂ or D leading to the release of arachidonic acid and its metabolites (Ford et al. 1989). Recently, it has been demonstrated the AII, through the AT₁ receptor, stimulates the Jak/STAT pathway (Marrero et al. 1995). Jak2 appears to associate intimately with the AT₁ receptor indicating that Jak2 activation in response to ligand occupancy of the AT₁ receptor is analogous to cytokine receptor activation. Angiotensin II has been shown to promote growth of smooth muscle cells and to induce proto-oncogenes such as *c-fos* (Geisterfer et al. 1988). It has been proposed in other cell types that the Jak/STAT pathway is used by cell surface binding cytokines responsible for transcriptional activation of early growth-response genes (Darnell et al. 1994). It is possible that the Jak/STAT pathway may play a similar role in the control of AII-mediated cell growth (Marrero et al. 1995).

1.8 Tissue Renin-Angiotensin Systems

The presence of renin activity in the blood vessel walls was demonstrated by Gould *et al* in 1964. Since then the possibility that there exists, outwith the circulating renal-derived RAS, tissue RASs in several tissues has provoked continued interest. These tissue RASs may have complementary and distinct functions from the classical, endocrine RAS. Locally synthesised vascular and cardiac tissue renin has been proposed in a number of biochemical and physiological reports (Dzau 1987; Dzau *et al.* 1987; Lindpaintner *et al.* 1991).

Other members of the RAS are produced, or present in a number of tissues. The production of hepatic angiotensinogen results in a relatively constant high level of renin substrate, close to K_m (half maximal velocity), in both intravascular and extravascular compartments. As previously mentioned, the angiotensinogen gene is also expressed in several tissues including the heart and kidneys (Stornetta *et al.* 1988; Ingelfinger *et al.* 1990). Converting enzyme is present in high concentrations in blood and interstitial fluid and systemic vascular endothelial cells.

Conversely, circulating renin levels are exquisitely regulated and subject to constant feedback mechanisms (Laragh *et al.* 1990). The kidneys are the only organs which have a demonstrated capacity to synthesise and process prorenin to active renin and to secrete renin into the circulation. Other tissues, such as the adrenal and the pituitary are capable of producing prorenin protein, but do not secrete active renin into the circulation.

There is no evidence that circulating renin is selectively taken up by any tissue other than the liver (Kim *et al.* 1987). Prorenin constitutes the vast majority of circulating renin yet there is no evidence that it is irreversibly converted, in any substantial amount, to active renin in any tissue (Nielsen *et al.* 1988). An hypothesis has been put forward by Sealey *et al.*, however, that prorenin could be reversibly activated on binding to a specific receptor that would ensure prorenin remained in an "open" conformation allowing it to cleave AI from angiotensinogen (Sealey *et al.* 1989). Despite this intriguing possibility there is no evidence of a prorenin binding protein or of any physiological reversible activation of prorenin.

The mode of action through which a vascular RASs may raise blood pressure has provoked several proposals. Most interest has focussed on the role vascular RAS has in determining vascular structure. Changes in resistance vessel structure, specifically a decrease in lumen to wall ratio could be a factor in chronically maintaining increased vascular resistance in hypertension (Folkow 1982).

There remains controversy over whether the heart and vasculature are examples of tissues with localised renin gene expression. Renin mRNA has been detected in these tissues in certain studies. If this were the case, these organs may be regulated by this local renin. If not, circulating renin would be the only determinant of Ang I production in the heart and vasculature. The analysis of studies proved problematical due to a number of considerations such as uptake of renin from the circulation and renin assay specificity. However, both local synthesis and uptake from circulation may be important mechanisms in tissue-specific RASs.

Unger *et al* (Dorer *et al.* 1978; Unger *et al.* 1985; Unger *et al.* 1989) found marked inhibition of ACE activity in the lung, kidney, aortic wall and myocardium, as well as in plasma following oral administration of ACE inhibitors to SHR. Treatment was discontinued after two weeks and plasma ACE activity promptly returned to pretreatment levels, whereas blood pressure remained low for 2-3 weeks following discontinuation of the ACE inhibitors. The lack of correlation of BP level with plasma ACE activity was not seen with tissue ACE activity, especially in the kidney, brain and aorta which were more closely correlated with blood pressure response. Assad and Antonaccio (Asaad *et al.* 1982) demonstrated that bilateral nephrectomy virtually eliminated plasma renin activity without reducing arterial renin concentrations over a 24 hour period after nephrectomy in both SHR and Wistar-Kyoto rats. The SHR showed a higher arterial renin concentration than that found in the Wistar-Kyoto rat and after bilateral nephrectomy Captopril still exerted an antihypertensive effect. Vascular renin, therefore, may respond to inhibition by Captopril and may be responsible for some of the increase in blood pressure level seen in the SHR.

Owens (Owens 1985) treated SHR with either hydralazine or Captopril at doses that gave equal blood pressure reduction. Smooth muscle mass was decreased with Captopril relative to hydrazine, suggesting a non-pressure related factor, possibly Ang II, may be an important mediator of smooth muscle cell growth. Captopril also significantly reduced smooth muscle cell size in the Wistar-Kyoto control rat, suggesting an Ang II role in smooth muscle cell growth in this normotensive strain.

As previously mentioned, Ang II also affects growth and gene induction in cultured cardiomyocytes and may play an important role in various disease processes in the heart as well as in the vessel wall. After demonstration by Pfeffer *et al* (Pfeffer *et al.* 1985) that ACE inhibitors prevent ventricular remodelling in rats after experimental myocardial infarction, groups in Boston (Pfeffer *et al.* 1988) and New Zealand (Sharpe *et al.* 1988) demonstrated that treatment with ACE inhibitors, following myocardial infarctions in humans, resulted in an increase in systolic volume index and an improvement in ejection fraction in both studies.

Although tissue RAS components may be either of local or circulating origin, these local compartments may generate Ang II with different enzyme-substrate concentrations and kinetics. Although circulating RAS is important for regulation of the cardiovascular system, in individual tissues production of Ang II can be altered by local activation of tissue ACE, which may not necessarily result in a systemic blood pressure effect but could result in altered local function such as cardiac and vascular remodelling and atherosclerosis.

1.9 Transgenesis

A transgene can be defined as a sequence of DNA introduced into a species by one of a number of methods. Following stable chromosomal integration, the transgene is transmitted through the germ line by Mendelian inheritance to subsequent generations (Hogan et al. 1986; Lang et al. 1994). Due to experimental constraints most transgenic animal work has been done in mice and more recently in rats. Transgenesis can be achieved in rabbits at a lower efficiency and with difficulty in sheep, goat, pig and cow, reviewed by Mullins and Mullins (Mullins et al. 1993). The effects of manipulating an individual gene, within a complex system, and the subsequent genetic and environmental interactions giving rise to the resulting phenotype can then be studied *in vivo*.

The genetic material forming the transgene construct may consist of DNA from the same species or from a different species. The promoter sequence of the gene of interest can be used to direct appropriate tissue specific and temporal expression. However, an heterologous promoter can be used to direct expression of a transgene to specific tissues, or allow expression to be stimulated in response to a defined stimulus. For example the metallothionein promoters can respond to dietary heavy metals mediated by heavy metal sensitive response elements within the promoter sequence (Yiangou et al. 1991). This can circumvent problems of transgene expression during foetal development or allow the study of the effects of transgene function starting at a defined time point. Regulatory elements within promoter sequences of genes can be studied using 'reporter' gene constructs where a promoter sequence is linked to a gene encoding an easily detectable enzyme (Hunter et al. 1993; von Harsdorf et al. 1993). These may be designed to express either a conveniently assayable product, e.g. the *Escherichia coli* Lac Z gene codes for β -galactosidase which produces a blue colour on cleavage of the substrate X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactosidase), and the green fluorescent protein (Prascher et al. 1992; Chalfie et al. 1994). Another marker of gene expression used is SV40 which by production of T antigen leads to

tumourigenesis (Sigmund et al. 1991). A series of constructs with systematic deletions of putative regulatory regions within promoter sequences ligated to the reporter gene enables identification of the specific sequences which are important for determining regulation of gene expression (Sigmund 1993).

The structure of the transgene makes an important contribution to the level and specificity of its expression. Locus control regions (LCRs) (Needham et al. 1992; Strouboulis et al. 1992), matrix attachment regions (MARs) (Stief et al. 1989) and co-integration strategies add to the level of complexity in applying transgenic technology. Both LCRs and MARs serve to insulate the transgene from any silencing effects seen in a region of integration and give position and copy-number independent gene expression. Often large constructs can be assembled by co-injection *in vivo* but it is preferable to manipulate genomic transgenes. This may ultimately take the form of artificial mammalian chromosomes, however presently transgenic animals have been produced using yeast artificial chromosomes (Montoliu et al. 1994).

For experiments that require large numbers of preimplantation embryos, such as microinjection of eggs, gonadotrophins are often administered to females prior to mating to increase the number of eggs that are ovulated (superovulation). Pregnant mare serum (PMS) is used containing follicle-stimulating hormone (FSH) and human chorionic gonadotrophin (hCG) containing luteinizing hormone (LH) is also used. The efficient induction of superovulation depends on several variables: the age and weight of the females, the doses of gonadotrophins and their time of administration, and the strain used.

Fertilised eggs can be collected several hours before microinjection. Once the oviducts are removed from the mated females they are placed in an embryological watch glass containing M2 medium (see table 2.1) at room temperature. The newly ovulated oocytes (~12 hours postovulation), surrounded by cumulus cells are found in the ampulla, the upper region of the oviduct. The oviduct is opened in M2 medium (see table 2.1) containing 300mg/ml hyaluronidase (Calbiochem) and incubated at room temperature for several minutes until the cumulus cells fall off. M16 medium is dispensed in 30 μ l drops on the bottom of a 35-mm sterile plastic tissue culture dish. Sterile pipettes are used to transfer the embryos into the drops of M16 medium and the dishes are placed in a 37°C, 5% CO₂ incubator.

The pronuclei of fertilised eggs can be most easily be injected when they are at their maximum size. The pronuclei swell progressively during the one-cell stage and are at an optimal state for injection for a period of 3-5 hours. An oocyte is prepared for injection by placing the tip of the holding pipette next to the oocyte and applying negative pressure through the holding pipette to keep the egg attached to the holding

pipette (see figure 3.2). The pronuclei are located under the microscope and ideally it should be in the hemisphere of the egg closest to the injection pipette, central to the axis of the holding pipette. Either male or female pronuclei may be injected, however, the male pronucleus is usually larger and easier to "hit".

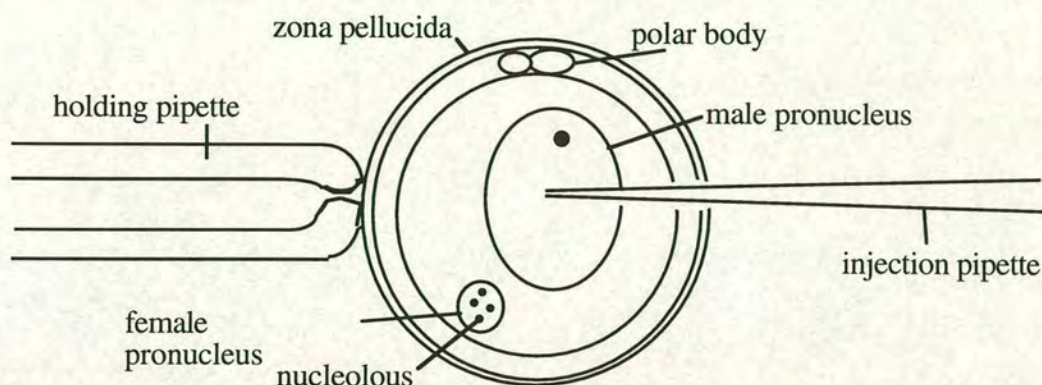


Figure 1.5

Injection of the pronucleus. The plasma membrane has been pierced and the egg returns to a spherical shape.

The zona pellucida of a fertilised rat egg is much more resilient than the mouse counterpart. This leads to problems in piercing the egg during injection. Typically 50-60% of the eggs survive the injection and they are then transferred into the oviduct of a 0.5 p.c. pseudopregnant female (see table 3.1).

Vasectomized or genetically sterile males are used to produce pseudopregnant females for oviduct transfer. After females in oestrus are mated with sterile males, the female reproductive tract becomes receptive for transferred embryos, despite her own unfertilised eggs degenerating. A pseudopregnant female will not resume her natural cycle for approximately 11 days.

The fertilised eggs are then removed, separated from surrounding cumulus cells by enzymatic digestion with hyaluronidase (Cal Biochem), 1U in 2.5 ml M2 media, and maintained in culture medium in a CO₂ incubator at 37°C. When the pronuclei are visible, microinjection of the DNA construct (1 mg/ml) is performed using a glass holding pipette and microinjection needle (<1 mm diameter) and approximately 2 pl of DNA solution is introduced, usually into the larger male pronucleus (see Chapter 3, figure 3.2). Fertilized eggs surviving this process are reimplanted into the oviducts of pseudopregnant females (mated with vasectomized males) and a variable proportion of the progeny born will then carry the transgene (Lang et al. 1994).

The transgene construct is first linearised by restriction enzyme digest which then forms concatamers of DNA and randomly integrates into the genome of the recipient. Integration is tested by Southern blot hybridisation and analysis of tail DNA from the resulting progeny using a transgene specific sequence as the probe or, alternatively, by polymerase chain reaction (PCR) using transgene specific sequences for primers. Multiple integration events can occur and this can be assessed by diagnostic Southern blot hybridisation. Integration may disrupt endogenous gene sequences or function, and hence any phenotype observed in transgene positive (TG+) animals must arise in more than one TG+ littermate to be certain that an insertional mutagenic effect is not responsible (Jaenisch 1988).

Expression of the transgene is then assessed by detecting mRNA by Northern blot analysis and *in situ* hybridisation determining both tissue distribution and temporal patterns of expression. The protein product or other intermediate phenotypes can be quantified and the resulting phenotype characterised. Breeding strategies can be developed to obtain animals homozygous for the transgene, segregate multiple insertion events or by crossing with a different transgenic line a new transgene can be introduced to create a double transgenic (Ornitz et al. 1991). The species into which the transgene is introduced may directly influence transgene expression. For example strain specific modifiers may affect transgene methylation and hence expression and phenotype (Boyes et al. 1991; Bird 1992).

Recent techniques for inhibiting or abolishing endogenous gene function have been developed. Selective inactivation or mutation of a targeted gene can be performed *in vitro* by homologous recombination in embryonic stem (ES) cells (Capecchi 1990; Doetschman et al. 1993). The gene of interest is interrupted by the introduction of a homologous sequence carrying a selectable marker, usually sequences which confer antibiotic resistance. Genetically altered ES cells carrying the mutation are then selected for by resistance to the relevant antibiotic, e.g. neomycin, in culture and are then introduced into a blastocyst. Resulting progeny will be chimaeric with coat colour being used to identify the relevant ES cell origin and confirm germ-line transmission of the mutated gene in subsequent generations. Gene knock-out technology and phenotype analysis are limited at present by the ability to culture ES cells only in certain strains of mice, predominantly mouse strain 129. Targeted disruption can be lethal during embryogenesis, but results of such studies are identifying new roles for some genes in development and apparent functional redundancy of others (Kessel et al. 1990; Chisaka 1991; Wright et al. 1991).

1.10 RAS Transgenics

Initial experiments using transgenesis in the RAS focused on generating a phenocopy of 2-renin gene mice, using a genetic background containing a single renin gene (*Ren-1c*) and a transgene consisting of the duplicated renin locus (*Ren-2*). Both Tronik *et al* (Tronik et al. 1987) and Mullins *et al* (Mullins et al. 1988) reported such models using different background strains, genetic origin of transgene and extent of transgene sequence used (2.5kb and 5.3kb of 5' flanking sequence respectively). The tissue-specific expression of the renin transgene differed in the two reports. Tronik *et al* reported qualitatively correct expression whereas Mullins *et al* reported low expression in some tissues yet elevated in others. These studies both observed that the transgenes used were hormonally regulated in the appropriate target tissues.

To assess more directly the location of important transcriptionally regulatory elements controlling mouse renin expression, transgenic mice were generated with constructs consisting of the mouse *Ren-2* promoter fused to the SV 40T antigen gene (Sola et al. 1989; Sigmund et al. 1990). Despite containing the same sequence as employed by Tronik *et al* inappropriate tissue-specific expression was observed and neither the tissues nor the tumours expressed renin endogenously. This suggested that the proximal 2.5kb of 5' flanking information was insufficient to direct expression of a reporter gene to renin expressing tissues *in vivo*.

Sigmund *et al* went on to generate transgenic mice with a construct containing 4.6kb of *Ren-2* 5' flanking sequence which exhibited qualitative tissue and cell specific expression (Sigmund et al. 1990). The transgene was also hormonally regulated in the submandibular gland, followed the normal developmental profile as the renin gene in the kidney and was responsive to the effects of angiotensin converting enzyme inhibitors during early postnatal life (Sigmund et al. 1991). These results suggest that important transcriptional regulatory elements lie between 2.5kb and 4.6kb, it does not rule out the possibility that redundant elements lie 3' of the gene or within the gene itself.

As with the mouse system, studies on human renin hRen expression began with large genomic clones. Both Fukamizu *et al* (Fukamizu et al. 1989) and Seo *et al* (Seo et al. 1990) first reported the tissue-specific expression of one hRen transgenic line, which contain the entire coding region and 3.0 and 1.2kb of 5' and 3' flanking sequences, respectively.

1.11 Transgenic Mouse Models of Hypertension

Initially studies into the genetic determinants of hypertension were limited to candidate genes. Polymorphisms in genes expected to be involved in hypertension were examined to determine if they cosegregated with an increase in blood pressure in F2 cohorts derived from hypertensive and normotensive strains. From these studies, renin was suggested to be linked to hypertension in some, but not all, hypertensive rat strains. To test the hypothesis that the renin-angiotensin system may be linked to the pathogenesis of hypertension, Ohkubo *et al* (Ohkubo et al. 1990) studied the result on blood pressure of elevated synthesis and release of components of the renin-angiotensin system. Two constructs were designed with expression of both the rat renin (rRen) and rat angiotensinogen (rAng) under the control of the mouse metallothionein (MT) I promoter. As the MT promoter is active in liver and is responsive to heavy metals such as zinc the aim of the experiment was to constitutively release the proteins into the circulation and examine the physiological effects. Transgenic mice were created containing either the rat renin (MT-rRen) or rat angiotensinogen (MT-rAng) and crossed to produce transgenic lines carrying both transgenes.

Systolic blood pressure was unchanged in any of the single transgenics compared with age matched nontransgenic littermates. Systolic blood pressure in the doubly transgenic males and females was increased by 30.5 mmHg and 25.2 mmHg respectively as compared with nontransgenic littermates. The increase in systolic blood pressure was reduced to normal levels, as anticipated, after treatment with Captopril. These results point out an important feature of the enzymatic interaction between renin and its substrate, namely it is species specific.

An analogous experiment to that of Ohkubo *et al* was carried out by Kimura *et al* (Kimura et al. 1992) generating transgenic mice containing the entire rat angiotensinogen, but not the rat renin gene. In some cases these singly transgenic exhibited a sustained increase in mean arterial pressure despite the absence of the rRen gene. The differences in design of this study as opposed to Ohkubo *et al* were 1) Kimura *et al* used a genomic rAng gene and 2.5 kb of flanking sequence, 2) the method of measuring blood pressure was performed by chronic intra-arterial catheter in conscious mice instead of tail cuff plethysmography employed by Ohkubo *et al* and 3) the level of rAng was twice that seen in the MT-rAng transgenic mice.

The species specificity of renin for its substrate was examined by Fukamizu *et al* by using recombinant mouse SMG renin and recombinant human renin with mouse

liver extract as the substrate. It was demonstrated the human recombinant renin had a 2.5 fold lower K_m and 23 fold lower K_{cat} than the mouse renin. This suggests that mouse angiotensinogen is not a suitable substrate for human renin. Fukamizu *et al* then examined transgenic mice containing the human renin and angiotensinogen genes (Fukamizu *et al.* 1993). As with the transgenic models containing both rat renin and angiotensinogen genes, the singly transgenic mice showed normal blood pressures whereas the doubly transgenic mice showed a sustained increase of 30 mmHg in systolic blood pressure. Despite all experimental groups showing a decrease in systolic blood pressure after administration of an ACE inhibitor only the doubly transgenic mice demonstrated a decrease with a human specific inhibitor.

1.12 Transgenic Rat Models of Hypertension.

Cardiovascular research has historically been carried out on animals larger than mice such as rats, rabbits and sheep. Despite techniques for measuring detailed physiology in mice improving there has been a great deal of interest in generating transgenic species of larger animals. The first rat model of hypertension was created by Mullins *et al* using the entire mouse Ren-2 gene (Mullins *et al.* 1990). The 24 kb *Xho*-1 fragment of mouse genomic DNA containing the *Ren-2d* gene as described above (Mullins *et al.* 1988) was microinjected into fertilised TG1 oocytes of a Sprague-Dawley/WKY cross. Thirty-seven eggs were re-implanted and eight progeny were delivered. By Southern blot hybridisation five were transgene positive (TG+), of which three (founders #25, #26, and #27) transmitted the transgene to the next generation. Four of the five TG+ founders were found to be hypertensive with SBP>200 mmHg by ten weeks of age confirming a transgene position-independent phenotype (Mullins *et al.* 1990). The normotensive TG+ founder #26 was found to be mosaic for the transgene, however, inheritance of the transgene in her offspring was associated with blood pressure elevation comparable to the other positive founders. Significant hypertension was sustained in subsequent generations in two lines, #26 and #27, though male infertility in line #26, a probable consequence of transgene insertional mutagenesis, prevented the subsequent generation of homozygotes in this line. The line TGR(mRen-2)27 as derived from founder #27 which has now been the subject of extensive investigation into detailed characterisation of the phenotype and the cause of the hypertension seen in these animals (Peters *et al.* 1991; Bachmann *et al.* 1992; Peters *et al.* 1993; Tokita *et al.* 1994; Vettor *et al.* 1994; Yasuo 1994).

Serial measurements of SBP by tail cuff plethysmography of TGR(mRen-2)27 rats (hemizygous for the transgene) showed an increasing blood pressure with age,

attaining a plateau at nine to ten weeks. Initial characterisation of the phenotype showed high plasma prorenin, and elevated adrenal tissue renin content (Mullins et al. 1990). Adrenalectomy with maintenance therapy of oral NaCl resulted in a fall in plasma prorenin to transgene negative (TG-) levels together with a fall in SBP suggesting that the adrenal was the principal source of the high circulating prorenin (Bachmann et al. 1992). *In situ* hybridisation and immunohistochemistry of the adrenal gland showed both over-expression of the mouse *Ren-2* transgene and renin protein to be principally present in the zona glomerulosa and outer zona fasciculata. Northern blot and ribonuclease (RNase) protection assays also identified high transgene mRNA transcripts in the adrenal gland, ~2500 fold higher than endogenous rat renin gene expression, and between 10 and 500 fold higher expression in this tissue compared with any other tissues assayed (Peters et al. 1991). Expression of the transgene in adrenal tissue, as shown by *Ren-2* mRNA levels was high from birth and progressively increased with age (Zhao et al. 1993).

Transgene expression in the kidney however, was highest at birth and decreased markedly at two to four weeks of age with further reductions occurring with developing hypertension. A similar profile was seen for endogenous rat renin expression with evident suppression in adult TGR compared to SD controls (Zhao et al. 1993). Suppressed kidney tissue renin and PRA was seen in TG+ compared to TG- littermates (Mullins et al. 1990).

Detectable transcripts were also found in brain (cortex/septum), pituitary, aorta, mesenteric vessels, lung, testes, ovary, coagulation gland, thymus, small intestine, stomach and uterus, all being sites of normal *Ren-2* expression in the mouse. However, *Ren-2* expression was not found in the rat SMG gland suggesting an absence of necessary *trans* acting factors. Other notable sites of absent expression as determined by Northern hybridisation included liver, heart, parotid gland, spleen and cerebellum (Peters et al. 1991). Others have reported *Ren-2* mRNA in the heart by RNase protection assay at the limits of detection (Zhao et al. 1993). In TG- controls, using a *Ren-2* derived probe that hybridised to rat renin mRNA, endogenous rat renin gene expression has been only detected in the kidney (Peters et al. 1991), but others have reported low levels of expression in adrenal, brain, pituitary and thyroid by RNase protection assay (Zhao et al. 1993).

The underlying cause of hypertension in the TGR(mRen-2)27 rat line is yet to be elucidated. Interest has focused on the adrenal gland in view of the high transgene expression and concomitant elevation of adrenal tissue renin and prorenin which were predominantly transgene derived, while renal tissue renin and plasma renin reportedly remained suppressed (Yamaguchi et al. 1992; Peters et al. 1993). In association with

the elevated adrenal transgene expression, mineralocorticoid levels were altered with increased 24 hour urinary excretion rates for 18-OH cortisol, deoxycorticosterone (DOC) and aldosterone in TG+ rats from six to eighteen weeks of age, but not at thirty weeks, compared with age matched SD rats. Conflicting results were found for plasma measurements with decreased DOC, in both transgenic homozygotes and hemizygotes compared to SD controls while plasma corticosterone levels were similar in SD and transgenic homozygotes but were significantly elevated in hemizygotes. No significant differences were observed for either plasma sodium or potassium levels in the three groups (Sander et al. 1992). Electron microscopy of adrenal tissue has revealed evidence of cholesterol accumulation in zona glomerulosa (ZG) cells. Studies on dispersed ZG cells from female transgenics showed a higher basal aldosterone secretion compared to cells from SD rats, but no difference between transgenics and controls in the net increase in aldosterone release in response to either Ang II or ACTH (adrenocorticotrophic hormone) stimulation was found (Rocco et al. 1994).

An increase in active renin release, from cultured adrenal cells from transgenic rats was seen in response to both Ang II and raised intracellular Ca^{2+} (Peters et al. 1993). Such mediators would normally be expected to elicit a negative feedback response on the kidney, suggesting that the normal negative feedback control does not operate on cultured adrenal cells derived from TG+ animals. Prorenin release *in vivo* was clearly stimulated by ACTH with a 10-fold rise in plasma levels compared with only a 4-fold rise in non-transgenics. However plasma renin concentration remained unaffected or fell respectively (Sander et al. 1992). If transgene expression in the adrenal does lead to deranged mineralocorticoid synthesis and metabolism, it seems unlikely that it is the sole factor inducing hypertension as spironolactone therapy did not lower blood pressure or prevent the development of high blood pressure in TG+ rats despite treatment leading to an elevation of plasma renin concentration in both SD and TG+ animals (Bader et al. 1992; Sander et al. 1992).

Measurement of free intracellular sodium in intact lymphocytes from TGR(mRen-2)27 using a sodium sensitive fluorescent dye method showed an increase relative to SD. This appeared to be secondary to reduced Na^{+} - K^{+} ATPase activity measured in erythrocytes, which also argued against a mineralocorticoid mediated effect (Tepel et al. 1994). It was not however established as to whether the animals studied had established hypertension or were pre-hypertensive. Dexamethasone suppression of pre-hypertensive TGR(mRen-2)27 did attenuate the BP rise, with a concomitant rise in mineralocorticoid levels, but a fall in plasma glucocorticoids (D. Ganten, unpublished observations) [Springate, 1994 #526].

No evidence of increased insulin resistance in TGR(mRen-2)27 hemizygotes has been demonstrated as shown by similar fasting plasma glucose and insulin levels together with equivalent fasting rates of hepatic glucose production and glucose utilisation during a hyperinsulinaemic euglycaemic clamp (Vettor et al. 1994).

Aims

The aims of this project were to:

1. Elucidate role of high levels of circulating prorenin in the TGR(mRen-2)27 rat.
2. Express prorenin, at a site distant from the kidney in transgenic rats.
3. Validate mouse mRNA and protein expression in the transgenic rats.
4. Investigate the phenotype of hypertension, with severe cardiac hypertrophy, apparent in the transgenic animals.

CHAPTER 2

Materials

2.1 Chemicals

The chemicals for general work were supplied by either Fisons (Loughborough), BDH (Poole) or Sigma (Poole), and were Analytical grade or equivalent. If a particular brand of chemical was required, this is noted in the text. Media for bacterial culture were supplied by Difco (East Molesley). Radionucleotides were supplied by Amersham International (Amersham). Double-distilled water was prepared using the PRIMA system from Elga (Bucks).

Polyacrylamide gels were prepared using the Sequagel reagents, TEMED and urea for gel electrophoresis were supplied by Sigma, as was ethidium bromide. Hexanucleotides for random priming DNA synthesis were supplied by Pharmacia (Milton Keynes). Phenol (containing hydroxyquinoline) was purchased redistilled and equilibrated with 10mM Tris-HCl (pH8.0) from Phi-Bio through Fisons.

2.2 Enzymes

Restriction endonucleases were purchased from Boehringer Mannheim, or New England Biolabs. T4 polynucleotide kinase was supplied by Gibco-BRL. DNA polymerase I (Klenow fragment) was supplied by Amersham International (oligolabelling and creating flush ends). T7 DNA polymerase and DNase I were supplied by Pharmacia. T4 DNA ligase was purchased from both New England Biolabs and Gibco-BRL. Proteinase K was purchased from Boehringer Mannheim (Lewes), and ribonuclease A from Sigma.

2.3 Animals

Animal husbandry and associated procedures were all performed in accordance with the regulations set down by the Animal (Scientific Procedures) Act 1986. All animals were housed in the Centre for Genome Research, and were maintained in controlled conditions with regulated temperature (18-20°C) and humidity (45-65%). A

12:12 hour light/dark cycle was used. Rats were kept with their mothers to three weeks of age. At three weeks of age the animals were tail biopsied and weaned from their mother. It was then that the animals were split, according to sex, into cages of no more than four. Founder animals and breeding animals were caged individually, except when mating with non-transgenic mates. Special breeding cages were used to house pairs of pregnant females that were 2.5 times larger than a standard rat cage. All transgenic animals were checked twice daily. Bedding was changed weekly, feed and water topped up daily. Standard rat chow (0.32% sodium, CRM diet, SDS Witham, Essex, UK) was used. Tap water was provided *ad libitum* to drink, and captopril was administered to the breeding animals (10 mgkg^{-1} per day) in the water.

Methods

2.4 General Methods

Restriction endonucleases and DNA modifying enzymes were used according to the manufacturers' instructions. Other enzymes were used according to published protocols, as indicated in the text. All solutions for use with RNA were made up with water to which 0.02% (v/v) diethyl pyrocarbonate (DEPC, from Sigma) had been added prior to autoclaving. The transcription buffers used in the synthesis of riboprobes were an exception to this, as DEPC inhibits T3 and T7 RNA polymerases.

2.5 Tail DNA Preparation

After application of local anaesthesia (ethyl chloride B.P. spray), the distal 1 cm of tail was cut off pups at weaning and placed immediately in an eppendorf tube on dry ice. To the resultant wound tissue adhesive (VetBond™) was applied. Tissue obtained from tail biopsy was digested overnight in 700µl of a buffer containing 50mM Tris-HCl pH 8.0, 100mM EDTA, 100mM NaCl, 1.0% SDS with 35µl of 10mg/ml proteinase K at 55°C. 700µl of tris-saturated phenol was added and the samples placed on a vertical rotator for 15 minutes. Samples were then spun in a microfuge for 5 minutes to separate the phases and the aqueous phase and interface were transferred to fresh 1.5ml Eppendorf tubes. Phenol/Chloroform/Iso Amyl Alcohol (25:24:1) (700µl) was added to each sample and again placed on a vertical rotator for 15 minutes. The samples were spun in a microfuge for 5 minutes to separate the phases and the aqueous phase and interphase were transferred to fresh 1.5ml tubes. Chloroform/Iso Amyl Alcohol (24:1) (700µl) was added and the samples were placed on a vertical rotator for 15 minutes. The phases were separated by spinning the samples in a microfuge then only the aqueous phase was transferred to a fresh tube. Isopropanol was added 700µl then the tubes were inverted several times and left at room temperature for 10 minutes. The samples were then spun at maximum speed for 10 minutes, the supernatant removed and the pellet washed in 70% ethanol. Finally, the samples were spun in a microfuge for 5 minutes, the ethanol removed, the pellet allowed to dry briefly and then resuspended in 200µl of Tris/EDTA pH8.0 and stored at -20°C.

2.6 Polymerase Chain Reaction

In order to identify transgenic positive animals as soon as possible a diagnostic PCR was developed taking advantage of the differences in renin genomic and transgene structure. The PCR reaction was carried out using three primers, all 21-mers, from exon 3 (CCATCCCAGACCTTCAAAGTC) exon 6 (CCTGGCAGATCACCATGAAGG) and exon 7 (GCTCCCTGCAGTTGATCATGC). There is a single base mismatch in primers exon 6 and exon 7 to compensate for a sequence difference between rat and mouse renin genes. This mismatch allows the primers to anneal at the same temperature to both DNA species. As the reaction conditions have not been optimised for generation of large PCR products, the exon 3 primer/exon 7 primer pair did not give rise to a product in the genomic situation. for Ren-2 sequence.

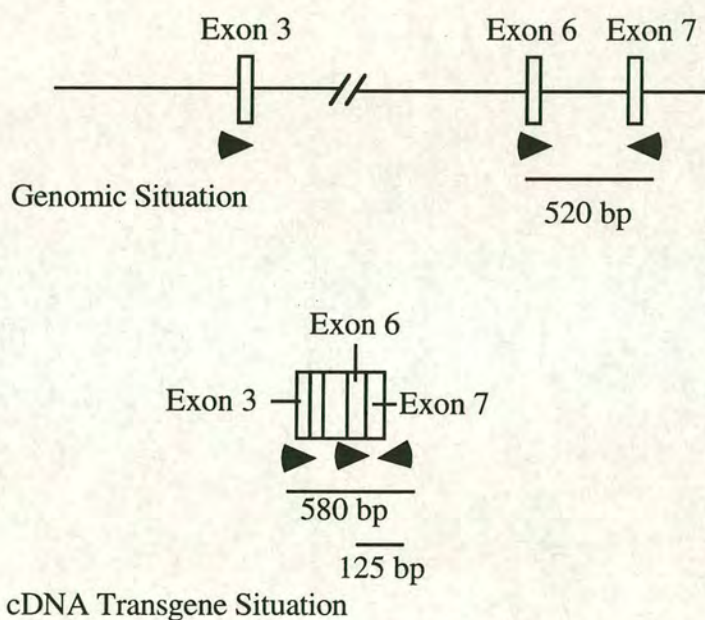


Figure 2.1 Schematic of diagnostic PCR reactions. See above for details of the PCR reaction and the oligonucleotides used.

Tail DNA, 2.5 μ l, was added to 40.5 μ l of double distilled H₂O (Elga autostill) and 5 μ l 10x Boehringer Mannheim (U.K.) Taq polymerase incubation buffer (Tris-HCl 100mM, MgCl₂ 15mM, KCl 500mM, gelatine 1mg/ml pH 8.3). To the reaction mix 0.5 μ l of 10mM dNTP and 1.25 μ l of each primer (10pM) was added. Finally 0.25 μ l of Taq polymerase, Boehringer Mannheim (U.K.) was added with 75 μ l of light white mineral oil, Sigma (U.K.) overlaid. The running conditions were as follows:

denaturing 94°C for 1 minute, annealing 58°C for 1 minute and extension 72°C for 1.5 minutes with thirty cycles of each. A final extension was performed at 72°C for 10 minutes followed by cooling to 18°C. The products were then resolved on a 2.5% agarose gel.

2.7 Electrophoresis

a) *Agarose*. Plasmid and genomic DNA was analyzed by electrophoresis through horizontal agarose slab gels (Seakem LE, NuSieve GTG, both purchased from Flowgen, Sittingbourne, Kent), which varied between 0.8% and 4% (w/v). Gels were run in 0.5 x TAE (20mM Tris-acetate; 10mM sodium acetate; 0.2mM EDTA, pH 8.3), or in 0.5 x TBE (45mM Tris-borate; 45mM boric acid; 1mM EDTA, pH 8.2) unless otherwise stated. Similarly, RNA samples were analyzed by agarose gel electrophoresis, except tone gel tank was set aside specifically for this purpose. This tank was cleaned regularly, treated with 3% H₂O₂ and rinsed with DEPC-treated water immediately prior to use.

The choice of size markers used on each gel was dependent upon the sizes of the DNA fragments to be resolved, and was one of the following: pUC18 x *Hpa*II; bacteriophage λ (Gibco-BRL) x *Hind*III/*Eco*RI + *Hind*III or λ x *Eco*RV.

b) *Polyacrylamide*. Polyacrylamide gels for resolving the products of RNase protection reactions were according to the method of Sanger and Coulson (1978), and were 6% polyacrylamide and 8M urea in a 0.5 x TBE to 1 x TBE buffer gradient. An alternative to this was the Sequagel Sequencing System (National Diagnostics, Bucks, England) where concentrate, buffer and diluent are ready-prepared and mixed in the appropriate ratios to produce gels of differing concentrations (typically 5%).

2.8 Southern DNA Blotting

Capillary transfer was used to transfer nucleic acids separated by gel electrophoresis onto positively charged nylon membranes (Boehringer 1417240). The method is based on that of Southern (1975). Gels were 0.8% agarose (Seakem LE) and were run in 0.5 x TAE (20mM Tris acetate; 10mM sodium acetate; 0.1mM EDTA, pH 8.3) unless otherwise stated in the figure legend. Ethidium bromide was added to the buffer to a final concentration of 0.5 μ g/ml. After visualising the DNA by ultraviolet light capillary transfer was set up according to Maniatis (Sambrook et al. 1989). The DNA was then cross-linked to the filter by baking the filter at 80°C for 30 minutes.

2.9 Preparation of plasmid DNA

2.9.1 Mini preparation of plasmid DNA:

An overnight 1ml culture of DH5 α *E. coli* cells was centrifuged for 15 seconds at full speed in an Eppendorf centrifuge. The supernatant was removed and the cell pellet resuspended in 100 μ l of lysis solution (lysozyme 2mg/ml, 25mM TMO pH 8.0, 10mM EDTA pH 8.0, 1% Glucose), vortexed briefly and left on ice for 5 minutes. To the lysis mix 200 μ l of alkaline/SDS solution (0.2M NaOH, 1% SDS) was added and again left on ice for 5 minutes after a gentle mix. The solution was centrifuged for 2 minutes and the supernatant passed through a polyallomer wool plug to remove chromosomal DNA into a fresh Eppendorf tube. Cold ethanol (1ml) was added to the filtered supernatant and the Eppendorf placed in a dry ice/ethanol bath for 5 minutes. The samples were centrifuged for 2 minutes and the ethanol removed. The pellet was resuspended in 200 μ l of Tris-Acetate solution (100mM NaAc pH 4.8, 50mM Tris-HCl pH 8.0) and 400 μ l of ethanol. After 5 minutes in a dry ice/ethanol bath the samples were microfuged for 2 minutes. The ethanol was removed and 100 μ l of 70% ethanol added prior to a further 2 minute centrifugation. After removal of the ethanol the pellet was resuspended in 40 μ l of H₂O.

2.9.2 Maxi preparation of plasmid DNA:

A 500ml culture was grown in L-Broth and 0.02% Glucose and ampicillin at 37°C. The cells were centrifuged in an HB101 rotor and Sartorius RC5 centrifuge at 6,000rpm for 10 minutes at 4°C. The cell pellet was resuspended in 7.5ml of ice cold sucrose solution (25% Sucrose, 50mM Tris-HCl pH 8.0) and 1.25ml of 10mg/ml lysozyme. The solution was inverted gently and left on ice for 15 minutes. To this solution 2.5ml of 0.5M EDTA pH 8.0 was added and again was mixed gently and left on ice for 15 minutes. The solution was transferred to a 50ml centrifuge tube and 10mls of Triton lysis mix (2% Triton, 0.05M Tris-HCl pH 8.0, 0.0625M EDTA pH 8.0) added. The tube was inverted and gently mixed then centrifuged in an SS-34 rotor at 15,000rpm for 30 minutes at 4°C. After decanting the solution the volume was made up to 25mls with a 1:1 mix of sucrose solution and Triton lysis mix. Cesium chloride was added (24g) as was ethidium bromide (912.5 μ l of 10mg/ml) and mixed well at room temperature. Beckman ultracentrifuge tubes (42mls) were used and mineral oil

was used to fill the unused space and balance tubes. The sample was spun in an NVT 65-1 rotor at 55K for 24 hours at room temperature. The plasmid band was removed using a G19 needle and 10ml syringe. The solution was extracted five times with an equal volume of isoprepanol saturated with CoCl_2 saturated TE pH8.0 to remove the ethidium bromide. The sample was then passed over a Pharmacia NAP-25 column to remove the salt and eluted with 3.0 μ l of 3M NaAc pH 5.6 at -20°C overnight. The sample was then placed in a glass Corex 33ml centrifuge tube and spun at 15,000rpm in the SS-34 rotor and RC5 centrifuge for 30 minutes at 4°C. After removal of the ethanol the pellet was washed with 70% ethanol (3mls) and recentrifuged at the previous conditions. When the 70% ethanol was removed the DNA was resuspended in 500 μ l of TE pH 8.0.

2.10 Electroelution of DNA from Gels

The Sall/HindIII fragment containing the entire Ren-2 cDNA was restricted from the plasmid p α 1AT/Ren-2 plasmid and electrophoresed to separate it from the plasmid DNA for use as a cDNA probe in the analysis of the offspring.

Gel slices were cut from agarose gels and the DNA eluted using two separate methods.

Samples were loaded into dialysis apparatus as shown (Figure 2.2). The buffer (1 x ISCO) used was 0.05M Tris-HCl, 2 x 10⁻⁴M EDTA, pH 7.7. The dialysis vessel was placed in the Little Blue Tank ensuring the bridge was dry. The gel slice was then put into place and the power adjusted to 15mA for 2 hours. The sample was collected by reversing the current for 10-15 seconds and then removing the buffer from the large well and from the small well level with the mesh. The buffer below the level of the mesh was placed into a 1.5ml Eppendorf tube, phenol:chloroform extracted and ethanol precipitated.

Another method used was to place the excised gel slice into dialysis membrane (Medicell International, London), secured at both ends (exclusion limit 1200 Daltons) with a minimum volume of electrophoresis buffer (200 μ l 0.5 xTAE). The dialysis membrane was then placed onto a Pharmacia gel tray and into the gel tank. The apparatus was run at 75V for 1 hour. The buffer/DNA was then ethanol precipitated and the DNA fragment resuspended in a suitable volume of TE pH 8.0.

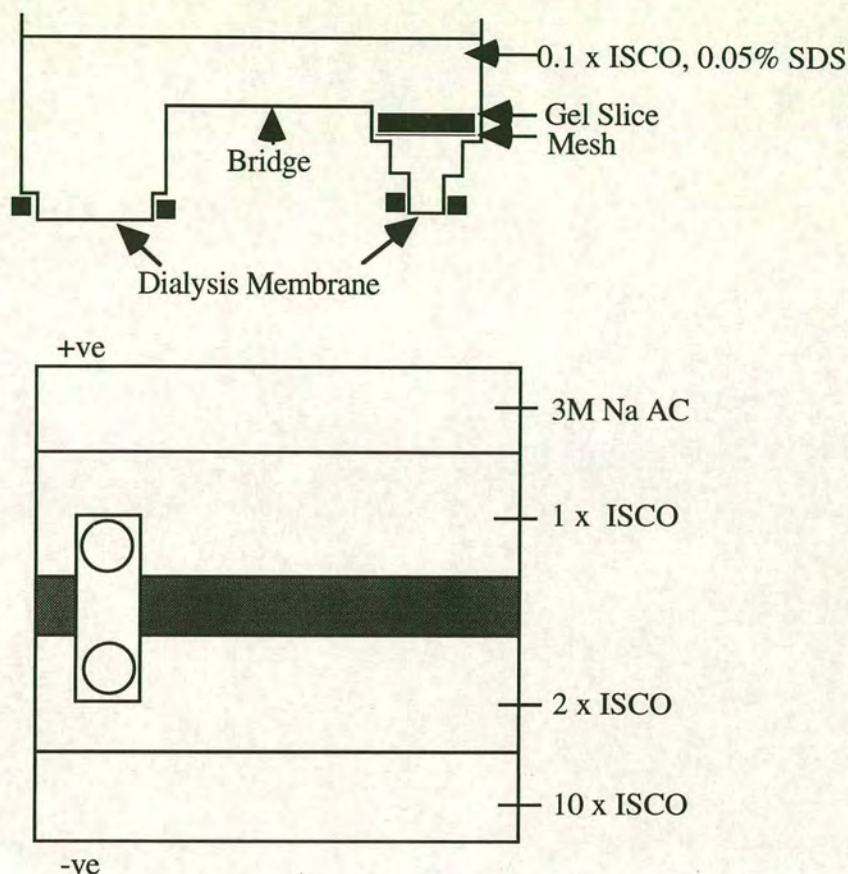


Figure 2.2

Dialysis apparatus for use with the "Little Blue Tank". Samples were loaded into dialysis apparatus as shown above. The buffer (1 x ISCO) used was 0.05M Tris-HCl, 2×10^{-4} M EDTA, pH 7.7. The dialysis vessel was placed in the Little Blue Tank ensuring the bridge was dry. The gel slice was then put into place and the power adjusted to 15mA for 2 hours.

2.11 Randomly Primed Probe

For both Southern blot hybridisation and Northern blot hybridisation, to detect the presence of the Ren-2 transgene or Ren-2 transcript, the *Sall/HindIII* fragment (nucleotides 1970-3219) from p α 1AT/Ren-2 was used (see Chapter 3, figure 3.1A for details of the p α 1AT/Ren-2 plasmid). The method used to radiolabel DNA restriction fragments was that of Feinberg and Vogelstein (1990) (Feinberg et al. 1990). An initial mix was prepared containing 1 μ l of template DNA (50ng/ μ l), 2 μ l of TE buffer pH 8.0 and 1 μ l of random primer (100ng/ μ l) in a screw-cap Eppendorf then heat

denatured at 100°C for 2 minutes followed by rapid quenching on ice. After a brief centrifugation, in a bench-top centrifuge, 2µl of oligo labelling buffer (0.5M - Tris-HCl pH 6.9, 0.1M MgSO₄, 1mM DTT, 1mM dATP, 1mM dTTP, 1mM dGTP), 5µl of ³²P-dCTP (10µCi/µl), 7.6µl H₂O, 1µl of Klenow (5U/µl) and 0.4µl of BSA (10µg/µl) were added and mixed. The reaction was incubated at room temperature (22-25°C) overnight and diluted to 100µl with TE pH 8.0.

Pharmacia NAP-5 columns were used to remove unincorporated nucleotides from the reaction mixture in order to lower background hybridisation on the Southern blot. The NAP-5 column was a prepacked disposable column containing Sephadex G-25 Medium of DNA grade. The columns were equilibrated with 10mls of 10mM NaPO₄ pH 6.8 and the sample added to the column in a volume of 500µl of buffer. After the sample entered the gel bed the randomly primed probe was eluted with 1ml of buffer. Any oligonucleotides less than 10bps in length are retained in the columns after elution.

The efficiency of incorporation of radiolabelled nucleotide was tested for each new aliquot of probe DNA. This was done by pipetting 2µls of the final probe mix (approximately 1/50) onto a 2cm circle of DE81 paper (Whatman, Maidstone), and Cerenkov counting in a Packard 1600CA Series scintillation counter (Pangborne, Berks), both before and after washing off the unincorporated nucleotides with 0.5M Na₂HPO₄. A typical reaction had 80-90% of the labelled nucleotide incorporated into high molecular weight DNA, but any probe containing greater than 50% incorporated labelled nucleotide was suitable with no further purification without producing any significant background interference during hybridisation.

2.12 Filter Hybridisation

Prehybridization was for a minimum of 2-3 hours at 65°C in 3 x SSC (1 x SSC is 150mM sodium chloride; 15mM trisodium citrate, pH 7.0), 5 x Denhardt's solution (100 x Denhardt's is 2% (w/v) each Ficoll 400 (Pharmacia); bovine serum albumin (fraction V, Sigma); polyvinyl-pyrrolidone), 0.1% (w/v) sodium dodecyl sulphate (SDS), 6% (w/v) polyethylene glycol 6000 and 200µg/ml denatured salmon testis DNA (Sigma).

Hybridization was performed in the same solution, except that the Denhardt's was reduced to 2 x concentration, for 16-40 hours at 65°C. Unless otherwise stated, 25mls of prewarmed hybridization or prehybridization solution was used for each set of filters in a sealed Techne hybridisation bottle.

2.13 RNA Extraction From Tissue

This protocol is for the preparation of total cellular RNA, and is modified from the method of Noyes *et al.* (1979) (Noyes et al. 1979). Frozen biopsy tissues were taken from liquid nitrogen, suspended in 5mls of TLES [0.2M Tris-HCl (pH9.0); 0.1M LiCl; 25mM EDTA; 0.1% SDS], and 5mls of phenol:chloroform:iso-amyl alcohol (25:24:1) in a Falcon 2059 tube and immediately homogenised with a Polytron homogeniser. The aqueous phase was then immediately overlaid on a caesium chloride pad (5.7M CsCl, 0.005M EDTA) and centrifuged in a Beckmann ultracentrifuge (5000 rpm, 18 hours, 25°C). The purified RNA was ethanol precipitated, air dried, redissolved in DEPC treated distilled water and stored at -70°C.

2.14 Purification of Poly(A) RNA

Messenger RNA (mRNA) was purified from total RNA using the Oligotex-dT system (Qiagen). 250µg of total RNA was incubated with 15µl of Oligotex-dT latex beads (10% w:v) in a binding buffer of 60mM tris-HCl (pH 7.5), 3M NaCl and 6mM EDTA at 65°C for three minutes. The samples were left at room temperature for ten minutes, to allow hybridisation between the oligo (dT)₃₀ linked to the latex particles and the poly (A) tail of the mRNA, then centrifuged at 14000 rpm for two minutes. The supernatant was removed and the Oligotex-dT beads washed twice in 600µl of wash buffer (10mM Tris-HCl (pH 7.5), 150mM NaCl and 1mM EDTA) and respun after each wash. The mRNA was then extracted from the Oligotex-dT beads by the addition of 2x20µl preheated (80°C) elution buffer (5mM Tris-HCl pH 7.5). The poly (A) mRNA was then ethanol precipitated and resuspended in the appropriate volume of DEPC treated distilled water.

2.15 Northern RNA Blotting

This method is modified from Thomas, 1980 (Thomas 1980). The required amount of RNA was ethanol precipitated on dry ice for 20 minutes. After centrifuging the samples at 14,000 rpm for 15 minutes at 4°C, the supernatant was removed and the pellet washed in 70% ethanol then respun at the same speed and temperature but for 5 minutes. The pellet was then dissolved in 3.7µl DEPC H₂O and 12.3µl 'Glyoxal mix' (160µl spectroscopic grade DMSO, 32µl 0.1M NaPO₄ : pH 7.0, 54µl deionised glyoxal). The samples were then denatured at 50°C for 1 hour and cooled on ice with

4 μ l of RNA loading buffer added (10mM NaPO₄; pH 7.0, 50% (v/v) glycerol, 0.4% (w/v) bromophenol blue).

The RNAs were resolved on a 1.2% (w/v) agarose gel containing 10mM NaPO₄. The gel was submerged in 10mM NaPO₄ and run at a constant current of 2-4mA/cm with buffer recirculation. The blotting procedure to transfer RNA onto positively charged nylon membrane was as per Southern blotting.

2.16 RNase Protection

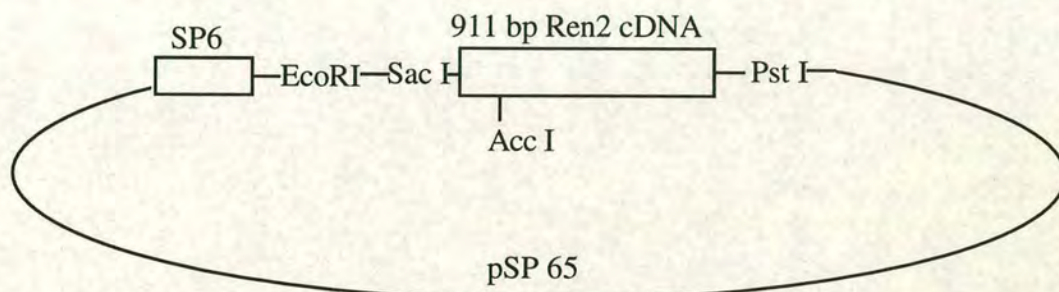


Figure 2.3 A cartoon representation of the pSLM plasmid used in the preparation of an RNA probe (see text below). The Ren2 specific sequence was a 911bp SacI-PstI fragment ligated into pSPG5 [Onoyama et al JapHeartJ 1978]. The plasmid (termed pSLM) was linearised with AccI and transcribed with SP6 polymerase to produce a 224 nucleotide cRNA (Field et al. 1984).

RNase protection studies (Zinn et al. 1983) used a sequence specific to the mouse Ren-2 transcript to determine the tissue specificity of the transgene. The riboprobe was produced by transcribing the desired sequence from linearised DNA. The 'transcription mix' contained 1 μ g of linearised DNA, 1.5 μ l of 0.1M DTT, 0.75 μ l of 2mg/ml BSA, 3 μ l of ATP/UTP/GTP (10mM each), 0.5 μ l of RNAsin, 6.25 μ l [³²P] CTP, 1.5 μ l 10 x Transcription buffer (400mM Tris-HCl pH 7.5, 60mM MgCl₂, 20mM Spermidine-HCl) and 1 μ l of SP6 polymerase. The reaction was incubated at room temperature for 1 hour. The template DNA was then digested by 1 μ l of DNase 1, incubated at 37°C for 20 minutes. After phenol/chloroform extraction the reaction mix was precipitated with NH₄Ac/Isopropanol and spun immediately. The supernatant was removed and the pellet resuspended in 100 μ l of DEPC H₂O. To ascertain the efficiency of the reaction 1 μ l of the probe was Cherenkov counted as per the randomly labelled probe protocol.

The probe was then diluted to $1\mu\text{l} = 150,000\text{cpm}$ and $1\mu\text{l}$ of probe was NaAc/ethanol coprecipitated with the total tissue RNA on dry ice for 20 minutes. The pellet was spun down at maximum speed in an Eppendorf centrifuge for 15 minutes, then rinsed in 70% ethanol. The 70% ethanol was removed and the damp pellet was resuspended in $30\mu\text{l}$ of RNase protection hybridisation buffer (RPH buffer, 87% v/v deionised formamide, 40mM PIPES pH 6.4, 430mM NaCl, 1mM EDTA). The samples were denatured at 85°C for 15 minutes then hybridised at 60°C overnight.

Each of the samples then had $350\mu\text{l}$ of digestion buffer added (8mM Tris-HCl pH7.5, 4mM EDTA pH8.0, 0.25M NaCl, 4000U RNase T₁, 240 μg RNase A) and were incubated at room temperature for 30 minutes. To the digestion buffer sample mix was added, $20\mu\text{l}$ of 10% SDS and $2.5\mu\text{l}$ of 20mg/ml Proteinase K which was incubated at 30°C for 10 minutes. The samples were then phenol/chloroform extracted and $5\mu\text{g}$ of yeast tRNA added before precipitation with ethanol on dry ice for at least 20 minutes. After centrifugation and the supernatant removed the pellets were washed in 70% ethanol and respun. The samples were air dried before being resuspended in $1\mu\text{l}$ of DEPC H₂O and $4\mu\text{l}$ of sequencing gel loading buffer. Following denaturation at 90°C for 10 minutes the samples were placed on ice and loaded onto a 5% polyacrylamide gel.

2.17 Photography

Gels were photographed with a Polaroid MP4 land camera, using Polaroid Type 57, Type 667 (positive only)(R. West. Edinburgh). Fixed tissue sections used for histology were photographed with an Olympus C-35AD-2 camera and an Olympus BHS microscope with PM-10ADS Automatic Photomicrographic System (Olympus, London) using Kodak T64 colour slide film or Ilford FP4 black and white print film.

2.18 Autoradiography

Autoradiography of [³²P]-labelled materials was performed at -70°C with intensifying screens (or for very short periods at room temperature), with either Kodak X-Omat, or Fuji RX X-ray film. RNase protection gels were dried under vacuum prior to autoradiographed at -70°C .

2.19 Phosphoimaging

Dried down gels on Whatman 3MM paper and covered in Saran wrap were placed in Molecular Dynamics Storage Phosphor Screens, which have the inner surface of the cassette plate coated in hygroscopic phospho-crystals. After a variable length of time the screens were laser scanned in a Molecular Dynamics phosphoimager and the results either quantitated with associated software, stored on optical disc or printed out.

2.20 Histology

Paraffin sections were taken by first placing the tissues in 4% formal saline for a minimum of 24 hours. The samples were then placed in a Shandon Citadel 2000 automatic processor where the tissues were successively dehydrated in 70%, 80%, 90%, & 3x100% ethanols for 1h each except for the final 2 ethanols which were 2h. The samples were then immersed in xylene three times for 1h, 2h and 2h respectively and the final stages were two wax baths for 3h and 4h respectively. The final wax bath was under vacuum.

The wax blocks were kept on ice for 30 mins prior to sectioning. The blocks were placed in a Shandon microtome and the block adjusted so that the face was flat to the blade. Preliminary sectioning at 25 μ m was used to cut into the sample until the desired area was reached. Sectioning was carried out at a maximum of 5 μ m. Several sections produced a strip which was removed from the microtome and placed in a 50°C waterbath. The slide was then submerged in the waterbath and the strip of sections floated onto it. The slide was then dried before staining.

The slides were then counterstained by rehydrating in 100%, 90%, 80%, 70% and 50% ethanol for five minutes each. The slides were then immersed in H₂O for five minutes and haemalum (Mayer's) BDH, Poole, England for three minutes. The slides were then washed in H₂O before staining with eosin, Sigma, Poole, England for two minutes. After dehydration in 2x95% ethanols for five minutes the slides were placed in isopropanol then xylene for five minutes each. The slides were then air dried and 500 μ l of DPX mountant applied and a cover slip placed on top.

2.21 Immunohistochemistry

Slides for immunocytochemistry were prepared by baking paraffin sections at 60°C for 30 minutes. The slides were then layered with 5% goat serum in phosphate

buffer solution (PBS) for 1h followed by aspiration of the goats serum. The first antibody, which is a 'total renin' antibody which cross reacts with mouse and rat renin, is then layered on and left overnight in a moist box. After removal of the renin antibody, again by aspiration, the slides were washed three times in 2% goat serum in PBS. The sections had 3% H₂O₂ added in 2% goat serum in PBS for 1 minute. After three more washes in 2% goat serum a goat anti-rabbit antibody was applied for 30 minutes. There were another three washes in 2% goat serum before approximately 0.2mg of diaminobenzidine tetrahydrochloride is applied and 0.1% H₂O₂ is added (See Chapter 4, figure 4.2). Once the samples were processed they were counterstained with eosin and heamallum as described above.

2.22 Ligation

Ligations were performed using T4 DNA ligase (Gibco-BRL) at 20°C for a minimum of 16 hours. For cloning small fragments into vectors, a molar excess of small fragment (up to 10-fold) was sometimes used, without the production of multiple-insert species. For the ligation of blunt-ended DNA fragments, a higher concentration of T4 DNA ligase was used (2.5U), and the reaction incubated for longer periods (48 hours or more).

Specific Procedures

2.23 Blood Recovery and Plasma RAS Determination

Two methods of blood recovery were employed, dependent on the size of the animal.

1. Cardiac Puncture: To obtain 500µl of serum, required for the RIA, tail bleeding was not appropriate. Three week old animals were lightly anaesthetised by halothane inhalation and shaved in the left lateral position. A 24G needle, with a 5ml syringe attached was inserted through the rib-cage, into the left ventricle of the heart. Negative pressure was applied slowly through the syringe and 1.0ml of whole blood was removed. The rat was then humanely sacrificed, still under anaesthetic, by cervical dislocation.

2. Retro-Orbital Bleeds: In older, larger, rats sufficient blood could be obtained by retro-orbital bleeding. Again, animals were lightly anaesthetised prior to the procedure with halothane. An heparinised microcrit tube was gently worked between the eyeball and the lachrymal glands until blood from the optical sinus flowed through the microcrit. In this case the animals recovered with no discomfort and could have the procedure repeated, after a suitable period to recover from the anaesthetic.

In both cases the blood was collected into a 1.5ml Eppendorf tube containing 350µl of inhibitor mix (0.25mM 1.10 O-phenanthraline, 1mM EDTA) and centrifuged immediately, at maximum speed for 5 minutes in a benchtop centrifuge. The serum was decanted into a fresh tube and frozen at -70°C until required for the RIA.

Total renin concentration was measured by activating a 10µl aliquot of plasma with 10µl of trypsin solution (400 units/ml trypsin, dissolved in TES buffer (0.1M N-Tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid; pH 7.2, 0.01% neomycin, 10mM EDTA)). Samples were incubated on ice for 10 minutes and trypsin-activation stopped by the addition of 5µl of egg white trypsin inhibitor (1600 units/ml, in TES buffer). Plasma active renin was measured by the addition of 10µl of TES buffer (without trypsin) to 10µl of plasma. Each sample was split into three aliquots before incubating each with radioactively labelled lyophilised renin substrate (¹²⁵I-angiotensinogen), isolated from nephrectomised rat plasma (final concentration; 80mg/ml, 0.11% 2,3-dimercapto-1-propanol, 1.15mg/ml 8-hydroxyquinolin in TES buffer). Reactions proceeded for 1-3 hours at 37°C and were stopped with RIA buffer (0.1M Tris-acetate; pH 7.4). The Ang I generated was measured by radioimmunoassay (Schelling et al. 1980; Hermann et al. 1988). Three measurements for each plasma were then averaged (for each assay) and if reactions and readings worked properly an error of ±5% was expected. Plasma prorenin concentration was determined as the difference between total renin concentration and plasma active renin concentration. Statistical significance was assessed using a Wilcoxon rank test and results are given as the mean ±1 SD.

To determine the contribution of mouse renin to the sum of mouse and rat renin, the samples were incubated for 2h at 4°C with a monoclonal antirenin antibody (No.120), which was found to be specific for murine renin with <3% cross-reactivity to purified rat renin (Peters et al. 1993). The incubation buffer was 0.1M Tris-acetate buffer pH 8.1. Incubation with antibody-free buffer served as a control. Antibody/renin complexes were precipitated with formalin fixed *Staphylococcus aureus* protein A. The renin activities were determined before the procedure and in the supernatant after immunoprecipitation.

Ang I and Ang II peptides were extracted from plasma with an equal volume of an acetone, 1M HCl and water mix (40/1/15 by volume). Samples were centrifuged (6,000 x g, 4°C, 10 min) and the pellet re-extracted with the same volume of the extraction medium and re-centrifuged. The supernatants were pooled, the acetone removed at 40°C in a stream of air, and the remaining aqueous phase was frozen and lyophilised. The dried samples were redissolved in 1ml of 1mM HCl, and centrifuged (10,000 x g, 4°C, 5min) before radioimmunological measurement of AI and AII.

Ang I and Ang II were iodinated by the Chloramine T method of Greenwood and Hunter (1963), and the ^{125}I -labelled angiotensins were purified on a 200 x 10mm plastic column packed with DEAE-Sephadex A 25 (Sigma). Antibodies were raised in New Zealand White rabbits immunised with angiotensin that had been coupled to bovine serum albumin by the carbodiimide method. Ang I and Ang II antibodies were used in the RIA in dilutions of 7.6×10^{-4} and 2.5×10^{-6} respectively.

2.24 Tail Cuff Plethysmography

Systolic blood pressure was measured with an programmable sphygmomanometer, IITC Life Sciences, Diss, Norfolk, using tail cuff methods. Animals were lightly anaesthetised (1-2% Halothane in oxygen using an Acoma vaporizer F (International Market Supply, Congleton, UK)) in a perspex chamber before being transferred to a thermostatically controlled pad and maintained under anaesthesia during measurement using a mask. The heating pads maintained the rats at 33°C which were placed prone during measurements being taken. The duration of anaesthesia was kept to a minimum and equal for all animals tested. Systolic blood pressure (SBP) was defined as the initial arterial flow pressure detected. Diastolic blood pressure (DBP) was calculated from the SBP and MBP values using the equation $\text{DBP} = (3 \times \text{MBP} - \text{SBP})/2$. Five readings were taken for each measurement (Whitworth et al. 1994).

2.25 Telemetric Blood Pressure Monitoring

With most methods of continuous blood pressure anaesthesia, restraint or tethering are required. These requirements have all been shown to induce stress responses in rodents. Even with training of the animal tail cuff plethysmography is associated with a rise in systolic blood pressure. This means that this method is not suitable for continuous measuring of blood pressure.

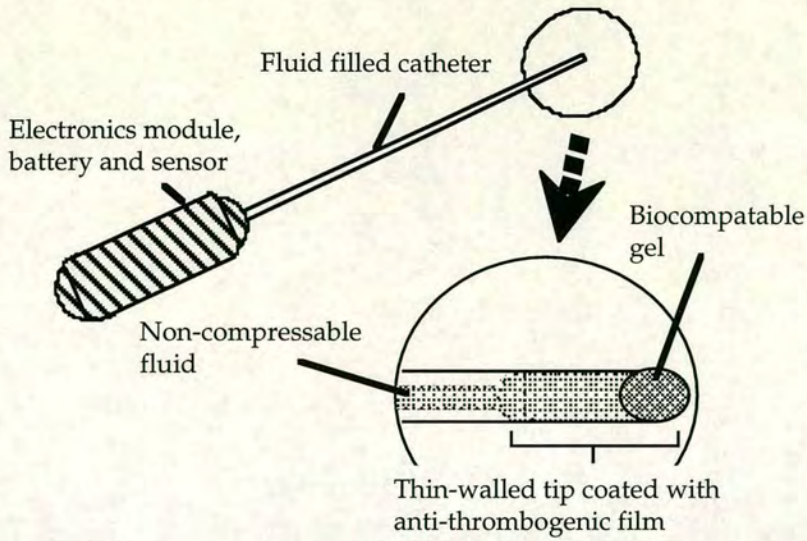


Figure 2.4

Diagram of the pressure transducer/transmitter implanted into the abdominal cavity of the rats monitored.

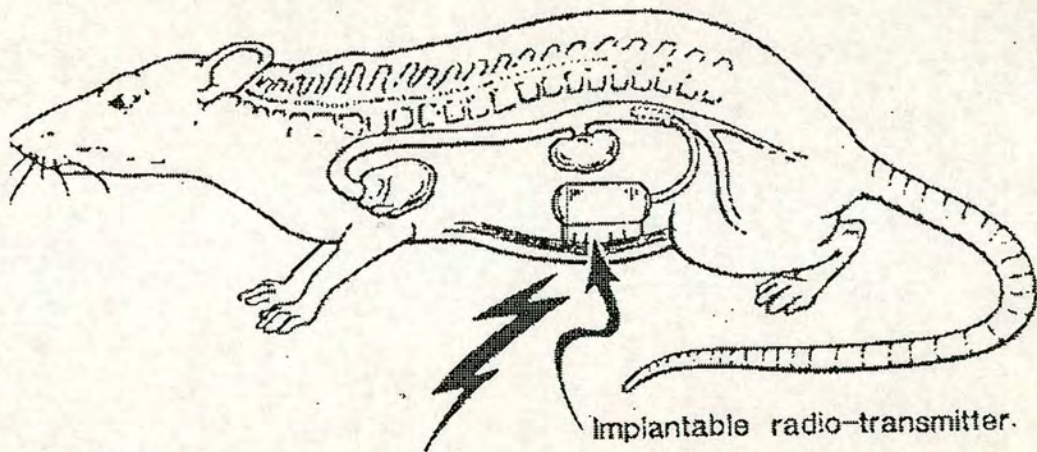


Figure 2.5

Diagrammatic representation showing the position of the telemetric implant relative to the descending aorta.

A radio-telemetry system allowed continuous intra-aortic recording of SBP, MBP, DBP, heart rate (HR) and motor activity with minimum stress placed on the animal. Direct blood pressure monitoring was performed using the Dataquest telemetric system from Data Sciences International, St. Paul, Minnesota, USA. Surgical implantation of a transmitter (TA11PA-C40) (see figure 2.4) containing a

strain gauge sensor resulted in arterial pressure conducted to the sensor via a 0.7 mm diameter catheter (see figure 2.5).

The rat was maintained on 2-4% Halothane/air mixture, under constant supervision on a 37°C heat pad throughout the surgical procedure. All four limbs were taped to a cork base-plate to give access to the chest and abdominal region. The fur was shaved from the entire chest and abdominal region and the area wiped with ethanol. An incision was made using a scalpel (shape 10A) extending from below the sternum to the base of the abdomen. Fat and other organs were cleared from the descending aorta using cotton swabs. A region of the descending aorta was then teased from the attached tissue. A suture was threaded under the descending aorta and held tight to prevent blood flow. Whilst the blood flow was halted the aorta was pierced with a specially designed needle. The catheter was placed in the opening in the aorta and a small swab (2.5mm x 2.5mm) placed behind the vessel. A drop of Vetbond was used to seal the catheter in place and the suture removed slowly in case of any bleeding from the aorta. All swabs and instruments were removed and the transmitter was sewn into the abdominal wall. Finally the wound was closed using staples that fell out once healing was complete. The animal was checked regularly for 2h post-operation.

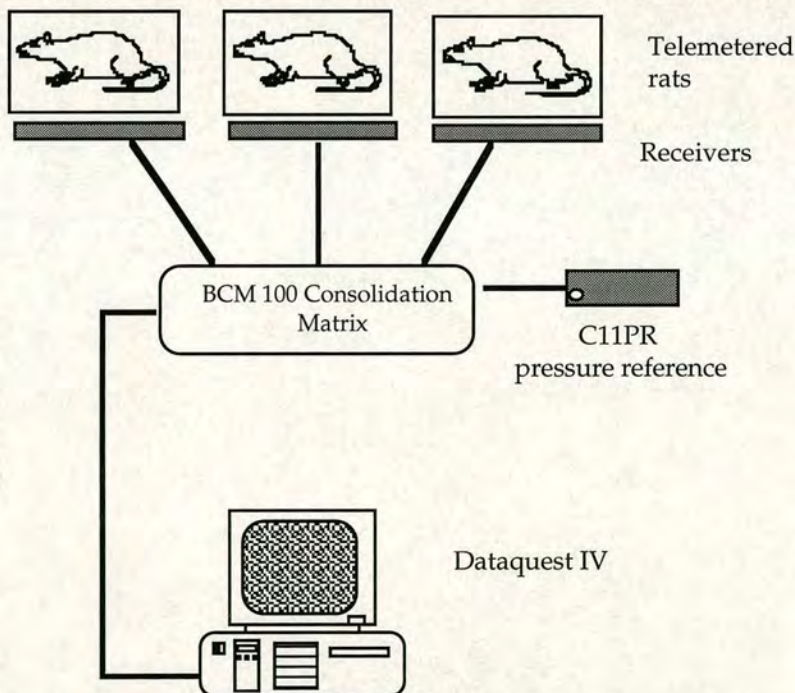


Figure 2.6 Diagrammatic representation of the telemetric equipment used to monitor blood pressure continuously.

The final 1cm of the catheter was coated with an anti-thrombic film and the tip filled with bio-compatible gel (see figure 2.4). The 5.5 - 6 week old male rats were



placed in a cage which sat upon a receiver panel (RA 1010) which sent the signals to a consolidation matrix (BCM 100) which is decoded by a card in an Everex 386/25 personal computer with accompanying software (Dataquest IV 2.2) (Bidani et al. 1993) and Microsoft OS/2 (see figure 2.6).

A dual ambient pressure monitor (C11PR) measured barometric pressure to correct for the implant pressure relative to a vacuum. These signals were also sent to the Everex 386/25.

2.26 Echocardiography

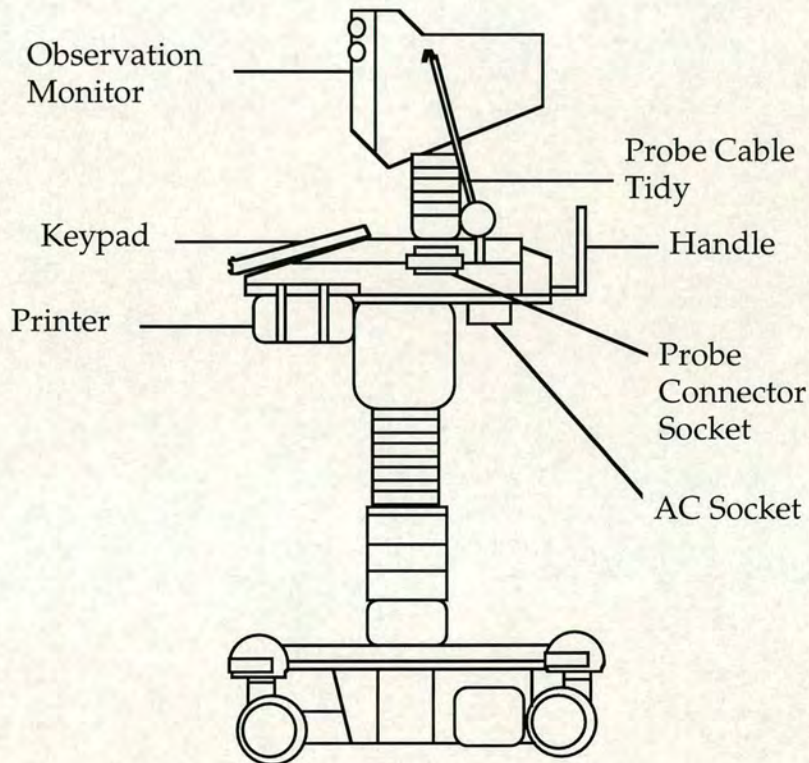


Figure 2.7 The Toshiba Capasee Diagnostic Ultrasound. The device could print copies of measurements taken or could send them directly to a personal computer.

All measurements were made using a convex 7.5 MHz probe on a Toshiba Capasee Diagnostic Ultrasound System (see figure 2.7) with Clear Image ultrasound couplant from Diagnostic Sonar, Livingston. Animals were lightly anaesthetised with halothane (1-2%) and shaved in the left lateral position. After application of the couplant a two-dimensional B-mode image was used to guide an M-mode scan through the left ventricle (Jones et al. 1992). The image displayed is dependent on how the probe is applied. Various probe angles were attempted to obtain the image best suited

for examination. The probe is provided with a mark (black arrowhead) that indicates the direction of the probe (see figure 2.8). End-diastole was identified as the widest diameter of the left ventricle during the cardiac cycle. Using on-screen digital calipers measurements (to 0.1mm) were taken of left ventricular end-diastolic diameter (EDD), diastolic interventricular septum (IVS) and diastolic peripheral wall thickness (PWT). Left ventricular mass (LVM) was then calculated using the cube formula: $LVM = 1.04 \times [(EDD + IVS + PWT)^3 - EDD^3]$ (Sahn et al. 1978) where 1.04 is the specific gravity of myocardium. Five measurements of the form above were taken and the average of the five LVMs calculated.

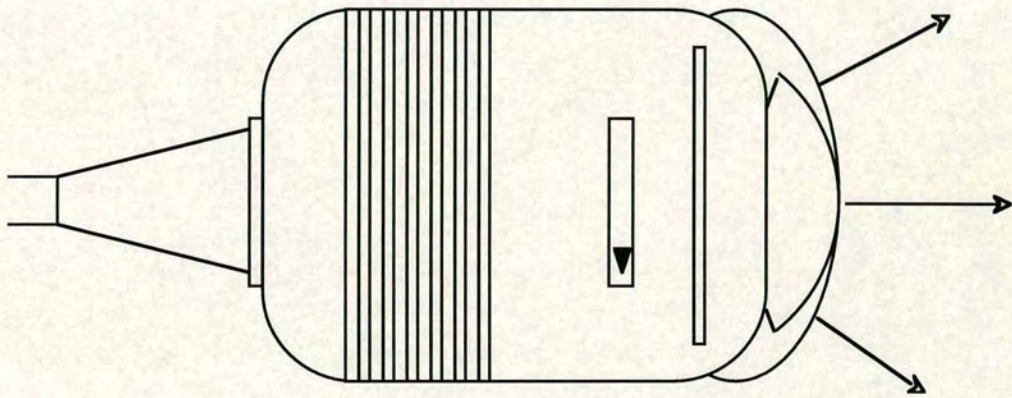


Figure 2.8 High frequency ultrasonic probe used with the Toshiba Capasee system.

CHAPTER 3

Construct Design and Production of Stable Transgenic Lines

3.1 Construct Design

In the rat, the renal juxtaglomerular (JG) apparatus is the only known site of prorenin conversion to active renin. To avoid any possibility of activation of prorenin-2 by the JG apparatus, a fusion transgene was constructed which consisted of an expression cassette, based on the human $\alpha 1$ anti-trypsin promoter, fused to the cDNA sequence encoding the mouse renin-2 protein. The design of the expression cassette (Jallat et al. 1990) enabled liver-specific expression of a heterologous transgene. It was therefore expected that an $\alpha 1$ AT/Ren-2 fusion transgene would be expressed in, and secreted from, the liver without the possibility of expression in renal juxtaglomerular cells. The expected specificity of expression should simplify interpretation of any phenotype observed in the transgenic animals.

One of the salient features of this vector was the provision of intronic sequences which are known to generally enhance the expression of transgenes (Wilson et al. 1990; Choi et al. 1991). The use of the 5' portion of the first intron of the $\alpha 1$ antitrypsin gene not only provided this function, but was known to contain elements important for appropriate liver-specific expression (M. Courtney, personal communication). The 3' portion of the intron was replaced by a c-myc-derived splice acceptor sequence and cloning sites for permitting the generation of fusion constructs (Bernard et al. 1983).

The p $\alpha 1$ AT/Ren-2 fusion construct, illustrated in Fig. 3.1, was initially prepared by amplifying the Ren-2d cDNA from plasmid pR2R2 by PCR (a gift from J. Mullins). The primers for the PCR contained the restriction sites *Sall*, which was found in the final construct at nucleotide 1970-1993 (all numbering is taken from the p $\alpha 1$ AT/Ren-2 where the *EcoRI* site is nucleotide 1), and *HindIII* (nucleotide 3224). A 1886bp *EcoRI/Sall* restriction fragment from pTG3925, containing the human $\alpha 1$ anti-trypsin promoter (GenBank Accession No., K02212) was ligated through

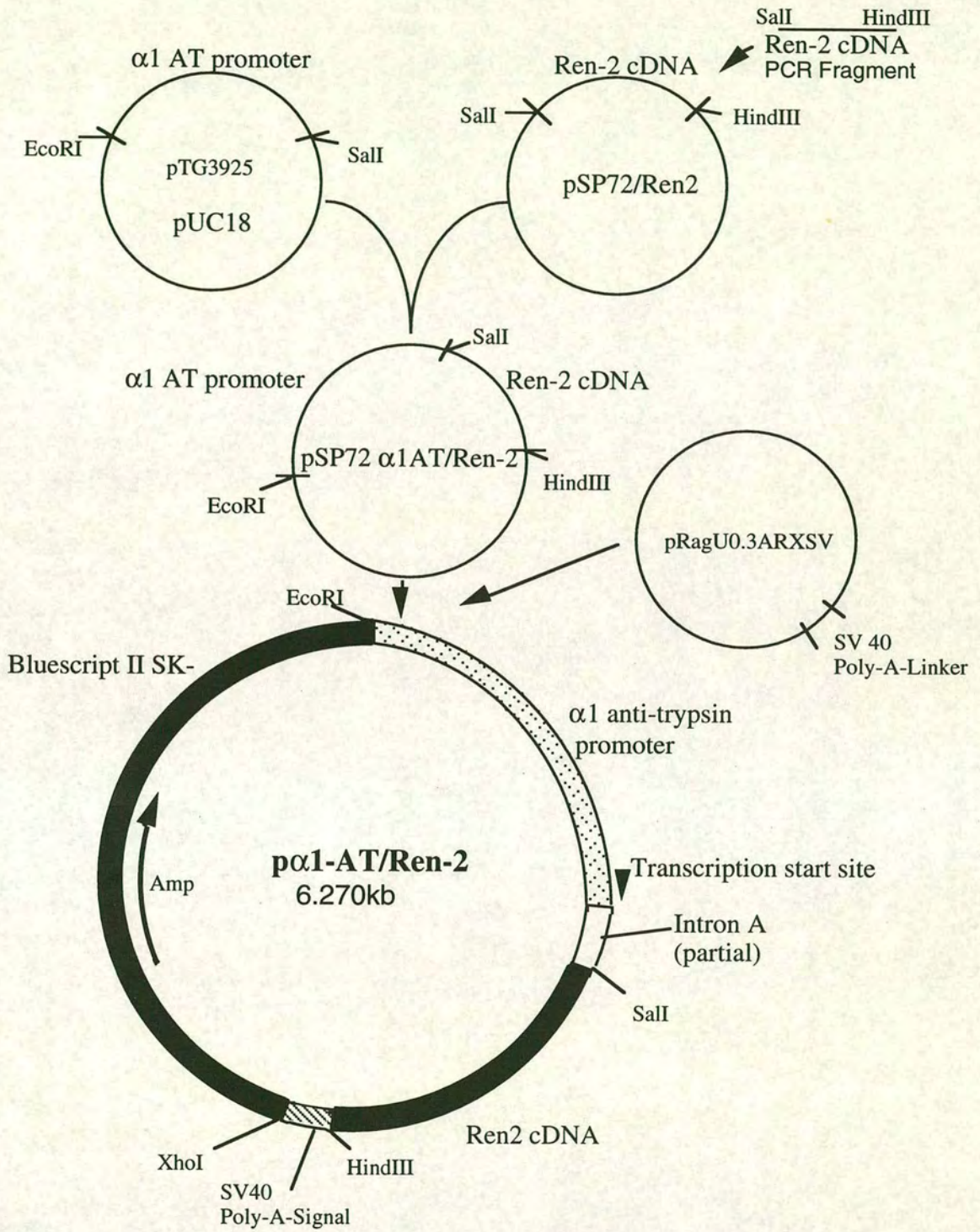


Figure 3.1

Schematic showing the structure of $p\alpha 1\text{AT/Ren-2}$. Only restriction sites in key steps of construct generation are shown. Amp^r: ampicillin resistance gene. ~: transcription start site for $\alpha 1\text{AT/Ren-2}$, → direction of transcription of the amp^r gene.

complementary *Sall* sites into pSP72/Ren-2, see figure 3.1. The entire *EcoRI/HindIII* restriction fragment of pSP72 α 1AT/Ren-2 was sub-cloned into Bluescript SKII-. Finally, *HindIII* and *XhoI* restriction sites were introduced to a SV40 poly A signal by PCR amplification of pRagU0.3ARXSV (GenBank Accession No., K02212 nucleotides 3219-3232 and nucleotides 3430-3418). The modified SV40 poly A signal restriction fragment was then ligated through complementary *HindIII/XhoI* sites in pSK α 1AT/Ren-2 to give the final construct, p α 1AT/Ren-2. The transgene was excised from p α 1AT/Ren-2 as an *EcoRI/XhoI* restriction fragment.

3.2 Transfection of α 1AT/Ren-2 Into HepG2 Cells

The p α 1AT/Ren-2 construct was transiently transfected into the human liver cell line HepG2 (Aden et al. 1979; Knowles et al. 1980) to validate its ability to be expressed and correctly spliced. RNA was prepared from the transfected cells and it was demonstrated by Northern blot hybridisation analysis that these cells produced a renin transcript of the predicted length (1.2kb), whereas sham-transfected cells did not produce a renin transcript (data not shown, Graham Barrett personal communication).

Stable HepG2 derived cell lines expressing the p α 1AT/Ren-2 construct were also produced in the laboratory. These cell lines were used to determine the proportion of prorenin to active renin secreted into the medium as determined by indirect radioimmunoassay for AI with and without trypsin activation (see Chapter 4, 4.5). The results showed that less than 1% of total renin was active and that there was 105 ± 17 ng AI/ml/h of prorenin present in the medium. Although a minor amount of activation cannot be excluded, the small amount of active renin detected in the medium is most likely due to proteolytic degradation of prorenin. Assuming that HepG2 cells are a representative model of rat liver cells, these results demonstrated, in principle, that the transgene would deliver high levels of prorenin directly into the plasma without activation in the liver.

3.3 Micro-Injection and Screening of Offspring

The α 1AT/Ren-2 transgene was introduced into both Sprague Dawley and Fischer/344 single cell embryos, in two independent series of micoinjections. Thirty-two and one hundred and eighteen healthy offspring were obtained from the injection and reimplantation of one hundred and fifteen and five hundred and eighty-eight

Sprague Dawley and Fischer embryos respectively. Similar success rates were obtained for both genotypes, as summarised in Table 3.1.

Table 3.1

| Strain | Embryos Injected | Live births | Transgenic Positive | % Transgenic Positive |
|----------------|------------------|-------------|---------------------|-----------------------|
| Sprague Dawley | 115 | 32 | 3 | 9.4% |
| Fischer/F344 | 588 | 118 | 13 | 11.0% |

All viable offspring were screened for the presence of the α 1AT/Ren-2 transgene by a transgene specific PCR analysis as described in Chapter 2, 2.7. Results from PCR screening of the offspring were confirmed by Southern blot hybridisation analysis. Sixteen tail DNAs gave the transgene-specific products during PCR analysis, and thirteen out of those sixteen proved positive by Southern blot hybridisation (see figure 3.2). Figure 3.2 shows the transgenic founders derived by microinjections as determined by Southern blot hybridisation. The plasmid p α 1AT Ren-2 was diluted to the equivalent of ten copies per haploid genome in 10 μ l of water. This was run as a transgene copy number control. The final percentage of offspring that were positive from the second microinjection series was 14.6%. Densitometric analysis demonstrated that there was a significant range of transgene copy number, from founder TG6 with approximately 5 copies per haploid genome, to founder TG8 with over 100.

3.4 Nomenclature for Transgenic Animals

A suggested nomenclature for transgenic animals is described in ILAR news (Institute of Laboratory Animal Research)(ILAR 1992). A transgene symbol, it suggests, should consist of three parts, all in Roman type, as follows: TGX (YYYY)#####ZZZZ, where TGX indicates the mode of insertion of DNA (N for non-homologous insertion, R for insertion via a retroviral vector, or H for homologous recombination). (YYYY) is the insert designation, indicating salient features of the transgene as determined by the laboratory. ##### is the laboratory-assigned number, a unique number the laboratory assigns to each stably transmitted insertion after germ-line transmission is confirmed. ZZZZ is the laboratory code, uniquely assigned to each laboratory that produces transgenic animals by ILAR (USA only). In our laboratory

this nomenclature has been modified. TGX indicates whether the transgenic animals is mouse (M) or rat (R). (YYYY) is the insert designation and ##### the animal number (see example below). There is no ZZZ code. In the case of a transgenic founder giving rise to more than one line of offspring, due to multiple integration sites (see TGR(α 1AT/Ren-2)7647, figure 3.9), after the founder number there is a slash and the subtype number, eg. TGR(α 1AT/Ren-2)7647/2.

eg. TGR(mRen-2)27 or TGR(α 1AT/Ren-2)3375

For the sake of brevity in my thesis, founder animals will be referred to as TG(founder number) and their offspring by founder number/animal number. Table 2.1 below provides animal number, genetic background and founder animal.

Table 3.2

| Animal Number | Strain (Sprague Dawley or Fischer) | Founder |
|---------------|------------------------------------|---------|
| 1694 | SD | TG2 |
| 2529 | SD | TG2 |
| 2532 | SD | TG2 |
| 2533 | SD | TG2 |
| 3373 - 3385 | SD | TG2 |
| 1270 | SD | TG3 |
| 8610 | F | TG7 |
| 8611 | F | TG7 |
| 8612 | F | TG7 |
| 790 | F | TG7 |
| 791 | F | TG7 |
| 626 | F | TG12 |
| 627 | F | TG12 |
| 3188 | F | TG12 |
| 3189 | F | TG12 |

3.5 Creation of Transgenic Lines

In order to study both expression of the α 1AT/Ren-2 transgene and any phenotype the transgenic animals may have had it was necessary to breed extensively both from founder animals and males from the G₁ generation. Transgenic males were used as they could be paired with more than one non-transgenic female at a time, thus producing more offspring.

One transgenic founder, TG1, died at five weeks of age, exhibiting extreme left ventricular cardiac hypertrophy. This suggested that the animal may have suffered from high blood pressure and therefore, as a precaution, the subsequent founders were maintained on Captopril at 10mg/kg per day to control their blood pressure. Angiotensin-converting enzyme (ACE) inhibitors, such as Captopril and Enalapril, block the conversion of angiotensin I to angiotensin II, promote vasodilation and decrease aldosterone levels. ACE inhibitors also block the degradation of bradykinin, a vasodilator, which results in increased levels of plasma bradykinin.

For each of the two founders, TG2 and TG3, three litters were produced and a male transgenic positive animal from each line (animals TG2/1694 and TG3/1270 respectively) was set aside for future breeding, see table 3.2. There were four litters in the G₂ generation of TG2 from which two males, TG2/2529 and TG2/3374, and one female, TG2/3377, were treated with Captopril and maintained as breeders (see figure 3.3 for an example of an TG2 Southern blot hybridisation analysis, 3.3E for an example of the results obtained from the transgene-specific PCR and figure 3.4 for a partial pedigree).

All offspring from TG2/2529 were sacrificed for tissue analysis or telemetry leaving offspring from TG2/3374 and TG2/3377 for future breeding. Scanned images and densitometric analysis showed transgenic positive animals from previous litters had a transgene copy number of approximately 30. The offspring of animals from TG2/3373-3386, however, showed a copy number of one consistent with non-transgenic animals, see figure 3.6. All animals from the other breeding litters had been used in expression analysis studies, resulting in the apparent loss of the transgene array in line TG2. There may have been a rearrangement that left fragments of transgene to produce a positive PCR signal or perhaps there was a complete deletion of the transgene locus and contamination of the tail DNAs. With second tail biopsies producing DNA which gave the same transgenic positive PCR products it is unlikely, but not impossible, there was some form of tail DNA contamination.

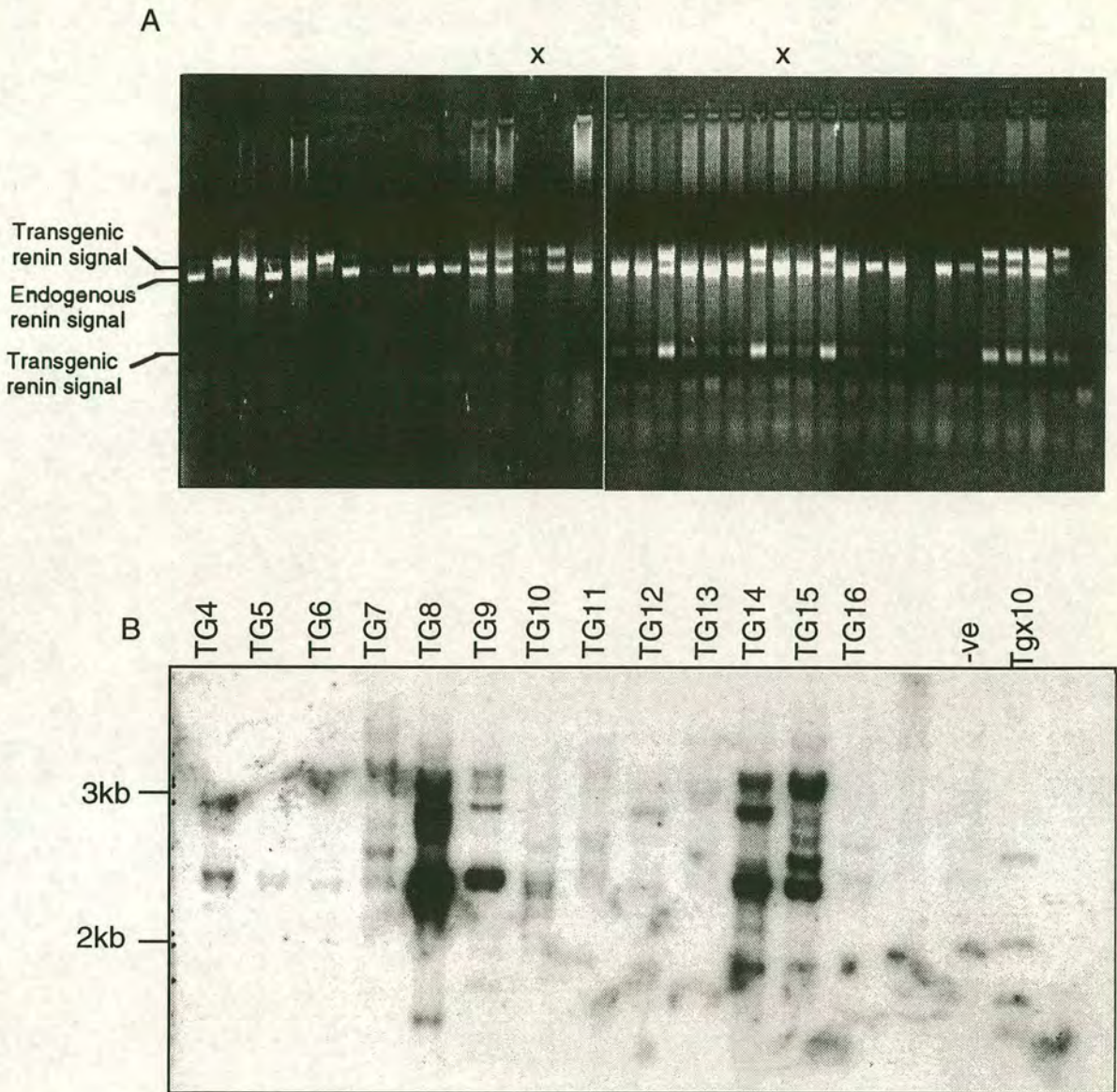


Figure 3.2

Panel A: PCR screening of potential founder animals, TG4 - TG16. Those PCRs that showed products of 580bp and 125bp were tested by Southern blot hybridisation analysis. The x marks denote possible PCR positive signals that were proved to be negative by Southern blot hybridisation. Panel B: Transgenic founders derived by microinjection as determined by Southern blot hybridisation. Genomic DNA (10 μ g) was digested with 2.5U of *Pst*I and Southern blotted as described in Chapter 2, 2.12. The probe used was the *Sall/HindIII* fragment of Ren-2 cDNA, see Chapter 2, 2.11. The plasmid p α 1AT/Ren-2 was diluted to the equivalent of ten copies per haploid genome in 10 μ l and run as an indication of the range of transgene copy number present in the Fischer founder animals in the Tgx10). The size markers used were λ HE, see Chapter 2, 2.8 and non-transgenic rat DNA was used as a negative control.

The line TG3 was bred to characterise the line (see figure 3.3B for an example of a TG3 Southern blot). Line TG3 did show an elevated blood pressure but this was not as severe as in line TG2. There was never a malignant phase to the blood pressure and the mortality rate of line TG3 was very low. Animals from the line TG3 did not show any abnormalities in their plasma RAS or exhibit any clinical signs of hypertension. This line was, therefore, not extensively bred or studied in detail.

Two litters from each of the founders TG4 - TG16 were required to produce enough transgenic positive animals for the initial physiological analysis of these lines (see figure 3.5 for pedigree). Four founders (TG5, TG8, TG9 and TG15) did not pass on the transgene to their progeny, see table 3.2, and founder TG10 did not produce any progeny. Founder TG11 produced offspring that showed two separate Southern blot hybridisation patterns with a *Pst I* restriction digest of tail DNA (see figure 3.7). The two Southern blot hybridisation patterns were treated as individual lines and bred separately. Founder TG16 only produced one litter and both transgenic pups died soon after birth. After the blood pressure data was collected from the offspring of the remaining founders it was decided to maintain the 6 lines that produced two litters and to breed extensively from founders TG7 and TG12 as they had demonstrated the highest blood pressures as measured by tail cuff plethysmography. Southern blot analysis was used to determine that the integrity of the transgene array was maintained between generations of both transgenic lines, TG7 and TG12, see figure 3.9. Figure 3.9 shows Southern blot hybridisation of three generations of transgenic lines TG7 and TG12.

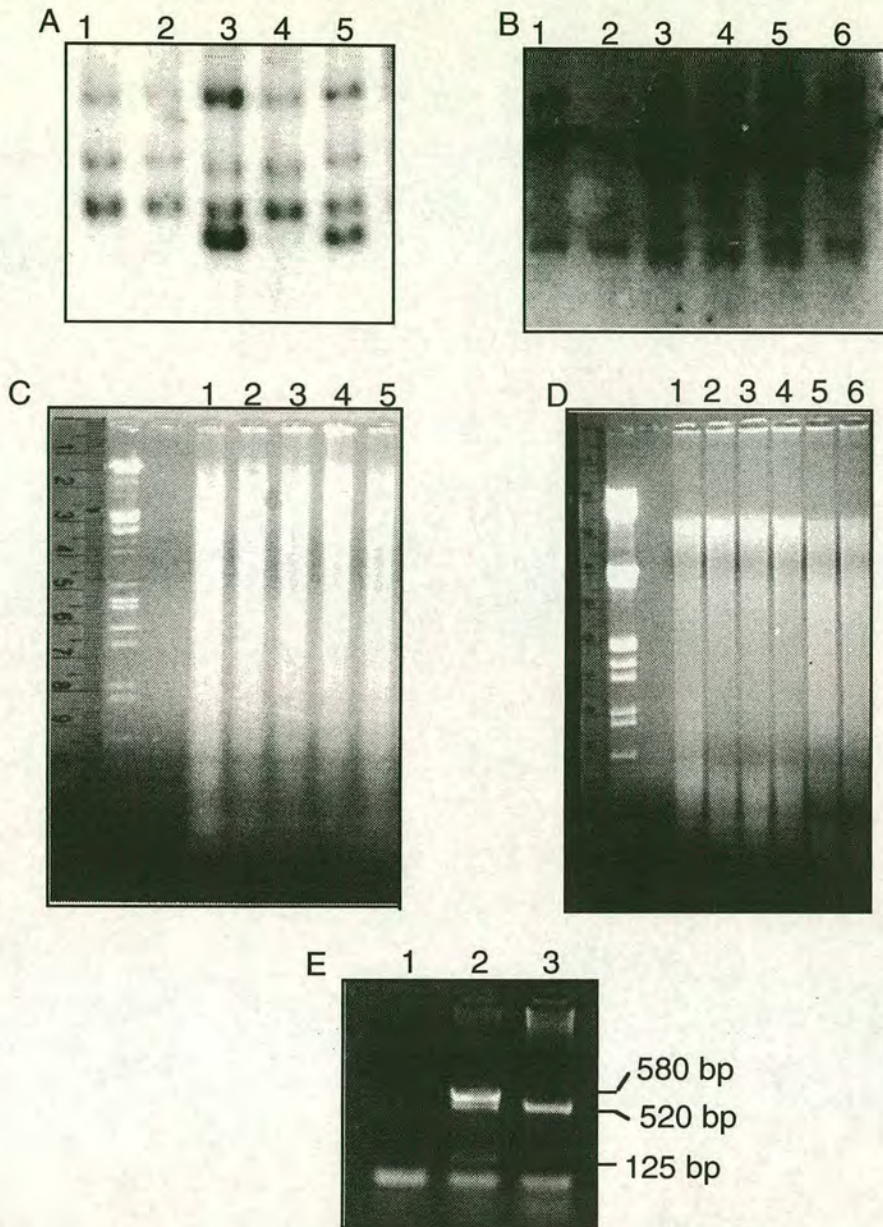


Figure 3.3

A: Southern blot hybridisation of 10 μ g of genomic DNA from progeny of TG2 digested with 2.5U of *Pvu II*. The probe used was the *SalI/HindIII* fragment of Ren-2 cDNA, see Chapter 2, 2.11. Lane 1, known non-transgenic; Lanes 2, & 4 non-transgenics ; Lanes 3 & 5 transgenic. B: Southern blot hybridisation of 10 μ g of genomic DNA from progeny of TG3 digested with 2.5U of *Pst I*. Lane 1 & 2 known non-transgenic; Lanes 3-6 transgenics. C: Genomic DNA from progeny of TG2 digested with 2.5U of *Pvu II*, visualised by ethidium bromide/U.V. light, prior to Southern blot hybridisation shown in panel A, markers used was λ H3/R1 (See Chapter 2, 2.87) D: Genomic DNA from progeny of TG3 digested with 2.5U of *Pst I*, visualised prior to Southern blot hybridisation shown in panel B, markers used were λ HindIII. E: PCR gel showing the results obtained from no DNA (Lane 1), transgenic positive DNA (Lane 2) and transgenic negative DNA (Lane 3) - See Chapter 2, 2.7 for details of PCR reaction.

Partial Pedigrees from TG Founders

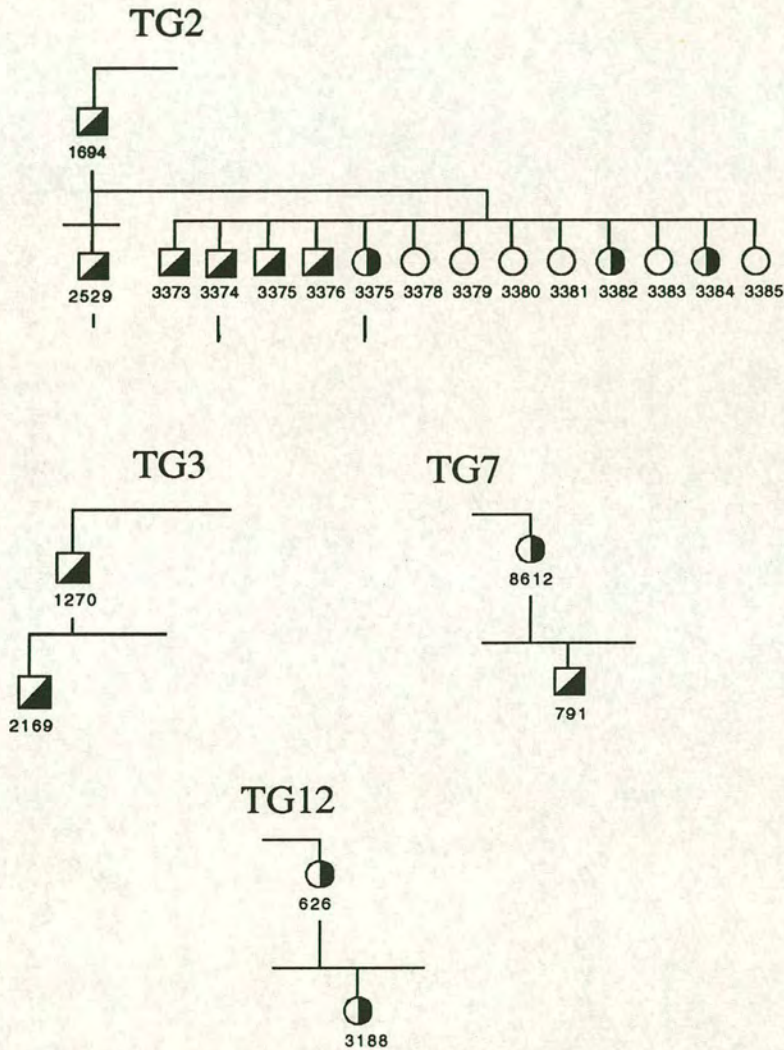


Figure 3.4

Partial pedigrees of the main transgenic lines. Only animals quoted in the text are shown here. Square symbols denote male animals and circular symbols denote female animals. Symbols that are half-shaded denote a hemizygous transgenic animal. Lines indicate where the pedigrees extend. Numbers below symbols are unique identifying numbers used in the laboratory.

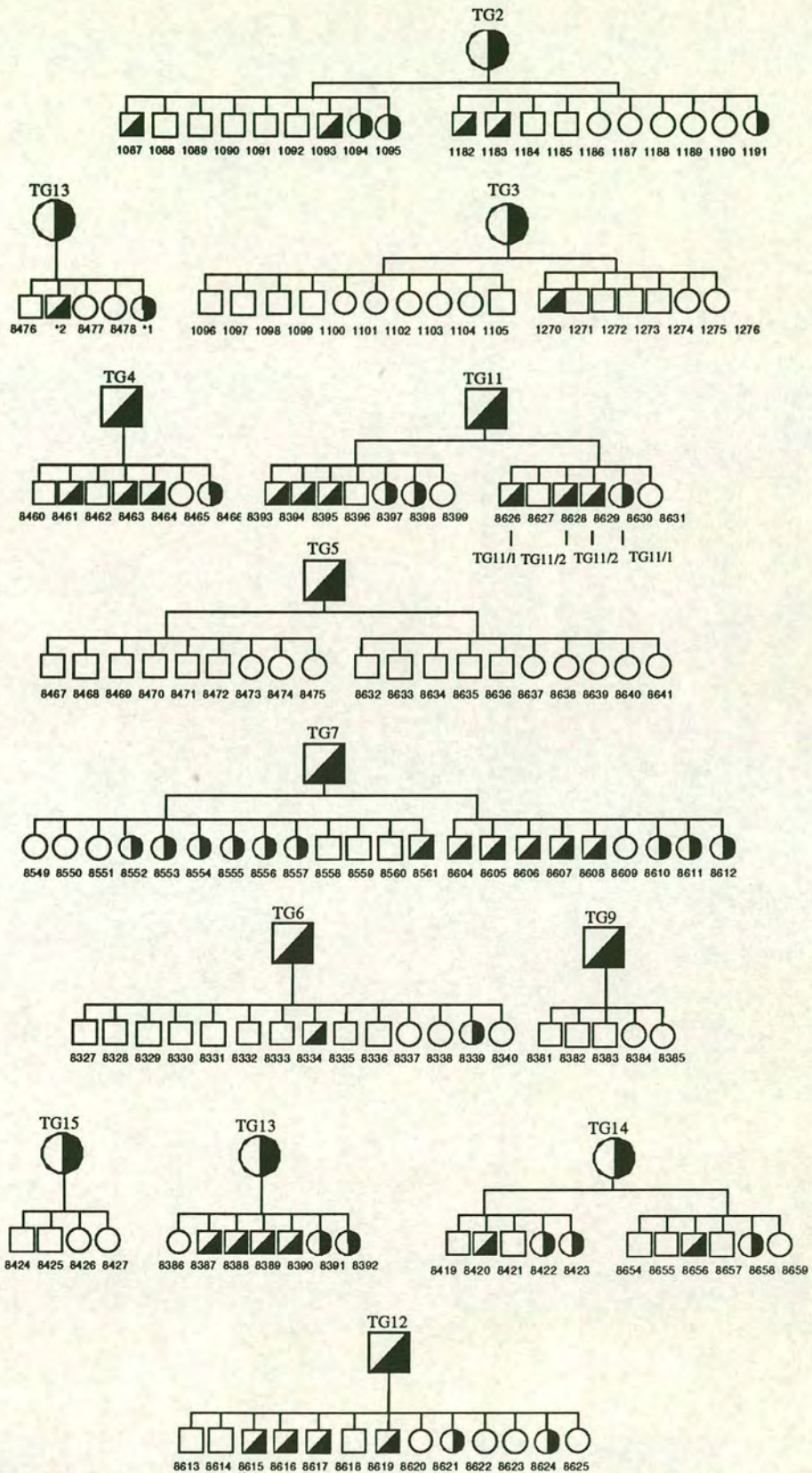


Figure 3.5

Initial pedigrees of transgenic lines TG2 - TG16. See table 3.2 for a summary of all founder animal breeding. TG11 sublines are shown under the litter numbers. Square symbols denote male animals and circular symbols denote female animals. Symbols that are half-shaded denote a hemizygous transgenic animal. Numbers are unique identifying numbers used in the laboratory.

Table 3.3

| Founder & Transgene Copy nos. | Litters Born From Founder | Phenotype | Outcome |
|-------------------------------|---------------------------|-------------------------------------|----------------------------------|
| TG1: 3 | - | Severe left ventricular hypertrophy | Founder died at 5 weeks of age |
| TG2: 5 | 6 | Chronic and malignant hypertension | Line established |
| TG3: 2 | 5 | No discernable phenotype | Line established |
| TG4: 14 | 2 | High blood pressure | Initial study only |
| TG5: 2 | 2 | - | Did not pass on transgene |
| TG6: 2 | 2 | High blood pressure | Initial study only |
| TG7: 8 | 6 | Chronic and malignant hypertension | Line established |
| TG8: 50 | 1 | - | Only 1 litter born and pups died |
| TG9: 25 | 2 | - | Did not pass on transgene |
| TG10: 6 | - | - | Did not breed |
| TG11: 3 | 2 | High blood pressure | Initial study only |
| TG12: 5 | 6 | Chronic and malignant hypertension | Line established |
| TG13: 2 | 2 | High blood pressure | Initial study only |
| TG14: 35 | 2 | High blood pressure | Initial study only |
| TG15: 40 | 1 | - | Did not pass on transgene |
| TG1: 26 | 1 | - | Transgenic pups died |

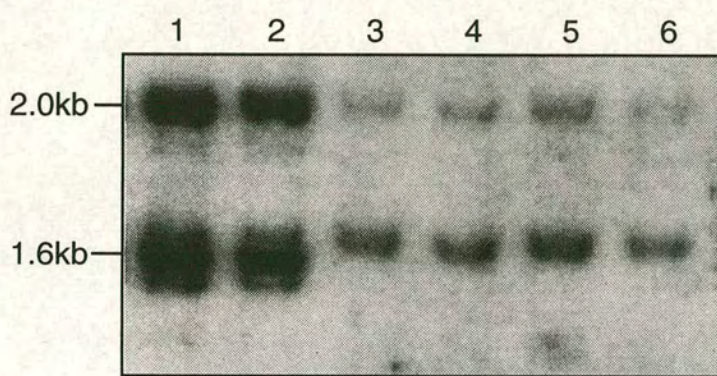


Figure 3.6

Southern blot hybridisation analysis of genomic DNA (10 μ g), digested with 2.5U of *Bam*HI, from animals from transgenic line TG2. Lanes 1-5 show samples that gave PCR results consistent with a transgenic animal, lane 6 is a sample of non-transgenic DNA. The probe used was the *Sal*II/*Hind*III fragment of Ren-2 cDNA (see Chapter2, 2.8). Size markers are indicated on the left of the figure.

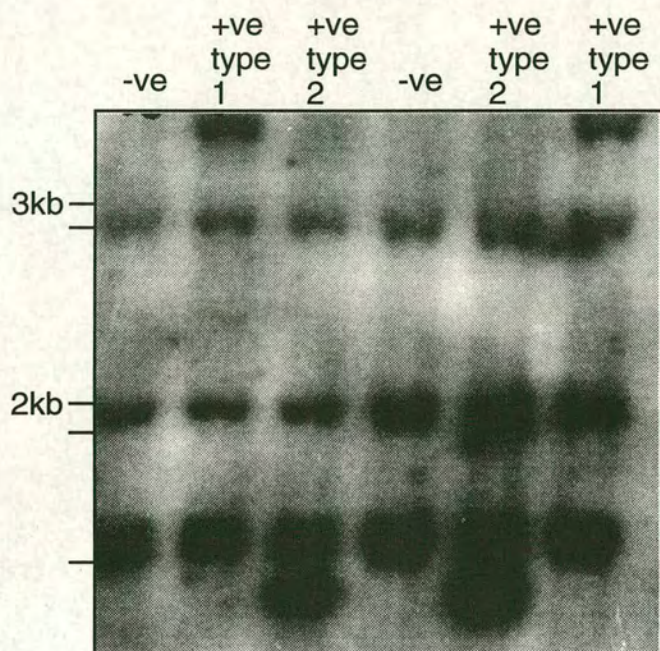


Figure 3.7
Southern blot hybridisation analysis of the sub-line divisions of transgenic line TG11. Genomic DNA (10 μ g) from the G2 generation of TG11 was digested with 2.5U of *Bam*HI. The probe used was the *Sal*I/*Hind*III fragment of Ren-2 cDNA. Two hybridisation patterns were noted in the transgenic samples and designated type 1 and type 2 (See Chapter 2, 2.4 for nomenclature).

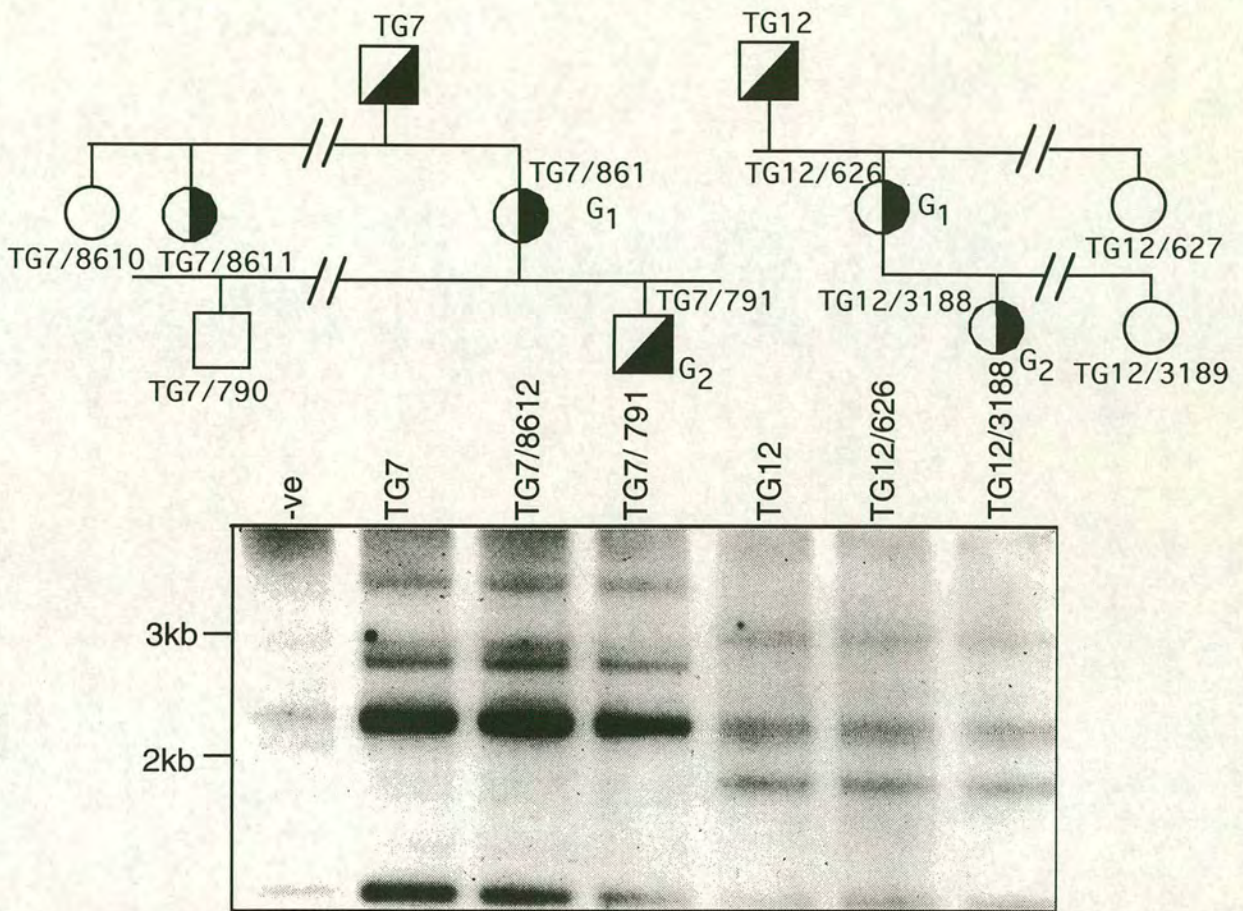


Figure 3.9

Southern blot hybridisation of three generations of transgenic lines TG7 and TG12. Genomic DNA (10 μ g) was excised with 2.5U of *Pst*I and Southern blotted as described in Chapter 2, 2.11. The probe used was the *Sal*II/*Hind*III fragment of the *Ren-2* cDNA as described in Chapter 2, 2.8. The negative sample was DNA from a non-transgenic animal. Markers used were λ HE as described in Chapter 2, 2.8. The linkage relationship between the animals used for the Southern blot are indicated above.

CHAPTER 4

Expression Analysis of Transgenic Offspring

4.1 Isolation of RNAs

Samples were taken from a range of tissues and processed as described in Chapter 2. The integrity of all RNAs was demonstrated by gel electrophoresis. Tissues taken for Northern blot analysis include, liver, kidney, adrenal gland, SMG, heart, lung, testes, thymus, whole brain, spleen and muscle. Figure 4.1B shows a representative gel with total RNAs extracted from a variety of tissues. Total RNA was used in Northern blot hybridisation analysis and RNase protection analysis. However, poly A+ mRNA was purified from total RNA and used in certain Northern blots, in an attempt to increase the sensitivity of the assay.

4.2 Northern Blot Analysis

In TG2 and TG3 transgenic rats the Northern blot analyses showed no detectable transgene transcript, see figure 4.1A. Total and mRNA Northern blot analyses were carried out on five male and female samples when the TG7 and TG12 transgenic rats were tested for expression. Again, no detectable transcript was seen with total RNA in any of the tissues tested, as shown in figure 4.2A. The Northern blots carried out on the same samples using mRNA, however, did reveal a renin-specific transcript in the liver samples of transgenic, but not wild type animals. In figure 4.2B renin RNA transcripts were detected in the liver and kidney samples. The absence of any renin-specific hybridisation signal, in the rat kidney samples, when subjected to total RNA Northern blot hybridisation may be due to the poor cross hybridisation of the mouse *Ren-2* cDNA probe to rat renin RNA. This was not seen when the renin message was enriched in poly (A)+ RNA Northern blot hybridisations.

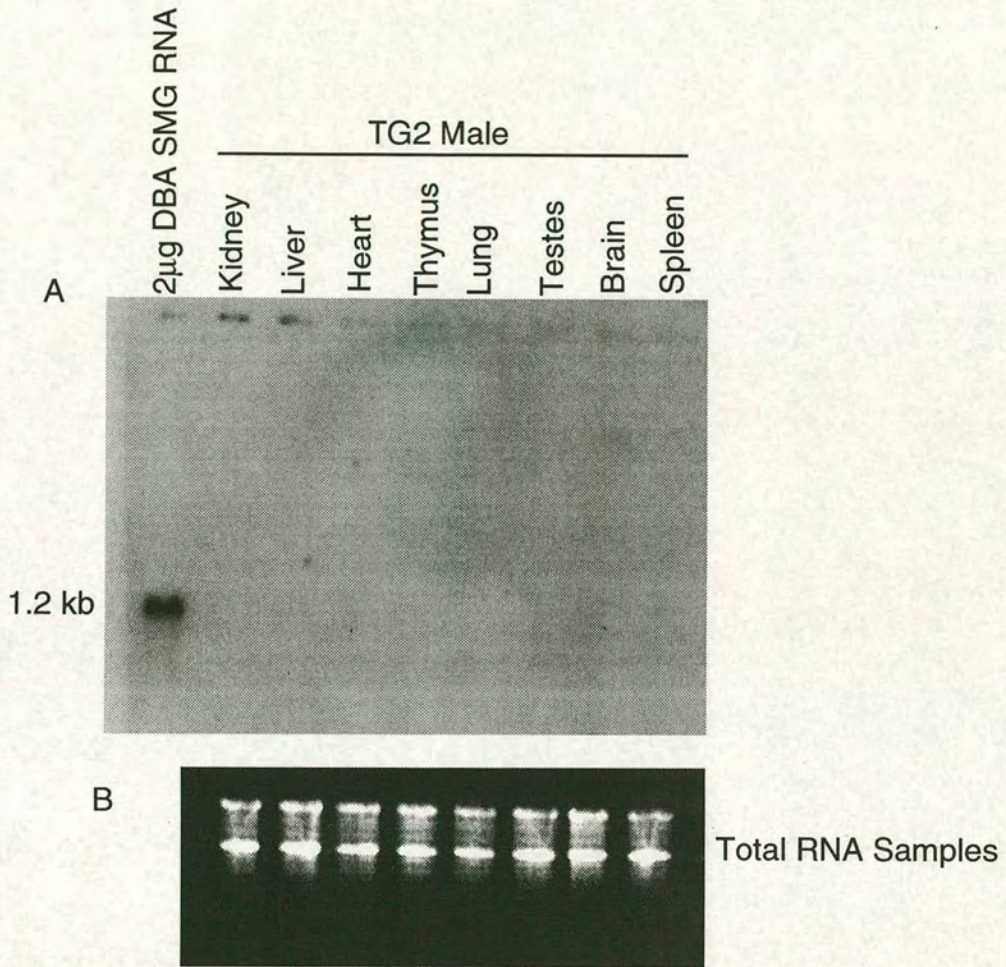


Figure 4.1

Panel A: Northern blot hybridisation analysis of total RNA (50µg) from a male from transgenic line TG2. Mouse (DBA/2J) SMG RNA (2µg total RNA) was used as a positive control. The probe used was the *Sall/HindIII* fragment of Ren-2 cDNA, see Chapter 2.11. Panel B: Ethidium bromide stained agarose gel demonstrating the integrity of the RNA samples in panel A.

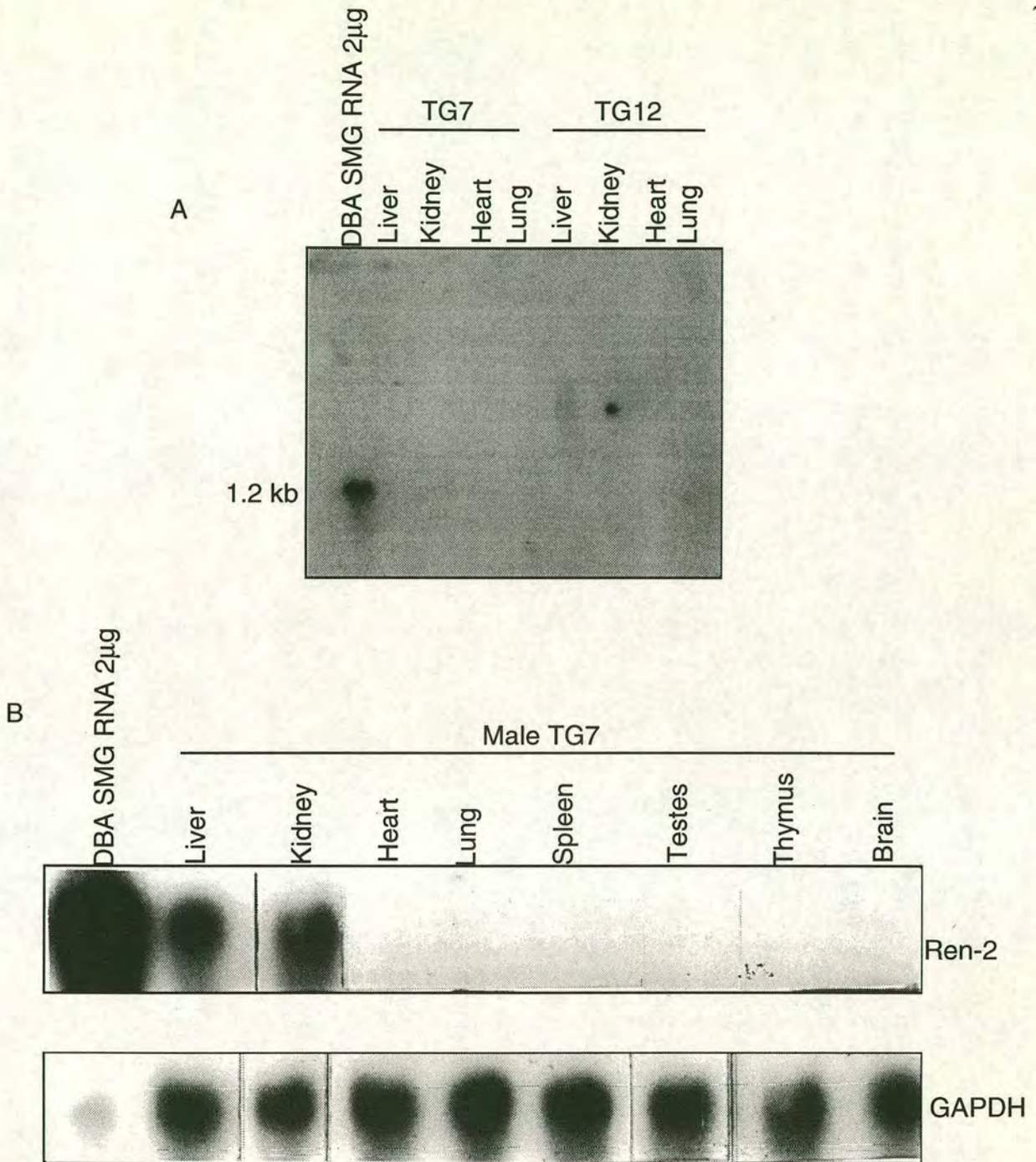


Figure 4.2

Panel A: Northern blot hybridisation analysis of total RNA (50 μ g) from transgenic line TG7 and TG12. Mouse (DBA/2J) SMG RNA (2 μ g total RNA) was used as a positive control. The probe used was the *Sall/HindIII* fragment of Ren-2 cDNA, see Chapter 2, 2.11. Panel B: Northern blot hybridisation analysis of poly (A)+ RNA. Samples were taken from a transgenic male from line TG7, poly (A)+ RNA was prepared as per chapter 2.13. RNA was loaded (5 μ g of sample poly(A)+ RNA) and the procedure was that followed for a total RNA Northern blot (chapter 2.15). The top panel in 4.2B shows the Northern blot hybridised with the *Sall/HindIII* fragment of Ren-2 cDNA, see Chapter 2, 2.11. The bottom panel in 4.2B shows the same Northern blot hybridised with an *EcoRI/BamHI* fragment of GAPDH cDNA.

Due to the availability and sensitivity of RNase protection analysis it was felt that it was suitable to ascertain whether small, but perhaps significant, expression of the transgene could be detected in any of the previously tested samples.

4.3 RNase Protection Analysis

The RNase protection assay used produces a specific protected fragment of 250 nucleotides. This assay is specific for *Ren-2* transcripts and does not detect *Ren-1* or rat renin transcripts (Field et al. 1984). In rat lines TG2 and TG3 it was shown that they produce the *Ren-2* transcript in the liver at relatively low levels. Previous $\alpha 1$ anti-trypsin derived transgenes have shown some expression in the testes, lung, and adrenal (Dalemans et al. 1990). The construct used here, however, only demonstrated expression in the liver as was reported by Sifers *et al* 1987 (Sifers et al. 1987). The RNase protection assays on the TG2 and TG3 transgenic rat lines confirmed the absence of *Ren-2* RNA transcript in any organ, other than the liver, as suggested by Northern blot hybridisation analysis. Due to the absence of any ectopic expression, physiological effects could be ascribed to the production of mouse *Ren-2* protein from a single organ of origin. It was anticipated that the hepatic cells were not capable of storing renin as human HepG2 derived cells could express the transgene, in a transient transfection, and that the protein was constitutively secreted into the media, see Chapter 3, 3.2.

The relative levels of *Ren-2* specific transcript, as detected by RNase protection analysis, in lines TG2 and TG3 was greater in line TG2 as shown in figure 4.3. This was thought to play a role in the subsequent differences in the phenotype seen between lines TG2 and TG3. Expression levels of transgenic transcript vary greatly depending on the site of integration and the construct used. Due to factors such as variations in mRNA stability, however, transcript levels do not always correlate with the observed protein levels. Expression levels did vary between lines, as did the observed blood pressure, and to a certain extent between the sexes. There appeared to be a higher level of a *Ren-2* specific transcript, and higher level of systolic blood pressure, seen in both TG2 and TG3 male samples than in their female counterparts. As there was no internal loading control, this may be not be significant.

The RNase protection assays performed on five male and female samples from TG7 and TG12 demonstrated similar results to those seen with the TG2 as shown in figure 4.5. Expression of the *Ren-2* transcript was only detected in the liver and no

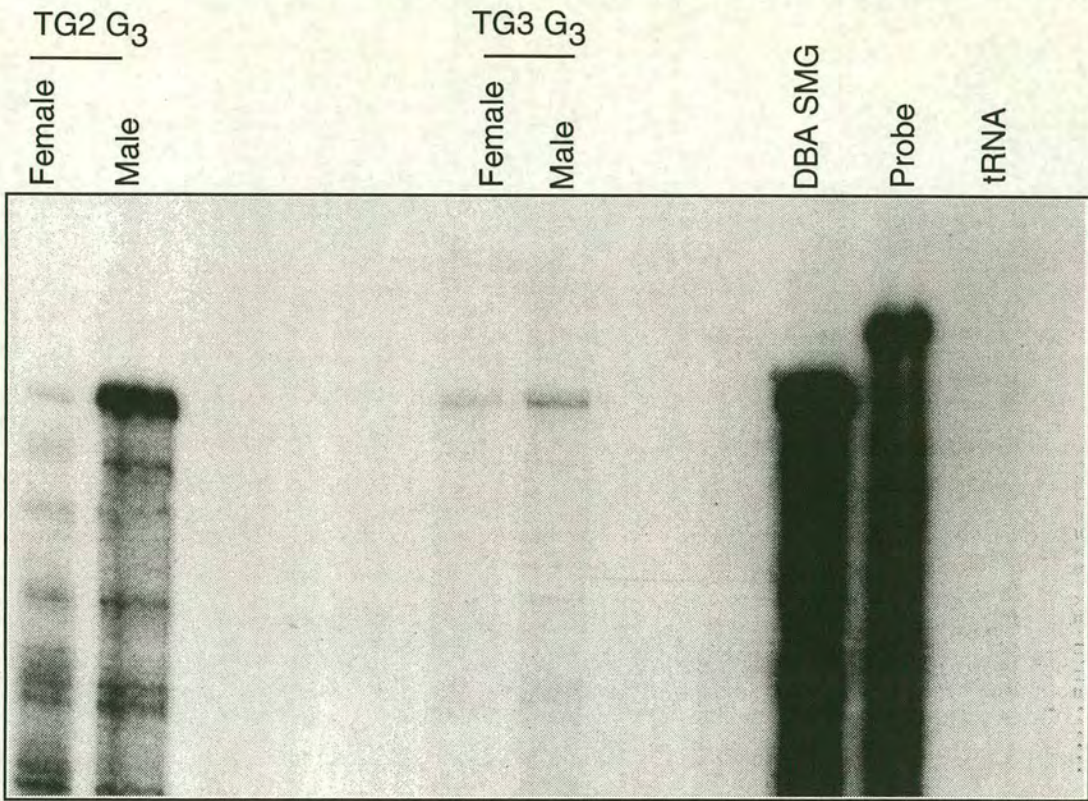


Figure 4.3

RNase protection analysis of samples from male and female transgenic animals from lines TG2 & TG3. Total RNA (20 μ g) was hybridised with a Ren-2 specific probe, see Chapter 2, 2.16. The samples were electrophoresed on a 6% polyacrylamide gel before drying the gel and placing it in an autoradiography cassette with Fuji autoradiography film. The film was exposed overnight at -70°C .

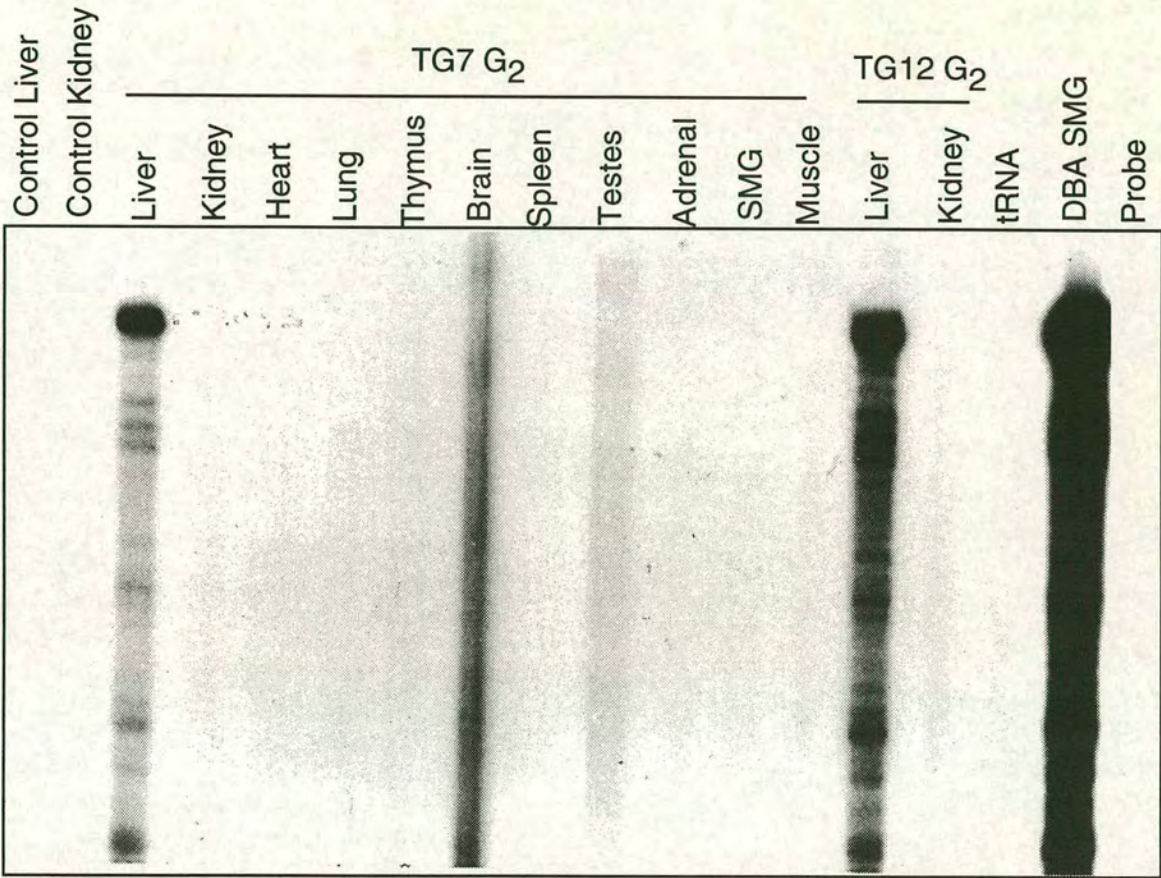


Figure 4.4

RNase protection analysis of samples from male transgenic animals from lines TG7 & TG12. Total RNA (20 μ g) was hybridised with a Ren-2 specific probe, see chapter 2, 2.16. The samples were electrophoresed on a 6% polyacrylamide gel before drying the gel and placing it in an autoradiography cassette with Fuji autoradiography film. The film was exposed overnight at -70°C.

other organ tested. There appeared to be a higher level of Ren-2 specific transcript, and higher level of systolic blood pressure, seen in both TG2 and TG3 male samples. As there was no internal loading control, this may be not be significant.

4.4 Immunohistochemistry in Sprague Dawley and Fischer Transgenic Rats

A polyclonal rabbit renin antibody raised against Ren-2 protein was kindly donated to the laboratory by Professor John Baxter. The antibody bound to all mouse prorenins, renins and cross-reacted with rat renin. It was used to detect the presence of prorenin or renin protein in small, localised areas. In general, little staining was seen in the transgenic line TG3. In wild type animals the brown diaminobenzidine (DAB) chromophore produced by the reaction of the Horse Radish Peroxidase antibody (see figure 4.5) was seen localised to the area of the juxtaglomerular apparatus of a few glomeruli as was expected of a normotensive sample see figure 4.6. Staining in the liver samples of all transgenic lines (22 samples taken) showed low-level diffuse staining in the small arteries as would be consistent with secretion of the protein into the blood stream. The staining in the kidney sections was qualitatively seen as comparable between the transgenic and non-transgenic samples. Intense staining of renal tubules was seen in a few cases, however, where animals were in the final stages of malignant hypertension. In these cases intense DAB staining was seen, not only in the JG cells, but at specific points of the renal tubules (see figure 4.7). It may be that due to the high blood pressure, interlobular arteries had become hypertrophic and occluded resulting in local hypotension causing a release of renin from the JG cells and through the renal tubules (see chapter 6 for a complete explanation). At high magnification there was seen a striated pattern of staining in the renal tubule. The functional significance of this remains unclear but no such staining pattern was recorded in non-malignant or control animals.

The only other area that was shown to contain renin protein was the small arteries of the heart in transgenic positive animals from lines TG8 and TG12 as shown in figure 4.8. It is often thought that a physiological consequence of the high blood pressure seen in these animals was cardiac hypertrophy (Messerli et al. 1992). In a few animals staining with the renin antibody was seen in the cells of the small arteries of the heart. This observation was not wide spread, not every transgenic animal demonstrated this staining and not every small artery was stained in those that did. Unlike the staining pattern seen in the renal tubules the staining was not restricted

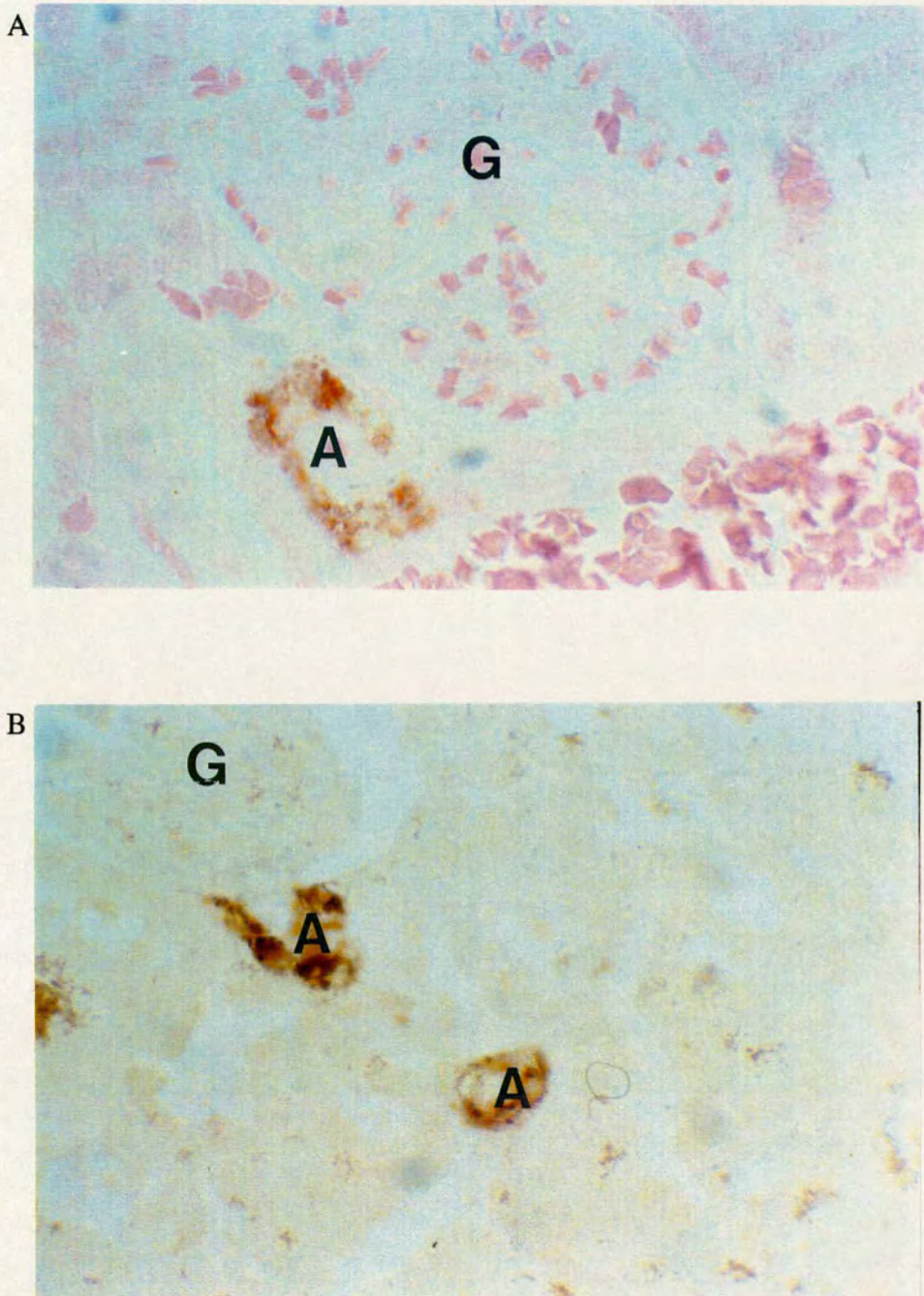


Figure 4.6 Immunohistochemical analysis of renal sections from 6 week old (A) non-transgenic and (B) transgenic TGR(α 1AT/Ren-2) rats using a total renin antibody. G: Glomerular Tuft, A: Afferent Arteriole. Panel (A) is a counterstained paraffin section (3 μ m thick) (x400); whereas panel (B) has no counterstaining (x400). Both panels shown DAB staining of the juxtaglomerular apparatus.

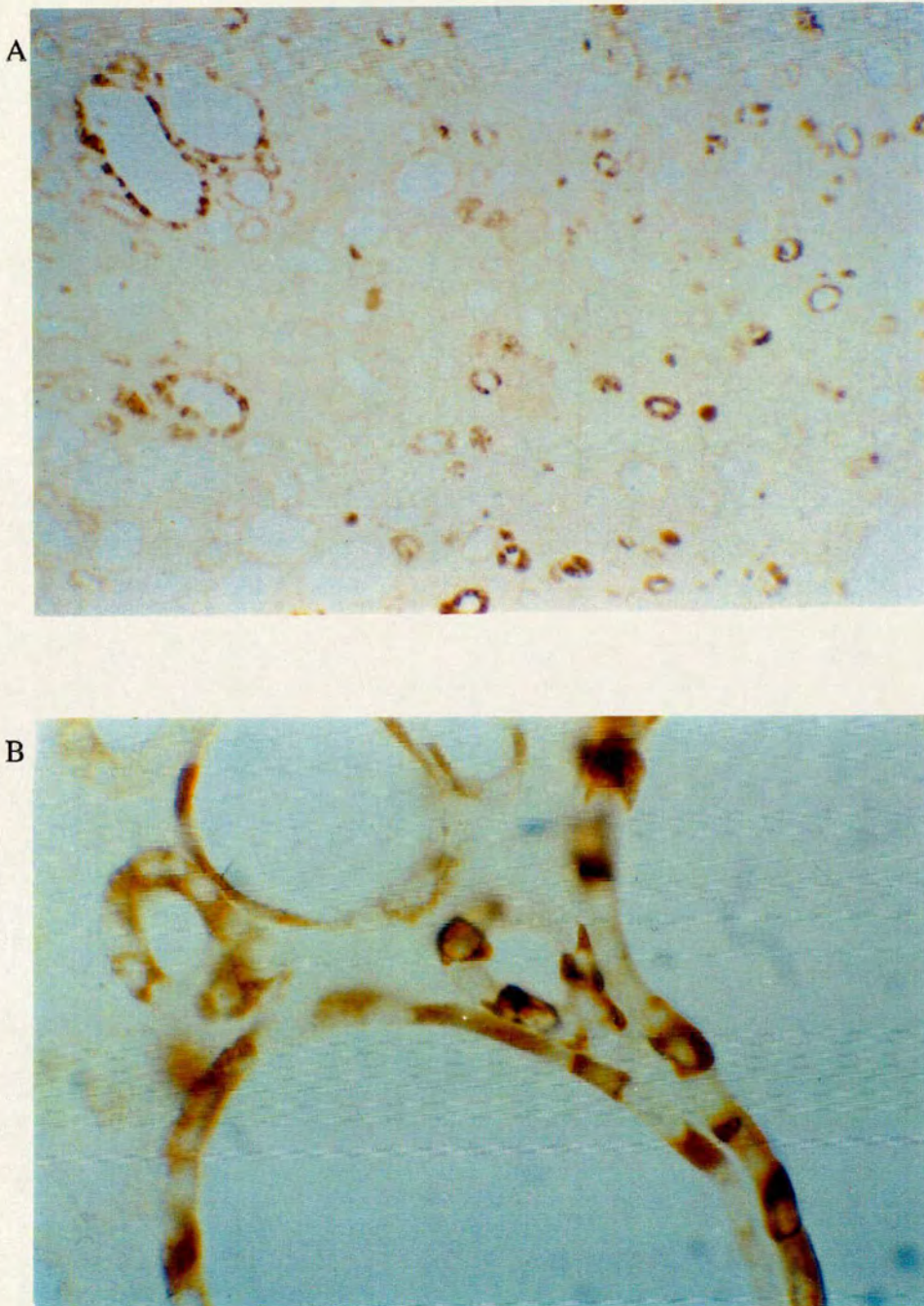


Figure 4.7 Immunohistochemical analysis of renal sections ($3\mu\text{m}$ thick) from a 7 week old transgenic rat which suffered from malignant hypertension. The intense staining was seen in the renal tubules. Panel (A) (x100), panel (B) (x400).

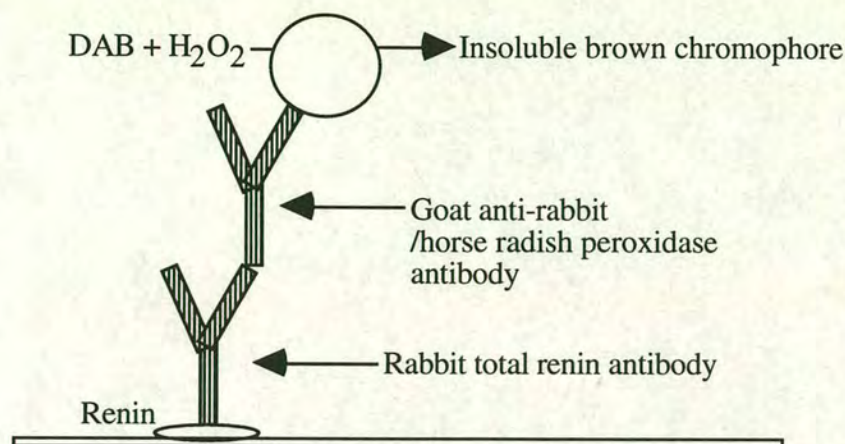


Figure 4.5

Diagrammatic representation of the Horse Radish Peroxidase (HRP)/DAB reaction. Diaminobenzidine is an exceptionally sensitive substrate for HRP that can be further enhanced by the addition of cobalt or nickel ions to the substrate solution. The drawback to DAB is that it reacts so quickly that it is easily overdeveloped giving high background (Harlow et al. 1988).

within the cells of the small arteries. Rather, there was a more diffuse staining in the epithelial cells of the small arteries. As the end product of the RAS is, however, implicated in hypertrophy of the heart, and renin is the only component of that pathway not conclusively demonstrated to be produced by the heart, it is possible that there is some form of uptake which plays a part in the hypertrophy seen here (von-Lutterotti et al. 1994).

4.5 Plasma Renin-Angiotensin System

Plasma samples were collected as EDTA plasma, obtained from the retroorbital plexus after light anaesthesia. The plasma active and inactive (prorenin) renin concentrations were determined according to the method of Glorioso *et al* 1983 (Glorioso et al. 1983). To activate prorenin 20 μ l of plasma was incubated with 40 μ l of trypsin (400 U/ml dissolved in TES buffer: 0.1M TES, pH 7.2 0.01% neomycin, 10 mM EDTA). Samples were incubated on ice for 10 minutes and the reaction stopped by addition of 40 μ l of soybean trypsin inhibitor (600 U/ml in TES buffer). To measure active renin 80 μ l of TES-buffer was added to the sample without trypsin. The pre-treated samples were incubated with lyophilised renin substrate isolated from

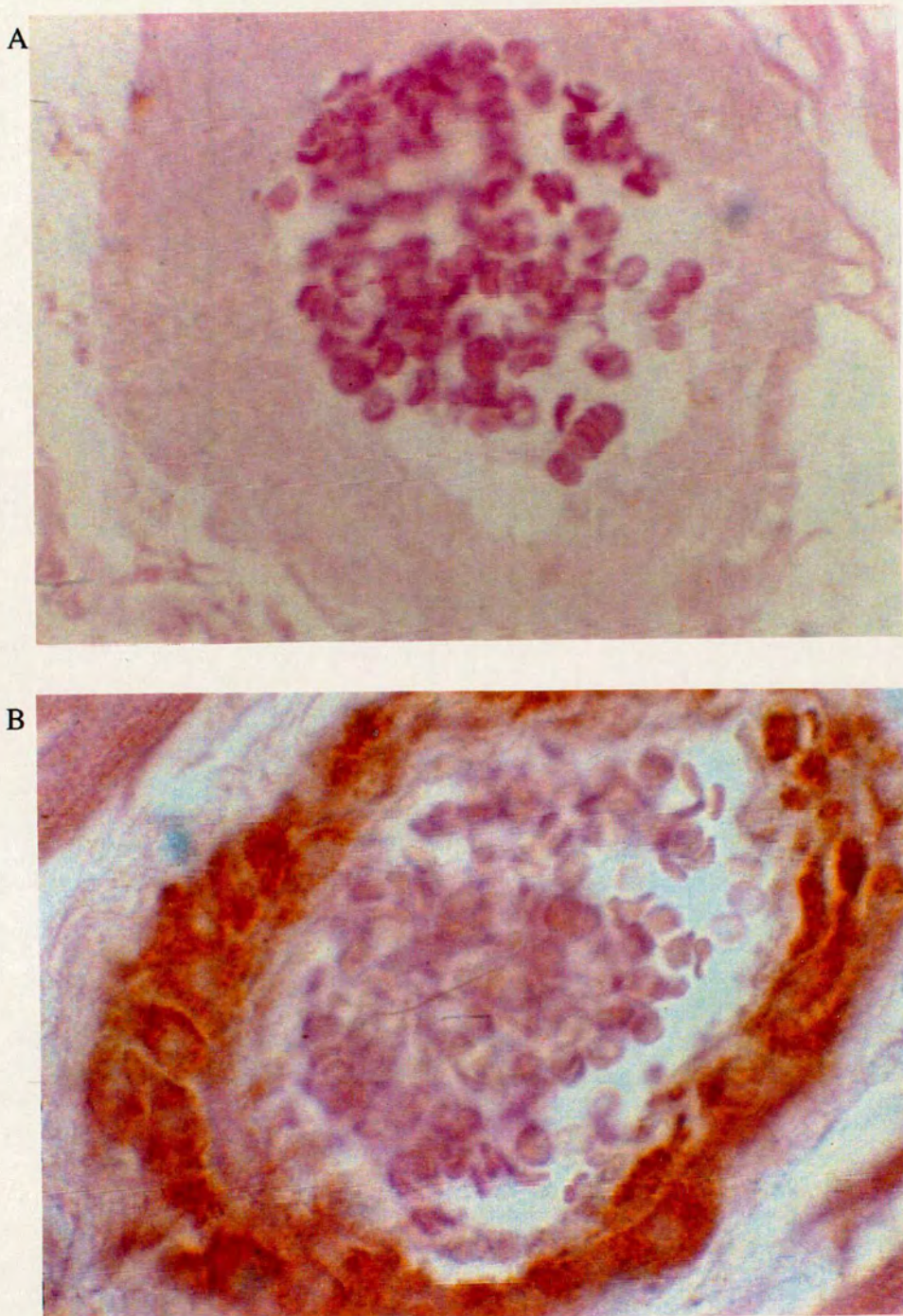


Figure 4.8 Immunohistochemical analysis of cardiac sections (3 μ m thick) from 6 week old (A) non-transgenic and (B) transgenic TGR(α 1AT/Ren-2) rats. Both sections show small cardiac arteries treated with a total renin antibody. Both panels are counterstained with eosin. Panel (A) (x100), panel (B) (x100).

nephrectomized rat plasma (80mg/ml, 0.11% 2,3 dimercapto-1-propanol, 1.15mg/ml 8-hydroxyquinolin in TES buffer). The reaction was stopped with RIA buffer (0.1M Tris-acetate, pH7.4). Generated ANG I was measured by a radioimmunoassay (Hermann et al. 1988). Samples were divided into male and female, transgenic and non-transgenic. The comparison between male transgenic and non-transgenic rats from lines TG2 and TG3 showed that line TG2 had an extremely high level of circulating prorenin whereas line TG3 had a smaller, but significant, increase in plasma prorenin level. This may reflect the differences seen in transgene transcript level as detected by RNase protection. The only other statistically significant result was seen between the circulating AII levels of line TG2 males and non-transgenic litter mates ($p < 0.05$, unpaired student t-test). The AII level seen in line TG2 males was marginally elevated when compared to controls. The results from the female transgenic and non-transgenic animals in both lines TG2 and TG3 were similar to those of the males. However, the level of prorenin detected was slightly lower in the female transgenic rats from line TG2 when compared to male transgenics from the same line. There was also no significant difference in the levels of circulating AII seen between female transgenic and non-transgenic animals from line TG2.

A similar sampling method was used for Fischer transgenic animals. Males from line TG7 showed significantly elevated prorenin levels. There were also no significant differences in levels of renin, AI or AII when compared with male non-transgenic litter mates. Line TG12 transgenic males also showed a characteristic elevation in plasma prorenin with no associated rise in plasma renin, AI or AII. Despite blood pressure and pathology data corresponding between TG2, TG3 and TG7, TG12 transgenic males, the Fischer males at 31 days of age demonstrated a much smaller rise in plasma prorenin of approximately 360ng AI/ml/h compared with approximately 17000ng AI/ml/h seen in the transgenic line TG2 males.

As well as initially choosing a single time point of 31 days to study the plasma renin-angiotensin system in the transgenic animals a time course was also undertaken. Both transgenic lines TG7 and TG12 showed plasma prorenin levels of approximately 100ng ANGI/ml/h as compared to 7ng ANGI/ml/h in non-transgenic controls at 4 weeks of age. The non-transgenic animals showed a very small increase in plasma prorenin levels by six weeks of age. In contrast, both sets of transgenic animals had elevated plasma prorenin levels of approximately 4000ng ANGI/ml/h at five weeks of age and 3500ng ANGI/ml/h at six weeks of age, which were not significantly different to the week five results. The plasma active renin levels for all three groups are shown in table 4.1. There were no significant differences in the results obtained for the transgenic and non-transgenic animals.

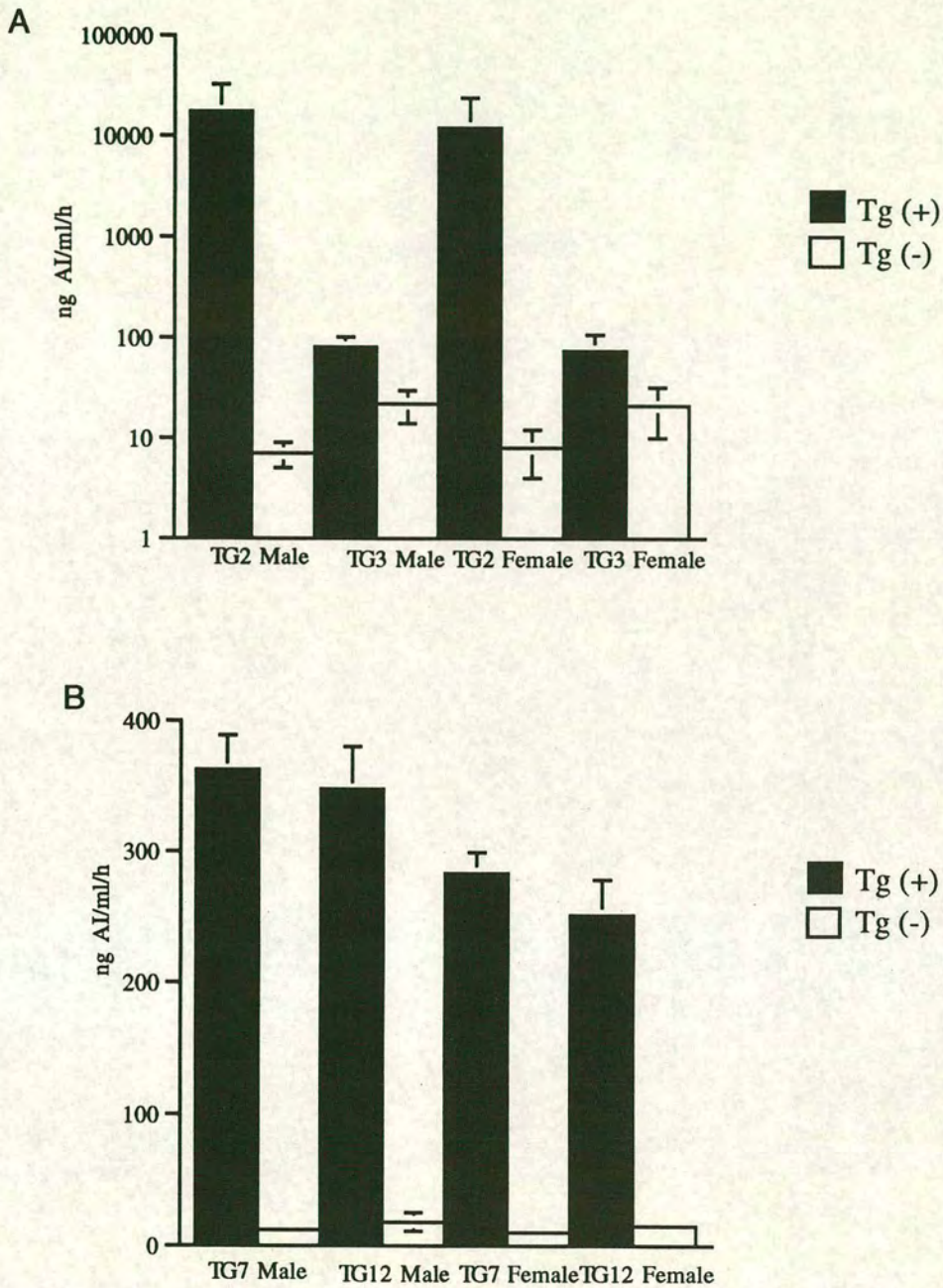


Figure 4.9

Panel A: Plasma prorenin levels in TG2 and TG3 transgenic and non-transgenic animals. Prorenin levels were measured by indirect RIA (see chapter 4, 4.5 for method) Five animals were tested in each group, variation is given as one standard deviation of the mean. Panel B: Plasma prorenin levels in TG7 and TG12 transgenic and non-transgenic animals. Five animals were tested in each group, variation is given as one standard deviation of the mean. All animals were five weeks of age.

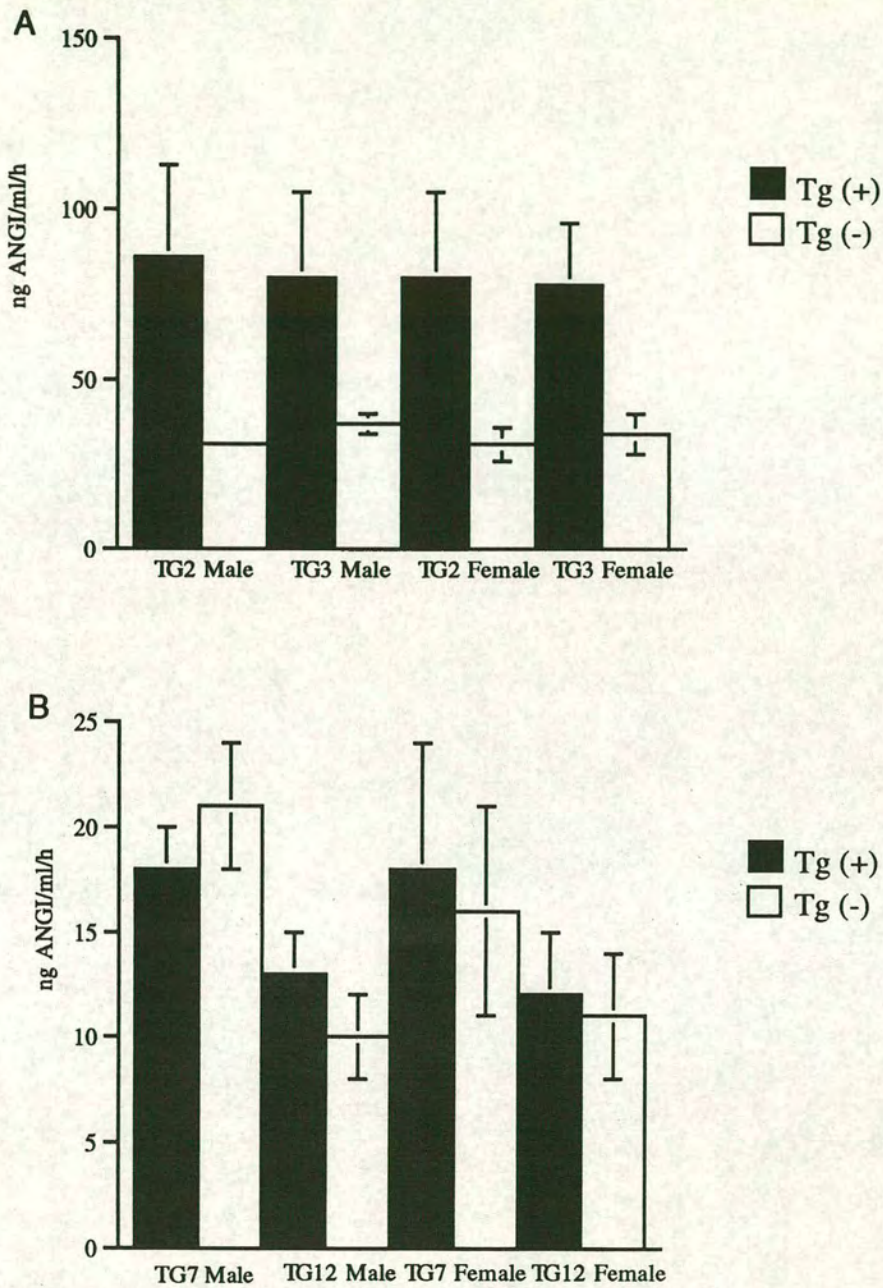


Figure 4.10

Panel A: Plasma renin levels in TG2 and TG3 transgenic and non-transgenic animals. Renin levels were measured by indirect RIA (see chapter 4, 4.5 for method) Five animals were tested in each group, variation is given as one standard deviation of the mean. Panel B: Plasma renin levels in TG7 and TG12 transgenic and non-transgenic animals. Five animals were tested in each group, variation is given as one standard deviation of the mean. All animals were five weeks of age.

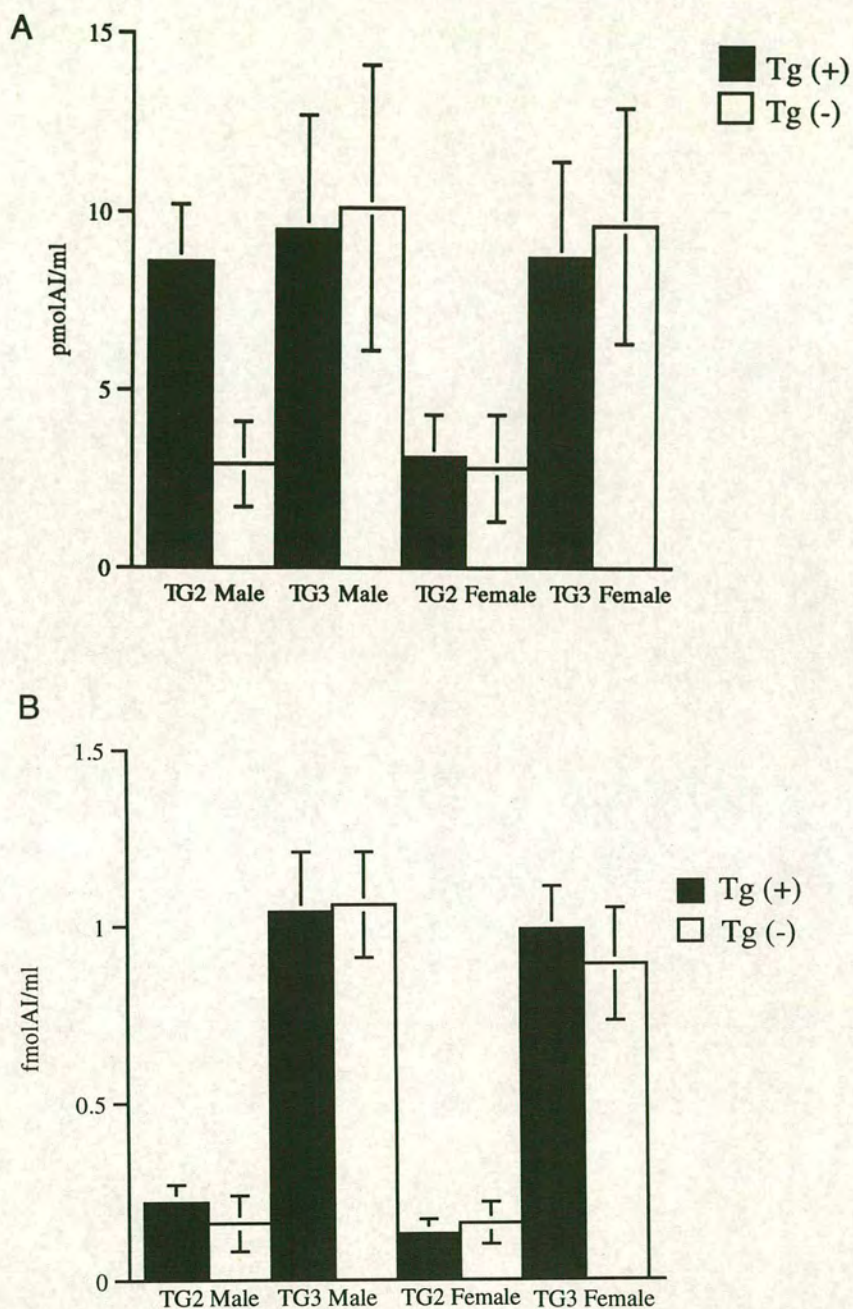


Figure 4.11

Panel A: Plasma angiotensin I levels in TG2 and TG3 transgenic and non-transgenic animals. Angiotensin I levels were measured by direct RIA (see chapter 4, 4.5 for method) Five animals were tested in each group, variation is given as one standard deviation of the mean. Panel B: Plasma angiotensin II levels in TG2 and TG3 transgenic and non-transgenic animals. Five animals were tested in each group, variation is given as one standard deviation of the mean. All animals were five weeks of age.

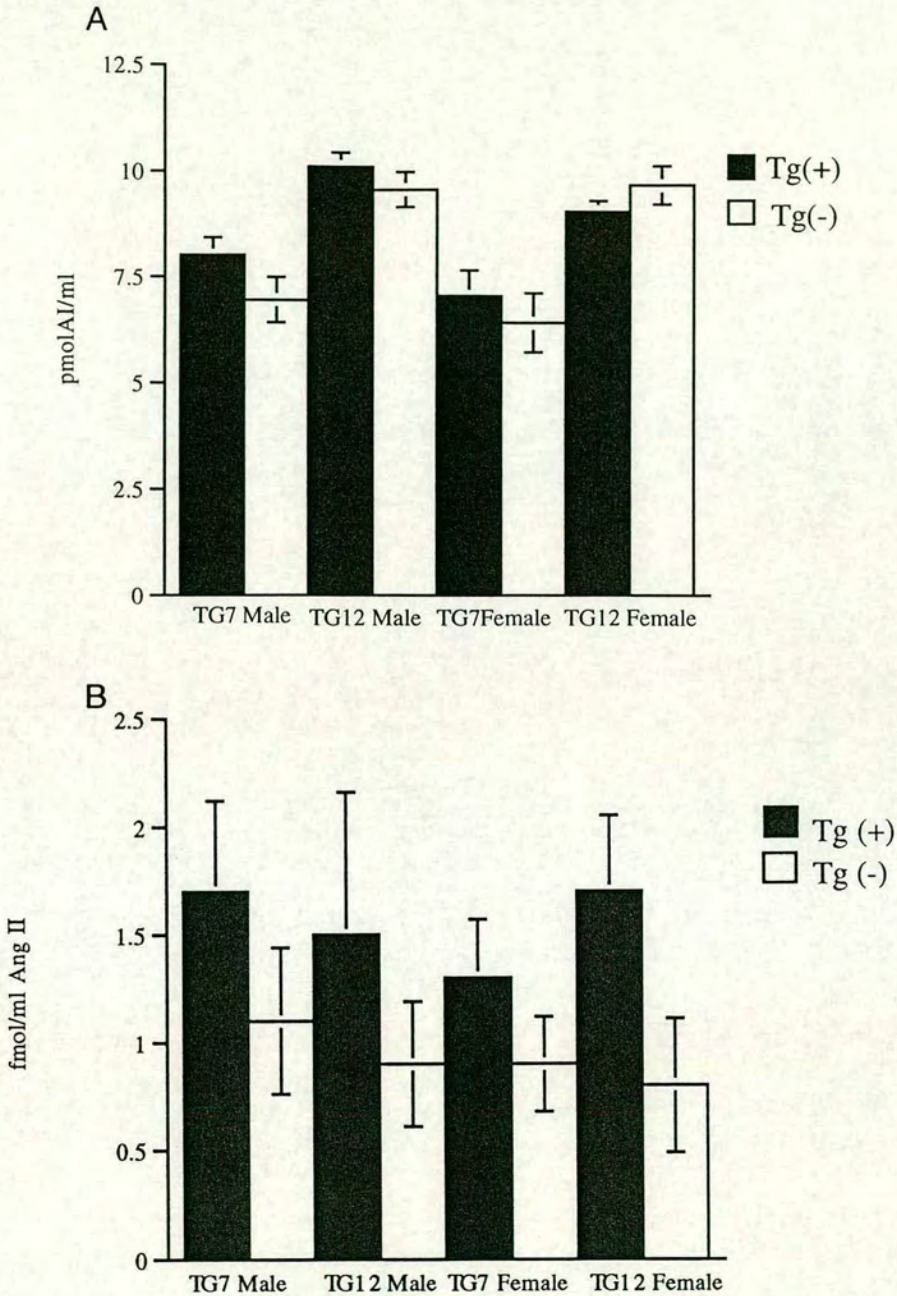


Figure 4.12

Panel A: Plasma angiotensin I levels in TG7 and TG12 transgenic and non-transgenic animals. Angiotensin levels were measured by direct RIA (see chapter 4, 4.5 for method) Five animals were tested in each group, variation is given as one standard deviation of the mean. Panel B: Plasma angiotensin II levels in TG7 and TG12 transgenic and non-transgenic animals. Five animals were tested in each group, variation is given as one standard deviation of the mean. All animals were five weeks of age.

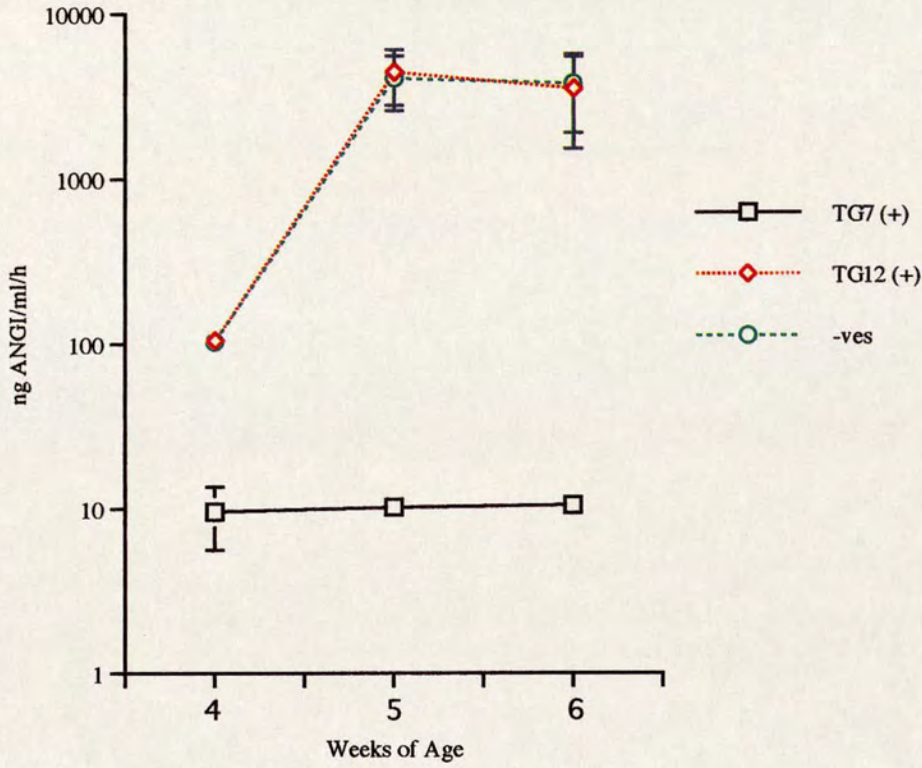


Table 4.1

| | Renin (ngANGI/ml/h) | | | Ang II (fmol/ml Ang II) | | |
|--------|---------------------|----------|----------|-------------------------|------------|------------|
| | Week 4 | 5 | 6 | 4 | 5 | 6 |
| TG7 | 14 ± 2.7 | 18 ± 3.3 | 18 ± 4.2 | 1.2 ± 0.52 | 1.7 ± 0.42 | 1.6 ± 0.51 |
| TG12 | 11 ± 3.9 | 13 ± 2.5 | 16 ± 3.7 | 1.1 ± 0.78 | 1.5 ± 0.60 | 1.8 ± 0.43 |
| (-ves) | 8 ± 2.4 | 10 ± 2.1 | 11 ± 3.0 | 0.8 ± 0.15 | 0.9 ± 0.29 | 0.9 ± 0.36 |

Figure 4.13

Plasma prorenin levels in TG7 and TG12 transgenic males over a three week period. Levels were measured by indirect RIA (see chapter 4, 4.5 for method). Five animals were tested in each group, variation is given as one standard deviation of the mean. Plasma renin and Ang II levels are given in table 4.1.

The transgenic female animals from lines TG7 and TG12 also showed prorenin levels that were elevated with respect to non-transgenic controls (280ng AI/ml/h and 10ng AI/ml/h respectively). All renin, AI and AII measurements showed no significant differences when compared to non-transgenic litter mates. By RNase protection there appears little difference between transcript levels but the RIA demonstrated a significant difference between protein levels. It was reported that in mice carrying a transgene consisting of this promoter and human Factor IX there was considerable variation in the levels of protein produced by different transgenic lines and as not all TG4 - TG16 lines produced were sampled it may be that other Fischer lines had plasma prorenin levels comparable to that seen in the lines TG2 or TG3.

4.6 Mouse and Rat Plasma Renins

To determine the contribution of mouse renin to the plasma renin content, the samples were incubated for 2h at 4°C with a monoclonal antirenin antibody, specific for murine renin with <3% cross-reactivity to purified rat renin (provided Dr. Jörg Peters from a donation by Dr. Celio, Universté Pérolles, Switzerland). Antibody/renin complexes were precipitated with formalin fixed *Staphylococcus aureus* protein A. The renin activities were determined before the procedure and in the supernatant after immunoprecipitation.

Transgenic male animals from line TG2 showed 75% of plasma active renin was derived from the $\alpha 1$ anti-trypsin/Ren-2 transgene. Despite demonstrating a lower plasma prorenin level, transgenic male animals from line TG3 also showed that 70% of plasma active renin was transgene derived. These results were reflected in the transgenic females from lines TG2 and TG3 where they showed 68% and 71%, respectively, of plasma active renin was transgene derived.

Despite a difference in genetic background from the Sprague Dawley animals the transgenic males from lines TG7 and TG12 also showed transgene-derived plasma active renin levels of approximately 83% (80% and 87% respectively). Consistent with these results, transgenic Fischer females from lines TG7 and TG12 showed levels of plasma active renin of 76% and 79% respectively. There is no clear reason for such similar results unless there is some form of feedback mechanism involved that can regulate mouse renin in rat plasma. Also, as there is an increased level of plasma

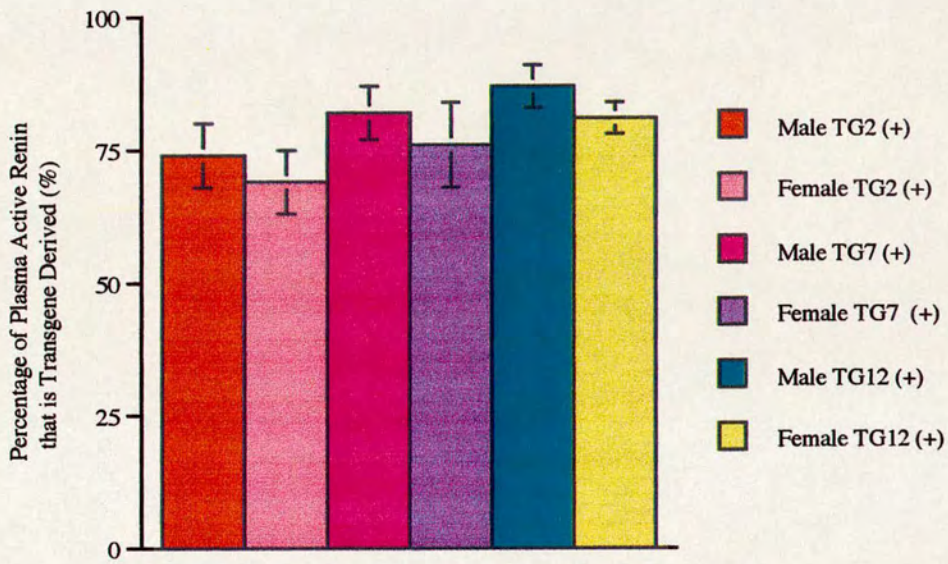


Figure 4.14

Percentage contribution of transgene derived active renin in the plasma as determined by immunoprecipitation and RIA. There were five animals in each group and variation is given as one standard deviation from the mean. All animals were five weeks of age.

prorenin in the transgenic animals it suggests that the difference between wild type and transgenic animals is due to transgene expression. Yet, despite extensively elevated levels of plasma prorenin in lines, TG7 and TG12 the percentage of plasma active renin in these lines is similar to that of line TG3 which demonstrated a much more modest increase in plasma prorenin. Therefore the percentage of plasma active renin which is transgene derived does not appear to be dependent on the absolute level of plasma prorenin.

CHAPTER 5

Physiological Data From Transgenic Lines

5.1 Growth Curves

Due to an obvious difference in apparent growth rate in the transgenic animals, weight measurements were taken weekly on groups of transgenic and non-transgenic animals. Transgenic males from line TG2 showed a significant difference in weight as early as three weeks of age when compared to non-transgenic controls, see figure 5.1A. These animals gained weight at a slower rate than the non-transgenic controls and continued to be significantly smaller than their non-transgenic counterparts. Males from transgenic line TG3 did not show a significant difference in weight at three to seven weeks of age when compared to control animals, see figure 5.1A. Studies were only carried out up to seven weeks of age due to the high mortality rate seen in the untreated transgenic animals (see following section). The lack of normal weight gain did not appear to be due to obstruction of the digestive tract or behavioural differences.

The trend of low weight gain was also seen in the male Fischer transgenic animals. At four weeks of age males from line TG7 weighed, on average, $74\text{g} \pm 5.7\text{g}$ as compared to $86\text{g} \pm 6.3\text{g}$ seen in the non-transgenic animals, see figure 5.2A. Fischer transgenic males from line TG12 also showed a lower weight at four weeks of age ($85 \pm 4.9\text{g}$) but it was not significantly different to non-transgenic animals, see table 5.1. By five weeks of age there were significantly lower average weights of both Fischer transgenic lines (TG7; $87 \pm 9.6\text{g}$, TG12; $112 \pm 3.3\text{g}$) when compared to the weight of the non-transgenic animals ($148\text{g} \pm 10.6\text{g}$).

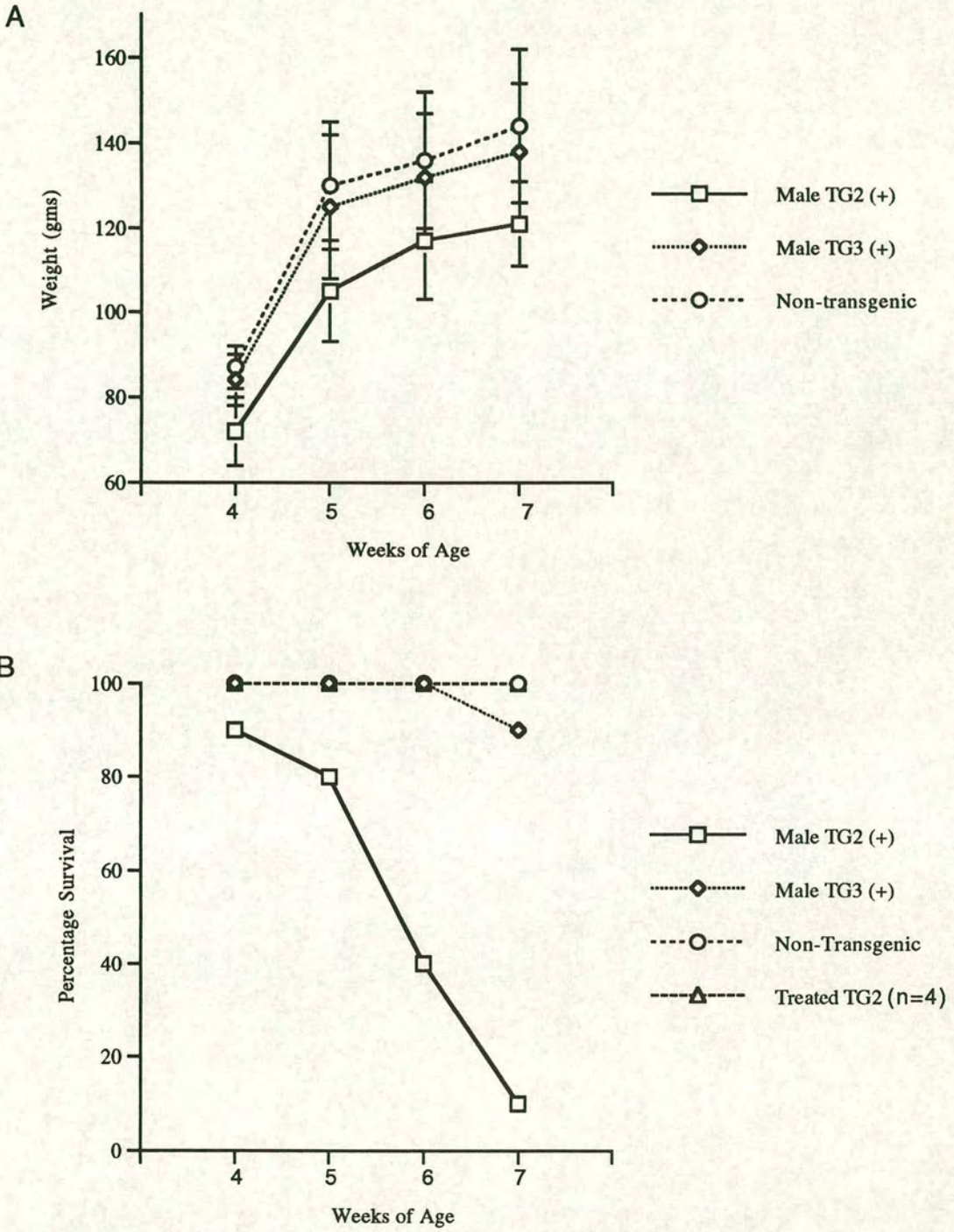


Figure 5.1

Panel A: Weight gain in the transgenic TG2 and TG3 groups. Panel B: Mortality rate in the transgenic TG2 and TG3 groups. Ten male animals were monitored in each group initially. Ten animals were tested in each group, variation is given as one standard deviation of the mean.

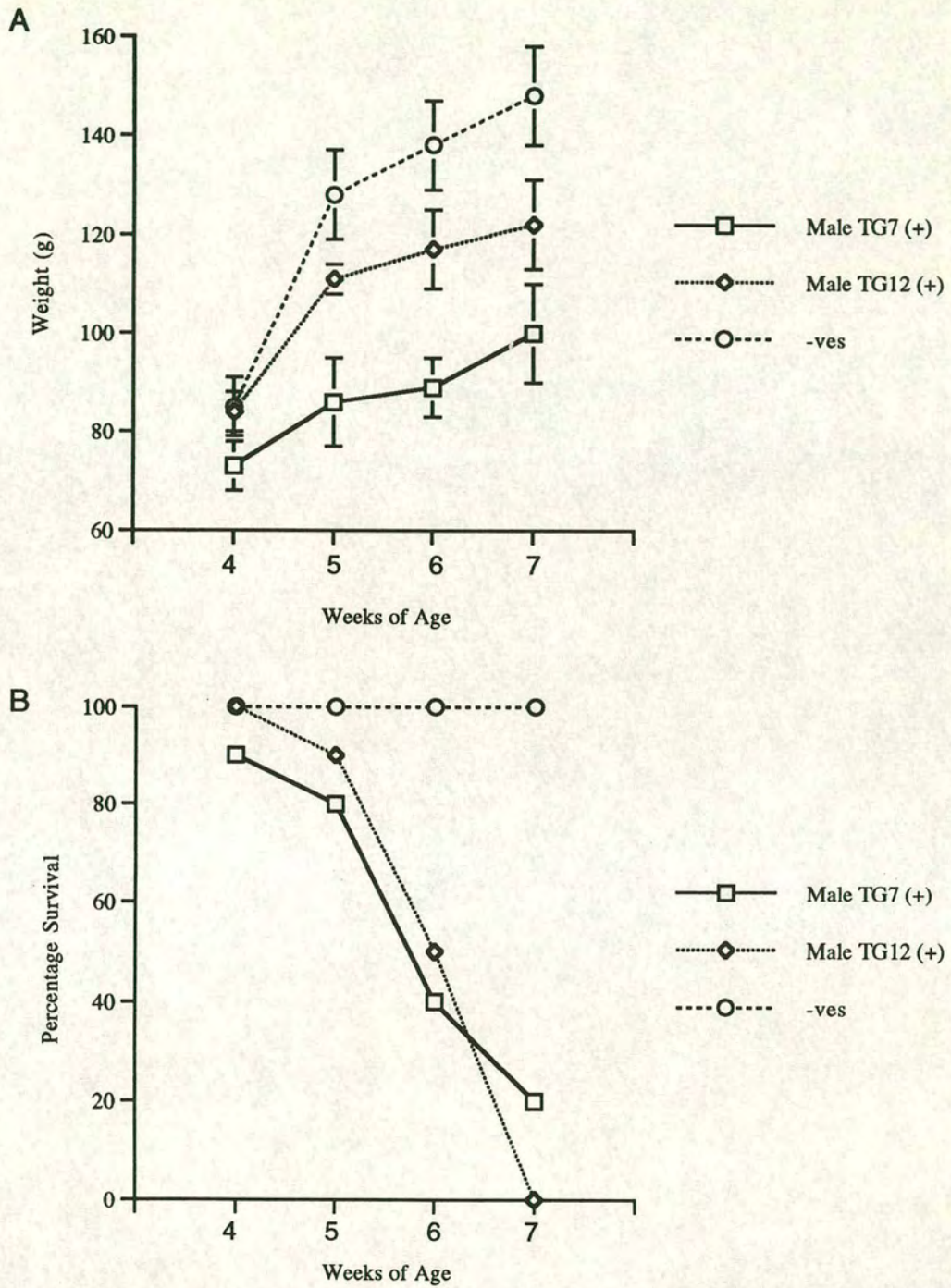
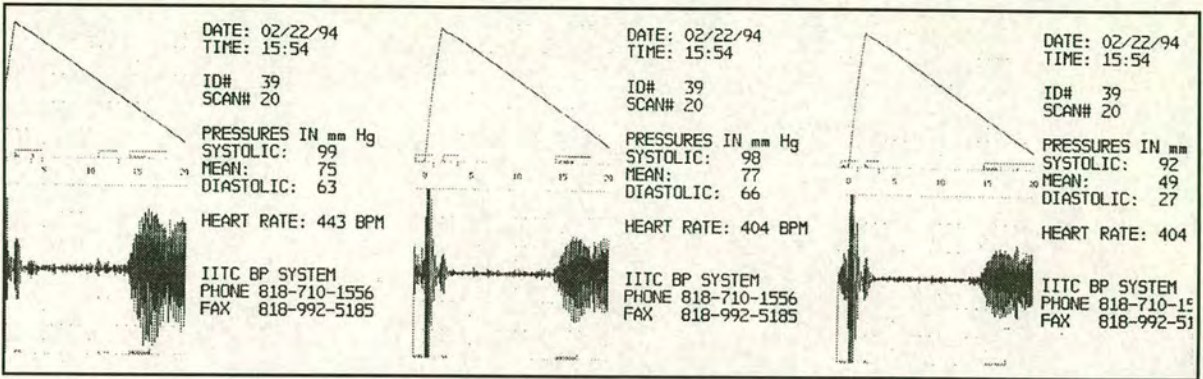


Figure 5.2

Panel A: Weight gain in the transgenic TG7 and TG12 groups, variation is given as one standard error of the mean. Panel B: Mortality rates in the TG7 and TG12 groups. Ten male animals were monitored in each group initially.

A Non-Transgenic



B Transgenic

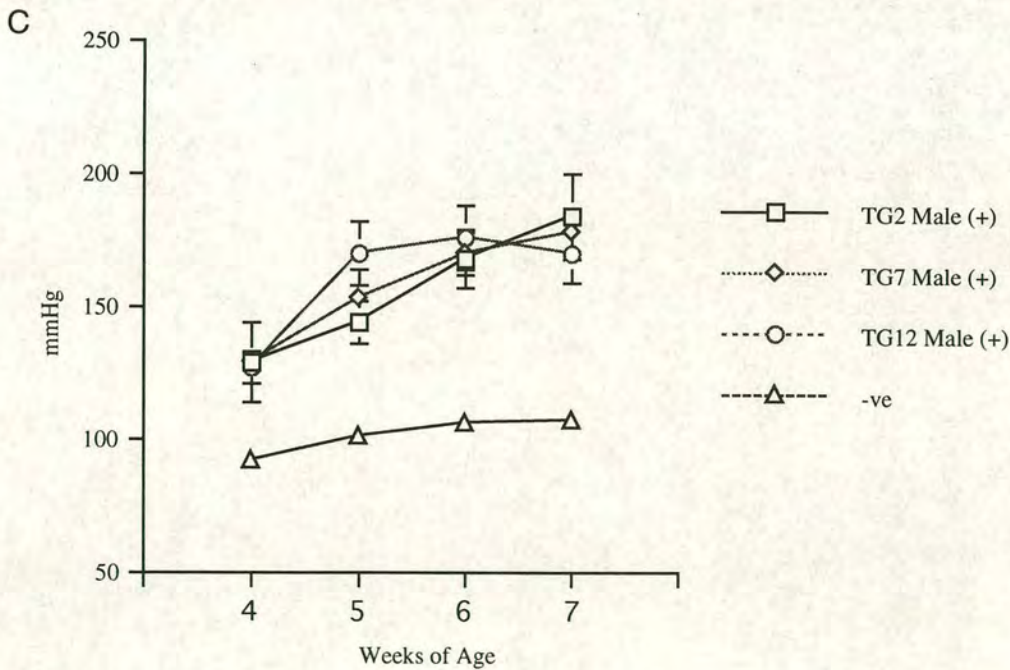
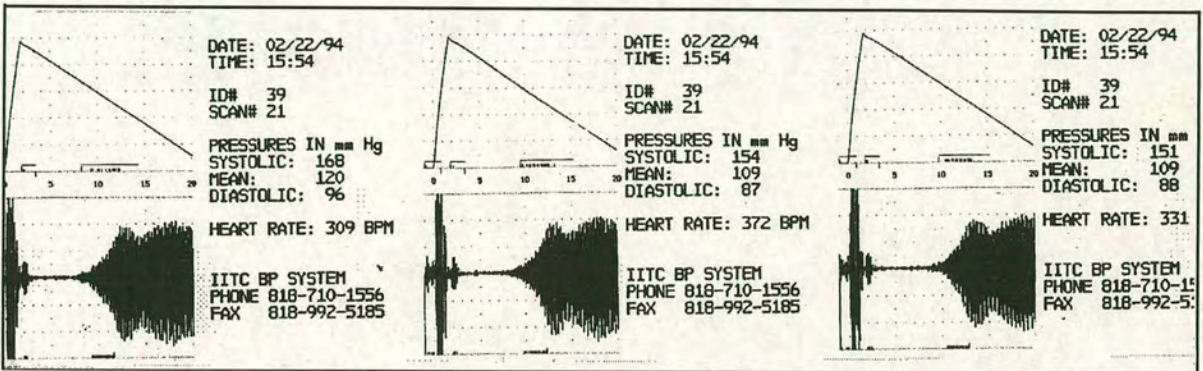


Figure 5.3

A: Output from the IITC Life Sciences Programmable Sphygmomanometer. Non-transgenic TG2 male 6 weeks of age trace. B: Transgenic TG2 male 6 weeks of age, trace. C: Tail cuff blood pressure measurements (systolic blood pressures) over a four week period. Ten male animals were monitored in each group and variation given as one standard error of the mean.

5.2 Mortality

As previously mentioned, transgenic rat lines TG2, TG7 and TG12 exhibited a high mortality rate in the first two months of life. Sprague Dawley founder transgenic male TG1 died at five weeks of age, before reaching sexual maturity. Animals from line TG2 demonstrated a very steep mortality curve with less than 50% survival of untreated males by six weeks of age, see table 5.2 and figure 5.1B. The cause of death in the transgenic animals was renal failure, cardiac hypertrophy or stroke, all associated with chronic or acute high blood pressure. Conversely, line TG3 demonstrated an almost normal mortality rate in the first seven weeks of life. As no severe pathology was ever seen in samples from line TG3 it was concluded that it was an altered phenotype from that seen in line TG2, perhaps due to the normal circulating prorenin levels seen.

Both Fischer lines showed mortality rates similar to that seen in line TG2. In the study of Fischer line TG7 one male died before four weeks of age and by seven weeks of age only two untreated males survived. The mortality rate for line TG12 demonstrated a higher rate with no surviving males at the end of the study, see figure 5.2B. No control animals died in the first seven weeks of life. It was noted that the three founder male animals from line TG2 and one each from lines TG7 and TG12 treated continuously with Captopril from weaning survived the seven week study.

5.3 Tail Cuff Plethysmography

Tail cuff plethysmography was carried out on both TG2 and TG3 lines at regular intervals. Only mean blood pressure and systolic blood pressure measurements were taken, as diastolic blood pressure was not measured, by the IITC sphygmomanometer, rather it was calculated using the formula;

$$\text{Mean Diastolic Pressure} = (3 \times \text{MBP}) - \text{SBP})/2.$$

Due to the requirement for anaesthesia in the protocol, four weeks was routinely used as the minimum age at which the animals were anaesthetised and the animals required a minimum of five days to recover. Both transgenic males and females from line TG2 demonstrated systolic blood pressures significantly higher than the non-transgenic control group. The TG2 males demonstrated a steep rise in systolic blood pressure from four to seven weeks of age ($129 \pm 3 \text{ mmHg}$ - $184 \pm 16 \text{ mmHg}$). This

rise was mirrored by the blood pressure measurements from the TG2 females (120 ± 6 mmHg - 185 ± 22 mmHg). Blood pressure measurements from line TG2 females were lower, but not significantly so, when compared to transgenic males from the same line. The Sprague Dawley transgenic animals from line TG3 showed no significant difference in blood pressures, at any age, when compared with the non-transgenic controls. It has been postulated that the normal blood pressures seen in transgenic males and females from line TGR TG3 are due to the normal levels of prorenin and other constituents of the renin-angiotensin system seen in these animals. Also it has been reported that in humans small variations in blood pressure, at an early age, may indicate larger variations to the norm in adult life. Systolic blood pressures for both males and females from line TG2 were elevated, but not significantly so, at four weeks of age. The large increase in blood pressure seen in the following weeks is consistent with the findings in humans. As blood pressure measurements required anaesthesia, as previously mentioned, readings were only taken once a week. This meant that any rhythm, diurnal or otherwise, could not be detected using this method. The maximal blood pressure reached by the male TG2 animals (~ 200 mmHg systolic) was similar to that reached by the transgenic line TGR(mRen-2)27. Despite lower plasma prorenin levels in the TGR(mRen-2)27 the maximal blood pressure was similar to that seen in line TG2 but TGR(mRen-2)27 demonstrated a more gradual increase in blood pressure. The TGR(mRen-2)27 rats reached a plateau at 200mmHg at 10-12 weeks of age whereas line TG2 animals had reached systolic blood pressure of 200mmHg by seven weeks of age. The difference in blood pressure increases between TGR(mRen-2)27 and the line TG2 animals could be due to their respective plasma prorenin levels.

Of the thirteen Fischer founder animals seven produced offspring that carried the transgene. Two litters were bred from each founder, and blood pressure measurements were taken by tail cuff at six weeks of age (Table 5.3). The range of systolic blood pressures exhibited by the G₂ offspring was between 141 ± 4.6 mmHg (lines TG11 and TG15) and 182 ± 6.3 mmHg (line TG7). Non-transgenic control animals exhibited systolic blood pressure of 113 ± 2 mmHg.

Both Fischer transgenic lines TG7 and TG12 showed increases in systolic blood pressure, which were statistically significant at four weeks of age, unlike the TG2 line. Transgenic line TG7 males showed systolic blood pressure of 129 ± 8 mmHg whereas males from line TG12 showed blood pressures of 127 ± 15 mmHg compared with non-transgenic males which demonstrated blood pressure of 96 ± 9 mmHg at four weeks of age. Both transgenic lines showed steep increase in blood pressure in the region of 40 mmHg in weeks four to six whereas the non-transgenic control animals demonstrated an increase of 15 mmHg over the same time period. The standard

Table 5.1

| Founder | TG4 | TG6 | TG7 | TG11 | TG12 | TG13 | TG14 | Non Tg |
|-----------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Nos. Transgenic (+ve) | 4 | 2 | 7 | 6 | 6 | 7 | 6 | 20 |
| Mean SBP (mmHg) | 148 ±5.6 | 166 ±8.1 | 182 ±6.3 | 141 ±4.7 | 177 ±4.3 | 155 ±5.2 | 141 ±3.9 | 113 ±2.2 |

Tail cuff plethysmography data from transgenic Fischer lines

deviation values for the transgenic groups are high due to sharp increases in blood pressure of individual animals at different time points and the high mortality rate which meant that at the end of the study period only four males remained in each of the transgenic groups.

The transgenic females from both TG7 and TG12 lines followed similar profiles for systolic blood pressure over the four week trial. As was the case for the female animals from line TG2, the female transgenic animals showed a lower systolic blood pressure at four weeks of age, when compared with transgenic males from line TG7 and TG12, however it was not statistically significant. By week seven both transgenic female groups had demonstrated blood pressures of approximately 190 mmHg and mortality was similar to that of the transgenic males.

Three male transgenic animals from line TG7 were given Captopril in their drinking water over the same four week period of blood pressure monitoring. There were no initial differences in blood pressure between the transgenic animals treated with Captopril and non-treated, non-transgenic animals. There was also very little increase in blood pressure and no mortality in the group. This suggests that, as expected, the hypertension seen in these animals was mediated through the renin-angiotensin system.

5.4 Telemetric Blood Pressure Monitoring

Direct blood pressure monitoring using the Dataquest telemetric system from Data Sciences International, USA, provided continuous blood pressure measurements on conscious, unstressed animals (Bidani et al. 1993), see table 5.5 for an example of the system output. Due to the sensitivity to anaesthesia and relatively low body weight of the transgenic animals, only two male Sprague Dawley line TG2 transgenic and two male non-transgenic animals underwent study by this method.

Both non-transgenic control males demonstrated normal mean blood pressure of approximately 100 mmHg and variation in blood pressure of ± 10 mmHg over the

period of study. Using this method both systolic and diastolic blood pressures were measured directly and output was expressed as mean arterial pressure. Both transgenic animals showed severe elevation of blood pressure post-operatively, and in the case of transgenic TG2/2532, an inability to reduce blood pressure following the operation. Transgenic TG2/2533 did show a steep drop in blood pressure of almost 90 mmHg in the 2 hours following the operation. This was followed by a steep increase in blood pressure over the subsequent 4-6 hours to return blood pressure to 210 mmHg. Whilst TG2/2533's mean blood pressure continued to fluctuate around 190 mmHg TG2/2532 had a steep rise in mean arterial pressure, peaking at 270 mmHg before going into a steady decline. Transgenic TG2/2533 survived 2.5 days after the operation, however, TG2/2532 only survived 40 hours post-operatively. Transgenic TG2/2533 also showed a steep increase in blood pressure, peaking at 220 mmHg before going into steep decline, 60 hours after the operation.

The telemetric analysis of the TG7 animals took the form of one male transgenic and one female transgenic from receiving implants, along with the appropriate non-transgenic controls. The transgenic male showed a drop of 50 mmHg, from 175 mmHg to 125 mmHg in the 12 hours following the implant operation. The male non-transgenic animal demonstrated a steady baseline measurement of 115 mmHg with ± 10 mmHg variation throughout the test period. The transgenic male showed some periodicity in the measurements taken which becomes more erratic as the trial continued. As rats are nocturnal animals, peak activity and blood pressure should coincide at approximately 5am. In TGR(mRen-2)27 rats the diurnal rhythm is reversed as is often seen in human essential hypertensive patients (Pickering 1990). The results obtained from telemetry were not over a sufficient time period for the diurnal rhythm to be ascertained.

The non-transgenic female had a similar baseline measurement to the non-transgenic male (115 mmHg) with a slightly higher degree of variation (± 15 mmHg). The transgenic female had an abnormally low mean blood pressure of 75 mmHg post-operatively but over the following 24 hours had an almost exponential rise in blood pressure plateauing 36 hours after the operation at 180 mmHg. Any oscillations in the blood pressure of the female are difficult to discern. Unlike transgenic animals from line TG2 males both transgenics from line TG7 survived for more than five days on telemetry. The male transgenic from line TG7 died 3.5 days, and the female transgenic also from line TG7 died four days, after the initial readings. Both demonstrated a steep rise in blood pressure (10-15 mmHg in 1-2 hours) prior to going into steep decline. The different traces produced for TG2 and TG7 were due to the different sampling times for automatic data.

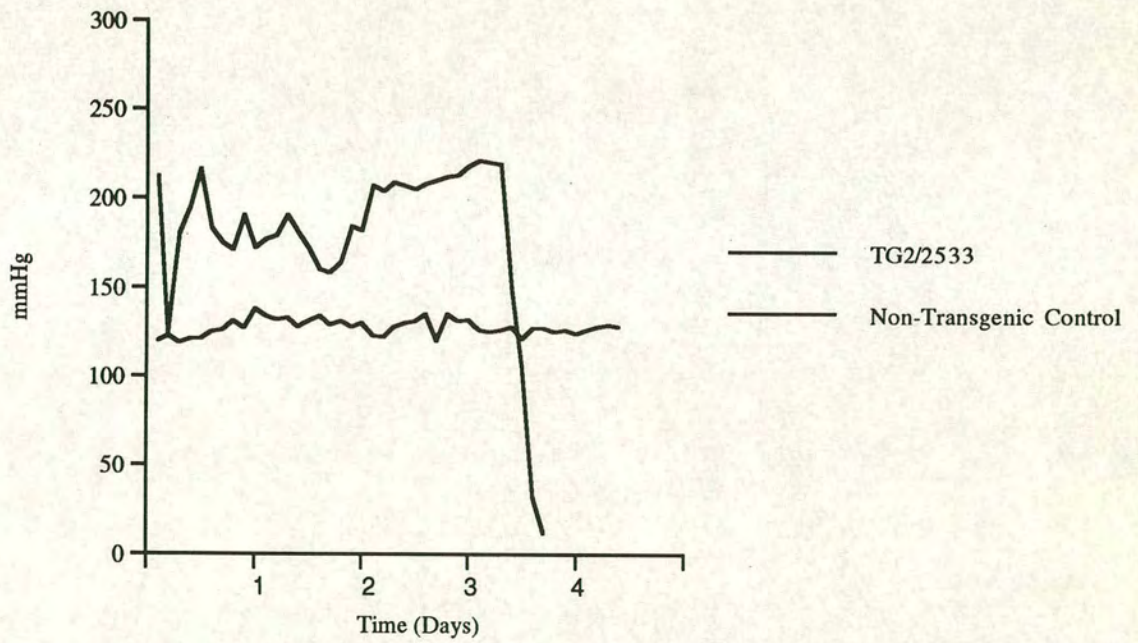
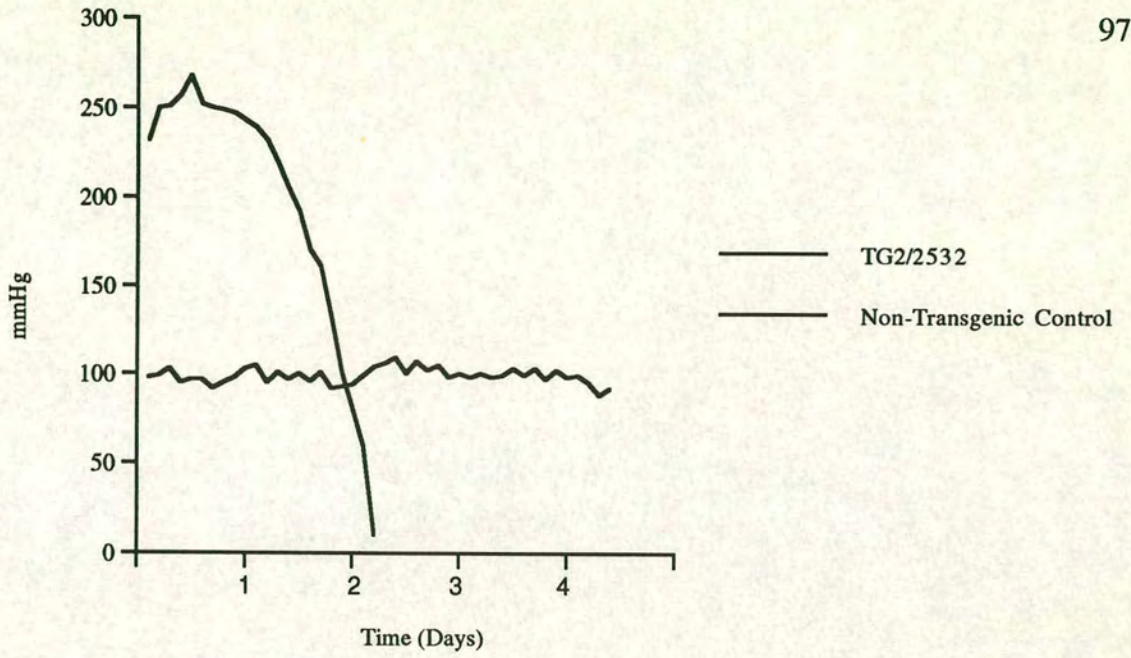


Figure 5.4

A: Telemetric data from transgenic and non-transgenic TG2 males (see chapter 2, 2.5 for methodology) over a three day time period. B: Telemetric data from transgenic and non-transgenic TG2 males over a three day time period.

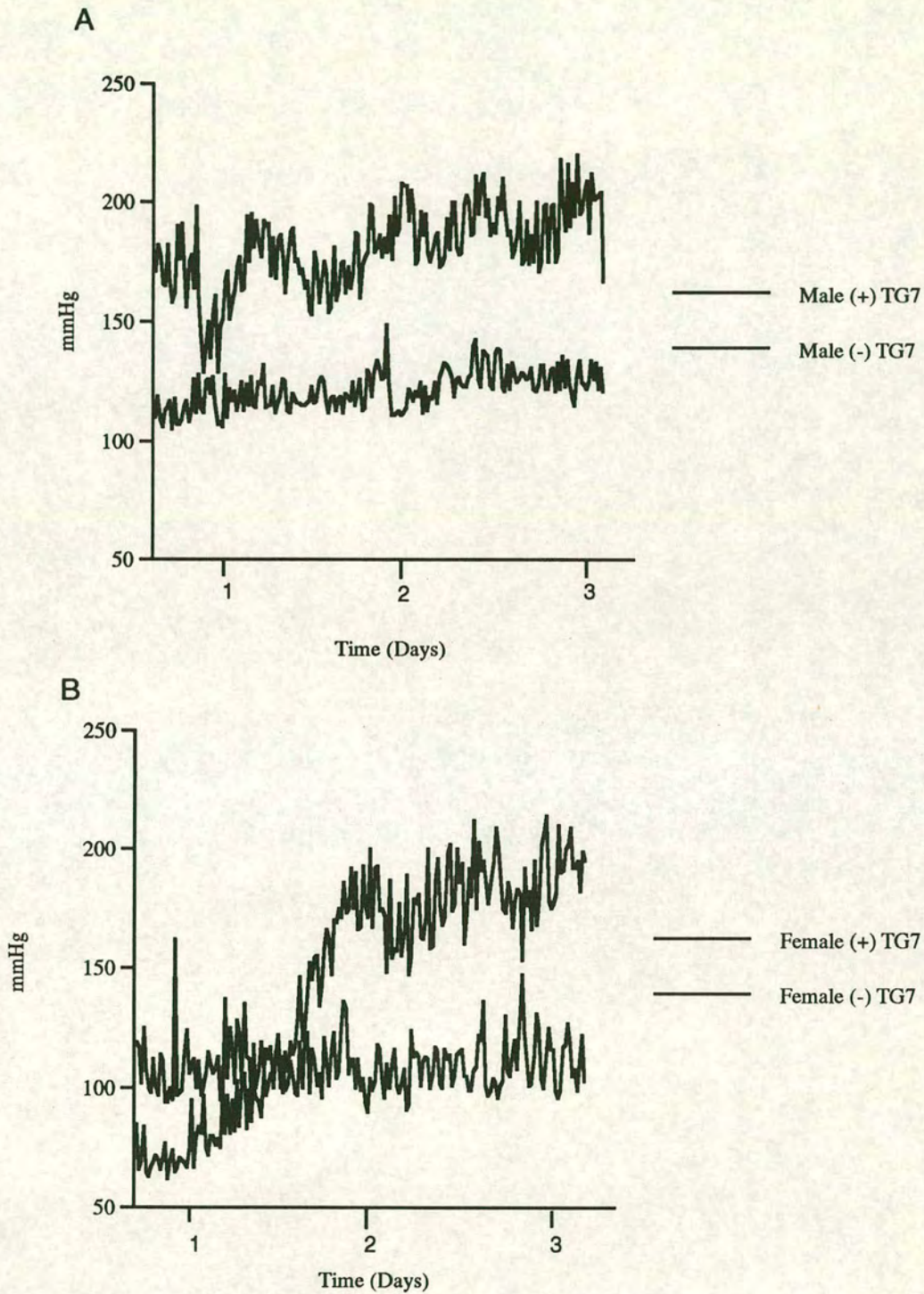


Figure 5.5

A: Telemetric data from TG7 transgenic and non-transgenic males (see chapter 2, 2.5 for methodology) over a three day time period. B: Telemetric data from TG7 transgenic and non-transgenic female over a three day time period.

The telemetric system allowed continuous blood pressure monitoring on conscious, untethered animals. Accurate systolic, diastolic and mean blood pressures were obtained using this method. However, due to the comparatively short life span of the transgenic animals after the implant operation this procedure did not prove as successful as was hoped. If smaller telemetric implants were available it may be possible to start the procedure on younger animals that have the potential to survive longer.

CHAPTER 6

Cardiac and Pathology Data

6.1 LV:BW Ratios In All Transgenic Lines

Angiotensin II has been implicated as a growth factor or growth modulator in the cardiovascular system and it has been suggested that it plays an important role in development and maintenance of cardiovascular hypertrophy (Sadoshima et al. 1993). Sadoshima and Izumo found that in culture medium conditioned by stretch cardiac myocytes there was at least one factor that caused induction of immediate early genes and activation of second messenger systems in non-stretched myocytes. This factor was subsequently found to be Ang II and the autocrine secretion was found to play a critical role in the stretch-induced hypertrophic response (Sadoshima et al. 1993). Animal studies into the role of the RAS hypertrophy have drawn parallels between the human condition and heart failure found in the transgenic rat strain TGR(mRen-2)²⁷ (Paul et al. 1994).

There are several indications as to the hypertrophic state of cardiac muscle, such as interstitial collagen volume fraction, ventricular interstitial fibrosis or perivascular collagen area. Such techniques require morphometric analysis, however, heart : body weight ratios can be used to give an indication as to the extent of hypertrophic growth. As left ventricular hypertrophy (LVH) is the major risk factor associated with myocardial failure it is usual to present data in the form of left ventricular weight : body weight ratios.

The animal was sacrificed, weighed, and the heart removed. All blood was taken out of the ventricles of the heart and total heart weight taken. The right ventricle was then removed from the left ventricle and the left ventricle weighed separately.

Transgenic males from line TG2 demonstrated an LV:BW ratio of $6.8 \pm 0.26 : 1$ which was statistically significant when compared to the male non-transgenic ratio of $4.4 \pm 0.21 : 1$. Again, the female transgenic animals from line TG2 also had a statistically significant ratio of $6.7 \pm 0.45 : 1$ when compared with the female non-transgenic ratio of $4.2 \pm 0.19 : 1$. Sprague Dawley line TG3 transgenic males and females demonstrated no statistical significance in the LV:BW ratios when compared with their non-transgenic counterparts. As there was no pathology to indicate cardiac

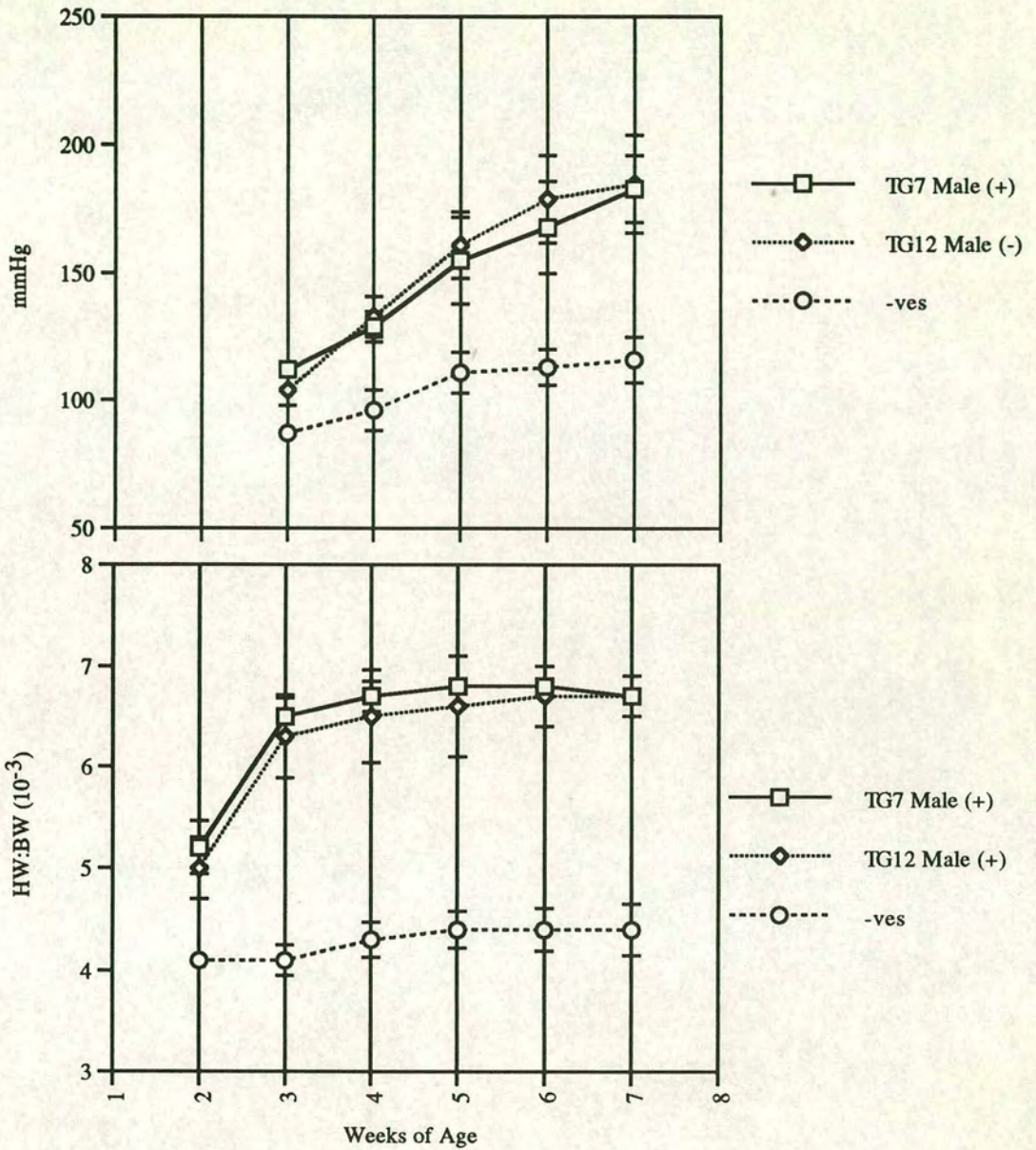


Figure 6.1

Comparison of blood pressure values and HW:BW ratios. The left hand Y axis shows systolic blood pressure as measured by tail cuff plethysmography. The right hand axis shows heart weight:body weight ratios ($\times 10^{-3}$). Each time point contained 3 male animals from the groups TG7, TG12 and negative controls.

hypertrophy in line TG3 it was not expected that the LV:BW ratio would differ from that of the control animals.

It was suggested by Paul et al (Paul et al. 1994) that the TGR (mRen-2)²⁷ rats developed cardiovascular hypertrophy at an age where blood pressure was not persistently elevated. In light of this, the transgenic TG7 and TG12 males (n=3) were measured for LV:BW ratios and BP at 3 weeks of age (see figure 6.1). Animals from both lines TG7 and TG12 demonstrated LV:BW ratios consistent with severe cardiac hypertrophy (5.25 ± 0.2 and 5.20 ± 0.48 respectively) when compared with non-transgenic, age matched, controls (4.15 ± 0.37). When systolic blood pressures were measured in all three groups there were no significant differences between TG7, TG12 or non-transgenic animals (107 ± 5.5 , 103 ± 6.45 and 86 ± 2.9 respectively) suggesting blood pressure may not have been a determining factor in the onset of cardiac hypertrophy.

Table 6.1

| Weeks of Age | TG7 HW (g) | TG7 HW:BW | TG12 HW (g) | TG12 HW:BW | -ves HW (g) | -ves HW:BW |
|--------------|-----------------|----------------|-----------------|----------------|-----------------|----------------|
| 2 | 0.32 ± 0.03 | 5.2 ± 0.26 | 0.39 ± 0.05 | 5.0 ± 0.30 | 0.36 ± 0.07 | 4.1 ± 0.12 |
| 3 | 0.40 ± 0.06 | 6.5 ± 0.18 | 0.45 ± 0.09 | 6.3 ± 0.41 | 0.44 ± 0.10 | 4.1 ± 0.15 |
| 4 | 0.49 ± 0.07 | 6.7 ± 0.15 | 0.56 ± 0.13 | 6.5 ± 0.46 | 0.55 ± 0.16 | 4.3 ± 0.17 |
| 5 | 0.61 ± 0.09 | 6.8 ± 0.11 | 0.74 ± 0.13 | 6.6 ± 0.50 | 0.61 ± 0.18 | 4.4 ± 0.18 |
| 6 | 0.61 ± 0.07 | 6.8 ± 0.13 | 0.79 ± 0.16 | 6.7 ± 0.31 | 0.61 ± 0.19 | 4.4 ± 0.21 |
| 7 | 0.67 ± 0.12 | 6.7 ± 0.11 | 0.82 ± 0.20 | 6.7 ± 0.22 | $0.65 \pm .23$ | 4.4 ± 0.25 |

Tabulated data of study into the development of hypertrophy. Data presented as a graph with blood pressure analysis in figure 6.1

6.2 Echocardiology Data From Transgenic Lines TG7 and TG12

The introduction of clinical echocardiology and its broad application in the past 20 years has greatly changed the ability to detect and quantify hypertensive left ventricular hypertrophy in humans. In a study by Gardin *et al*, echocardiology detected left ventricular hypertrophy in nearly 50 per cent of hypertensive patients, whereas no more than 5 per cent were identified by electrocardiography and chest radiography (Gardin *et al*. 1979). The routine measurement of left ventricular mass by echocardiography is best accomplished using M-mode (one-dimensional) tracings with careful placement of the ultrasound beam under two-dimensional (B-mode) guidance. With simple measurements of interventricular septal thickness (IVST) and posterior wall thickness (PWT) and end diastolic diameter (EDD, the minor axis of the ventricle), accurate measurements of the ventricular muscle mass are obtained. This involves the use of wall thickness and internal dimensions in an empirically derived regression formula developed from echocardiography - autopsy correlations:

$$\text{LVmass (LVM)} = 1.04[(\text{EDD} + \text{PWT} + \text{IVS})^3 - \text{EDD}^3] / \text{Body Weight}$$

(Sahn *et al*. 1978; Devereux *et al*. 1986) and validated in rats by Jones *et al* 1992 (Jones *et al*. 1992), where 1.04 is the specific gravity of cardiac muscle.

In humans multivariate analysis revealed that the left ventricular mass measurement was a more powerful predictor of prognosis than blood pressure or age (Laragh 1992). It should be noted that in patients with hypertension, hypertrophy is not an inevitable consequence. Hypertensive left ventricular hypertrophy does not appear to be a single morbid process as it is expressed as different geometric patterns in different patients.

In a study by Bruckschlegel *et al* (1995) (Bruckschlegel *et al*. 1995), which looked at the role of the renin-angiotensin system in chronic pressure-overload hypertrophy in rats, it was found that Losartan and Ramipril significantly reduced left ventricular mass, twelve weeks after aortic constriction. Hydrazine treated rats, however, demonstrated a marked elevation in LVM over the same time period. These results were consistent with results found in the renin-dependent model spontaneously hypertensive rat [Pfeffer 1982].

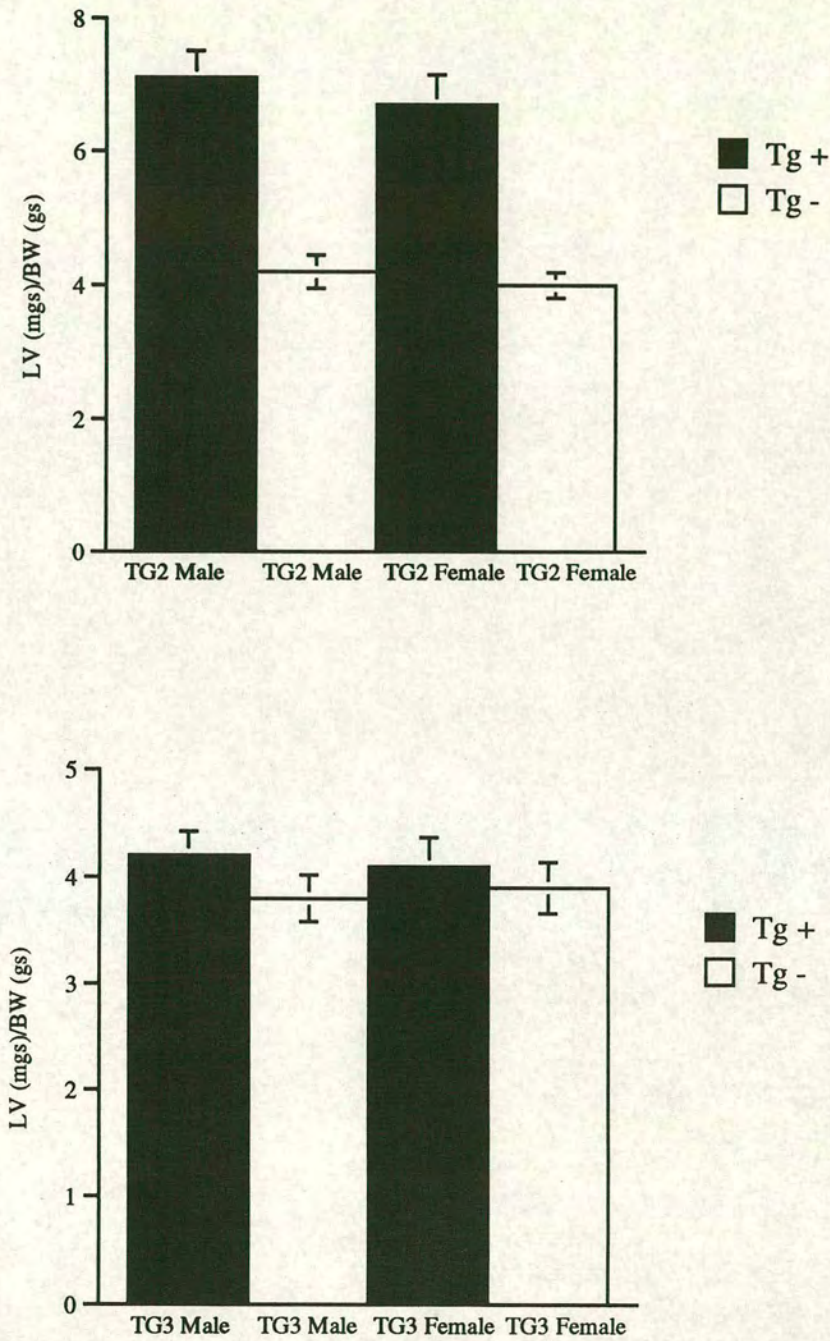


Figure 6.2

Panel A: Left Ventricular : Body Weight ratios for transgenic line TG2, four animals were tested in each group and the error given as one standard deviation from the mean. Panel B: Left Ventricular : Body Weight ratios for transgenic line TG3, four animals were tested in each group and the error given as one standard deviation from the mean. All animals were six weeks of age when tested.

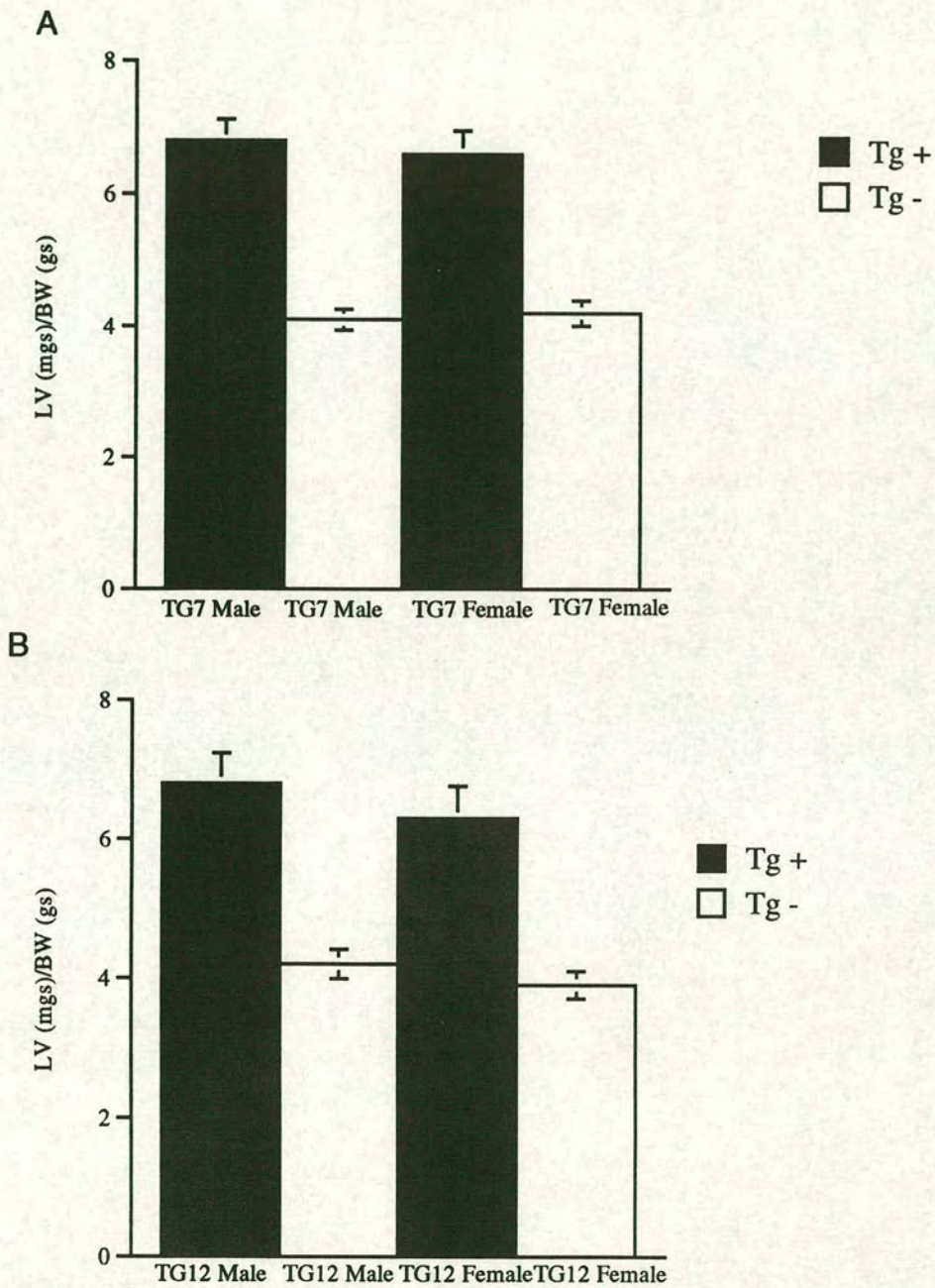


Figure 6.3

Panel A: Left Ventricular : Body Weight ratios for transgenic line TG7, four animals were tested in each group and the error given as one standard deviation from the mean. Panel B: Left Ventricular : Body Weight ratios for transgenic TG12, four animals were tested in each group and the variance is given as one standard deviation from the mean. All animals were tested at six weeks of age.

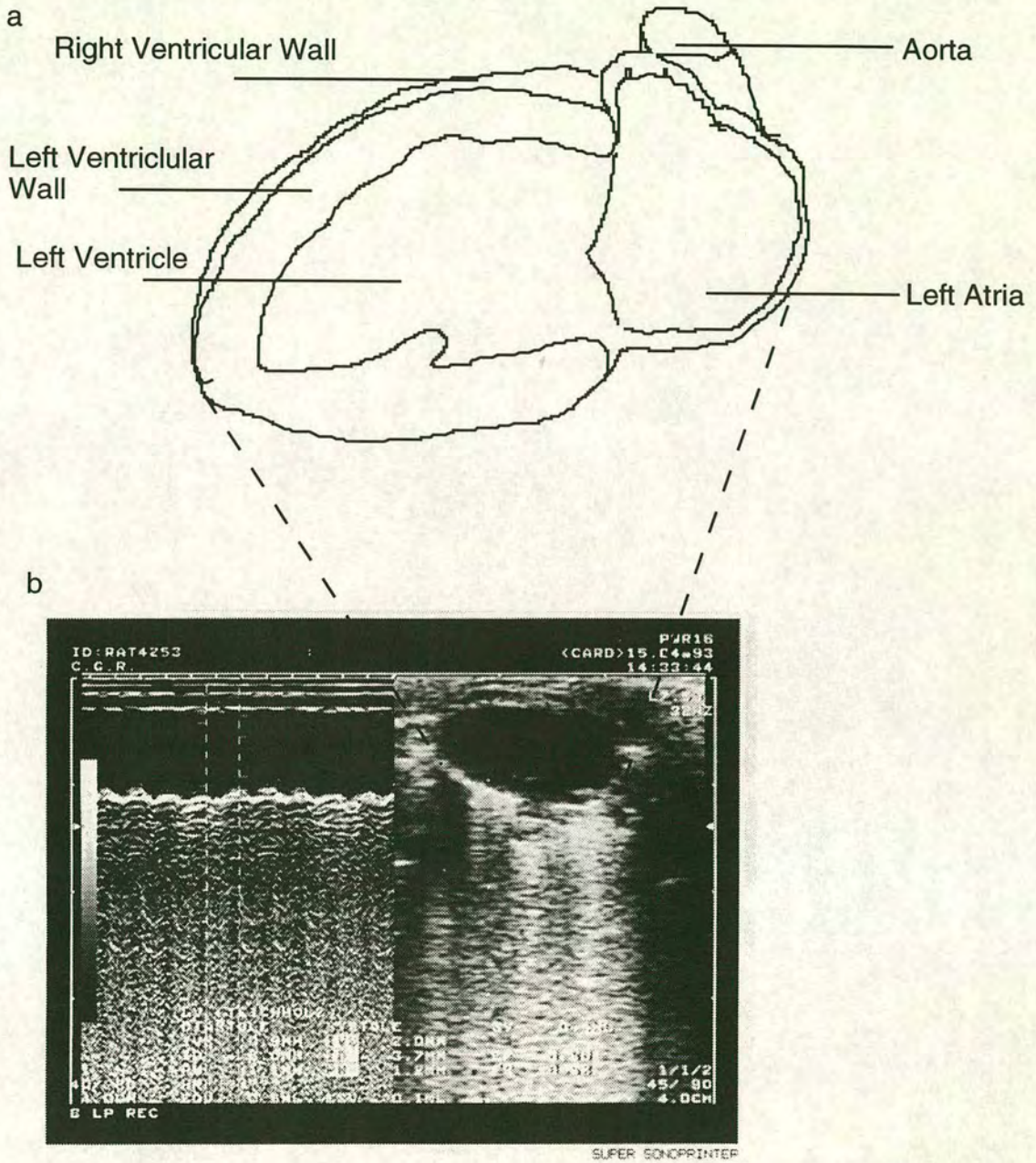


Figure 6.4

A: Diagrammatic representation of the heart as seen during echocardiographic analysis. B: Output from the Toshiba Capasee system, left hand panel M-mode (one dimensional), right hand panel B-mode (two dimensional).

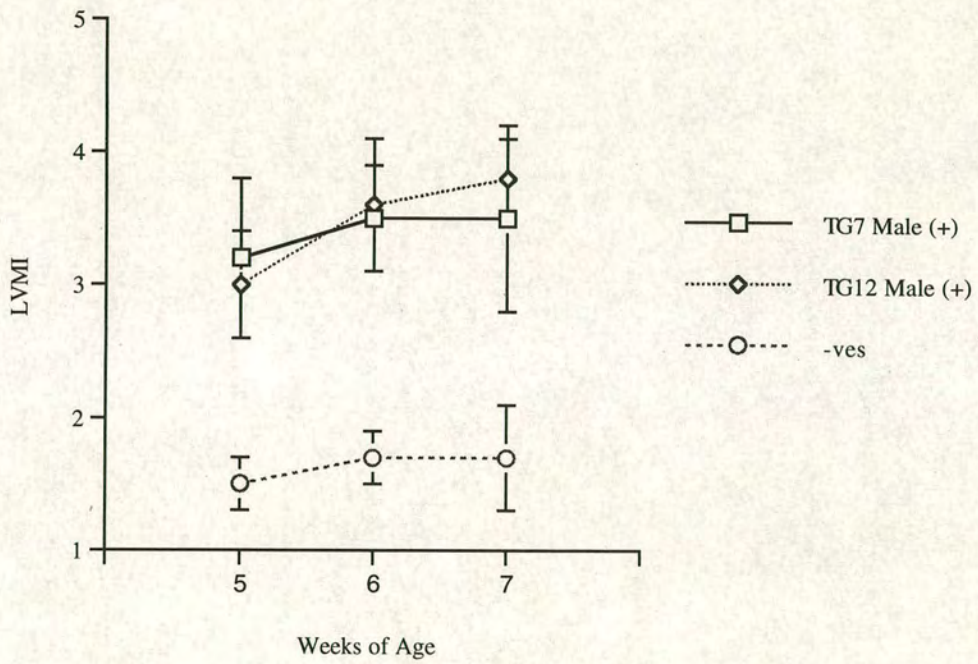


Figure 6.5

Left Ventricular Mass Indices for transgenic lines TG7 and TG12 over a period of three weeks. Six animals were tested in each group and the error given as one standard deviation from the mean.

The use of the ultrasound monitor to measure *in vivo* left ventricular thickness was validated in our laboratory by Dr. Whitworth (M.D. thesis) and Dr. Veniant. The measurements were taken at various stages of development and compared to the actual wet weight of the subjects' left ventricle. There was no statistical difference between methods (ANOVA). The study by Jones *et al* (1992) (Jones *et al.* 1992) showed the use of echocardiography in rats and compared LVM Indices (LVMI) of hypertensive and normotensive animals. The LVMI of the spontaneously hypertensive rats (SHR) at 9 weeks of age was 3.029 and normotensive Donyru rat (DRY) showed an LVMI of 2.054 at the same age. Between 4 - 7 weeks of age the non-transgenic control animals demonstrated LVMI of 1.5 ± 0.23 - 1.7 ± 0.41 . These results were not statistically different from the results obtained by Jones *et al* on the normotensive DRY rats. Both Fischer transgenic lines TG7 and TG12 demonstrated LVMI at 5 weeks of age comparable to those seen by Jones *et al* in the SHR animals at 9 weeks of age. Males from line TG7 showed an LVMI of 3.2 ± 0.43 increasing to 3.5 ± 0.47 at six weeks of age, with a similar mean value of LVMI at 7 weeks of age. Despite a slightly lower LVMI (3.0 ± 0.33), males from line TG12 showed a steep rise in LVMI by week 6 of 3.6 ± 0.45 , reaching a maximum of 3.7 ± 0.41 at 7 weeks of age. It was apparent that left ventricular hypertrophy was established by 5 weeks of age in the transgenic animals and was maintained throughout the study period.

Table 6.2

| Weeks of Age | TG7 LVMI | TG12 LVMI | -ve LVMI |
|--------------|---------------|---------------|---------------|
| 4 | 3.2 ± 0.6 | 3.0 ± 0.4 | 1.7 ± 0.2 |
| 5 | 3.5 ± 0.4 | 3.6 ± 0.5 | 1.7 ± 0.2 |
| 6 | 3.5 ± 0.7 | 3.8 ± 0.3 | 1.7 ± 0.4 |

Tabulated data of study into the development of hypertrophy by echocardiography. Data presented as a graph in figure 6.5

6.3 Pathological Analysis of All Transgenic Lines

The first pathological alteration seen in the TGR(α 1AT/Ren-2) rats was concentric left ventricular cardiac hypertrophy. The extent of the hypertrophy has been quantified above. Histological examination of cardiac sections from twenty-five transgenic animals revealed that it was indeed hypertrophy, not hyperplasia, as the

cardiomyocytes had become enlarged. Histology also showed that the hypertrophy was concentric and restricted to the left ventricle, consistent with involvement with hypertension, see figures 6.11.

During the course of essential hypertension the larger arteries in the kidneys, as is the case elsewhere, become rigid and thickened, but their lumina are not seriously reduced. These changes also occur in the arcuate arteries but due to their smaller calibre, thickening of the wall may result in reduction of the lumen. Ischaemia, lack of oxygen due to reduction in circulation, often results from changes in the smaller vessels: the interlobular arteries become elongated, with medial fibrosis and fibro-elastic thickening of the intima resulting in significant narrowing of the lumen. The afferent glomerular arteriolar walls become acellular, eosinophilic with varying degrees of luminal narrowing. This results in glomerular ischaemia, the glomerular tuft becomes shrunken and the capillaries are gradually replaced by pale staining homogeneous material, eventually the entire tuft is converted to an acellular sphere (see figures 6.6 and 6.7). Despite the loss of individual nephrons renal function is not significantly impaired.

In malignant (accelerated-phase) hypertension the interlobular arteries show extensive intimal thickening, due to the formation of fine, concentric layers of connective tissue and smooth muscle cells, with severe reduction in the lumen. The afferent arterioles show fibrinoid necrosis, the wall being thickened, brightly eosinophilic and granular. The necrotic material gives staining reactions of fibrin and the lumen is often completely obliterated, or occupied by thrombosis which merges with the necrotic wall. The fibrinoid necrosis can extend into the glomerulus, involving part or all of the tuft (see figure 6.8). This form of damage was only seen in 25-30% of the TG7 and TG12 animals. Other glomeruli are less severely damaged and show intense capillary dilatation and congestion. The glomerular changes are the direct result of acute ischaemia resulting from fibrinoid necrosis of the afferent arterioles. These vascular changes of malignant hypertension also occur in other organs although the effect is not usually so devastating as in the kidneys.

The considerable thickening of the intima produces severe narrowing of the lumen. The diffuse damage to the vascular tree may be accompanied by intravascular coagulation which accentuates the endothelial cell damage. The passage of blood at high pressure through a damaged vascular bed with intravascular coagulation causes red cell fragmentation - microangiopathic haemolytic anaemia. An example of this condition in transgenic rats was shown by Whitworth *et al* (1995) in a genetic model of malignant hypertension. The blood films from malignant phases TGR(mRen-2)27 rats

all exhibited microangiopathic haemolytic anaemia with red cell fragments, spherocytes, microspherocytes and reticulocytosis. Also in the study by Whitworth *et al* it was reported that in TGR(mRen-2)27 rats microscopic myocardial infarcts were found, occasionally related to fibrinoid necrosis of cardiac arterioles. The early lesions were cardiomyocytes with focal necrosis, but granulation tissue and focal fibrinotic scars suggested a healing response.

A rise in intraluminal pressure can overcome the normal reflex vasoconstriction of an artery. This is more likely to occur when the rate of rise is rapid. The failure to resist the pressure causes focal dilatation. There is an increase in permeability and plasmatic vasculosis; this is associated with histological evidence of smooth muscle cell necrosis resulting in fibrinoid necrosis. The proliferative endarteritis is the healing stage of many types of arterial damage, all of which have in common endothelial cell injury. The cellular intimal thickening is caused by migration of smooth muscle cells through fenestrae in the internal elastic lamina. The cells then orientate themselves circumferentially and secrete matrix proteins; the intima is initially oedematous and rich in proteoglycans, but later becomes collagenized.

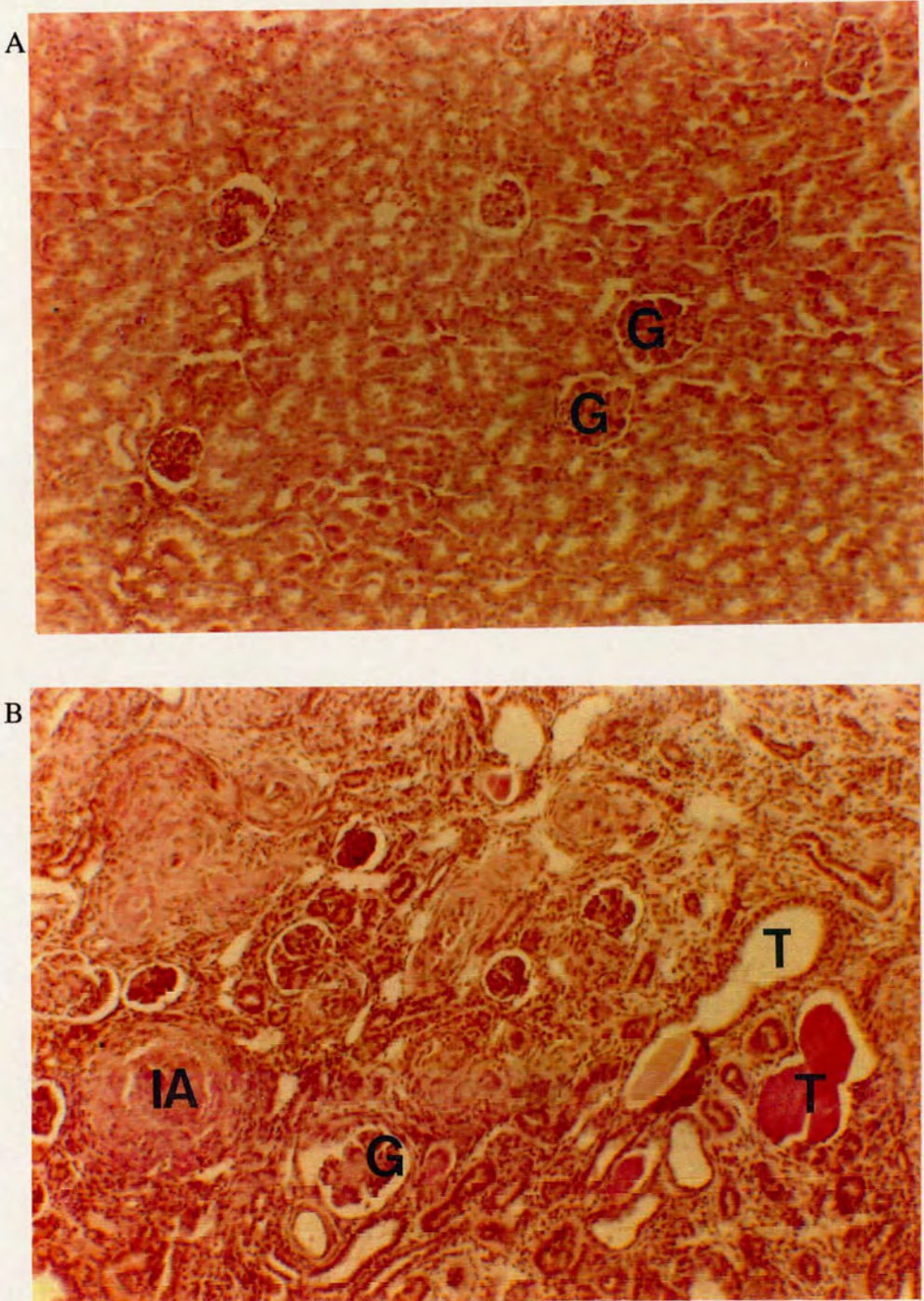


Figure 6.6

Histochemical analysis of renal sections ($3\mu\text{m}$ thick) from 6 week old (A) non-transgenic and (B) transgenic rats. G: Glomerulus, T: Tubule, IA: Interlobular Artery. Both panels are stained with eosin and haemalum. Panel (A) (x100), panel (B) (x400).

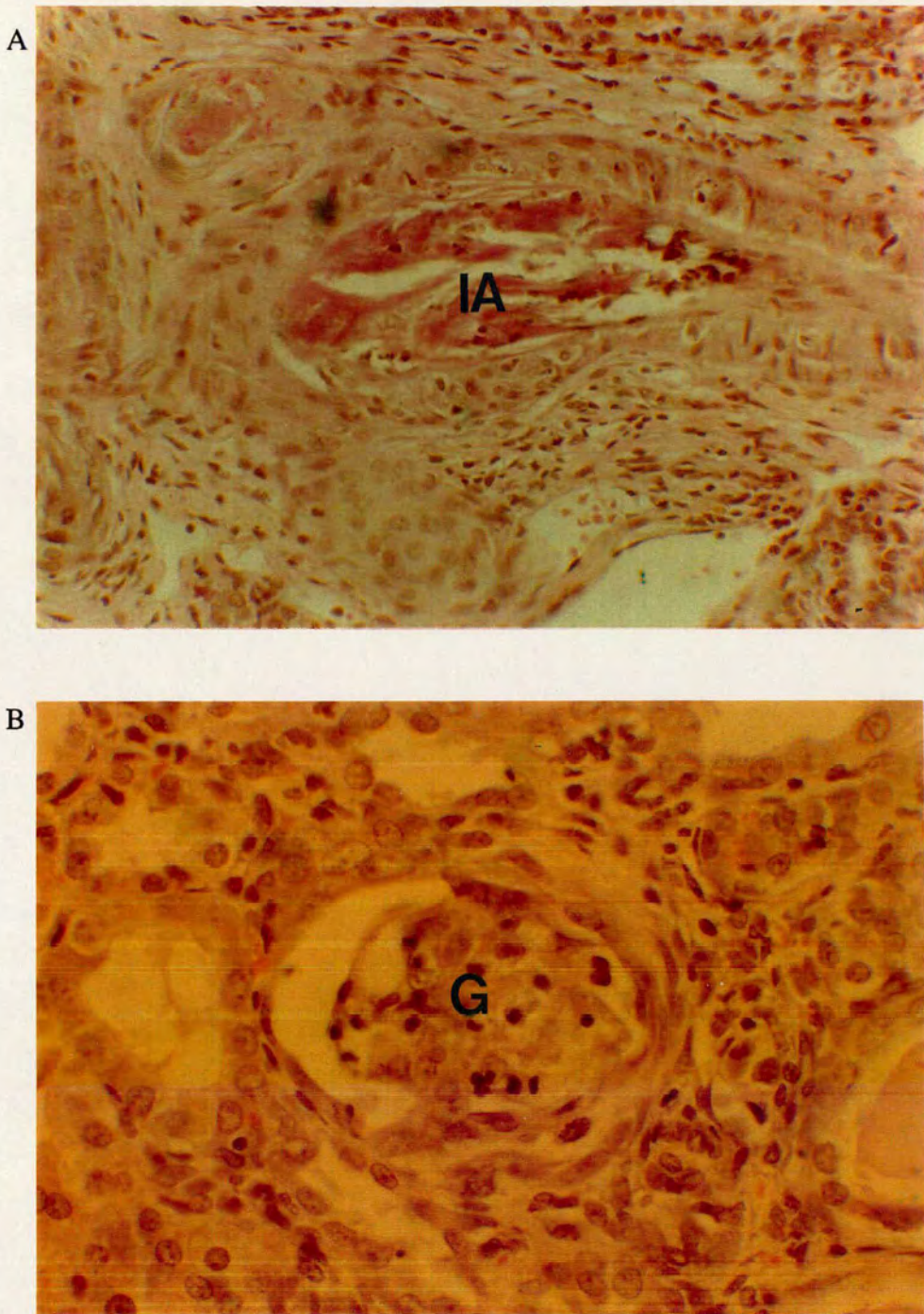


Figure 6.7

Histochemical analysis of renal sections (3 μ m thick) from 6 week old (A) Interlobular artery thickening (B) Cresential Damage to the Bowman's Capsule. IA: Interlobular Artery, G: Glomerulus. Both panels are stained with eosin and haemallum. Panel (A) (x100), panel (B) (x400).

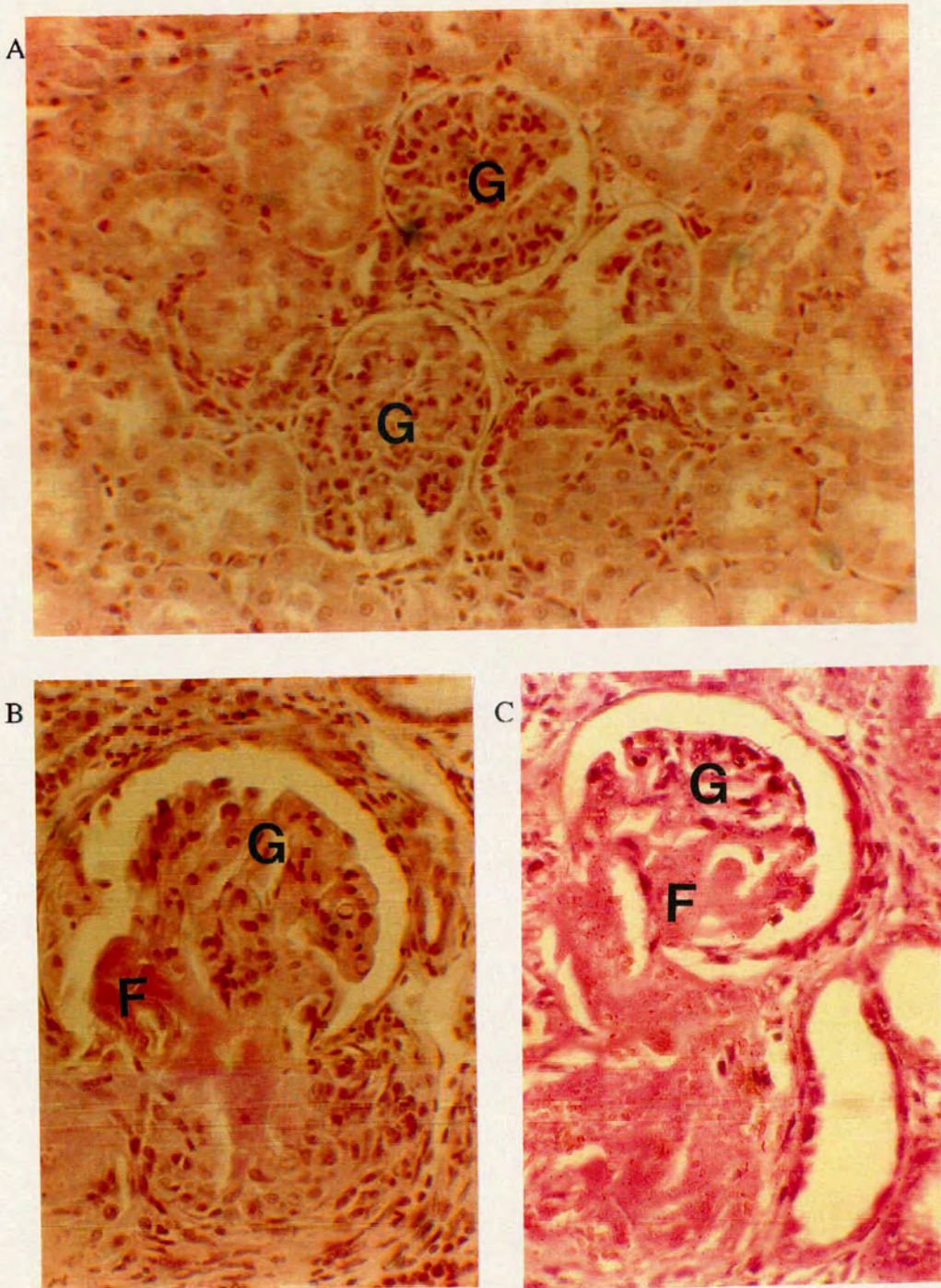


Figure 6.8

Histochemical analysis of renal sections ($3\mu\text{m}$ thick) from 6 week old (A) non-transgenic and (B & C) transgenic rats. Both panels are stained with eosin and haemallum. G: Glomerulus, FN: Fibrinoid Necrosis. Panel (A) (x100), panel (B & C) (x400).

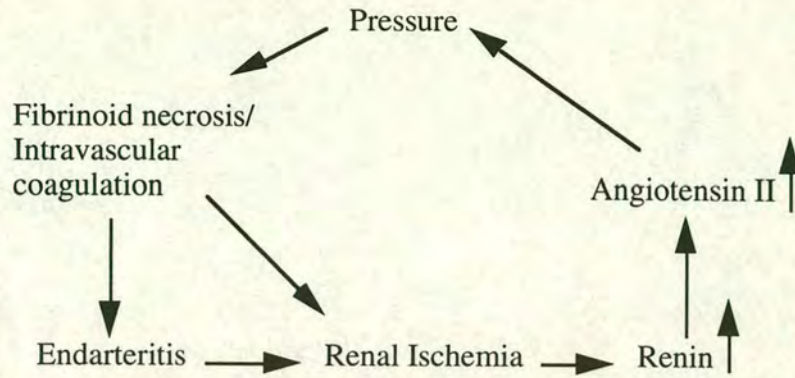


Figure 6.9 The circle of malignant hypertension. The vascular damage induced by hypertension causes renal ischaemia which leads to high plasma levels of vasoconstrictors such as angiotensin II, further increasing blood pressure.

As previously mentioned, there was no abnormal pathology in the transgenic line TG3. This was most likely due to the lack of expression of the transgene and as a consequence the normal blood pressure demonstrated in these animals. In the transgenic transgenic line TG2 there were lesions associated with essential and malignant phase hypertension. Despite the variation in severity of the lesions seen, in both male and female transgenic animals, all demonstrated pathology consistent with high blood pressure. In some cases only interlobular artery thickening or tubule dilatation was seen in a few instances, yet in others the renal ischemia was much more widespread and pronounced with a few examples of fibrinoid necrosis being recorded. The cardiac hypertrophy already measured by LV:BW ratio was first noted in the pathology of transgenic founder TG1. Target organ damage was also seen in the form of liver arteriolar necrosis.

The renal pathology seen in transgenic lines TG7 and TG12 was as severe as that demonstrated in transgenic line TG2. There was extensive ischemic damage, shrunken and damaged glomerular tufts in both male and female samples. The interlobular artery thickening and dilated tubules shown in line TG2 were also present in the TG7 and TG12 samples. Fibrinoid necrosis was present in a number of samples extending from the afferent arterioles into the glomeruli on a number of samples. As seen in samples from line TG2, the extent of renal damage varied from very few glomeruli damaged to very few glomeruli intact. Other target organ damage included occasional microscopic myocardial infarcts similar to those seen in the sub-population of TGR (mRen-2)²⁷. Again, as previously quantitated by echocardiography, one of the most prominent pathologies was severe cardiac hypertrophy. All pathology seen in the transgenic samples was consistent with either chronic or acute blood pressure effects.

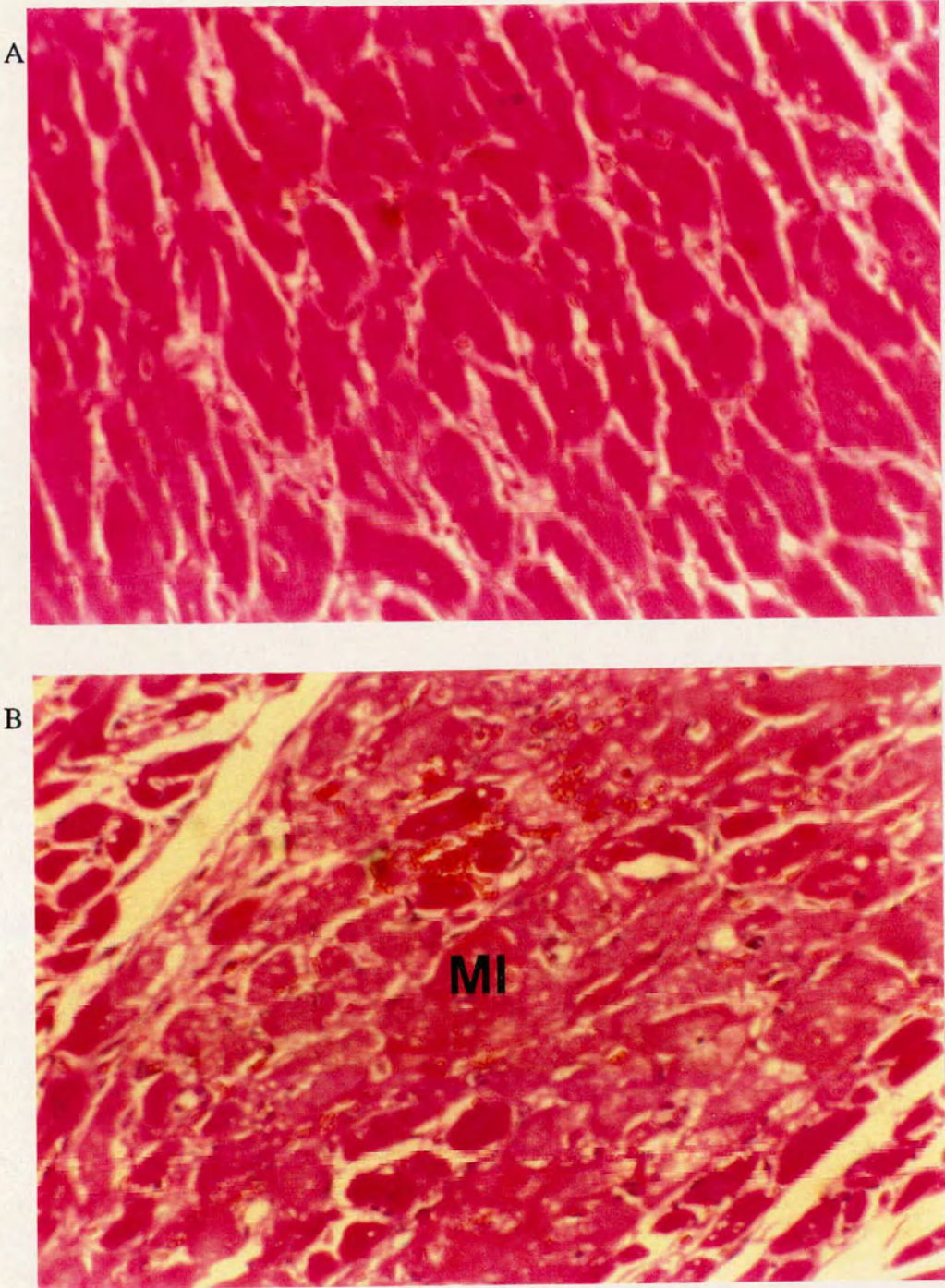


Figure 6.10 Histochemical analysis of cardiac sections ($3\mu\text{m}$ thick) from 6 week old (A) non-transgenic and (B) transgenic rats. MI: Microinfarct. Both panels are stained with eosin and haemalum. Panel (A) ($\times 100$), panel (B) ($\times 100$).

CHAPTER 7

Discussion

Despite essential hypertension remaining one of the main reasons for initiating long-term medical treatment a clear understanding of the causes of high BP in the majority of cases has not been reached. Transgenesis has facilitated the study of the contribution of individual genes in the models of hypertension, such as the TGR(mRen-2)²⁷ rat which resulted in a focus on the extra-renal RAS activity and the role of prorenin in hypertension. It is the role prorenin has in hypertension that was the focal point for the study undertaken. Refinement of the transgene used in the TGR(mRen-2)²⁷ rat meant the role extra-renal prorenin played in the hypertension seen in the TGR(mRen-2)²⁷ rat could be addressed directly.

The expression pattern of the α 1AT/Ren-2 transgene was similar to the expression pattern of the α 1AT/FIX shown by Jallat *et al* (Jallat *et al.* 1990). Despite the fact that the α 1AT promoter was of human origin, and the experiments performed by Jallat *et al* were in mice, there did not appear to be any species difference when used in transgenic rats. In the first series of microinjections three founder rats were produced and TG2 was shown to express the transgene, founder TG1 presumably expressed the transgene to caused its premature death and TG3 did not to express the transgene as highly as TG2. All the transgenic mice produced by Jallat *et al* contained an endogenous intron from the human FIX gene, with the exception of pTG4915 and the minigene constructs. The transgenes under the control of the α 1AT promoter but lacking any intronic sequence did not produce expressing transgenic mice.

The progeny from the founder mice containing the α 1AT-genomic FIX construct produced low litter numbers. This may have been a problem unique to the C57 BL6 x SJ2 hybrid female mice used. The litter numbers in the α 1AT/Ren-2 transgenic rat lines appeared normal. Despite low expression levels of the human FIX protein Jallat *et al* reported a personal communication from W. Dalemans which stated high level expression of a heterologous protein using the same α 1AT regulatory sequence.

Expression in the transgenic lines presented here appeared low, only detectable by poly A+ Northern blot hybridisation and RNase protection, yet the protein levels of

plasma prorenin were similar, or greater, than that seen in the TGR(mRen-2)27 rats. The genomic Ren-2 transgene expression, however, in the TGR(mRen-2)27 rats was detected by total RNA Northern blot hybridisation.

Measurement of renin is performed with a kinetic assay where it cleaves Ang I from angiotensinogen. Angiotensin I is subsequently measured by direct RIA. The concentration of renin is expressed as the hourly rate of AI generation. In this quantitation one molecule of renin can generate many molecules of AI. However, the lysosomal enzyme cathepsin D is capable of cleaving AI and AII *in vitro* (Hackenthal et al. 1978), and is released by cell death. Cathepsin D has a lower activity at the pH used for the renin assay (pH 6.0-7.4) but it is not completely inactive (Dorer et al. 1978).

The measurement of plasma renin activity is a convenient method for estimating the action of the renin system, however, its interlaboratory reproducibility is poor. Alternative methods exist such as direct immunoradiometry (Morganti et al. 1995) and HPLC of AI and AII. The methodology used in this thesis is prone to variation when measuring small changes in plasma RAS but if comparing large differences is valid.

As mentioned the plasma RAS parameters of the α 1AT/Ren-2 rats were similar to that reported in the TGR(mRen-2)27 rats, with the notable exception of the plasma prorenin levels in the transgenic line TG2. This Sprague-Dawley line showed plasma prorenin levels 4-5 times greater than those measured in the Fischer lines TG7 and TG12. As expression of the transgene at the RNA level appears to be relatively equivalent on the lines TG2, TG7 and TG12 the reason for the higher protein level in line TG2 is unclear. Despite the high plasma prorenin in all the α 1AT/Ren-2 lines, plasma renin was not significantly elevated. The percentage of transgene derived renin was 70->80% in all α 1AT/Ren-2 lines, however, transgene derived prorenin was in excess of 95%. These data suggest that there may be a form of feedback mechanism to explain the differences in the transgene derived pro and active renin. It also shows that the prorenin to renin "convertase" in rat can activate mouse Ren-2 prorenin. It would be very informative to determine whether blood pressure in transgenic rats correlated with transgene expression levels. Taking blood pressure measurements and RNA samples on a weekly basis, from a number of transgenic lines it would be possible, by RNase protection, to show any relationship between transgene expression and blood pressure.

As all rats in this study were anaesthetized before tail cuff plethysmography it has been suggested that the halothane may have affected the blood pressure readings. The anaesthetic was the minimum required to keep the animal still during the procedure (1-2% halothane in O₂). If it had been feasible, however, to train every rat to remain still,

in a Perspex cylinder the results would have been more physiologically relevant and possibly lowered the variation within groups.

The blood pressure increase seen in the α 1AT/Ren-2 rats was markedly different to the TGR(mRen-2)27 rat. The TGR(mRen-2)27 rat reached a plateau of systolic blood pressure of 210mmHg at ten weeks of age before entering an accelerated phase hypertension, whereas the α 1AT/Ren-2 rats reached a maximum blood pressure of 200mmHg by seven weeks of age. The plasma prorenin levels on the lines TG7 and TG12 were significantly elevated at four weeks of age and continued elevation to five weeks of age, whereas blood pressure continued to rise to seven weeks of age. This suggests there is no direct correlation between plasma prorenin and blood pressure. It was suggested by Lemmer *et al* (Lemmer et al. 1993) that there was an alteration to the circadian rhythm of blood pressure in the TGR(mRen-2)27 rat. In secondary forms of hypertension the normal pattern of circadian variation is frequently disturbed with nocturnal decrease in blood pressure failing to occur (Pickering 1990). Despite the short period of telemetric monitoring of the TGR(α 1AT/Ren-2) rats the periodicity in the blood pressures that is present does not appear to be disturbed. The telemetric analysis does reveal to what extent the blood pressure increase in the transgenic lines TG2 and TG7.

It has been suggested that as all transgenic founders were maintained on Captopril, female founders may have passed on a protective effect, caused by the ACE inhibitor. To answer this males from lines TG13 and TG14 could be bred with non transgenic females and a comparison made between blood pressure measurements taken from offspring derived from transgenic and non-transgenic mothers.

In humans obesity is possibly one of the best predictors for risk of development of hypertension. The Framingham Study showed data on the tracking of body weight and blood pressure (Gordon et al. 1976). It demonstrated the relationship between degree overweight and risk of hypertension also that an individual who was initially hypertensive had a greater chance of gaining weight over time than in normotensives. Rats fed a high fat diet, and allowed to overeat and become obese, also developed hyperinsulinaemia, elevated sympathetic activity and hypertension (Hwang et al. 1987).

This does not address the strong genetic disposition in human obesity. The Zucker strain represents an animal model that combines heredity with obesity, insulin resistance and hyperinsulinaemia (Kasiske et al. 1992). Kurtz *et al*, however, suggested only 50% of studies showed development of hypertension in Zucker obese rats (Kurtz et al. 1989). Despite elevated blood pressure at three weeks of age in lines TG2, TG7 and TG12 they showed no increase in body weight when compared to

normotensive controls. In fact all three transgenic lines demonstrated a significantly lower weight gain (TG2 $p < 0.05$, TG7 $p < 0.01$ and TG12 $p < 0.01$) than normotensive controls. There was no significant difference in food intake by the transgenic and non-transgenic animals. There may have been a difference in metabolic rate and certainly the transgenic animals displayed some polyuria in the late stages of the hypertensive phenotype, possibly contributing to the apparent difference in weights.

The immunohistochemical data presented showed DAB/H₂O₂ staining in only a few JG cells in non-transgenic and transgenic animals as is consistent with low endogenous renin production in the normotensive situation. If hypertension occurs gradually endogenous renin production decreases. If, in the case of malignant hypertension, severe occlusion of the interlobular arteries occurs in can produce local areas of hypotension in the kidney and endogenous renin production increases as a result of the baroreflex response.

Despite pathological evidence that some transgenic samples demonstrated malignant hypertension (fibrinoid necrosis in the afferent arteriole) this was not shown immunohistochemically. This could be due to the extent of renal damage already present with upwards of 75% of glomeruli damaged in some cases, obscuring any response. It could be that serial sections through a kidney with pathological evidence of malignant hypertension would be necessary to be certain of observing this phenomena. The immunohistochemical staining seen in the small arteries of the heart in transgenic samples from the line TG7 may provide a link between the hypertrophy and hypertension seen. If, as suggested by von Lutterori, renin is the only constituent of the RAS not synthesised in cardiac tissue this may be evidence of uptake of pro/active renin from the circulation. Without *in situ* data it is impossible to know whether this is uptake or *de novo* synthesis of prorenin. If either case is true AII could be formed locally in the heart and could therefore lead to hypertrophy before hypertension.

Certainly the hypertrophy, as measured by echocardiography, seems to be present in both TG7 and TG12 transgenic lines before the hypertension is fully established. The heart:body weight data from, two and three week old TG7 and TG12 transgenic males also supports the finding that hypertrophy may be established before the onset of elevated blood pressure. Although hypertrophy is often thought of as an adaptive process in response to high blood pressure it may be in this case that the abnormal levels of prorenin in the plasma can lead to locally generated cardiac AII. This AII could then cause a hypertrophic response that consequently would have an effect on blood pressure. It would be possible with the use of AT1 receptor antagonists, eg. Mibefridil to determine whether or not the hypertrophic response in

this case was mediated through AII. By administering enalapril at doses that lower blood pressure it would be possible to ascertain if the hypertrophy was purely a result of high blood pressure.

The renal lesions seen in all transgenic lines are consistent with high blood pressure effects either chronic or acute. There is a hypertrophic thickening of the interlobular arteries, damage to glomerular tufts and crescentic thickening seen in some Bowman's capsules. As with the transgenic line TGR(mRen-2)27, in certain cases fibrinoid necrosis was seen in the afferent arterioles as an indication of malignant hypertension in the α 1AT/Ren-2 lines. However, despite malignant phase hypertension having been demonstrated with a specific genetic cross of Sprague Dawley, TGR(mRen-2)27 rats, fibrinoid necrosis was seen in both Sprague Dawley and Fischer backgrounds for the α 1AT/Ren-2 lines. This suggests that the malignant phase hypertension seen in the α 1AT/Ren-2 lines is independent of genetic background.

The genetic background of individual α 1AT/Ren-2 transgenic lines does not appear to have any effect on the phenotype seen. The TGR(mRen-2)27 rats were generated on an outbred Sprague-Dawley background as were TG1, TG2 and TG3 presented here. As the Sprague-Dawley strain was outbred there were some genetic differences between individual animals. The TG4-TG16 animals were generated on an inbred Fischer/F344 background making it possible to perform organ transplants and more classical genetic experiments on these lines.

The hypertension seen in the α 1AT/Ren-2 transgenic animals was most likely a result of activation of the high plasma prorenin. The evidence for this is the fact that the majority of plasma active renin in the transgenic animals was derived from transgene expression. As the transgene expressed prorenin in the liver, any transgene-derived plasma renin must be the result of activation of this prorenin in organ or organs unknown.

Investigation of the specific role elevated plasma prorenin has in these transgenic animals could employ site directed mutagenesis to look at uncleavable prorenin. Alteration of the cleavage site of prorenin may determine the physiological role of prorenin. Similarly alteration of the glycosylation sites in the Ren-2d molecule may produce evidence as to the role of glycosylation in renin proteins.

Vertebrate aspartyl proteases, such as chymosin, pepsin and renin are synthesised as inactive zymogens and are then converted to active enzymes by limited proteolysis. This enzymatic activity is expressed by the exposure of the zymogen to acidic conditions. This activation is a reversible, unimolecular reaction (Leckie et al.

1980), suggesting a conformational change leading to an opening of the substrate binding cleft as a result of acidification.

Site-directed mutagenesis (SDM) has been used to investigate the role positively charged residues (Arg 10, Arg 15, Arg 20) in the prosegment play in acid activation [Yamauchi, J Biochem 1990]. When these residues were changed to noncharged residues the prorenin mutants were active without acid treatment. The hypothesis that was proposed was that the positively charged residues stabilise the inactive prorenin structure through electrostatic interaction between the prosegment and the active enzyme.

In order to determine the physiological role uncleaved prorenin has, if any, in the $\alpha 1$ anti-trypsin/Ren-2 transgenic animals it seems reasonable to alter the amino acid sequence at the pro/renin cleavage site in such a way that it would no longer be activatable to renin by any endogenous enzyme (see figure 7.1 for possible strategies). Possible strategies to mutate the pro/renin cleavage site may include substitution of the Lys-61, Arg-62 recognition site with Ala-Ala which would probably provide little chance of cleavage. However, there would be an alteration to the local charge ratio around these residues which could alter the structure of the cleavage site. Another possibility if the local charge difference caused by the Ala-Ala mutation proved to alter the structure of the protein is to produce a more conservative mutation such as the Lys Arg site mutated to Lys Ser.

Other possibilities for extending the transgenic project could include a chimeric gene using segments from the mouse Ren-2d cDNA fused with sequence from rat renin cDNA, making a complete renin cDNA. It may then be possible to identify the functional unit(s) of the mouse Ren-2d cDNA that cause a rise in blood pressure, when rat renin cDNA in transgenic rats does not. Also useful would be a tissue specific, inducible promoter. This would allow an increase in plasma prorenin at any given time by the addition of an inducing agent. It may also allow study of the effects prorenin has on individual organs as in the case of cardiac hypertrophy. It may also be possible to titrate the level of plasma prorenin required for a given rise in blood pressure, if the link between prorenin and blood pressure in these animals is a direct one.

The aim of the project was to elucidate the role that circulating prorenin has in the TGR(mRen-2)²⁷ rat. To that end these transgenic lines show that prorenin, irrespective of the site of origin, can produce a hypertensive phenotype. They also raise a number of important questions as to the role prorenin, and tissue RASs play in the phenotype. If the immunohistochemical staining has a relevance then there may be transport mechanisms seen in the renal tubules and small cardiac arteries. Prorenin as a physiological significant molecule is a contentious subject yet there is no evidence here

that any other constituent of the RAS is significantly altered. The results demonstrate that in the α 1AT/Ren-2 rat extra-renal expression of Ren-2 prorenin, directly or indirectly, results in hypertension with extreme cardiac hypertrophy. This model also further refines our understanding of the basis of hypertension in the classic TGR(mRen-2)-27 rat.

Appendix 1

Media used in transgenic generation

Table 2.2 Components of M2 and M16 Media

| Compound | M2 | | M16 | |
|--|-------|--------------------|-------|--------------------|
| | mM | / gl ⁻¹ | mM | / gl ⁻¹ |
| NaCl | 94.66 | | 94.66 | |
| KCl | 4.78 | | 4.78 | |
| CaCl ₂ .2H ₂ O | 1.71 | | 1.71 | |
| KH ₂ PO ₄ | 1.19 | | 1.19 | |
| MgSO ₄ .7H ₂ O | 1.19 | | 1.19 | |
| NaHCO ₃ | 4.15 | | 25.00 | |
| HEPES | 20.85 | | — | |
| Sodium Lactate | 23.28 | | 23.28 | |
| Sodium Pyruvate | 0.33 | | 0.33 | |
| Glucose | 5.56 | | 5.56 | |
| BSA | | 4.0 | | 4.0 |
| Penicillin G, K Salt (Final Conc., 100U/ml) | | 0.06 | | 0.06 |
| Streptomycin sulphate (final conc., 50 mg/ml) | | 0.05 | | 0.05 |
| Phenol Red | | 0.01 | | 0.01 |
| ddH ₂ O | | to 1l | | to 1l |

Adapted from (Hogan et al. 1986).

The above media are used in the microinjection procedure (see Chapter 1.3).

Appendix 2

Tabulated Data

Table 5.2

| Week | TG2 | TG3 | TG2 & TG3 Control | TG7 | TG12 | TG7 & TG12 Control |
|------|-----------------|-----------------|----------------------|-----------------|----------------|--------------------------|
| 4 | 71.2 ± 7.8 | 82.0 ± 4.1 | 85.4 ± 4.6 | 73.5 ± 5.7 | 84.7 ± 4.9 | 85.6 ± 6.3 |
| 5 | 103.7 ± 8.6 | 123.9 ± 13.6 | 132.7 ± 9.3 | 86.9 ± 9.6 | 111.8 ± 3.3 | 128.3 ± 9.0 |
| 6 | 118.3 ± 14.4 | 131.7 ± 15.6 | 137.1 ± 11.1 | 89.8 ± 4.1 | 117.4 ± 8.4 | 138.5 ± 9.2 |
| 7 | 120.1 ± 11.5 | 138.9 ± 12.4 | 143.0 ± 13.5 | 100.3 ± 10.5 | 122.1 ± 9.2 | 148.2 ± 10.6 |

Tabulated results of weight gain (in grammes) in TGR(α 1AT/Ren-2) rats. See figures 5.1B and 5.2B for graphs.

Table 5.3

| Week | TG2 | TG3 | Treated TG2 | TG2 & TG3 Controls | TG7 | TG12 | TG7 & TG12 Controls |
|------|-----|-----|----------------|--------------------------|-----|------|---------------------------|
| 4 | 90 | 100 | 100 | 100 | 90 | 100 | 100 |
| 5 | 80 | 100 | 100 | 100 | 80 | 90 | 100 |
| 6 | 40 | 100 | 100 | 100 | 40 | 50 | 100 |
| 7 | 10 | 90 | 100 | 100 | 20 | 10 | 100 |

Survival rates of TGR(α 1AT/Ren-2) rats given as a percentage, see figures 5.1A and 5.2A for graphs. There were ten animals in each group.

Table 5.4

| Week | TG2 | | | TG7 | | | TG12 | | | Non-transgenic Controls | | |
|------|-----|-----|-------------|-----|-----|-------------|------|-----|-------------|-------------------------|-----|-------------|
| 4 | 122 | 137 | | 128 | 134 | | 134 | 121 | | 88 | 91 | |
| | 129 | 124 | | 124 | 129 | | 116 | 130 | | 90 | 85 | |
| | 127 | 121 | | 127 | 133 | | 119 | 125 | | 86 | 86 | |
| | 135 | 127 | x= 128.3 | 131 | 125 | x= 129.0 | 128 | 129 | x= 125.9 | 87 | 88 | x= 87.5 |
| | 131 | 130 | SD= 5.2 | 130 | 129 | SD= 3.2 | 126 | 131 | SD= 5.7 | 87 | 87 | SD= 1.8 |
| 5 | 148 | 149 | | 141 | 151 | | 195 | 165 | | 90 | 96 | |
| | 140 | 136 | | 158 | 149 | | 168 | 176 | | 92 | 94 | |
| | 137 | 143 | | 162 | 147 | | 159 | 192 | | 95 | 94 | |
| | 145 | 140 | x= 143.1 | 153 | 150 | x= 152.1 | 167 | 171 | x= 174.8 | 91 | 93 | x= 93.3 |
| | 149 | 146 | SD= 4.7 | 155 | 156 | SD= 5.9 | 180 | 175 | SD= 11.5 | 95 | 93 | SD= 1.9 |
| 6 | 168 | 171 | | 166 | 168 | | 185 | 180 | | 98 | 106 | |
| | 166 | 171 | | 162 | 164 | | 172 | 176 | | 102 | 105 | |
| | 177 | 170 | | 172 | 169 | | 173 | 179 | | 101 | 99 | |
| | 179 | 167 | x= 171.6 | 175 | 165 | x= 167.6 | 172 | 182 | x= 178.2 | 104 | 102 | x= 101.9 |
| | 181 | 166 | SD= 5.5 | 168 | 167 | SD= 3.8 | 184 | 179 | SD= 4.5 | 100 | 102 | SD= 2.6 |
| 7 | 177 | 202 | | 168 | 184 | | 166 | 165 | | 107 | 100 | |
| | 189 | 181 | | 192 | 172 | | 169 | 165 | | 108 | 102 | |
| | 194 | 176 | | 185 | 177 | | 172 | 173 | | 108 | 104 | |
| | 182 | 182 | x= 183.7 | 171 | 168 | x= 176.5 | 169 | 176 | x= 170.4 | 110 | 105 | x= 105.1 |
| | 173 | 187 | SD= 8.9 | 176 | 172 | SD= 8.1 | 177 | 173 | SD= 4.5 | 104 | 103 | SD= 3.1 |

Tabulated results (in mmHg) from four week tail cuff plethysmography studies. Each individual value is the result of five systolic blood pressure measurements. See figure 5.3 for graphical representation of these results.

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