Disruption of the *Ren-1^d* Gene

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I declare that this thesis was compiled by myself and that all this work is my own, except where otherwise stated.

Allan Clark

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Abbreviations

Standard abbreviations and symbols recommended by the IUPAC-IUB Commission on Biochemical Nomenclature have generally been used. Standard abbreviations for nucleotides and three-letter abbreviations for amino acids were used throughout the text.

Most non-standard abbreviations used are described in full in brackets after their first use in the text. Exceptions to this are listed below.

ATP	adenosine-5'-triphosphate
bp	base pair(s)
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
cpm	counts per minute
ddNTP	2',3'-dideoxynucleoside-5'-triphosphate
	N=A: adenosine
	N=C: cytidine
	N=G: guanosine
	N=T: thymidine
dH_2O	distilled water
DIA	differentiation inhibiting activity
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-dideoxynucleoside-5'-triphosphate
	N=A: adenosine
	N=C: cytidine
	N=G: guanosine
	N=T: thymidine
DTT	1,4-dithiothreitol
E	embryonic day
EDTA	ethylenediaminotetraacetic acid
g	gram
G418	gentamycin
GMEM	Glasgow modified eagle medium
kb	kilobase

kD	kilodalton
LIF	Leukaemia inhibitory factor
m	metre
M	molar (moles/litre)
mol	mole
mRNA	messenger ribonucleic acid
neo	*Escherichia coli* neomycin phosphotransferase II gene
PCR	polymerase chain reaction
PEG	polyethylene glycol
RNA	ribonucleic acid
RNase	ribonuclease
ROP H_2O	reverse osmosis purity water
rpm	revolutions per minute
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
Tris	tris (hydroxymethyl) aminomethane
UHP H_2O	ultra high purity water
UV	ultraviolet
v	volume
w	weight
x*g*	times gravitational force

Standard prefixes used were

c	centi (10^{-2})
m	milli (10^{-3})
μ	micro (10^{-6})
n	nano (10^{-9})
p	pico (10^{-12})
f	fento (10^{-15})

Abstract

The secretion of renin from granules stored in renal juxtaglomerular (JG) cells plays a key role in blood pressure homeostasis. The synthesis of renin and the extent of granulation is regulated by several mechanisms including signaling from the macula densa, neuronal input and blood pressure. Physiologically, JG cells are the most important sites of renin expression since they are the only cells known to convert prorenin to the active enzyme, renin, and to secrete it into the plasma in large amounts. Present data indicate that most mammals possess a single renin gene, however in most strains of mice there exists an additional gene, *Ren-2*, encoding a highly homologous but physically distinct enzyme Renin-2. The two genes have different but often overlapping expression patterns, with both being expressed at equal levels in the JG cells of the kidney (mRNA level). A major difference between the two enzymes is their capacity for *N*-linked glycosylation, Renin-1^d being glycosylated at one or more of its three potential glycosylation sites, whereas Renin-2 is not glycosylated, lacking any *N*-linked glycosylation consensus sequences.

To facilitate studies of the physiological significance of the two renin genes in mice, we have disrupted the *Ren-1^d* gene by gene targeting, leaving Renin-2 as the only functional renin isozyme capable of participating in the renin-angiotensin system. The targeting construct used to disrupt the *Ren-1^d* gene was assembled using homology arms of 3.7 and 3.6kb generated by PCR. *Ren-1^d -/-* animals are viable, display no gross, visible abnormalities and express *Ren-2* as the only renin mRNA. The kidneys of all adult homozygous mutant animals display altered morphology of the macula densa and complete absence of JG cell granulation. Blood pressure homeostasis in these animals displays a sexual dimorphism, with female, but not male, *Ren-1^d -/-* animals showing a reduced blood pressure. These results prove that Renin-1^d and Renin-2 are not functionally equivalent enzymes. *Ren-1^d* being required for normal macula densa cell morphology, granulation of JG cells and the maintenance of normal blood pressure in female *Ren-1^d -/-* animals.

CHAPTER 1

Introduction

1.1 The Renin-Angiotensin System

The renin-angiotensin system (RAS) is one of the most studied biochemical pathways involved in blood pressure regulation (Figure 1.1). Perturbations in the RAS have been seen in many human conditions including hypertension, cardiac hypertrophy and vascular pathologies associated with diabetes and renal disease (1-4). The first step in this pathway is the conversion of angiotensinogen to angiotensin I (Ang I) by renin, the activity of which is rate limiting in humans (5). In contrast, it is the substrate angiotensinogen which is the limiting factor in the mouse (6). Angiotensin I is re-cleaved by angiotensin I converting enzyme, (ACE) to produce the active octapeptide, angiotensin II (Ang II), a peptide hormone which exerts its effects in a receptor-mediated manner. Two pharmacologically distinct types of Ang II receptors have been identified so far - AT_1 and AT_2. There are two subtypes of AT_1 receptor -AT_{1A} and AT_{1B} - which have wide tissue distribution of expression and are derived from different, although highly homologous genes (7-10). It is believed that Ang II mediates blood pressure via the AT_1 receptors (11-13). The function and significance of the AT_2 receptor type in blood pressure regulation is as yet unknown (13-15). Included in Ang II function are direct stimulation of vasoconstriction and increasing secretion of aldosterone from the adrenal gland causing an increase renal sodium reabsorption (16-18).

1.1.1 Evidence for the Renin Angiotensin System In Blood Pressure Regulation

Evidence for the RAS's implication in blood pressure dysregulation comes from linkage studies in rats and humans (Table 1.2). Polymorphisms in or linked to renin, angiotensin I converting enzyme and angiotensin receptors have all been associated with hypertension in rat models of hypertension. In contrast, the only constituent of the RAS which consistently

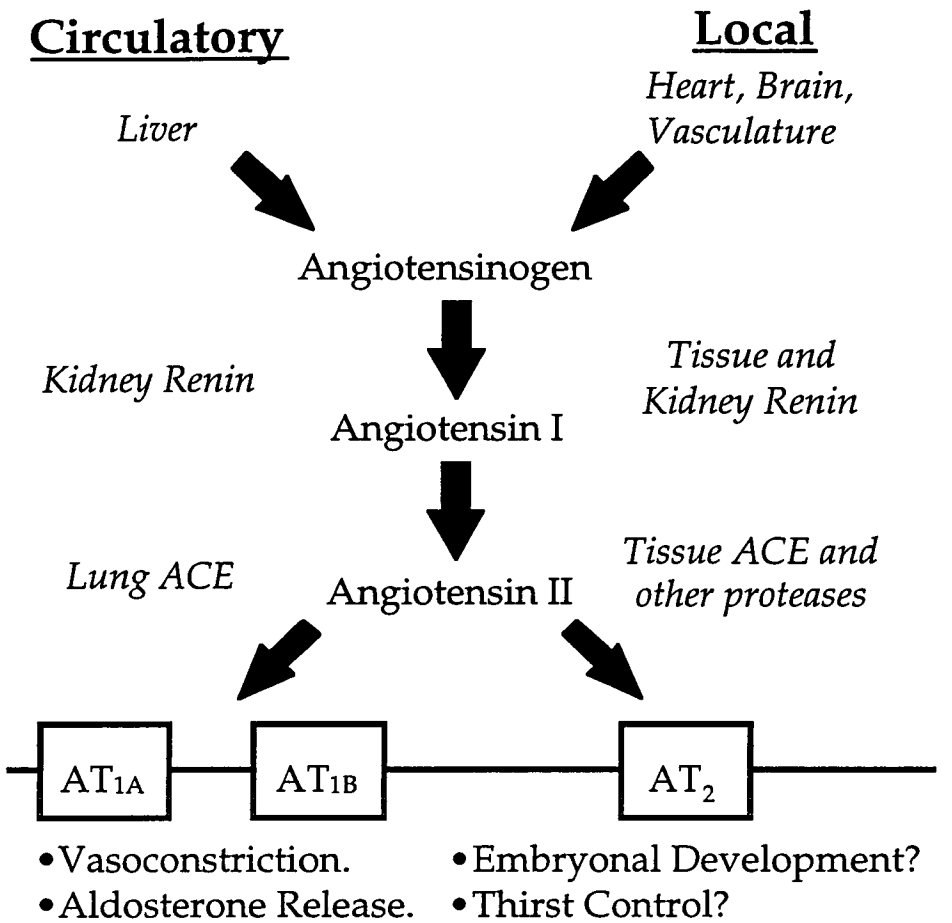


Figure 1.1: Key elements of the renin-angiotensin system: Angiotensinogen is converted to angiotensin I by renin followed by cleavage to produce the active octapeptide angiotensin II (Ang II) by the enzyme angiotesin I converting enzyme (ACE). Ang II then exerts its range of physiological effects via the Ang II receptors.

shows linkage with hypertension in humans is angiotensinogen. No linkage has been found in humans between hypertension and renin or angiotensin receptors. In some cases, but not others, a polymorphism associated with the angiotensin I converting enzyme is associated with hypertensive phenotypes (Table 1.2).

The results of several transgenic animal studies have implicated renin and the other constituents of the RAS in the regulation of blood pressure (discussed in section 1.3). Other evidence comes from work involving angiotensin II, the active octapeptide. It has been shown that infusion of Ang II can increase blood pressure in rats (55), whereas infusion of Ang II antibodies causes a decrease in blood pressure (56). Renin has also been directly implicated in blood pressure in a series of experiments using renin

Gene	Linkage in Rats	Linkage In Humans	No Linkage In Humans
Angiotensinogen		(19-28)	(29-32)
Renin	(33, 34)		(35-41)
ACE	(42-45)	(46, 47)	(41, 48-51)
Ang II Receptors	(52, 53)		(54)

Table 1.2: Linkage studies of RAS in humans and inbred rats: The publications listed have shown linkage between hypertensive phenotypes and constituents of the RAS in rats or humans. Negative results are also listed.

antibodies. Upon introduction of renin antibodies into salt depleted dogs (57, 58), marmosets (59), Goldblatt hypertensive dogs (58, 60) and spontaneously hypertensive rats (61), a reduction in arterial blood pressure was observed.

1.1.2 Localised RAS Activity

The traditional view of the RAS is of a biochemical reaction occurring in the circulation. Angiotensinogen is produced in the liver and secreted into the plasma where it is cleaved by active renin derived from the kidney. The product, Ang I, is then cleaved to produce Ang II by the membrane-bound angiotensin I converting enzyme present in the lung and other vascular beds. This view is now being challenged because of the mounting evidence which suggests the presence of local RAS's operating in a paracrine fashion independent of circulating enzyme or substrate levels (62-66). Renin, angiotensinogen and ACE expression have all been detected in the heart, adrenal, testis and brain (65, 67-69). There is even evidence of a local RAS in the kidney, possibly acting to stabilise the glomerular filtration rate by regulating the renal vascular tone (69, 70).

1.2 The Molecular Biology of Mouse Renin

1.2.1 Organisation of the Renin Genes in Mice

Inbred strains of mice can be split into two groups-those that have only one renin gene ("one-renin gene" mice), termed *Ren-1^c* (*e.g.* C57BL/6J) or those strains of mice where there are two renin genes present ("two-renin gene" mice), termed *Ren-1^d* and *Ren-2* (*e.g.* DBA/2J and 129/Ola; see Figure 1.3) (71-79). All wild *Mus Musculus* subspecies and many, but not all

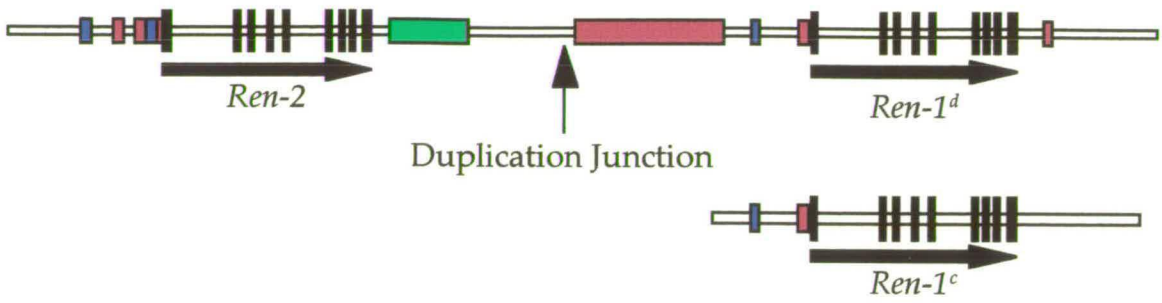


Figure 1.3: Structure of the three renin genes showing introns and exons (black boxes) with the insertions found in each gene also shown: The *Ren-1c* gene contains two insertions, a B1 repetitive element (blue boxes) approximately 2kb upstream of the first exon and an M3 element (red boxes) 80bp upstream of the transcription start site (both of these insertions are common to all three genes). Four insertion elements are present in the *Ren-1d* locus, these being a 7kb insertion so called M1 element approximately 3.8kb upstream of the *Ren-1d* gene, an M4 element 1.5kb downstream of the *Ren-1d* locus and M3 and B1 elements (see *Ren-1c* for details). The *Ren-2* locus contains several insertions - one in the 3' flanking region, a 3.0kb proviral intracisternal A particle (green box), 0.9kb downstream of exon 9 and four insertions in the 5' promoter sequence. The 5' insertions are an M2 insertion approximately 0.7kb upstream of exon 1, M3 and B1 elements (see *Ren-1c* for details) and a B2 element (mouse homologue of a human Alu-2 sequence) 300bp upstream of exon 1 (inserted within the M3 element).

of the other species in the *Mus* genus also contain two renin genes (79). *Ren-1d* and *Ren-2* are tightly linked and are thought to be the result of a duplication of a 21kb fragment from a *Ren-1c*-like ancestral locus (80, 81). This duplication event is believed to have occurred between 2.5 and 7 million years ago (79, 82).

Two repetitive insertion sequences are found in the 5' promoter regions of the renin genes, these being a B1 element, present in all mouse, rat and human genes and a B2 element, only found in the mouse *Ren-2* gene. Since the duplication event, several insertions have occurred in the *Ren-1c*, *Ren-1d* and *Ren-2* loci. These include a partial intracisternal A particle 1kb downstream of the *Ren-2* gene (83) and four insertions specific to the mouse genome (M1-M4) (75, 81, 84-87). All three renin genes share the same overall genomic organisation (nine exons and eight introns) with all intron/exon boundaries also conserved (80, 81, 88-90). The nucleotide (or amino acid) sequences of all three cDNAs (or proteins) have been determined (77, 88, 89, 91-97) and a comparison shows that all three coding regions are highly conserved (98.5% identity between *Ren-1c* and *Ren-1d* and 95.8% between *Ren-1d* and *Ren-2*). At the amino acid level *Ren-1c* and *Ren-1d* show a 98.5% similarity and *Ren-1d* and *Ren-2* show a 93.3% similarity.

1.2.2 Mouse Renin Proteins

Renin is a glycosylated peptide that belongs to the aspartyl protease family. Unlike other members of this family (*e.g.* pepsin, chymosin and lysosomal cathepsins) renin shows a high substrate specificity (the only known substrate is angiotensinogen) and is active at neutral pH (98-101). One important difference between *Ren-1^c/Ren-1^d* and *Ren-2* is that *Ren-2* has no potential sites for *N*-linked glycosylation, whereas *Ren-1^c* and *Ren-1^d* can be glycosylated in any of three asparagine positions (88, 93, 102). The proteins also display different thermostabilities, Renin-1^c (and presumably Renin-1^d) being relatively stable at 60°C compared to the thermolabile Renin-2 protein (73).

Many groups have concentrated their research on the translational processing of renin (92, 93, 95, 103-112). Mouse renin is synthesised as preprorenin (45kD, sizes refer to mouse *Ren-2*) with the leader sequence (pre segment) being rapidly hydrolysed by signal peptidase during processing in the rough endoplasmic reticulum where any glycosylation also occurs (112, 113). Sizes given are for the Renin-2 protein, and although the Renin-1^d protein differs in length by only one amino acid, it typically migrates on protein gels at a much larger size because of it's glycosylation (93, 95, 96, 102).

In the juxtaglomerular (JG) cells and submandibular gland of the mouse, prorenin (43kD) is then packaged into granules where it undergoes a second cleavage step to produce active renin. This involves removal of the first 45 amino acid pro segment (92, 95). In the kidney the endogenous maturase enzyme(s) which convert prorenin to active renin (38kD) is unknown, but is most likely to be cathepsin B (114-118). In the submandibular gland the prorenin processing enzyme has been identified as epidermal growth factor-binding protein type B (mouse kallikrein gene 13). Interestingly though, this enzyme cannot process the Renin-1^d protein, and is not the processing enzyme in other tissues (119, 120).

Activation, when it occurs, is rapid and is followed by a slower additional hydrolysis step between amino acids 290 and 291 (positions given are for active renin) to give a heavy and light chain (33 and 5kD respectively) (93, 93, 95, 107-109). The heavy and light chains generated are held together

by a disulphide bridge between cysteines at positions 320 and 357 (92, 93, 95, 106). Figure 1.4 shows the maturation pathway of mouse *Ren-2* derived renin.

The structure of mouse and human renin has been inferred by homology to other aspartyl protease family members (88, 121-125) and has also been determined by X-ray crystallography (126-128). These studies show that renin is a bilobular molecule with an active site containing two aspartate residues (Asp104 and Asp 292 in human prorenin) required for catalytic activity (125-129). X-ray crystallography also shows that prorenin is maintained in an inactive state because the pro segment occupies the active site in the substrate binding cleft preventing the cleavage of angiotensinogen (125). A similar mechanism is responsible for the inhibition of renin by

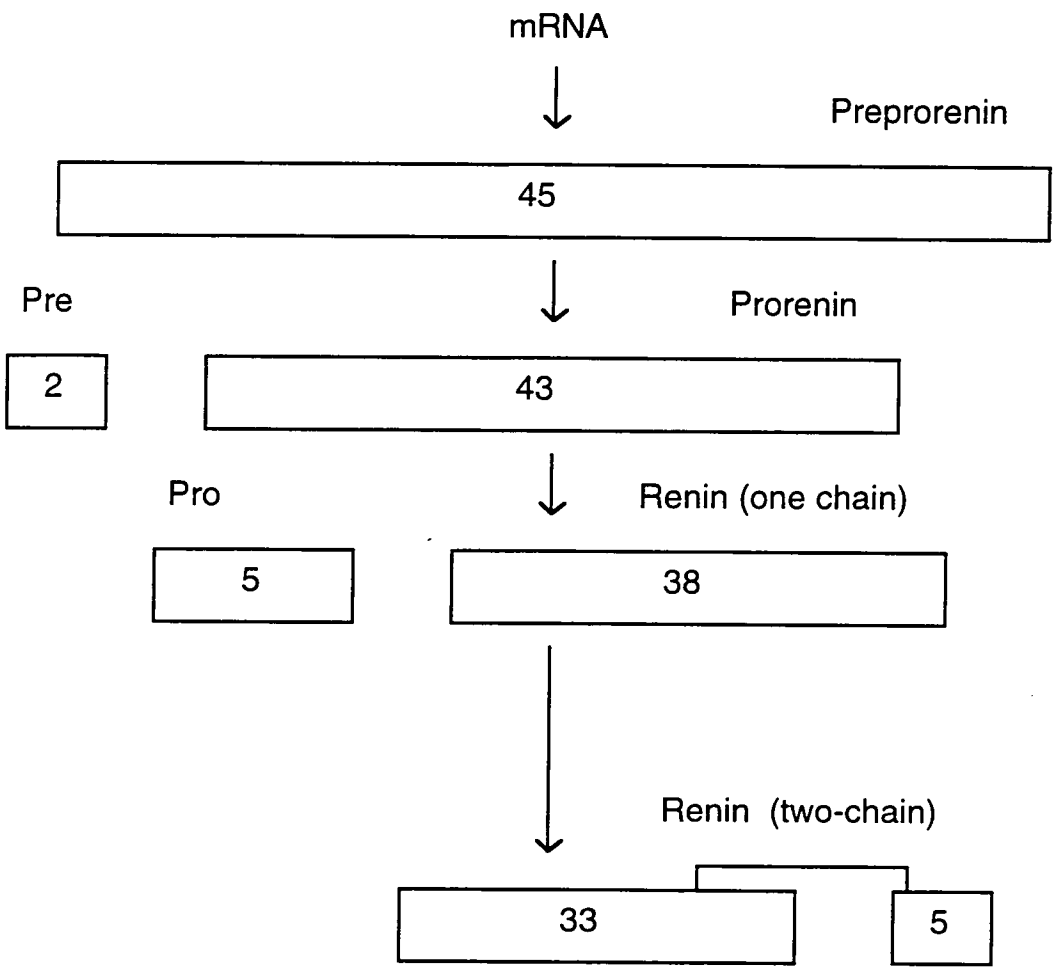


Figure 1.4: Structure and processing of preprorenin: The processing of preprorenin to active renin shown is based on the sizes (kD) observed for the nonglycosylated Renin-2 protein.

various renin-inhibitors (127, 128). X-ray crystalography also shows that the prorenin structure has larger loops adjacent to the active site and substrate binding cleft and this is proposed, at least in part, to be responsible for the substrate specificity of renin (126-128).

1.2.3 Expression Patterns

1.2.3.1 General Expression Patterns

The primary sites of renin synthesis are the JG cells of the kidney (Figure 1.5), although other sites of renin expression have been identified. Historically, the kidneys of "two-renin gene" mice were believed to express only *Ren-1^d*, based on the failure to isolate *Ren-2* cDNAs from kidney RNA (77, 96) or to detect the protein using Renin-2-derived antibodies (73). However, primer extension assays have been used to show that both genes are expressed at roughly equivalent levels in the kidneys of "two-renin gene" mice (130, 131).

Extrarenal tissues known to express renin (detected by RNA blot and ribonuclease protection) are the testis, anterior prostate, ovaries, heart, adrenal gland, and in mice, the submandibular gland (SMG) (16, 132). In addition, by using Reverse Transcriptase-PCR (RT-PCR) it has also been possible to detect renin mRNA expression in the liver, spleen, thymus, lung, prostate, hypothalamus and whole brain (133).

1.2.3.2 Expression of Renin in the Kidney

In the kidney, renin expression is restricted to the JG cells (Figure 1.5), which are modified smooth muscle cells of the afferent arteriole containing dense secretory vesicles (modified lysosomal granules) in which renin is matured by removal of the pro segment to produce active renin (110, 113, 135). Active renin is then released from these granules by exocytosis in response to physiological stimuli, a mechanism termed regulated secretion (112). The kidney, however, secretes both active renin and prorenin, the latter believed to be released through another route, namely the constitutive secretion pathway, by which prorenin is secreted continuously, without storage (112). One important difference between the two pathways is the stage at which they can be regulated. Constitutive secretion can only be controlled at the transcriptional level, whereas regulated secretion can also

be controlled postranslationally (reviewed in King (136)). Prorenin is generally thought of as inactive and is activated by removal of the pro peptide (reviewed in Baxter *et al.* (5)). In rats and humans prorenin cleavage is thought to occur only in the JG cells of the kidney (137), whereas in mice it also occurs in the submandibular gland where active renin is secreted into the saliva (little, if any, of this renin is believed to enter the circulation under normal physiological circumstances) (6, 138).

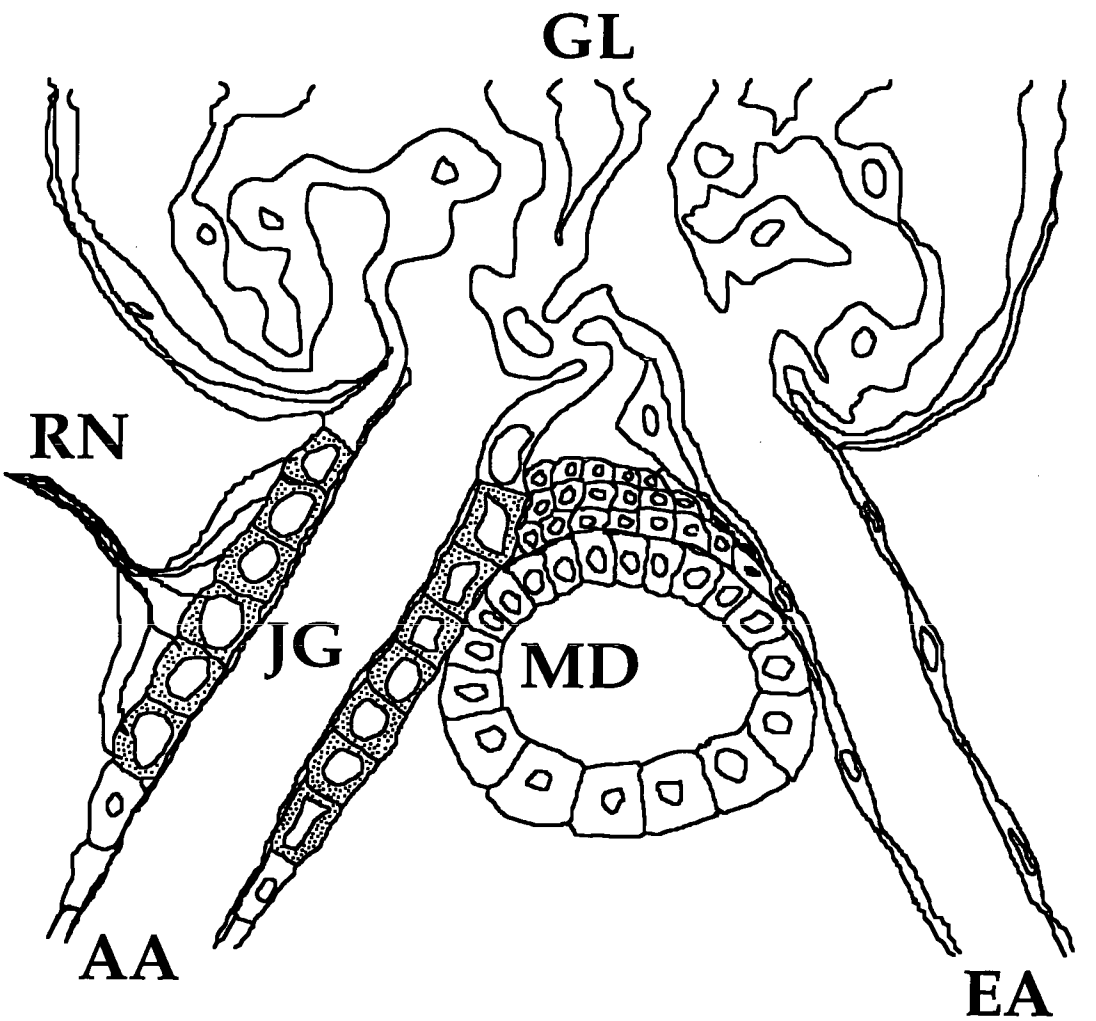


Figure 1.5: The juxtaglomerular apparatus of the kidney: Blood flows into the glomerulus (GL) in the afferent arteriole (AA) and leaves through the efferent arteriole (EA). The juxtaglomerular cells (JG) are the modified smooth muscle cells of the afferent arteriole closest to the glomerulus. These cells contain larger nuclei and mature storage granules which contain active renin. Release of this renin is in a regulated manner controlled by several factors. The cells are believed to possess "stretch receptors" allowing them to sense pressure changes in the afferent arteriole and adjust renin secretion accordingly. The neighbouring macula densa cells (MD) of the proximal tubule are also thought to signal to the JG cells to release more renin when a low sodium concentration is detected in the distal proximal tubule. JG cells are also in contact with renal nerves (RN) suggesting a neural input in the control of renin secretion. Finally it is thought that Ang II also acts directly on JG cells to suppress renin secretion. This figure is adapted from Davis (134).

1.2.3.3 Extrarenal Differential Expression Patterns

In mice, the expression patterns of all three renin genes have been studied. These results show that the expression patterns are not the same for the three genes (See Table 1.6 for a summary of the expression patterns of the three genes).

Adrenal Gland

In adult "two-renin gene" mouse strains, *Ren-1*^{d} and *Ren-2* are expressed at roughly equivalent levels in the adrenal gland, whereas in an adult "one-renin gene" mice, *Ren-1*^{c} is not detectable (139). *Ren-1*^{c} expression is developmentally restricted and expression can be seen in foetal adrenal glands. In "two-renin gene" mice expression is restricted to two zones, the X-zone (a fourth zone of the adrenal gland, the size and presence of which varies according to age and reproductive status) and the zona fasciculata (140). In rats and humans expression is less restricted and can be seen in the outer cortical zone and the zona glomerulosa (67). Genetic crossing of mice strains containing one or two renin genes has shown that the differences in expression in adrenal glands are probably due to *cis*-acting elements (139). In F_1 hybrids the parental expression patterns are maintained, suggesting that *trans* activating effectors are not downregulating *Ren-1*^{d} expression.

Submandibular Gland (SMG)

The renin expression detected in the SMG of mice is peculiar to this species (141) (no renin transcripts have been detected in the SMG of rats by RT-PCR (87, 133)). In "two-renin gene" mice, *Ren-2* is found in large

Organ	Ren-1^c		Ren-1^d		Ren-2^d
Kidney	1c	=	1d	=	2d
Submandibular Gland	1c	>	trace	«	2d
Foetal Adrenal Gland	1c	=	1d	=	2d
Adult Adrenal Gland	ND	<	1d	=	2d
Testis	1c	<	1d	>	2d
Ovary	1c	?	1d	=	2d
Anterior Prostate	1c	»	ND		ND
Foetal Subcutaneous	1c	=	1d	>	2d

Table 1.6: Tissues showing a differential expression pattern of the three renin genes are listed: In this table "expression" means detection of the appropriate mRNA (ND= Not Detected, ?= No comparison made).

quantities in the SMG (71, 73, 79, 96, 103, 130, 131, 139, 142-146) whereas *Ren-1^d* has only been detected at trace levels (131). *Ren-1^c* is also expressed in the SMG but at a level two orders of magnitude lower than *Ren-2* (much higher than *Ren-1^d*) (139). At birth, expression in the SMG is low but increases with age (142, 143, 147) and is restricted to the glandular epithelium, which represents 20% of the tissue, with renin representing 2.0% of the total SMG protein (90, 142, 148-151).

Reproductive Tissues

Differences in renin expression in the testis between mouse strains are not striking, with *Ren-1^d* being expressed at slightly higher levels than *Ren-1^c* or *Ren-2* (139). In the testis, expression is restricted to the interstitial Leydig cells (152). In "two-renin gene" mice, expression in the ovary from each gene is equal although the specific renin expressing cells in mice have not yet been determined (133). No studies of *Ren-1^c* expression in the ovary tissue have yet been carried out. Expression of *Ren-1^c* has been detected in the anterior prostate (a coagulating gland) with no *Ren-1^d* or *Ren-2* expression detectable (139). Like the SMG, expression is restricted to the glandular epithelium. Analysis of congenic lines (*Ren-1^d* and *Ren-2* genes on a "one-renin gene" genetic background, Balb/c) showed that expression is controlled in *cis* as the "two-renin gene" mouse expression pattern was observed (139).

Subcutaneous Tissue

Transcripts from *Ren-1^c* and *Ren-1^d* can be detected in subcutaneous tissue in equal quantities and in excess of *Ren-2* during foetal development (132, 153). Expression is restricted to fibroblasts near the developing skeletal muscle. Rats also show subcutaneous expression suggesting that this is not specific to mice and may be common to other species (153).

1.2.3.4 Expression During Development

Expression of renin in both 'one' and 'two-renin gene' mice has been found to start at 14.5 days *post coitum* (pc) throughout the adrenal gland (except in the outer cortical region and the zona glomerulosa) with expression reaching a maximum at 15.5 days pc (154). Expression in "one-renin gene" mice becomes progressively more restricted with no renin expression detectable in the adrenal glands of new born mice (139, 154). In

"two-renin gene" mice, expression becomes progressively more restricted with renin only being detectable in a zone of cells surrounding the developing medulla in the adrenal glands of new born mice (154).

All three renin genes have been shown to be expressed at equal levels in the developing kidneys of mice (154). In the mouse and rat, renin expression becomes more restricted as the embryo develops. Expression is first detected at 14.5 days pc in the intrarenal branches of the renal artery, with expression being restricted to the interlobular arteries and afferent arterioles by day 16.5 pc (154-159). By adulthood renin expression is found only in the JG cells (154-159). These results show that during the development of the kidney, the walls of the atrial tree go through a stage of expressing renin. This has led to speculation that renin expression may be important for the control of vascular growth, cell migration and differentiation (160-163). It has also been suggested that renin may serve as a marker for the development of the renal vasculature (154).

In adult kidneys, if renin secretion is chronically stimulated, *e.g.* by sodium depletion (164) or inhibition of ACE (165), smooth muscle cells of the afferent arteriole are found to undergo metaplastic transformation and become immunohistochemically positive for renin. This increased pattern of renin expression mirrors the pattern observed in the developing kidney, indicating some kind of de-differentiation towards a more foetal like expression pattern (154).

1.2.4 Regulation of Renin Secretion

1.2.4.1 Regulation of Renin in the Kidney

There are believed to be four major systems operating to regulate renin secretion and therefore maintain normal blood pressure. These are the renal baroreceptor and the macula densa mechanism which act at the single nephron level, and the renal nerve and Ang II receptor responses which act across the whole kidney. This topic has been reviewed in great detail elsewhere (134, 166-171).

Renal Baroreceptor

Tobian *et al.* first postulated the existence of a renal baroreceptor mechanism: because of the JG cells position (Figure 1.5) the authors proposed that they were ideally situated to act as a receptor, sensing changes in renal arterial perfusion pressure (172-176). This was supported by other data in which renin secretion was found to be altered in response to changes in the mean arterial pressure and not the mean pulse pressure (177, 178). This is thought to be because the pressure in the afferent arteriole is significantly dampened. More conclusive evidence of a baroreceptor present in JG cells came from cell culture experiments. A layer of JG cells were grown on a latex grid which, when stretched to 112%, resulted in a drop in renin secretion (179-181). This then rose again when the stretch force was removed. However, direct tests of this *in vivo* have produced conflicting results (182, 183).

Macula Densa Mechanism

Vander and Miller recognised that sodium delivery to the macula densa may also be involved in the regulation of renin secretion (Figure 1.5). He postulated that an increased delivery of sodium to the macula densa of the juxtaglomerular apparatus (JGA) would result in a reduction in renin secretion and vice versa (166, 184). By isolation and studying single nephrons (JGA, tubule and macula densa) Skott and Briggs were able to increase renin secretion by reducing the sodium concentration (by perfusion) at the attached macula densa (185). Precisely what is being sensed by the macula densa cells is controversial. Lorenz *et al.* found that sodium chloride concentration was more important than sodium chloride delivery (186). Lorenz *et al.* and Kotchen *et al.* found that chloride was more likely to be the signal than sodium (187, 188). However, the consensus opinion is that sodium chloride is involved and, for most practical purposes, it makes little difference if sodium or chloride is the actual signal (189).

The nature of the signalling molecule between the macula densa cells and JG cells has been the subject of much research. Early contenders for the molecule were Ang II, prostaglandins, calcium or adenosine (reviewed in Churchill (190), Katz (170) and Malvin (191) and Skott and Jensen (192)), but at present it seems most likely to be nitric oxide (NO) (193-195).

Nerve Stimulation/Cardiopulmonary Receptors

The kidney is an innervated organ (Figure 1.5) and stimulation of renal nerves causes an increase in renin secretion (166, 175, 196-198). This has been found to be mediated mainly through the ß-adrenoceptors (199, 200), although some positive (201-204) and negative (205) α-adrenoceptor input has been detected. The distribution of these receptors may not be exclusive to the kidney and there is evidence for an extrarenal location (206-208).

As one might expect the heart has been found to play a role in renin secretion and therefore in blood pressure regulation. Brennan *et al.* found that stretching the left atrium resulted in a reduction in renin release (209). Decreases in right atrial pressure was also found to increase renin secretion (210). Similar findings are also true of the left atrium (211, 212). This agrees with data suggesting that vagal afferent nerves also affect renin secretion (213, 214). Signalling could occur by stimulation of the ascending nerve traffic (possibly the vagal afferents) following detection of atrial expansion/stretch by a receptor resulting in decreased synaptic activity to the kidney and therefore a fall in renin secretion (215).

This cardiac influence on renin secretion could however be a hormonal mechanism (216), e.g. by atrial natriuretic factor (ANF). ANF has been found by some groups to inhibit renin release (169, 217, 218), but there is evidence to suggest that this is not consistent (169).

Direct Action of Ang II

As Ang II acts to increase blood pressure, this will indirectly cause a decrease in renin secretion. Circulating Ang II has also been found to suppress renin secretion directly in a negative feedback manner (166, 219, 220). In this system, high levels of Ang II in the plasma would be expected to cause a down-regulation of renin secretion and vice versa. This has been demonstrated using ACE inhibitors to stop the formation of Ang II resulting in an increase in renin synthesis and secretion (221). Ménard *et al.* have used renin inhibitors for similar investigations and reported comparative results (222).

1.2.4.2 Regulation of Renin in the Submandibular Gland

Regulation of renin secretion in the SMG is under hormonal control, being vastly upregulated upon treatment with testosterone (145, 143-146, 203, 223-227) or thyroid hormone (226-229). Expression levels are lower in females but can be increased by administering testosterone (79, 143-146, 223, 225, 230, 231). SMG renin levels in males can be reduced by castration and, in turn, rescued by testosterone (143, 225, 230).

SMG renin is active and under normal circumstances is secreted in the saliva (230-234) and not into the plasma (138, 235). However, upon stimulation by testosterone or thyroid hormone (see above), aggressive behaviour (138, 236), or gentle manipulation of the SMG (237, 238) increased levels of active renin are found in the plasma, most of which is derived from the SMG (increased saliva levels are also observed). This extrarenal circulating active renin has no effect on blood pressure regulation as the rate limiting factor in angiotensin I generation is angiotensinogen which is present in mice at very low levels in the plasma (renin is present in excess) (6). As there is no obvious role for SMG-derived renin in blood pressure regulation (138, 151, 235, 237, 239) it has been postulated that mice with high renin concentrations in their saliva have a selective advantage (6, 138). This is because the delivery of doses of renin to other animals (where renin is rate-limiting) will cause a rapid increase in blood pressure, local tissue damage and possibly death (6, 236). This is supported by the finding that injection of saliva from "two renin-gene" mice into rats causes an increase in blood pressure (240). All aspects of SMG renin production, regulation and function are reviewed in Bing *et al.* (151) and Nielsen and Poulsen (241).

1.3 Probing the Role of the RAS in Blood Pressure Regulation Using Transgenics

1.3.1 Renin Genes

Several transgenic experiments have helped to explain the different expression patterns of the three mouse renin genes, as well as giving an insight into blood pressure regulation in mice and rats. By introducing the *Ren-1*^{d} gene onto a *Ren-1*^{c} background it was possible to show that the different expression patterns of these two genes were due to DNA elements

working in *cis* and not *trans* acting factors (131). Similarly, by introducing the *Ren-2* gene onto a *Ren-1*^{c} background it was possible to show that differences in *Ren-2* expression were also due to *cis*-acting elements (140, 242, 243). The transgenic mice developed by Mullins *et al.* did not display high blood pressures (J. J. Mullins *et al.*, unpublished data), no blood pressure data are reported for the other transgenic mice. These results suggests that differential expression patterns are due to differences in the promoters of the three genes, for example the presence of several insertion elements (section 1.2.1). That is, in the kidney, all three promoters are functionally equivalent (*i.e.* all three promoters contain the elements required for renal transcription), whereas in extrarenal tissues other control elements which vary between the three different genes lead to varying expression patterns.

Transgenic mice developed by Ohkubo *et al.* containing the rat renin gene under control of the metallothionein promoter did not develop a hypertensive phenotype (244). This was to be expected because in the mouse renin is not thought to be rate-limiting (6) and it has been shown that rat renin is unable to cleave mouse angiotensinogen (245). Transgenic mice have also been produced using the human renin gene (246). Here only 900bp of promoter was used and resulted in regulated expression in the kidney and secretion of active renin into the plasma. The extrarenal expression pattern reflected that of the mouse *e.g.* expression of the human gene in the submandibular gland (renin expression is not detected here in the human). In a similar experiment using 3kb of 5' promoter sequence the human renin gene was found to be expressed predominantly in the JG cells of the kidney (247). Although renin was detected in extrarenal tissues it was not found in the submandibular gland suggesting that this transgene's expression pattern was more human-like (248).

Transgenic rats containing the *Ren-2* gene have been generated which developed a hypertensive phenotype (249). It is postulated that this is because in rats renin is rate limiting and because mouse renin can convert rat angiotensinogen to Ang I (245). In these animals the RAS is generally depressed although levels of expression in the adrenal glands are high resulting in increased plasma prorenin levels. Interestingly, these animals showed the same qualitative expression pattern of renin as is observed for the *Ren-2* gene in mice, except for the submandibular gland where no renin was detected. Transgenic rats have also been created using the human renin

gene (250). These animals are normotensive, probably due to the inability of human renin to convert rat angiotensinogen to Ang I (250).

1.3.2 Other RAS Constituents

Transgenic mice carrying the rat angiotensinogen gene have been shown to exhibit an increase in blood pressure (251). Ohkubo *et al.* who also made transgenic mice carrying the human angiotensinogen gene under the control of the heterologous metallothionein promoter showed no increase in blood pressure in their transgenic mice (244). No increase in blood pressure was observed, probably because of the inability of mouse renin to produce Ang I from human angiotensinogen (245). Takahashi *et al.* produced transgenic mice containing the entire human (promoter and gene) angiotensinogen gene (252). These animals expressed angiotensinogen in the liver, heart and, at much higher levels than expected, in the kidney. No investigation of the blood pressure in these animals is reported, however, conversion of human angiotensinogen to Ang I was not expected due to the inability of mouse renin to process human angiotensinogen (253, 254). This has been confirmed by Yang *et al.* who observed no increase in blood pressure upon the introduction of a similar human angiotensinogen transgene (255). It is postulated by the authors that the upregulation of expression in the kidney is caused by a negative regulatory element from the human gene which is not present in the transgene (252).

Ganten *et al.* have made transgenic rats carrying the human angiotensinogen gene which did not develop hypertension (250). This was due to species specific enzyme kinetics, human angiotensin not being a good substrate for rat renin.

When Ohkubo *et al.* cross-bred their transgenic mice, the resulting animals, which contained both the human renin and angiotensinogen transgenes, displayed a hypertensive phenotype confirming the species specificity of the human and mouse substrate and enzyme (244). Similar observations were made by Fukamizu *et al.* (256) when transgenic mice expressing human renin (248) were crossed with other transgenic mice expressing human angiotensinogen (252).

1.4 Generating Null Mutations and Gene Targeting

Gene targeting is a method used to introduce specific mutations into the mouse genome. This is achieved by introducing a mutation into embryonic stem (ES) cells in cell culture by homologous recombination. ES cells are totipotent pre-implantation embryo-derived cells which can be maintained in an undifferentiated state in cell culture (257, 258). Once genetically modified, these cells can be injected into recipient blastocysts and may then contribute to the germ-line of the mouse (Figure 1.7). These chimaeras can then be bred and if the mutation is passed through the germ-line, new mouse lines can be generated in which all tissues are derived from the genetically-manipulated cell line, allowing the effects of the alteration to be studied (259).

Targeting constructs typically consist of two arms of homology and a selectable gene and are designed to disrupt the gene, although alternative, more subtle, mutations can be achieved. The two homology arms are identical to DNA sequences within the gene to be disrupted and it is these regions which mediate the homologous recombination event.

When a targeting construct is introduced into ES cells (normally by electroporation) it can integrate into the genome either randomly or into it's homologous site. As the frequency of targeted events is generally much lower than random integration, it is best to develop stringent screening strategies for detecting targeted events (homologous recombination at the targeted locus). The standard screening procedure for detecting targeted events is Southern blotting (although PCR based strategies can also be used). Several factors are known to affect targeting efficiency and will be discussed below.

1.4.1 Types Of Gene Targeting Vector

There are two distinct vector types used for gene targeting experiments, these being replacement and insertion vectors (Figure 1.8). Replacement vectors contain two stretches of homologous sequence interrupted by a selectable gene *e.g. neo.* These vectors can be used to create null alleles by

deleting exons of the endogenous gene, deletion of exons depends upon the location of the arms of homology. The vector is linearised at one end of the homology arms and transfected into ES cells. These vectors are thought to insert into the targeted gene by a double reciprocal recombination event or by a single recombination event followed by gene conversion (259). Complete removal of the plasmid vector by cleaving at the end of both arms of homology may give increased efficiency and removes the possibility of plasmid sequences inserting in the genome, the effects of which are unpredictable.

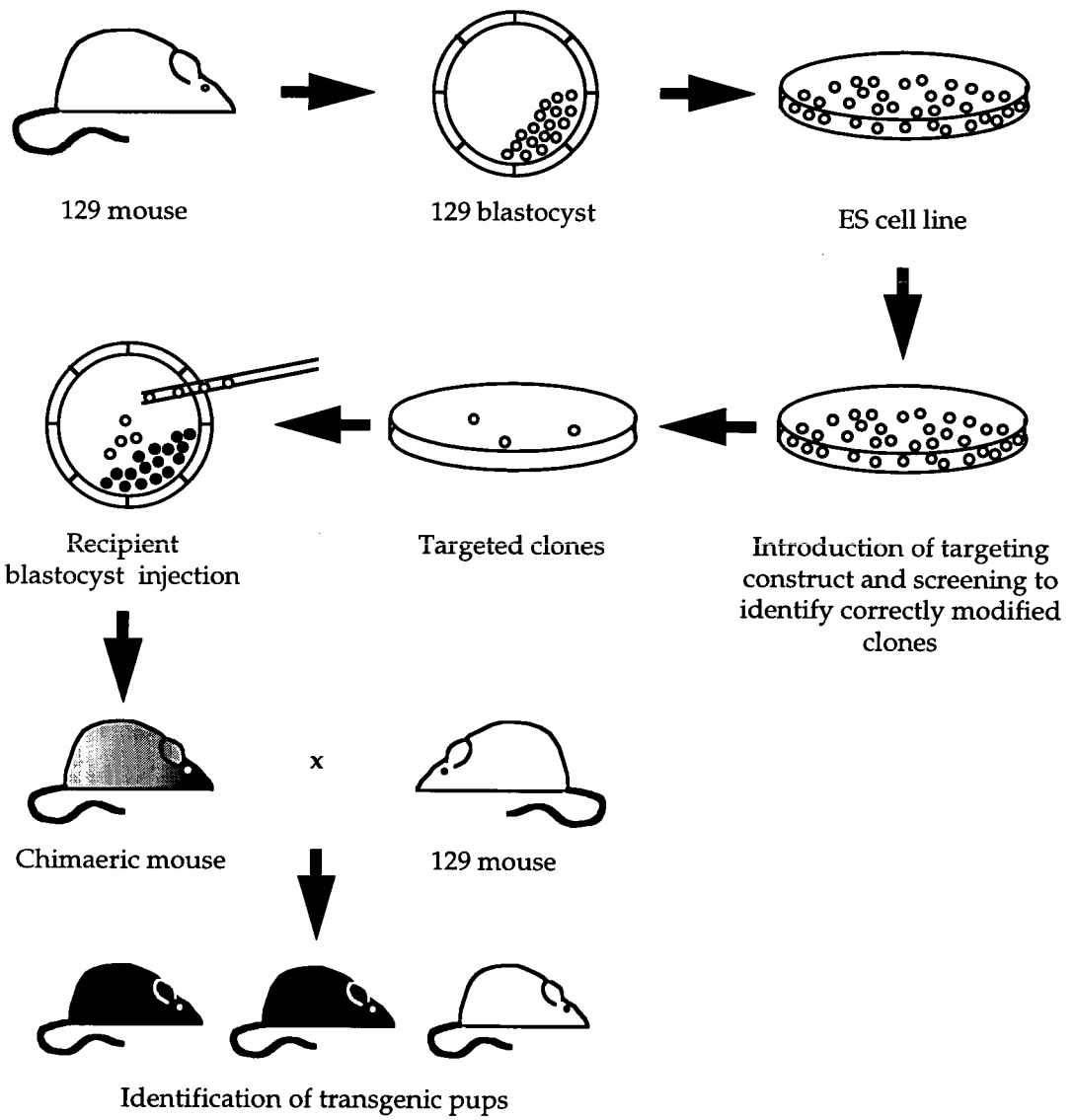


Figure 1.7: Generation of novel mouse lines from gene targeted ES cells: ES cell clones are derived from pre-implantation mouse embryos and maintained in cell culture. Targeting construct DNA is introduced into the cells and clones containing the construct selected. These cells can then be re-introduced into mouse blastocysts resulting in chimaeric mice which can be bred to generate new mouse lines.

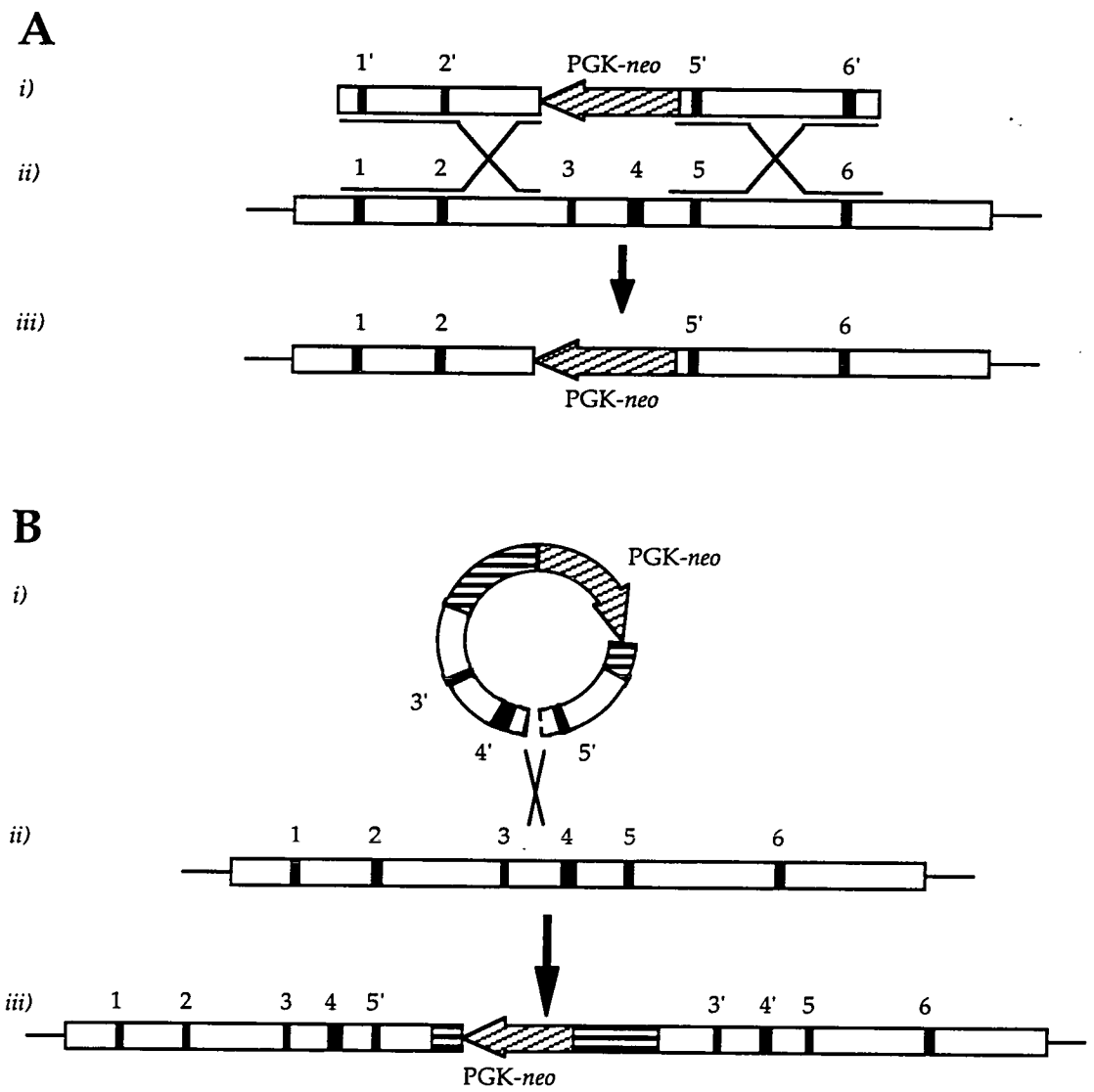


Figure 1.8: Gene targeting using replacement or insertion vectors: Ai) Shows a sequence replacement targeting vector including a PGK-*neo* selectable marker (diagonally stripe arrow indicates the direction of transcription) flanked by two homology arms (Black bars= exons, white box= introns, '= exon derived from targeting construct). Aii) Shows the genomic locus against which the targeting construct has been designed. Aiii) Shows the targeted locus after homologous recombination has occurred. Crosses indicate the sites at which recombination has occurred. This is only an example and recombination could have occurred anywhere in the two homology arms. Bi) Shows a sequence insertion targeting vector including a PGK-*neo* selectable marker linearised within the homology region (horizontal stripes= plasmid vector sequences). Bii) Shows the genomic locus against which the targeting construct has been designed. Biii) Shows the targeted locus after homologous recombination has occurred.

In contrast, when insertion vectors are used the entire construct is inserted into the region of homology. Insertion vectors contain one region of homology and a plasmid vector containing a selectable gene. To transfect ES cells, the construct is cut once within the region of homology, generating two arms of homology with the plasmid backbone and selectable marker between them. By a slight modification of the insertion vector it is possible to duplicate a region of the targeted locus, as has been reported for the angiotensinogen-encoding gene, *Agt* (260). One possible problem with insertion vectors is the fact that no part of the endogenous gene is deleted, and that it would be possible to generate a wild-type messenger RNA by exon skipping. This has been shown to happen when an insertional vector was used to disrupt the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene (261). In this instance, the generation of some wild-type activity is thought to be critical for the viability of the mice. In other *CFTR* mouse models created using replacement vectors, the homozygous mice die at a very young age and do not reflect the human cystic fibrosis phenotype as well as the insertional mutant mouse model (262-264). When *CFTR* expression was studied in the insertion mice, trace levels of wild-type product were detected and it is postulated that this is enough to increase the viability of these mice (261).

In a study of targeting at the *hprt* locus by Hasty *et al.* (1991) insertion vectors were found to be seven times more effective at targeting than replacement vectors (265). In a later study by the same group a similar increase in efficiency (6-fold greater) was observed when using a insertion vector (266). In both of these studies one of the arms in their replacement vectors was always limited to 1.2kb or less (265). Another study by Deng and Capecchi, also looking at gene targeting in the *hprt* locus, suggested that replacement vectors could be used with a targeting efficiency equal to that obtained using an insertional vector if sufficient lengths of homology were used on both arms of a replacement vector (267). A third study in 1994 by *Escherichia coli* neomycin phosphotransferase II gene (*neo*), which renders transfected cells eHasty *et al.* showed a 4-to 8-fold increase in targeting efficiency using an insertional vector at the *fgr* locus while no differences in efficiencies were detected between vector types when the *fah* locus was targeted (268).

1.4.2 Methods of Selection

Central to any gene targeting strategy is the selection of ES cells into which the targeting construct has been inserted. This is achieved using a selectable marker, such as thexpressing the gene resistant to the mammalian antibiotic G418. This strategy is termed positive selection and typically results in a low targeting efficiency (relative frequency of homologous recombination to total drug resistant colonies) since the expression of drug resistance is independent of the integration site. This is still, however, a perfectly adequate and commonly used strategy.

In order to reduce the number of colonies which need to be screened, other selection procedures have been developed. A powerful method for selection of targeted events is the use of vectors lacking promoter (269-272) or polyadenylation sequences (273). Here, in order for the selectable marker to be expressed, the construct must be inserted into the coding sequence (exons or 3' untranslated region) of a gene. In the case of random insertions this will be relatively rare and will therefore increase the relative number of targeted events isolated. A limitation of this system is that it relies on expression of the targeted gene in ES cells.

Positive/negative selection can also be used to enrich for targeted events (274). This method relies on the fact that random integrations tend to insert via their ends, whereas homologous recombination events are internal to the homology. Thus, any non-homologous sequences flanking these regions are lost. Cells can be positively selected for expression of *neo* and selected against using the thymidine kinase gene from the herpes simplex virus (HSV-*tk*), which when expressed, renders cells sensitive to the base analogue gancyclovir. If an HSV-*tk* gene is placed outside the region of homology, only random integrants should contain HSV-*tk* and will therefore be selected against in gancyclovir.

1.4.3 Regions of Homology

Historically, non-isogenic DNA was used in targeting experiments but it has been shown that when isogenic DNA is used to make targeting vectors the frequency of targeted events is greatly increased relative to equivalent non-isogenic constructs. Isogenic DNA is the term given to DNA derived

from the same strain *i.e.* if the ES cells are 129/Ola-derived, isogenic homology arms are derived from 129/Ola DNA. One such example is by te Riele *et al.* in which the same flanking arms of homology were used, derived from either 129/Ola or Balb/c DNA to target the *Rb* gene in 129/Ola ES cells (275). By using the same pMC1*neo*polyA vector (Stratagene) for both constructs, any differences in efficiency could not be due to the method of selection. Using 129/Ola DNA, a targeting frequency of 35% (33/94 G418 resistant colonies) was obtained, whilst the frequency when Balb/c DNA was used was only 1 in 144 (0.7%) G418 resistant colonies. This represented a 50-fold increase in targeted events when isogenic DNA was used. When the degree of homology between the two inbred strains of mice was studied, it was found that in a stretch of 1687bp, the longest stretch of perfect homology was only 278bp. The authors suggest that the decrease in targeting efficiency reflects an insufficiency of long, perfectly matched regions of homology. These data only apply to the *Rb* locus and care must be taken in extrapolating these results to other loci. However, in general, it is believed that using isogenic DNA significantly increases targeting efficiency.

It is well known that the extent of homology in a targeting construct influenced the targeting efficiency. It has been shown that increasing the homology used in insertion or replacement vectors increases the targeting efficiency (259, 266). Deng and Capecchi addressed this question more thoroughly and found that both types of vector had a strong dependence on the length of homology between the targeting vector and the targeted locus (267). Here, an exponential relationship was found between targeting efficiency and the extent of homology between the vector and targeted sequence, up to a plateau observed when 14kb of homology was used. A comparison between isogenic and non-isogenic DNA was also carried out and frequencies using isogenic DNA were found to be 4-5 times higher.

Gene targeting constructs are usually built using regions of homology subcloned from an isogenic genomic library. This step can be time consuming as clones must first be identified and then extensively characterised, often not resulting in the identification of suitably sized fragments. A possible solution to this problem would be to PCR amplify large DNA fragments from isogenic genomic DNA for use in targeting constructs, thus removing the need to screen genomic libraries. Other advantages of this system include the ability to position PCR primers

anywhere within a gene and the introduction of restriction sites (in a PCR primer) for use in cloning and/or screening. This strategy also makes the targeting of poorly characterised genes easier. All that is required is some sequence data for the design of PCR primers (*e.g.* a cDNA sequence) and a preliminary genomic map for the prediction of amplicon size and the development of a screening strategy. As exonic sequences are known to undergo mutation at a low rate (compared to intergenic or intronic regions), PCR primers designed against a cDNA sequence from a mouse strain other than that of the ES cells used (129/Ola) should suffice for the amplification of homology arms.

1.4.4 The Application of Gene Targeting to the RAS

Gene targeting has had a large impact on our understanding of many single gene disorders such as cystic fibrosis, but the technology can just as easily be turned to the dissection of multigene disorders such as hypertension by disrupting candidate genes. Several constituents of the RAS have been disrupted by gene targeting, resulting in valuable insights into the significance of each protein, as well as confirming the essential role of the RAS in maintaining normal blood pressure.

1.4.4.1 Angiotensinogen (*Agt*)

An elegant series of studies involved the duplication of the entire angiotensinogen locus (260), and the complementary disruption of the gene by conventional targeting (276). The duplication of the angiotensinogen gene was achieved by using an insertion-type targeting vector. Sequences from upstream and downstream that are believed to encompass all control sequences of the gene were used as the homology in the targeting construct. The 8kb 5' homology arm included 3kb of promoter and exon 1, and the 1.8kb 3' homology arm included exons 4 and 5 and extended 200bp beyond the polyadenylation site. The 8kb gap between 5' and 3' homology arms, spanning exons 2 and 3, was repaired (filled in) by cellular mechanisms during the recombination event resulting in duplication of the targeted locus (260).

By breeding animals possessing either the duplicated or disrupted locus it was possible to generate mice containing 0 to 4 copies of the

angiotensinogen gene (260, 276). This resulted in mice expressing 0 to 145% of normal plasma angiotensinogen levels, increasing in a non-linear but gene copy number-dependent manner. This relationship between gene copy number and angiotensinogen levels was also extended to include blood pressure, where mean arterial pressure was found to be proportional to gene copy number. Linear increases in blood pressure of 8.3 ±2.3mm Hg (mean arterial pressure) were reported for each additional copy of the *Agt* gene (276).

Of particular interest in the experiments of Kim *et al.* (276) is the null phenotype *i.e.* the complete absence of angiotensinogen. No data has been reported for the blood pressure of homozygous mutant mice-probably because the viability of these mice is severely reduced. Surviving homozygous mutant (*Agt* -/-) animals were found to have no obvious defects at birth although adult mice displayed pathological changes in the kidney. *Agt* -/- animals were found to have thickening of the medial layers of vessel walls, caused by an increase in cell number and loss of structural organisation, being most noticeable in the interlobular arteries. The mechanism underlying the wall thickening is unknown but may reveal a novel response to the low blood pressure or to the complete absence of angiotensinogen. General cortical thinning with foci of severe atrophy was also observed in the kidneys of *Agt* -/- animals. The areas of atrophy consisted of shrinkage and loss of tubules, interstitial fibrosis and interstitial infiltration of chronic inflammatory cells, and were postulated to be caused by ischaemic damage as a result of reduced blood flow through the arteries. The kidneys of all other animals (1 to 4 copies of *Agt*) appeared to be normal.

The relationship between copy number and plasma angiotensinogen levels was not linear. The values which one would have expected are 50% of wild-type activity for every copy of *Agt* present, as opposed to the 0, 35, 100, 124 and 145% observed for mice containing 0, 1, 2, 3 or 4 copies of the *Agt* gene respectively. There are two explanations for these results. Firstly the lower level of angiotensinogen present in the *Agt* 1/- animals is postulated by Kim *et al.* (276) to be related to the increased expression of renin in these animals. Secondly as the level of renin is much higher, a greater percentage of angiotensinogen would be expected to be converted to Ang I and ultimately to Ang II, therefore resulting in an increase in blood pressure. However, the expected increase in blood pressure is not observed. The lower

than expected levels of angiotensinogen observed in the 3 (*Agt* 2/1) and 4 (*Agt* 2/2) copy animals could be explained by negative feedback on *Agt* expression when elevated levels of angiotensinogen protein are present, or could be related to the structure of the *Agt* locus. Although all known *cis*-acting elements required for normal *Agt* levels were duplicated, additional, more distant, sequences may be essential for recapitulating expression *in vivo*. This can be investigated by studying the *Agt* 1/1 and *Agt* 2/- animals, both of which have two angiotensinogen genes. When levels in these groups of mice were compared, the *Agt* 2/- were found to have only 55% of the *Agt* 1/1 (wild-type) levels suggesting that not all the regulatory elements were duplicated in this experiment or that the close proximity of the two gene copies is inhibitory. Interestingly, this difference in angiotensinogen level had no statistical effect on blood pressure in the two groups of animals.

In an independent experiment in which the angiotensinogen gene was disrupted (277), no difference in blood pressure was observed in heterozygous (*Agt* +/-)animals, but a significant reduction in systolic blood pressure in the homozygous mutant (*Agt* -/-) animals was reported, (66.9 ±4.1mm Hg compared to 100.4 ±4.4mm Hg in wild-type animals). Similarly, diastolic and mean blood pressures were also reduced. When renin levels in both homozygous mutant and heterozygous animals were studied, renin levels in heterozygotes did not differ from wild-type values whereas null animals showed a 600 to 800% increase in renin expression levels. A third group have also reported the disruption of the *Agt* gene (278). These authors also report altered kidney morphology and hypotension in *Agt* -/- animals.

1.4.4.2 Angiotensin I Converting Enzyme (*Ace*)

In addition to the full-length transcript widely expressed from the *Ace* gene, a truncated transcript encoding a testis specific form of ACE is also expressed in postmeiotic spermatogenic cells (the latter being expressed from an alternative, testis-specific promoter). The precise function of this testis-specific isoform is unknown. However, the gene targeting strategy used by Krege *et al.* (279) to inactivate the *Ace* gene resulted in the disruption of both transcripts permitting the role of ACE in blood pressure regulation and fertility to be studied.

Male heterozygotes showed a significant reduction in blood pressure of

15-20mm Hg with female heterozygotes being indistinguishable from their wild-type litter mates. The reason for this sexual dimorphism is unclear, since in both sexes serum ACE activity was reduced. When homozygous mutant animals were studied, it was found that both males and females were hypotensive with a reduction of 35mm Hg in mean arterial pressures. Homozygous mutant mice also displayed histological changes in the kidney similar to those of the angiotensinogen mutant mice described earlier, with thickening of artery walls caused by an increase in the number of disorganised cells, as well as cortical thinning with focal areas of atrophy.

When the fertility of homozygous mutant animals was assessed, female fertility remained normal whereas male fertility was severely reduced with only one out of five males tested being fertile. A more detailed study of the males showed that they were still capable of mating and that testis pathology, sperm count and sperm morphology were all normal, suggesting that homozygous mutant males may have a reduced ability to fertilise ova.

Another independent group have disrupted the *Ace* gene, this time leaving the testicular isoform intact, in a bid to improve the fertility of the animals for breeding purposes (280). Blood pressures were not measured in these animals, but similar histological kidney abnormalities reported by Krege *et al.* (279) were observed. In addition, the *Ace* -/- mice generated by Carpenter *et al.* (280) were uraemic with mean blood urea nitrogen levels increased by over 3-fold (0.52 mg/ml compared to 0.15mg/ml in wild-type animals). This agrees well with the much reduced ability of the *Ace* -/- animals to concentrate their urine - the wild-type animals concentrating their urine to 4,000mOsm/l compared to 820mOsm/l in the *Ace* -/- animals. Urine is concentrated in the collecting ducts of the kidney which in the *Ace* -/- animals appear disorganised, the anatomy of the medulla and pelvis being distorted. The medulla, which contains the collection system, lacks the fan-like medullary rays seen in wild-type animals and the pelvis, where the collecting ducts empty into the ureter is mis-shapen and reduced in size. These mice display a more severe phenotype than the *Ace* -/- of Krege *et al.* (279) and most homozygous mutant mice die by three weeks of age. The authors suggest that this difference is down to "modification by other genes", which presumably means these two mouse lines are on different genetic backgrounds (not stated). *Ace* -/- mice that survived passed weaning age were found to be fertile confirming the need for expression of the testis-

specific *Ace* transcript for fertility.

1.4.4.3 Angiotensin II type 1A Receptor (*Agtr1a*)

Angiotensin II exerts its vasopressive effects via the AT_1 receptors. Ito *et al.* (281) disrupted the AT_{1A} receptor gene (*Agtr1a*) by gene targeting using a replacement vector. The resulting homozygous mutant mice were viable and displayed no outwardly visible abnormalities. Binding of Ang II was studied in homozygous mutant mice (*Agtr1a* -/-) using radiolabelled Ang II which, in general, was found to be reduced. Using the receptor antagonists Losartan (DuP 753) and PD123319, which block binding to type 1 and type 2 receptors respectively, it was possible to show that the Ang II binding observed in the kidneys of homozygous mutant mice was mediated through the AT_2 receptor. The response to Ang II injection was also studied with the direct infusion of Ang II into wild-type mice resulting in an increase in blood pressure which lasted 20 seconds followed by a delayed depressor effect lasting longer than 500 seconds. Infusion into heterozygous mice resulted in a similar short-lived rise in blood pressure, but the depressor effect was shortened to about 400 seconds whilst Ang II infusion into homozygous mutant mice had no effect on blood pressure.

Systolic blood pressure was measured in the heterozygotes which displayed a 12mm Hg decrease, with the homozygotes showing a blood pressure reduction of 24mm Hg when measured by the tail-cuff method or 17 and 43mm Hg respectively when measured by cannulation of the carotid artery. Contrary to the observations in the *Agt* and *Ace* targeting experiments already discussed, the homozygous mutant animals displayed no abnormal histopathology in the kidney.

Taken together, these results tell us that the AT_{1A} receptor is not essential for normal development and survival, or for normal kidney development. However the AT_{1A} receptor is essential for the pressor and depressor effects observed on infusion of Ang II. The AT_{1A} receptor is also responsible for almost all of the Ang II binding occurring in the kidney, and is involved in the regulation of blood pressure under normal conditions. An independent report on the disruption of the AT_{1A} receptor (282) also found systolic blood pressure decreases, with 10 and 22mm Hg reductions being observed in heterozygotes and homozygotes respectively (similar changes

were observed in diastolic blood pressures). Expression of renin was found to be upregulated in the kidneys of homozygous mutant mice resulting in a 7- to 8-fold increase in plasma renin. The study used a modification of the gene targeting strategy to express *lacZ* under the control of the *Agtr1a* promoter. After incubation with a β-galactosidase substrate (Bluo-Gal), blue staining was observed in the glomerulus and JG cells. To confirm that this expression was equivalent to that of the endogenous gene, antisense probes for the AT_{1A} receptor mRNA were also used and shown to mirror the expression pattern of *lacZ*. Using an AT_{1B}-specific probe it was shown that no AT_{1B} receptor expression was present in the kidney.

A third report on the disruption of the *Agtr1a* gene has been reported by Matsusaka *et al.* (283). *Agtr1a* +/- and *Agtr1a* -/- animals had reduced blood pressures comparable with those observed by Ito *et al.* (281) and Sugaya *et al.* (282). However Matsusaka *et al.* (283) report kidney abnormalities in *Agtr1a* -/- animals similar to those observed in the *Agt* and *Ace* knock-out experiments. In particular, hypertrophy of the interlobular arteries and JG cell hypertrophy, together with an increase in the number of renin-positive staining cells in the afferent arteriole (similar observations to those in the *Agt* -/- animals (278)). Again reporter gene expression was observed primarily in the JG cells, although the proximal tubule, glomerulus and afferent and efferent arteriolar smooth muscle cells adjacent to the glomerular pole also stained positively.

To investigate the possible role of Ang II in signalling renin production in the JG cells of the kidney (section 1.2.4.1), chimaeric mice were produced from wild-type and *Agtr1a* -/- ES cells. In these animals the degree of JG cell hypertrophy/hyperplasia was proportional to the degree of chimaerism, with wild-type and mutant JG apparatuses showing equal degrees of hyperplasia. Similarly, the renin positive portion of the afferent arteriole from wild-type and mutant JGA were also the same. These results prove that local Ang II actions via the AT1 receptors (AT_{1B} is not expressed in the kidney) were not responsible for controlling JG production of renin. The authors suggest, therefore, that in this regard a renal baroreceptor or macula densa mechanism is likely to control this production of renin.

The results of the first two AT_{1A} receptor knock-out experiments (decrease in blood pressure was observed in the absence of histopathological

alterations in the kidney), compared to the changes reported for the *Ace* and *Agt* knockouts, suggest that the kidney abnormalities may not be mediated through the AT_{1A} receptor. The results of the third *Agtr1a* knock-out experiment contradict this theory as similar phenotypes are seen in animals lacking the *Agt*, *Ace* or *Agtr1a* genes.

1.4.4.4 Angiotensin II type 2 Receptor (*Agtr2*)

Hein *et al.* (284) reported the disruption of *Agtr2* using a replacement-type targeting construct. Since the *Agtr2* gene is located on the X chromosome, only females were capable of inheriting the disrupted *Agtr2* gene from the chimaeric father in the first generation. After 3 generations (F_3) it was possible to study homozygous females and hemizygous males, both of which developed normally. These animals showed no abnormal organ or skeleton development and produced litters of comparable sizes to wild-type animals. To confirm the absence of AT_2 receptors, 18.5 day pc embryos were examined for RNA expression and ligand binding. RNA blot analysis revealed no expression in hemizygous mutant males or homozygous mutant females and using the radioligand CGP42112 it was possible to show the absence of AT_2 receptors in the membranes of E18.5 embryos. This removal of AT_2 receptors did not affect the expression of the AT_{1A} receptor. When blood pressure was studied in these animals no difference was observed between wild-type mice and hemizygous mutant males. Similarly, injection of Ang II into both groups of mutant mice resulted in the expected pressor effects previously shown to be mediated, at least in part, by the AT_{1A} receptor.

As Ang II is also important in the central control of many physiological responses, including thirst, the drinking habits of the mutant mice were also studied. When wild-type and mutant mice were given drinking water *ad libitum* no differences were observed. However, if water was withdrawn for 40 hours and then returned, the two groups of animals responded differently. In the subsequent 3 hour period the water intake of the mutant mice was significantly lower than the wild-type control animals. The AT_2 receptor is highly expressed in the locus corneleus, a part of the brain involved in integration of sensory information and arousal. Since the intracerebroventricular injection of Ang II stimulates exploratory behaviour, the locomotive activity of AT_2 receptor-deficient mice was evaluated.

Activity was measured on two consecutive days, in a light or dark environment. In the light periods activity was not significantly different, however in the dark the mutant mice displayed a lower activity compared to their wild-type controls. The physiological significance of the reduced activity is not known and it should be noted that this may be directly responsible for the alteration in water intake in the mutant mice, rather than a direct effect of ablation of *Agtr2* expression.

A second report describing the disruption of the AT_2 receptor used a replacement-type vector (285). In this study third generation (F_3) hemizygous mutant males or homozygous mutant females also developed normally, showed no abnormal organ or skeleton development and produced litters of comparable sizes to wild-type animals. Mutant mice were found to display lower levels of exploratory behaviour (reduced ambulation) however, contrary to the findings of Hein *et al.* (284), basal blood pressure was found to be elevated in the mutant animals with systolic blood pressure being 118.2 ±5.0mm Hg in hemizygous mutant males compared to 94.2 ±1.7mm Hg in control males. Administration of Captopril, an ACE inhibitor, reduced the blood pressure of both groups of animals to similar values. These animals were then subjected to increasing doses of Ang II and, at all doses tested, the mutant mice exhibited elevated blood pressures compared to the wild-type controls. Finally, the administration of Losartan, an AT_1 receptor antagonist, reduced blood pressure to the same levels as the original Captopril treatment in both groups of mice. Ichiki *et al.* (285) suggest that since basal blood pressure remains higher even when the AT_1 receptors are blocked, the AT_2 receptor may act to limit the response of the AT_1 receptors to Ang II. The theory is further supported by the fact that infusion of Ang II into Captopril-treated mutant mice resulted in a larger increase in blood pressure compared to controls. In light of these results and the reduction in blood pressure observed in other RAS gene targeting experiments Ichiki *et al.* (285) postulate that the *in vivo* regulation of blood pressure is dependent on a balance between AT_1 and AT_2 receptor activation.

Hein *et al.* (284) also report a similar altered response to infusion of low doses of Ang II into mice pretreated with Captopril. Unlike the findings of Ichiki *et al.* (285) no significant differences were observed in baseline blood pressure between wild-type and mutant mice. These apparent discrepancies

may be due to differences between the background strains onto which the mutations have been crossed (FVB/N as opposed to C57BL/6J).

1.4.4.5 Renin (*Ren-1^c*, *Ren-1^d*, *Ren-2*)

Since most ES cells used in gene targeting are derived from the inbred mouse strain 129/Ola which contains two renin genes, any attempt to ablate renin expression must take this into account. Three possible strategies exist for the ablation of renin production: disruption of the *Ren-1^c* gene in ES cells derived from "one-renin-gene" mice; disruption of each gene consecutively in ES cells derived from "two-renin-gene" mice; or the use of a targeting construct to simultaneously disrupt both genes by deleting part of each gene and all the sequence between them. The availability of mice totally lacking a renin structural gene will complement the existing gene knockout studies and determine whether angiotensins can be produced *in vivo* by alternative enzymatic pathway.

However, the use of ES cells from two-renin gene mice (129/Ola) presents a unique opportunity to dissect renin function. The individual roles of renin-1 and renin-2, during development and in the adult, can be defined. Studies on the renin genes in our laboratory have shown that *Ren-2* -/- animals are viable, fertile and have no histological abnormalities in the kidney, adrenal gland or submandibular gland (286). *Ren-2* -/- animals are normotensive despite an increase in circulating active renin and reduced plasma prorenin concentrations. To date, no function of renin has been identified which cannot be wholly fulfilled by the *Ren-1^d* gene and resultant protein, Renin-1^d. In combination with the *Ren-2* knockout, additional targeting studies at the *Ren* locus will further elucidate some of the remaining questions. To date no one has published a phenotype for mice containing a disruption of the *Ren-1^d* gene. However Miller *et al.* have reported the disruption of the gene in ES cells, but they did not report germ-line transmission of the disrupted allele (287).

1.5 General Aim

Since the expression patterns of the two renin alleles in 'two-renin gene' mice are different, it is difficult to dissect their relative importance or to see whether they are indeed functionally redundant. A factor suggesting that the two genes may not be functionally redundant is the maintenance of all consensus sequences, the maintenance of the open reading frames and the functionality of both proteins. If these genes were functionally redundant, one would expect that one gene would undergo random mutation at a high rate because there would be no selective constraints upon it. This does not appear to have happened, suggesting that each gene product may be important. Wild species of mice possessing two renin genes always have two active genes suggesting a selective advantage of having two renin genes, *i.e.* an inactive form of either enzyme has never been identified in wild mice containing the duplication event. The gene targeting experiment described here will help to address this question.

Although the basic strategy of gene targeting is well defined and can be routinely performed, the amount of time and resources required for such an experiment is high. Alternative strategies which reduce the time taken to generate knock-out animals, or increase the frequency of gene targeting therefore reducing the number of colonies which need to be screened are always being developed. We have therefore chosen to build the construct using homology arms generated by PCR rather than subcloned fragments from genomic libraries. This was because we wished to reduce the time taken to construct targeting vectors and to develop a general strategy for the construction of targeting vectors directed against genes about which little information is known.

The aim of this project is to generate transgenic mice in which the *Ren-1^d* gene has been mutated to produce a *Ren-1^d* null allele. Ablation of *Ren-1^d* will permit the study of *Ren-2* in isolation from *Ren-1^d*. This will allow the investigation of the ability of Renin-2 protein to mediate blood pressure regulation and kidney development. The following chapters describe the disruption of the *Ren-1^d* gene in ES cells, their to use to generate *Ren-1^d* -/-mice and the subsequent analysis of this new mouse line.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals/Solutions

All stock solutions were prepared using reverse osmosis water (Elgastat Prima Reverse Osmosis Water Machine; Elga Ltd, High Wicham, UK), filtered (0.22μm cellulose acetate membrane), and autoclaved before use. Solutions were treated with 0.01% diethyl pyrocarbonate, DEPC (Sigma-Aldrich Company Ltd, Poole, UK) or made up using DEPC-treated H_2O. Chemicals used were from BDH Laboratory Supplies (Merc Ltd, Lutterworth, UK) and were of analytical quality.

Acids, alcohols and solvents were supplied by BDH Laboratory Supplies or Fisons Scientific Equipment (Loughborough, UK), except absolute ethanol (Hayman Ltd, Litham, UK). Phenol was purchased redistilled and buffered with Tris-HCl from Fisons Scientific Equipment (Product code T/P633/05). Standard grade and Genetic Technology grade agarose was supplied by FMC (Flowgen, Sittingbourne, UK) or Boehringer Mannheim (Lewes, UK). Radioisotopes were supplied by Amersham International Plc (Little Chalfont, UK). Kodak XOMAT XAR-5 film was supplied by (IBI Ltd, Cambridge, UK). Oligodeoxynucleotide primers were synthesised by Oswell DNA Service (University of Southampton, Southampton, UK) or Pharmacia Biotech (St. Albans, UK). Random hexamers and dNTPs were supplied by Pharmacia Biotech.

2.1.2 Enzymes

All enzymes were supplied by Boehringer Mannheim except Perfectmatch® DNA polymerase enhancer (Stratagene, Cambridge, UK), *UlTma* DNA polymerase and the Amplitaq FS dye terminator sequencing kit (Applied Biosystems, Warrington, UK), T4 DNA ligase and T4

polynucleotide kinase (Gibco-BRL, Paisley, UK) and AMV Reverse transcriptase (Promega, Southampton, UK).

2.1.3 Bacterial Strains

All plasmids were maintained in *Escherichia coli*, strain DH5 cells (Genotype= supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 deoR F-lambda-) (288).

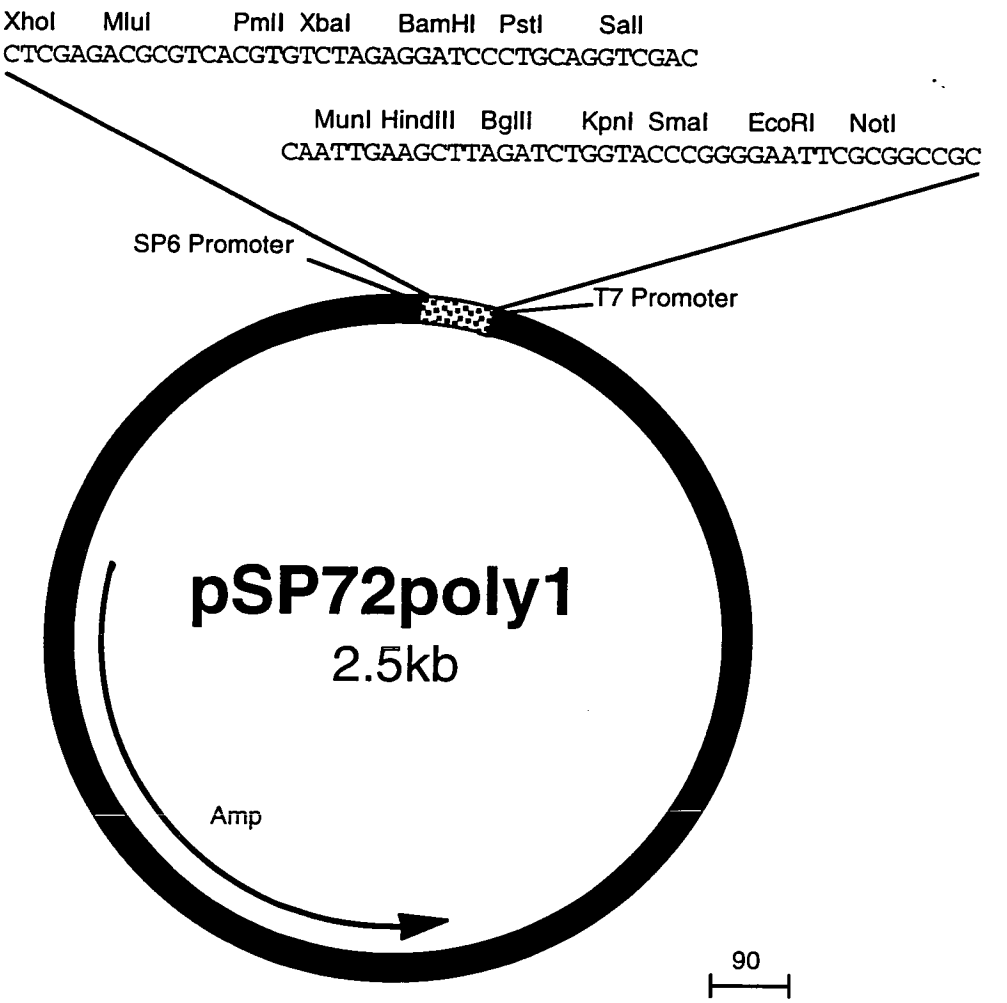
2.1.4 Cloning Vectors, Plasmids and Probes

pSP72poly1 and pSP72poly4 were constructed by removing the polylinker from pSP72 (Promega) and inserting annealed, complementary oligodeoxynucleotides (Figure 2.1 and Figure 2.2 respectively; S. Morley *et al.*, unpublished data). pBluePGK-*neo*pA (Figure 2.3), a PGK-*neo* selection cassette for use in gene targeting vectors was a gift from Austin Smith (University of Edinburgh, Edinburgh, UK).

The P1 clones used as templates for PCR reactions have all been identified as containing renin sequences defined by the presence of a PCR product using exon 6 and 7 directed PCR oligodeoxynucleotides (L. Mullins *et al.*, unpublished data).

The "external" DNA probes used to screen ES cells and tail DNAs for homologous recombination or inheritance of the *Ren-1^d* disrupted allele were derived from plasmid clones containing either *Ren-1^d* or *Ren-2* sequences. The 5' probe, a 297bp *Pvu* II/*Bam*H I *Ren-2* genomic fragment containing exon 1 was prepared from a larger plasmid, pR2D8 (K. Gross and W. Brammar, unpublished data). The 746bp *Hind* III/*Nco* I *Ren-1^d* genomic fragment used as a 3' probe containing exon 8 and part of exon 9 was prepared from a larger plasmid, pRn12 (D. Burt and W. Brammar, unpublished data).

Two plasmids were used to confirm that the 5' "external" probe (*Ren-2* derived) hybridised to *Ren-1^d* sequences. Plasmid pRn34 contains a 6.7kb *Bam*H I *Ren-1^d* genomic fragment (total plasmid size of 9.4kb). The plasmid pRen2-5'XK contains a 9.7kb *Xho* I/*Kpn* I *Ren-2* genomic fragment (total plasmid size of 12.3kb).

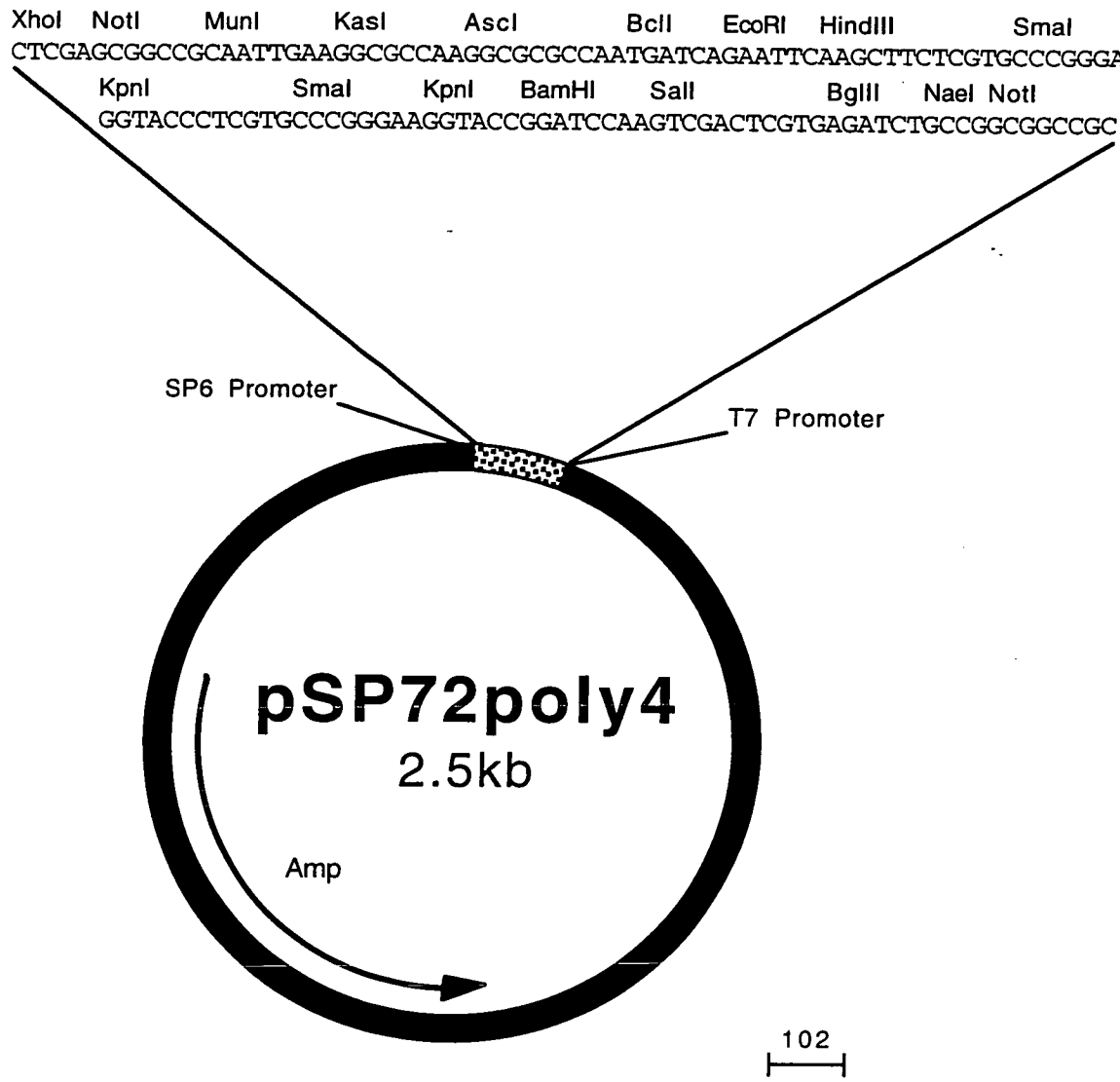


LAB/ I.D. No. JJM-60

VECTOR : pSP72poly1 is a pSP72 (Promega) based vector.
INSERT : Altered polylinker.
REFERENCE/SOURCE : Constructed by S. Morley, see also the Promega catalogue.

SELECTION : Ampicillin
STORAGE : Glycerol Stocks @ -70°C HOST: DH5
DNA: Stocks in database @ -20°C

Figure 2.1: pSP72poly1: This plasmid is a vector into which PCR products were subcloned. The polylinker of pSP72 was excised via the *Xho* I and *Bgl* II sites and replaced with a pair of self complementary 83mer oligodeoxynucleotides with 4 base overhangs at the 5' ends designed to recreate the *Xho* I site but to destroy the *Bgl* II site of the original vector. Correct insertion and the presence of restriction sites was verified by sequencing. The polylinker is flanked by SP6 and T7 promoter sites which can be used for sequencing through insert fusion points.

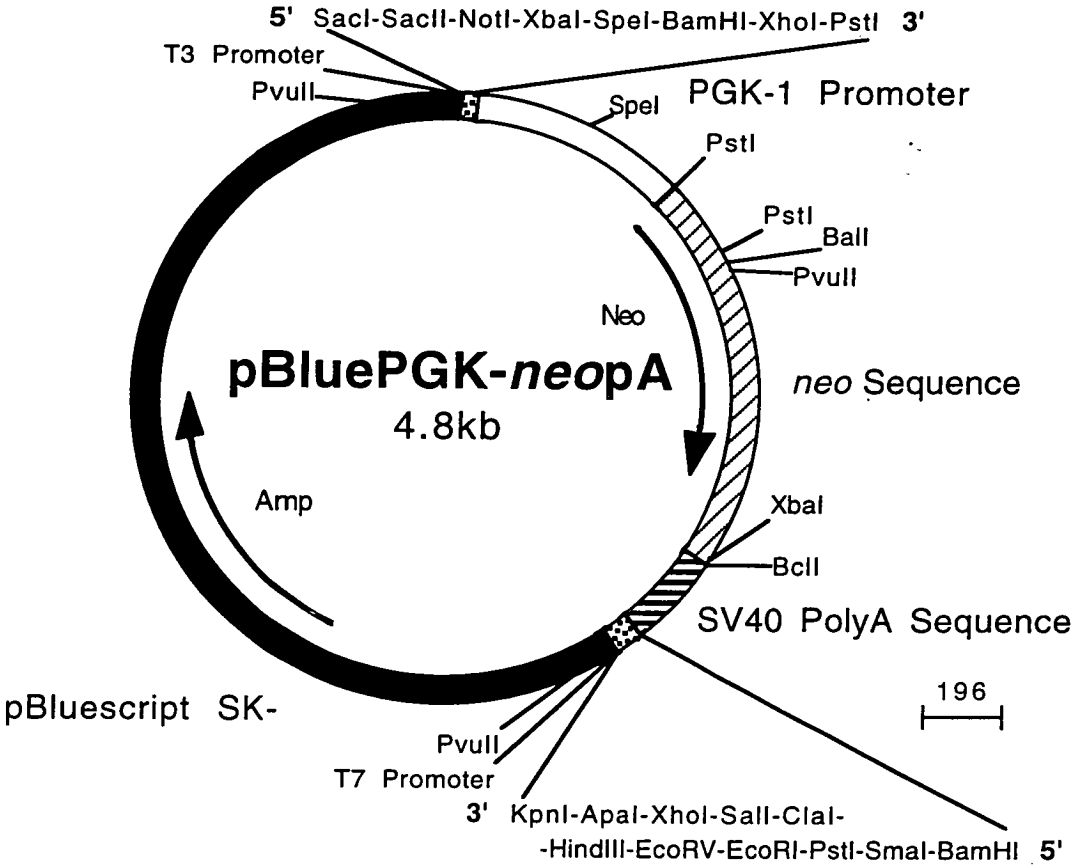


LAB I.D. No. JJM-105

VECTOR : pSP72poly4 is a pSP72 (Promega) based vector.
INSERT : Altered polylinker.
REFERENCE/SOURCE: S. Morley Results V, p65-80 and the Promega catalogue.

SELECTION : Ampicillin.
STORAGE : Glycerol Stocks @ -70°C HOST: DH5
DNA: Stocks in database @ -20°C

Figure 2.2: pSP72poly4: This plasmid is a vector into which PCR products were subcloned. The polylinker of pSP72 was excised via the *Xho* I and *Bgl* II sites and replaced with a pair of self complementary 111mer oligodeoxynucleotides with 4 base overhangs at the 5' ends designed to recreate the *Xho* I site but to destroy the *Bgl* II site of the original vector. Correct insertion and the presence of restriction sites was verified by sequencing. The polylinker is flanked by SP6 and T7 promoter sites which can be used for sequencing through insert fusion points.



LAB. I.D. No. JJM-133

VECTOR: pBluescript SK- (Stratagene).
INSERT: A PGK*neo* cassette with SV40 polyA sequence.
REFERENCE/SOURCE: Gift from Ian Chambers (Austin Smith Lab).

SELECTION: Ampicillin
STORAGE : Glycerol Stocks @ -70°C HOST: DH5
DNA: Stocks in database @ -20°C

Figure 2.3: pBluePGK-*neo*pA: This plasmid was created by inserting a *Sac* I/*Bal* I fragment from pBS-PGK*neo*2 (Austin Smith) which includes the PGK-1 promoter and 5' end of the *neo* gene into *Sac* I/*Bal* I digested pBS ßA*neo*15 (Austin Smith), a step which generates the wild-type *neo* gene (the base-pair variant which confers high phosphotransferase activity and therefore higher G418 resistance (289)) and places it under the control of the PGK-1 promoter. The PGK-1 promoter corresponds to a 0.5kb *Eco*R I/*Taq* I fragment of the PGK-1 gene (290) (GenBank Accession No. M18735). The 5' *neo* fragment (upstream of the *Bal* I site) is derived from a *Pst* I/*Hinc* II fragment of pMC1*neo*polyA (Stratagene). As the *Pst* I site is still present at the 5' end of the fragment it was assumed that no other alterations have been made, (confirmed by sequencing). The 3' end of the *neo* fragment was sequenced in the same run as the SV40 polyA signal fragment and overlapped with neo sequences. This was used to construct the above figure.

2.1.5 Embryonic Stem Cell Culture

All ES cell work was performed using E14Tg2a ES cells (291). Medium was made using filter-sterilised UHP water (Elgastat Prima Reverse Osmosis Filter followed by an Elgastat UHP water purifier). Prior to use, batches of foetal calf serum that supported optimum growth of established ES cell lines were selected (tested by D. Rout and D. Colby). DIA/LIF was also included in medium at a 1:1000 (100 units/ml) dilution to maintain the ES cells in an undifferentiated state (292, 293). DIA/LIF preparations were titred by D. Rout and D. Colby as described in Smith (294).

2.1.6 Mouse Strains and Housing

The inbred mouse line, 129/Ola, was used in all experiments except for derivation of chimaeric mice where blastocysts from the inbred mouse line C57BL/6J, and foster mothers from the MF1 Swiss albino outbred strain (MF1) were used. Mice were maintained in a stabilised environment on a regime of 14 hours light/10 hours dark (midpoint of the dark cycle being at 12.00, midnight), at a constant temperature (21°C± 2.0°C) and constant humidity (50%± 10%). The mice were supplied with food and water *ad libitum*. All animals were housed and bred within the Centre for Genome Research, Edinburgh, UK according to the provisions of the Animals (Scientific Procedures) Act (UK) 1986.

2.1.7 Computer Analysis

DNA analysis was performed using the DNAstar Lazergene 1.60 package (Lazergene, London, UK), ABI Prism™ 377 DNA Sequencer Data Collection 1.1 or ABI Prism™ DNA Sequencing Software 2.1.1 (both from Applied Biosystems). Plasmid representations were created using GeneConKit v1.18 (Textco Inc, West Lebanon, New Hampshire, USA). Statistical analysis was performed by hand (Student t-test) or using the Crunch version 3 program (Wilcoxon Rank Test) supplied by Crunch Software Cooperation (Oakland, California, USA), with graphical representations created using CA-Cricketgraph III v1.5 (Computer Associates International Inc., New York, USA). Southern gel autorads were scanned into a computer using a Canon CLC10 colour copier/scanner (Canon (UK) Ltd., Chessington, UK) and the Canon CLC10 scan 2.0.9

package (Canon (UK) Ltd.) and the contrast/brightness altered using the Adobe Photoshop 2.5.1 package (Adobe Systems Europe BV, Amsterdam, The Netherlands). RNA primer extension assay gels were exposed to phosphoimager screens (Molecular Dynamics Ltd., Sevenoaks, UK) and the data collected on a Molecular Dynamics Phosphoimager using the Image Quant v3.3 program (Molecular Dynamics Ltd.). Gels were then quantitated using the MD-Image 1.44MD.1 program (National Institute of Health, USA; public domain software).

2.2 Molecular Biology Methods

All molecular manipulations were carried out by standard techniques (288).

2.2.1 Bacterial Cell Culture

Bacterial liquid cultures were performed in Luria-Bertani medium, (L. Broth; 10g/l bacto-tryptone, 5.0g/l bacto-yeast extract, 10g/l NaCl; pH 7.2) supplemented with 0.2% (w/v) glucose and, where appropriate, 100µg/ml ampicillin (Sigma-Aldrich Company Ltd; Product code A-9158). Cultures were incubated at 37°C for 16-24 hours in an orbital shaker at 225-250rpm. Where large cultures, (>100ml), were required, these were inoculated at a 1:100 dilution using a starter culture, typically a 5.0ml culture started from a single colony. Where necessary, cultures were streak plated on L. Agar (L. Broth plus 15g/l bacto-agar) including 100µg/ml ampicillin, where appropriate, and these were then grown in an incubator overnight at 37°C. For storage bacterial strains were grown overnight in L. Broth containing 8.25g/l $K_2HPO_4.3H_2O$, 1.8g/l KH_2PO_4, 0.45g/l sodium citrate, 0.09g $MgSO_4$ and 44g/l glycerol (295). Cultures were stored in 1.8ml aliquots at -70°C. These conditions were also used for storing bacterial cell preparations containing plasmid DNAs where a clone was grown under the previously described conditions in the presence of 100µg/ml ampicillin.

2.2.2 Quantitation of Nucleic Acids

DNA and RNA concentrations were determined by measuring the optical density at 260nm (OD_{260}) on a spectrophotometer (Pharmacia Biotech; Model no. 80-2092-26). Briefly, plasmid preparations were diluted

in TE (10mM Tris-HCl; pH 8.0, 1mM EDTA) at 1:50, 1:100 and 1:200 dilutions, each in a final volume of 100µl. Optical densities of plasmid dilutions were measured after zeroing at 260 and 280nm with 100µl TE. The cuvette was always rinsed with TE between samples. DNA concentrations were calculated by multiplying the OD_{260} by the dilution factor and then by 50 before multiplying by 1000 to give the concentration in µg/µl. To quantify DNA fragment preparations, the sample volume was made up to 50µl and then quantified, without dilution, after treating the cuvette with 3.0% (v/v) H_2O_2 to inactivate contaminating nucleases and rinsing with nuclease free H_2O. RNA was quantified in a similar manner to DNA fragment preps, calculating the concentration by multiplying the OD_{260} by the dilution factor and then by 40 before multiplying by 1000 to give the concentration in µg/µl.

2.2.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate DNA molecules according to their size. Unless otherwise stated, all agarose gels were 0.8% (w/v) containing 0.5x TAE (20mM Tris-acetate, 0.5mM EDTA) and 0.5µg/ml of ethidium bromide (Sigma-Aldrich Company Ltd; Product code E-8752). Before loading, 6x "Maniatis" type IV loading buffer (0.25% bromophenol blue, 40% (w/v) sucrose, 60mM EDTA; pH 8.0) was added to each sample to give a final concentration of 1.0x buffer (288). Samples were always run in conjunction with size markers. These were 0.25-1.0µg lambda *Hin*d III/*Eco*R I digested DNA (sizes in base pairs are 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564 and 125) or lambda *Hin*d III digested DNA (sizes in base pairs are 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125). DNA in gels was visualised under UV light (302nm; UVP Transiluminator TM36; UVP Inc., San Gabriel, California, USA) and photographed using a Polaroid MP4 Land Camera (Polaroid (UK) Ltd, St. Albans, UK) with Polaroid 667 (ISO3000/36°) film (Polaroid (UK) Ltd; Product code 617798).

2.2.4 Restriction Digests

The basic guidelines for restriction digests in Sambrook *et al.* (288) were followed. DNA was digested at a concentration of 0.1-0.5µg/µl with less than 10% (v/v) enzyme (5.0% (v/v) glycerol) in the solution. After the

required incubation time (1-5 hours) at 37°C, 0.1-1.0µg of DNA was checked on an agarose gel for complete digestion. Digests were stopped by the addition of 10mM excess (compared to Mg^{2+} concentration) EDTA; pH 8.0 followed by denaturation at 65°C for 15 minutes. One exception to this was digests of miniprep DNA (0.1-1.0µg), when the whole digest (10µl) was run on a gel.

2.2.5 Nucleic Acid Precipitation

Typically DNA was precipitated with 0.5 volumes 4M ammonium acetate and 2.0 (final) volumes 100% isopropanol at -20°C for 15-60 minutes. DNA was pelleted by centrifugation at maximum speed for 15-30 minutes. The DNA pellets were then washed with 150µl 70% (v/v) ethanol and respun for 5-10 minutes (288), repeating the wash step for high purity DNA. Pellets were then air dried and resuspended in a suitable volume of TE. Where small quantities of DNA were being precipitated, samples were left at -20°C for longer and all spin times were increased in length.

2.2.6 PCR Methods and Conditions

2.2.6.1 Steps To Prevent Contamination

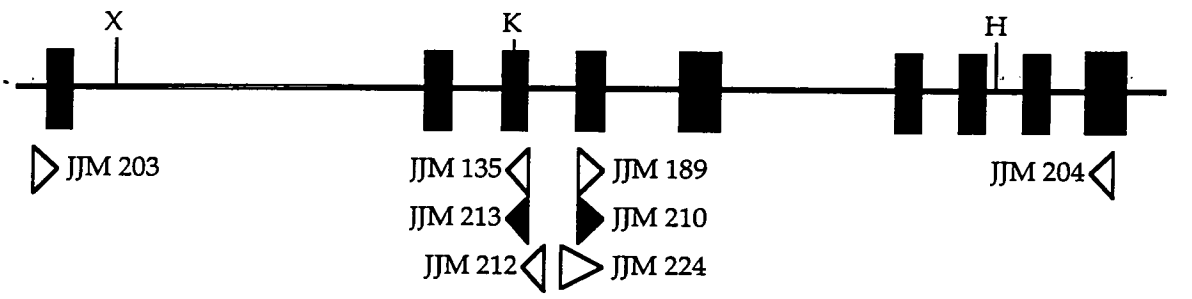
Pipette barrels and O-rings were routinely soaked in 1.0M HCl for 15 minutes before rinsing in reverse osmosis water and drying at 50°C. Reagents were pipetted using plugged tips and reactions were set up in a separate laboratory. Oil and ROP H_2O used in PCR reactions were UV treated by placing tubes on a transilluminator (wavelength= 302nm) for 20 minutes (296).

2.2.6.2 PCR Conditions

PCR reactions were either 25µl or 100µl in volume and were performed on a Hybaid Omnigene Thermal Cycler (Hybaid, Teddington, UK; Product code TR3 CM220). DNA was subjected to PCR amplification using specific oligodeoxynucleotide primers directed against the appropriate regions of the DBA/2J *Ren-1^d* gene (89) (refer to Figure 2.4 for primer binding site and primer sequences). In all experiments the proof-reading enzyme *UlTma* was used.

P1 DNA (1.0 to 100ng) or H_2O (negative control) was aliquoted into 0.5ml tubes, reaction "master mixes" (all components, excluding DNA/H_2O and enzyme) were made up for each experiment and aliquoted and finally each reaction was overlaid with 3 drops of light mineral oil (Sigma-Aldrich Company Ltd; Product code M-3516). The "master mix" was as follows:- 1.0x *UlTma* buffer (10mM Tris-HCl; pH 8.8, 10mM KCl and 0.002% (v/v) Tween 20®), 1mM $MgCl_2$, 40µM (each) dNTPs and 0.1µM (each)

A



B

Lab ID	Primer Description	Sequence
JJM 203	-162bp *Ren-1^d* Promoter (F)	CCGCTCGAGT CTGGACAGCC TACATGAC |*XhoI*|
JJM 135	*Ren-1^d* and *Ren-2* Exon 3 (R)	AAGGTCTGGG GTGGGGTACC |*KpnI*|
JJM 213	Biotinylated *Ren-1^d* and *Ren-2* Exon 3 (R)	B-AAGGTCTGGG GTGGGGTACC |*KpnI*|
JJM 212	*Ren-1^d* Intron C (R)	GTGCTAAAGA GGATTCTGGG CAC
JJM 189	*Ren-1^d* Exon 4 (F)	GCGGTACCAG CTACATGGAG AACGGGTC |*KpnI*|
JJM 210	Biotinylated *Ren-1^d* Exon 4 (F)	B-GCGGTACCAG CTACATGGAG AACGGGTC |*KpnI*|
JJM 224	Extended, *Ren-1^d* Exon 4 (F)	GCCGCTCGAG GTACCAGCTA CATGGAGAAC GGGTC |*XhoI*| |*KpnI*|
JJM 204	*Ren-1^d* and *Ren-2* Exon 9 (R)	GCAAGCTTGA CAAAATGGCC CCCAGGAC

Figure 2.4: Location and sequence of PCR primers for the amplification of *Ren-1^d* homology arms: A) Position of PCR primers used to amplify *Ren-1^d* homology arms. Schematic representation of the *Ren-1^d* gene (filled boxes are exons) with location and orientation of PCR primers shown as arrow heads (primers shown as filled arrowheads represent Biotinylated primers). To amplify the 5' arm JJM203 was used in conjunction with one of three primers (JJM135, JJM213 and JJM212). The 3' homology arm was amplified using reverse primer JJM204 along with one of three forward primers (JJM189, JJM210 and JJM224). B) PCR primer sequences. Table lists all PCR primers used by lab ID number, gives a description of where each primer binds (orientation is either F (forward) or R (reverse)) and lists the nucleotide sequence. B refers to a Biotin molecule attached at the 5' end of the primer. Where present, restriction sites are shown underneath the oligodeoxynucleotide sequence.

oligodeoxynucleotides. Reactions were "hot started" (enzyme added to reactions equilibrated at 95°C) with 1.2 units (25µl reactions) or 4.5 units (100µl reactions) of *UlTma*. The temperature of the block was controlled using a tube containing an equal volume of oil.

Template DNA was generally amplified as follows:- initial denaturation at 95°C for between 1 second and 5 minutes, followed by 95°C on HOLD (enzyme was added at this point for "hot start"). Amplification was for 40 cycles of denaturation for 1 minute at 95°C; annealing for 1 minute at 68°C (5' arm) or 66°C (3' arm); extension for 6.5 minutes at 72°C. This was followed by a final extension period of 10 minutes at 72°C before holding reactions at 20-25°C until removed from the machine. One exception to this was the use of a 2-step PCR amplification which was performed as follows; 95°C for 5 minutes followed by 40 cycles of 95°C for 1 minute, and 68°C for 10 minutes with a final stage of 72°C for 10 minutes before cooling to 20°C.

2.2.6.3 Expand™ Reverse Transcriptase and PCR Reactions

RT-PCR was used to show the absence of transcript from the *Ren-1^d* gene in knock-out animals using a primer from exon 3 (JJM56; CCAGCCCAGACCTTCAAAGTC) contained within the deleted region of the targeted allele and a reverse primer situated in exon 9 (JJM141; CCAGACAAATGGCCCCCAAG). Neither primer was specific to either renin transcript so the identity of amplified fragments was determined by *Ear* I digesting the product resulting in three bands from *Ren-1^d* or two bands from *Ren-2*.

RT-PCR reactions were set up using the Expand™ Reverse Transcriptase and Expand™ Long Template PCR System (Boehringer Mannheim). The Expand™ Reverse Transcriptase enzyme is a genetically manipulated version of the Moloney murine leukaemia virus reverse transcriptase enzyme engineered to have a reduced RNase H activity. This enzyme uses single stranded RNA as a template and generates a DNA strand synthesised from an oligodeoxynucleotide primer complementary to part of the mRNA (polyT or random hexamer priming is recommended, although priming with a specific primer is possible). The Expand™ Long Template PCR System utilises a mixture of two thermostable DNA polymerase, *Taq* and *Pwo*, to amplify larger fragments than can be obtained using *Taq* alone.

First strand cDNA synthesis was performed from kidney and testis RNAs and included the following controls:- H_2O plus reverse transcriptase (to show that reagents are not contaminated with DNA) and each RNA minus reverse transcriptase (to show that RNAs are not contaminated with DNA). Total RNA (1.0-2.0µg) and 5.0pmol random hexamer were denatured in a final volume of 11µl at 65°C for 10 minutes before quenching on ice. Expand™ Reverse Transcriptase buffer (4.0µl of 5.0x buffer), 2.0µl 100mM DTT, 2.0µl of 10mM dNTP stock (10mM each dNTP) and 50 units (1.0µl) of Expand™ Reverse Transcriptase were then added to denatured RNA/primer mix on ice. These reactions were incubated at 30°C for 10 minutes followed by 42°C for 45 minutes (performed on a Hybaid Omnigene Thermal Cycler). Reactions were stopped on ice before proceeding to the PCR step or stored at -20°C.

PCR reactions were set up firstly by making a master mix containing primer and dNTPs. The master mix was made according to the following "per reaction" contents- 2.0µl 10mM dNTP stock and 1.5µl of each primer (10µM stocks) made up to 25µl with dH_2O. Aliquots of this (25µl) were added to tubes on ice which already contained 5.0µl 10x PCR buffer 1 (17.5mM $MgCl_2$, 500mM Tris-HCl; pH 9.2 and 140mM $(NH_4)_2SO_4$), 2.0-3.0µl of each RT reaction (or a negative control using 2.0-3.0µl H_2O) and 0.8µl enzyme mix (3.5 units/µl) in a final volume of 25µl (made up to volume with dH_2O). This resulted in the following 50µl reaction conditions:- 1.0x PCR buffer 1 (1.75mM $MgCl_2$, 50mM Tris-HCl; pH 9.2 and 14mM $(NH_4)_2SO_4$), 400µM each dNTP, 0.3µM forward and reverse oligodeoxynucleotide primers and 2.6 units of enzyme mix. Reactions were overlaid with oil and underwent 1 cycle at 94°C for 2 minutes followed by 10 cycles of 94°C for 10 seconds, 64°C for 30 seconds and 68°C for 45 seconds. A further 30 cycles of 94°C for 10 seconds, 64°C for 30 seconds and 68°C for 45 seconds (increasing this elongation period by 5 seconds in every subsequent cycle) before a final period at 68°C for 5 minutes and holding at 25°C. After PCR reactions were completed, 5.0µl of each reaction was run on an agarose gel.

2.2.7 Preparation of PCR Products for Restriction Digestion

PCR products were subjected to restriction digestion to facilitate cloning according to one of three protocols.

2.2.7.1 Restriction Digestion

PCR product from three 100µl reactions were combined and ammonium acetate/isopropanol precipitated (section 2.2.5). DNA was then further purified by passing it through a Wizard PCR Prep DNA Purification System clean up column (Promega; Product code A7190) according to the manufacturers instructions. In brief, DNA was bound to 1.0ml of Magic PCR Purification resin in the presence of 100µl Direct Purification buffer. This was loaded onto a Magic Minicolumn before washing the resin with 80% (v/v) isopropanol and eluting the DNA in 50µl TE. This was digested with the appropriate restriction enzymes and a 3.0µl aliquot was checked on an agarose gel to confirm that complete digestion had occurred before gel purification (section 2.2.8) of the desired fragment.

2.2.7.2 Klenow, Kinase, Ligase Reaction Followed by Restriction Digestion

The Klenow, Kinase, Ligase (KKL) reaction is designed to allow efficient restriction digestion at sites at the ends of PCR products by ligating the products into large concatemers so that sites are now internalised and can be digested with appropriate restriction enzymes (297). Firstly, DNA from three 100µl PCR reactions were precipitated (section 2.2.5) and resuspended in 43.8µl ROP H_2O and then 6.0µl 10x KKL buffer (300mM Tris-HCl; pH 7.9, 100mM $MgCl_2$, 100mM DTT, 5.0mM ATP), 1.2µl 10mM dNTPs, 4.0µl 2.0 units/µl Klenow polymerase, 1.0µl 10 units/µl T4 polynucleotide kinase and 4.0µl 1.0 units/µl T4 DNA ligase were added. The reaction was incubated at room temperature overnight before diluting to double the volume in 1.0x restriction buffer and digesting with the appropriate restriction enzymes. After digestion, a 5.0µl aliquot was run on a gel to check for complete digestion. The desired restriction fragment was then gel purified (section 2.2.8).

2.2.7.3 Biotin/Streptavidin Purification Followed by Restriction Digestion

Here one of the oligodeoxynucleotides used in a given PCR reaction was biotinylated (JJM213 and JJM210) to produce a biotinylated PCR product which can be bound to magnetic streptavidin beads (Dynal (UK) Ltd, Bromborough, UK) allowing isolation of PCR product and restriction digested material.

Remaining oligodeoxynucleotides present after a PCR reaction will interfere with binding of the PCR product to beads therefore they were removed by three rapid precipitations at room temperature. Two large PCR reactions were combined and 20µg of calf thymus tRNA added as a carrier. DNA was ammonium acetate/isopropanol precipitated and incubated at room temperature for 5 minutes. DNA was spun down (10 minutes) and resuspended in 100µl of TE (288). After the third precipitation the pellet was washed twice, air dried and resuspended in 200µl TE.

Dynabeads (100µl) were prepared for washing by magnetising in a Dynal MPC (Magnetic Particle Concentrator) and leaving for 5 minutes before removing the supernatant. Beads were then washed in 100µl 1.0x Bind and Wash buffer (BW buffer is 10mM Tris-HCl; pH 7.5, 1mM EDTA and 2.0M NaCl), remagnetised, aspirated and resuspended in 200µl 2.0x BW buffer. Resuspended PCR product was added to the washed beads and incubated for 15 minutes at room temperature (or 37°C) to allow binding of the biotinylated PCR product to the beads. Beads were kept in suspension by flicking the tube at 2 minute intervals.

After binding of the PCR product, the beads were remagnetised before removal of the supernatant and resuspending in 100µl TE. PCR product was digested at an internal site (the desired fragment retaining its biotin moiety) and 5.0µl was checked on an agarose gel for complete digestion. The beads were then rebound by adding an equal volume of 2.0x BW buffer and remagnetising. After removal of the supernatant the beads were resuspended in 100µl TE and the second digest performed. This released the fragment from the beads allowing the fragment to be eluted in the supernatant after the addition of 2.0x BW buffer and remagnetisation.

As the supernatant, now containing the fragment of interest, was a 1.0M NaCl solution, a precipitation step was used to remove the salt. To aid the precipitation of the DNA 1.0µl of 20mg/ml glycogen (298) was added and the solution was diluted to 0.6M NaCl before adding 2.0 volumes of 100% ethanol and incubating at -70°C for 1 hour (288). DNA was centrifuged (4°C, 20 minutes) and the pellet was washed twice before air drying and resuspending in 20µl TE.

2.2.8 Gel Purification of Fragments

Digested DNA was loaded on a 0.8% agarose gel (genetic technology grade agarose gel run in a 3.0% (v/v) H_2O_2 treated tank). Under long-wave (366nm) UV light (UVP Inc.; Model UVGL-58) the band of interest was excised and stored at 4°C. DNA was then recovered from the gel by one of three methods.

2.2.8.1 Electroelution

Fragments of DNA were electroeluted from agarose gels using the ISCO "Little Blue Tank" apparatus (ISCO Inc, Lincoln, Nebraska, USA), according to the manufacturers instructions. Prior to use, the electroelution tank, chamber, collars and screens were soaked in 3.0% (v/v) H_2O_2. The electroelution tank and chamber were then set up as shown in Figure 2.5.

DNA was electroeluted at 10-15mA per chamber for 2 hours, at the end of which the current was reversed for 15 seconds. The 0.1x ISCO, 0.05% SDS buffer was removed down to the grid, the precipitate underneath being resuspended in the remaining buffer before transferring to a tube. The lower chamber was then rinsed with a further 200µl of 0.1x ISCO, 0.05% SDS. This was then extracted with 400µl buffered phenol, 400µl chloroform; isoamyl alcohol (24:1) and 40µl water saturated isobutanol to remove the contaminating SDS and ethidium bromide. The phases were separated by centrifugation for 1 minute, the aqueous phase being transferred to a clean 1.5ml tube before re-extracting with chloroform/isoamyl alcohol (24:1) (288). DNA was ammonium acetate/isopropanol precipitated overnight (section 2.2.5), pelleted, washed and resuspended in 50µl TE, pH 8.0. Fragments were quantitated (section 2.2.2) and checked on an agarose gel.

2.2.8.2 Qiaex Preparation of DNA Fragments

This method (see manufacturers instructions) is based on the solubilisation of agarose with sodium perchlorate and selective adsorption of DNA onto the QIAEX silicagel particles (Qiagen Ltd, Crawley, UK; Product code 20020). Briefly, gel was solubilised in QX1 buffer (3.0M NaCl, 4.0M sodium perchlorate, 10mM Tris-HCl; pH 7.0, 10mM sodium thiosulphate) and the DNA bound to QIAEX beads. These were repeatedly pelleted and washed firstly in QX2 wash buffer (8M sodium perchlorate, 10mM Tris-HCl;

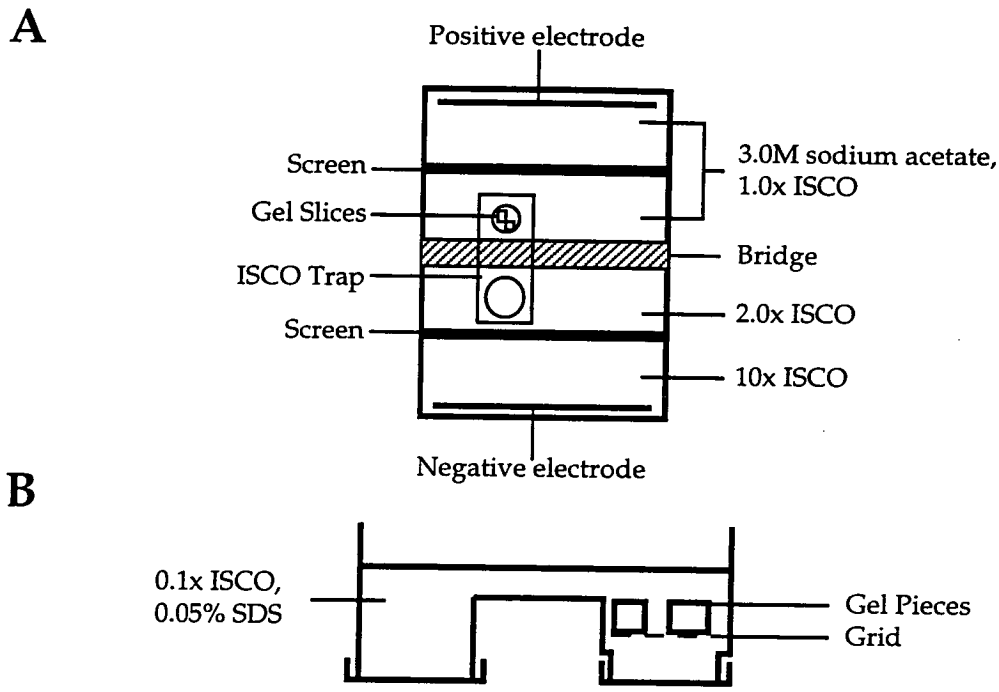


Figure 2.5: Electroelution using the ISCO "Little Blue Tank" apparatus: A) Electroelution tank. This is split into two sides by a bridge in the middle, each side is also split by a membrane screen creating four compartments which are filled with the following solutions; 3.0M sodium acetate, 1.0x ISCO buffer (25x ISCO is 1.25M Tris-HCl; pH 7.7, 5.0mM EDTA) was placed on the positive side of the tank on both sides of the screen, 2.0x ISCO buffer was placed between the bridge and the screen on the negative side and 10x ISCO buffer was placed between the screen and the negative electrode. The two buffers on the negative side of the tank were poured simultaneously to minimise leakage through the screen. For electroelution, a chamber was placed in the tank with the smaller well on the positive side of the tank (DNA was collected in this well). B) Electroelution chamber. This contains two wells and straddles the bridge of the electroelution chamber. When set up, this completes an electrical circuit allowing DNA to be electroeluted. Presoaked dialysis membrane (Gibco-BRL; Product code 15961-022) was used to cover the bottom of the wells of the electroelution chamber and held in place with collars. Chambers were rinsed in nuclease free water and 0.1x ISCO, 0.05% SDS buffer was then dripped into the narrower well to a level above the position of the screen. Pieces of agarose were placed on the grid and the chamber was filled with 0.1x ISCO, 0.05% SDS buffer until the depth was 5.0mm from the top.

pH 7.0) to remove the sodium thiosulphate and then in QX3 wash buffer (70% (v/v) ethanol, 100mM sodium chloride, 10mM Tris-HCl; pH 7.5) to remove the sodium perchlorate. DNA was eluted in 2x 20µl TE and the 2 supernatants pooled. Fragments were then quantitated (section 2.2.2) and checked on an agarose gel.

2.2.8.3 Amicom Spin Columns

In this method (see manufacturers instructions) a MICROPURE 0.22µm insert (Amicom Ltd, Stonehouse, UK) was used to filter out particular matter allowing the DNA solution to pass through the 0.22µm polysulphone membrane and be collected in the MICROCON 100 apparatus. The DNA then bound to the YM low-binding ultrafiltration membrane of the MICROCON unit allowing the DNA to be cleaned as required, before inverting the apparatus and recovering the DNA in 10µl TE. DNA concentration was estimated by comparing the fragment intensity to known quantities of a similarly sized, linear DNA fragment on an agarose gel.

2.2.9 Ligations

2.2.9.1 General Ligations

Ligation reactions were used to clone different ended DNA restriction fragments into plasmids. Ligations were set up with insert:vector molar ratios of 2:1 and 5:1. For each ligation 10-40ng of vector was used in a total reaction volume of 10-20µl. Parallel reactions were set up using vector alone, with and without ligase (to determine whether the vector used was capable of self ligation or not digested at all). Ligation reactions were performed using one of two buffer systems:- 1.0x Maniatis Ligation buffer (20mM Tris-HCl; pH 7.6, 5.0mM $MgCl_2$, 5.0mM DTT, 50µg/ml BSA and 5.0mM ATP) (288) or 1.0x Gibco-BRL Ligation buffer (50mM Tris-HCl; pH 7.6, 10mM $MgCl_2$, 1.0mM ATP, 1.0mM DTT and 5.0% (w/v) polyethylene glycol-8000). In a typical ligation reaction 1.0 units (1.0µl) of T4 DNA Ligase was used. Reactions were incubated at room temperature overnight and heat denatured at 65°C for 15 minutes before using 5.0-10µl of the reaction for transformation.

2.2.9.2 Polylinker Ligations

Complementary 71 (JJM146) and 79mer (JJM147) oligodeoxynucleotides (see Figure 2.6 for sequence of annealed oligodeoxynucleotides and restriction sites introduced) were annealed to each other using a "Touch-Down Lift-Off" program on a Hybaid Omnigene Thermal Cycler (initial denaturation at 98°C for 5 minutes, followed by annealing at 50°C for 1 minute, this cycle was repeated dropping 2°C in each denaturation cycle,

```
  ΔKpn I Sac I      Not I          Cla I        Sal I
5'      A GAGCTC AA GCGGCCGC AACA ATCGAT ACAA GTCGAC...
3' CATGT CTCGAG TT CGCCGGCG TTGT TAGCTA TGTT CAGCTG...

          Pvu II     Kpn I      Spe I      EcoR I ΔNot I
...AA CAGCTG TT GGTACC AA ACTAGT CC GAATTC TA     3'
...TT GTCGAC AA CCATGG TT TGATCA GG CTTAAG ATCCGG 5'
```

Figure 2.6: Sequence of the new polylinker for insertion into one of the plasmid intermediates: A 71mer oligodeoxynucleotide (JJM146) was annealed with a second oligodeoxynucleotide, a 79mer (JJM147) to generate the above double stranded polylinker fragment. Overhangs at each end were included to allow the directional cloning of the polylinker into *Kpn* I and *Not* I digested plasmid DNA such that neither site was recreated. Extra bases were included between each restriction site to act as a buffer allowing more efficient digestion at two sites within the polylinker.

finally reactions were held at 25°C). Reactions consisted of 400pmol of each oligodeoxynucleotide in 0.5x STE (50mM NaCl, 5.0mM Tris-HCl; pH 8.0 and 0.5mM EDTA; pH 8.0) in a total volume of 100µl overlaid with oil. Annealed oligodeoxynucleotides were ligated into digested plasmid DNA at molar insert:vector ratios of 5:1, 50:1 and 500:1 (previously determined to be suitable ratios for insertion of annealed oligodeoxynucleotides; Steve Morley, unpublished data). Reactions were performed in Maniatis buffer as described for General Ligations using 1.0 units (1.0µl) of T4 DNA Ligase.

2.2.10 Competent Cells and Transformations

Competent cells ($1.0x10^7$ transformants/µg DNA) were produced by a modification of the method of Chung *et al.* (299). Briefly, a 1ml aliquot of fresh overnight bacterial culture was inoculated into 100ml L. Broth supplemented with 10mM $MgSO_4$ and 0.2% (w/v) glucose. This was cultured until an OD_{600} of 0.2-0.3 was obtained at which point cells were cooled by swirling flasks in ice/water (all subsequent steps were performed at 4°C to achieve maximum competence). Cells were transferred to 250ml sterile Sorvall centrifuge bottles and centrifuged in a precooled swing-out rotor at 4°C for 10 minutes at 1,000x*g* (about 2,200rpm in a Heraeus Omnifuge 2.0RS; Heraeus Instruments Ltd, Brentwood, UK). The resulting cell pellet was resuspended in 10ml ice cold "Miller transformation solution" (L. Broth containing 10% (w/v) PEG, 5.0% (v/v) DMSO, 50mM $MgCl_2$) by swirling gently and aliquots (450µl) were snap-frozen in 1.5ml tubes in a dry ice/ethanol bath before storing at -70°C until required.

For transformations, frozen aliquots of competent cells were thawed in an ice/water bath and used immediately. Once thawed, 100µl of cells were pipetted into a cold Falcon 2059 tube containing 5.0-10µl (10-100ng) of plasmid DNA, mixed by swirling gently and incubated for 30 minutes at 4°C before adding 0.9ml of fresh, prewarmed SOC medium (20g/l bacto-tryptone, 5g/l bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose) and incubating at 37°C, 225rpm for 1 hour. Control transformations of 0.2ng and 2.0ng pBluescript SKII (+) were also performed to estimate transformation efficiency. Transformants were selected by plating 200µl of the transformation solution onto L. Agar plates containing 100µg/ml ampicillin. If necessary, the remaining 800µl was also plated out on additional plates.

2.2.11 Plasmid DNA Preparation Methods

All plasmid DNA preparations were carried out by a modification of the alkaline lysis method of Birnboin & Doly (300). DNA was recovered by alkaline lysis of lysozyme treated cells followed by neutralisation with potassium acetate. This lysis mixture was then pelleted to remove cell debris and chromosomal DNA, with the plasmid DNA remaining in the supernatant or purified by passing through a Qiagen column.

2.2.11.1 Minipreps

Single bacterial colonies were grown to saturation overnight (section 2.2.1) and 1.5ml aliquots were pelleted by centrifugation for 1 minute. Cells were resuspended in 100µl lysis buffer (25mM Tris-HCl; pH 8.0, 10mM EDTA, 10% (w/v) glucose, 2.0mg/ml lysozyme (Sigma-Aldrich Company Ltd; Product code L-7651)), left for 10 minutes at room temperature after which 200µl freshly made 0.2M sodium hydroxide, 1.0% (w/v) SDS solution was added, mixed gently by tapping the tube and left on ice for 5 minutes. The lysis mixture was neutralised by adding 150µl potassium acetate; pH 4.8 solution (3.0M potassium acetate, 2.0M acetic acid) and each tube vortexed thoroughly before placing on ice for 5 minutes. Lysates were centrifuged for 1 minute and supernatants were transferred to clean 1.5ml tubes. Samples were precipitated at -20°C for 15 minutes after the addition of 0.9ml isopropanol. Plasmid DNA and RNA were pelleted by centrifuging for 2 minutes and resuspended in 40µl TE. To purify the DNA

further, an ammonium acetate/isopropanol precipitation (section 2.2.5) was performed followed by washing and air-drying the pellets before resuspending each in 20µl TE containing 50µg/ml DNase-free RNase. This method routinely gave 5.0µg plasmid DNA which could then be used in restriction enzyme digestion analysis (4.0µl per digest).

2.2.11.2 Qiagen DNA Preps

This method involved the alkaline lysis of a bacterial culture (300), as described in the Miniprep method, followed by binding of plasmid DNA to the resin in QIAGEN columns. RNA and proteins were then removed during a medium salt wash and the plasmid DNA was eluted in a higher salt wash and precipitated, resulting in high quality DNA. The protocol provided with each kit was used to prepare up to 500µg DNA using a QIAGEN-tip 500 (Qiagen Maxiprep Kit; Product code 12162) or 100µg DNA using a Qiagen-tip 100 columns (Qiagen Midiprep Kit; Product code 12143). The DNA concentration was determined (section 2.2.2) and the identity of the plasmid was confirmed by restriction mapping.

2.2.11.3 Alkaline Lysis Maxipreps

A single bacterial colony was used to grow a 500ml culture as described in section 2.2.1. Cells were pelleted by centrifugation for 10 minutes at 4°C, 2,700x*g* (4,000rpm, Sorvall GS-3 rotor and Sorvall RC5C centrifuge), resuspended in 4.0ml of Lysis buffer (2.0mg/ml lysozyme, 10mM EDTA, 50mM glucose and 25mM Tris-HCl; pH 8.0) and left on ice for 10 minutes before lysing cells with 10ml of 0.2M NaOH, 1.0% (w/v) SDS solution. After 10 minutes the lysate was neutralised by the addition of 7.5ml potassium acetate; pH 5.5 solution (3.0M potassium acetate, 1.18M formic acid) and was left on ice for 10 minutes before centrifugation for 45 minutes at 4°C, 30,000x*g* (16,000rpm, Sorvall SS34 rotor and Sorvall RC5C centrifuge). DNA in the supernatant was precipitated with 1.0 volumes of isopropanol at -20°C for 1 hour. Precipitated DNA was centrifuged for 10 minutes at 3,200rpm (about 2,200x*g* in the Heraeus Varifuge 3.2RS), resuspended in 9.0ml of TE and any remaining insoluble material pelleted by centrifugation (as above). This supernatant was then used in the Caesium Chloride /Ethidium Bromide Equilibrium Centrifugation Method described below.

2.2.12 Caesium Chloride/Ethidium Bromide Equilibrium Centrifugation

This method, used for isolation of covalently closed circular plasmid DNA, is similar to that described by Radloff *et al.* (301) and Sambrook *et al.* (288). To achieve an appropriate CsCl density, 9.9g of CsCl (Sigma-Aldrich Company Ltd; Product code E-8751) and 0.45ml 10µg/ml ethidium bromide were added to 9.0ml of the supernatant from an alkaline lysate maxiprep before transferring the solution to a 11ml Beckman polyallomer Quickseal tube (Beckman RIIC Ltd, High Wycombe, UK; Product code 342413). The tubes were then filled to the top with a TE/CsCl/ethidium bromide solution (9.0ml TE, 9.9g CsCl and 0.45ml 10mg/ml ethidium bromide), balanced, sealed and centrifuged (Beckman L-60 or L-7 ultracentrifuges, NVT-65.1 rotor) for 16 hours at 20°C and 55,000rpm (approximately 260,000g), with the brake set to slow. After centrifugation the plasmid DNA was removed as shown in Figure 2.7.

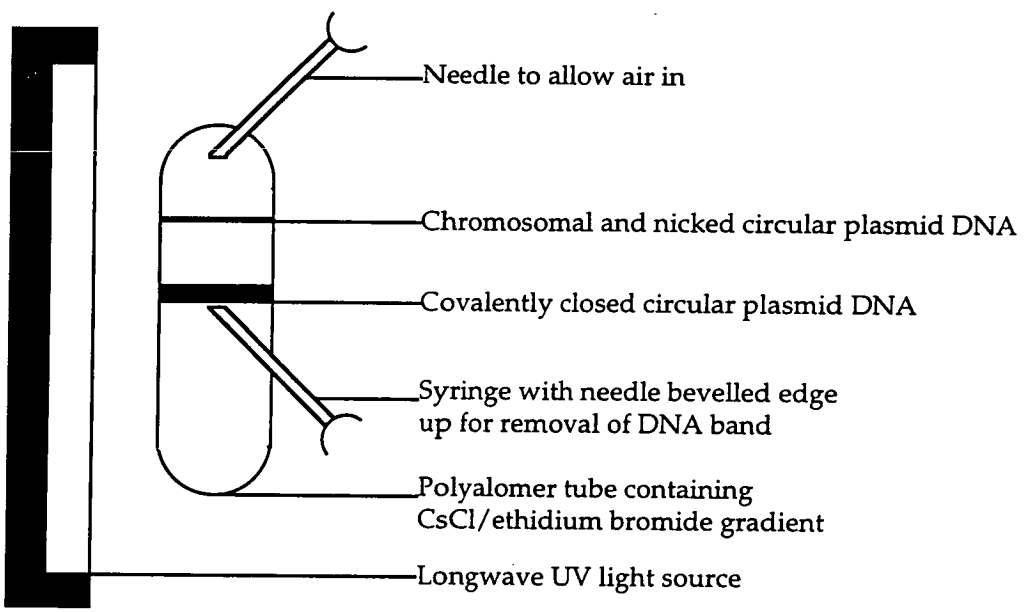


Figure 2.7: Removal of covalently closed circular DNA from a caesium chloride/ethidium bromide gradient: Upon examination under longwave UV light (366nm) two bands were seen; the smaller, upper band contained nicked plasmid and bacterial chromosomal DNA while the larger, lower band contained covalently closed circular plasmid DNA. A 19G needle was used to pierce the top of the tube to allow air in before a second needle (and 5.0ml syringe) was inserted 1.0cm below the lower band. With the bevelled edge facing upwards, the covalently closed circular plasmid DNA was removed in a total volume of about 2.0ml.

The plasmid DNA was recentrifuged (as above) and then removed. Ethidium bromide was then removed by repeatedly extracting with 2.0 volumes water-saturated isobutanol until the DNA solution was clear. After making up to 2.5ml with TE and loading on to an exclusion chromatography column (NAP-25 column; Pharmacia Biotech), previously equilibrated with TE, plasmid DNA was eluted by the application of 3.5ml TE to the column followed by an ammonium acetate/isopropanol precipitation step (section 2.2.5). DNA was pelleted by centrifugation for 45 minutes at 12,000g, 4°C (8,550rpm in Sorvall HB-4 rotor and Sorvall RC5C centrifuge), washed, air dried and resuspended in 200-1000µl TE. The DNA concentration was determined by spectrophotometry (section 2.2.2) and the identity of the plasmid was confirmed by restriction mapping.

2.2.13 Fragment Preparation for Electroporation

Targeting construct DNA (150µg) was *Asc* I digested and ammonium acetate/isopropanol precipitated (section 2.2.5) before digesting with *Mlu* I. Complete digestion was confirmed by checking 0.5µg of digested DNA on an agarose gel. After reprecipitation the DNA pellet was washed twice before transferring to the tissue culture labs under 150µl 70% (v/v) ethanol (for sterility).

2.2.14 Southern Blot Analysis

Southern blotting was performed as described in Sambrook *et al.* (288), a modification of the original method by Southern (302). The prehybridisation and hybridisation solution used in this method were taken from Church and Gilbert (303).

Restriction digests were set up using 20µl of ES cell DNA or 5.0-10µg tail DNA in a 40µl reaction volume with 15 units of the appropriate restriction enzyme and incubated overnight at 37°C. Samples were prepared for gel electrophoresis by adding 5.0µl of 6.0x Maniatis IV loading buffer and run on a 0.8% (w/v) agarose gel. Gels were depurinated in 0.25M HCl for 30 minutes with gentle agitation (followed by a brief rinse in ROP H_2O), before denaturation in 1.5M NaCl, 0.5M NaOH for 30 minutes and neutralising in 1.5M NaCl, 1mM EDTA, 0.5M Tris-HCl; pH 7.2, also for 30 minutes.

A Pyrex dish containing 20x SSC (3.0M NaCl, 0.3M trisodium citrate) with a glass plate over the top was set up with 2 sheets of Whatman 3mm paper acting as a wick into the 20x SSC solution. Two smaller sheets, cut to the gel size, were also placed on top. Gels were then placed upsidedown on the Whatman papers over the dish and surrounded with Parafilm. Nylon membrane (Boehringer Mannheim; Product code 1417240) was placed on top of the gel and two Whatman papers were individually wetted in 20x SSC and placed on the membrane. Finally, this was topped with paper towels and a glass plate, before the whole apparatus was sealed using Saran Wrap. An 800g weight was placed on top of this and the gel left blotting overnight. After blotting, DNA was cross-linked to the membrane by baking at 120°C for 30 minutes.

Filters were placed in a prewarmed Techne hybridisation bottle (Techne (Cambridge) Ltd, Cambridge, UK; Product code FHB-12) and 25ml of prewarmed Church and Gilbert prehybridisation solution (0.25M Na_2HPO_4, 7.0% (w/v) SDS, 1.0mM EDTA and 200mg/ml heat denatured sheared salmon sperm DNA) was added and incubated at 68°C for 2 hours to overnight (303) in a Techne hybridisation oven (Techne (Cambridge) Ltd; Product code HB-1). After prehybridisation, a heat denatured random primed probe was added and the blots hybridised overnight at 68°C. On completion of hybridisation, the probe solution was discarded and the blots were washed at 68°C, twice in 75ml of 2.0x SSC, 0.1% (w/v) SDS and twice in 75ml of 0.2 or 0.5x SSC, 0.1% (w/v) SDS (288). Blots were briefly air dried, wrapped in Saran Wrap and placed in a cassette for autoradiography with two enhancement screens. After 24 hours at -70°C, films were developed before re-exposing, if required, for a suitable amount of time to detect the desired signal.

Probes were labelled by a modification (304) of the original Feinberg and Vogelstein method (305, 306). Gel purified template DNA (50ng), 250ng random primer (100ng/µl) and TE to a final volume of 10µl were denatured at 100°C for 5 minutes before placing on ice. The remaining reagents were then added; 5.0µl 10x oligodeoxynucleotide labelling buffer (0.5M Tris-HCl; pH 6.9, 0.1M $MgSO_4$, 1.0mM DTT, 1.0mM dATP, 1.0mM dGTP, 1.0mM dTTP), 5.0µl α^{32}P-dCTP (3000Ci/mmol), 24µl sterile ROP water, 1.0µl 10mg/ml nuclease free BSA (Gibco-BRL; Product code 15561-012) and 2.0µl 2.0 units/µl Klenow enzyme. DNA was labelled at 37°C for 1 hour before

purifying the labelled probe from unincorporated nucleotides by passing through a NAP-5 column (Pharmacia Biotech) according to the manufacturers instructions. A 1.0µl sample was spotted onto a Whatman DE81 filter disc and used to determine the specific activity of the probe. Aliquots containing 2.5-5.0x10^7cpm were denatured at 100°C for 10 minutes before quenching on ice and adding to prehybridised blots.

2.2.15 DNA Sequencing

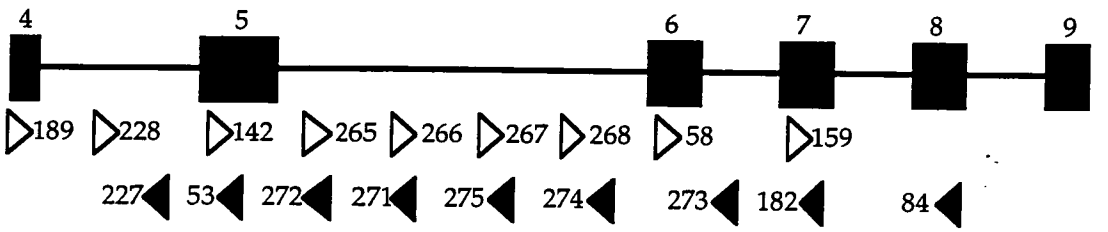
To determine how many, if any, errors were introduced by *UlTma* during the PCR amplification of the *Ren-1^d* homology arms, the entire 3' arm of the targeting construct and the corresponding region in the amplified product were sequenced. Binding sites of the sequencing primer used are shown in Figure 2.8 and the primer sequences are given in Table 2.9.

Sequencing was performed using the Amplitaq FS Kit from Applied Biosystems. Using this kit, dideoxynucleoside chain termination reactions were performed(307), a new DNA strand being synthesised from an oligodeoxynucleotide primer with extension terminating when a 2',3'-dideoxynucleoside-5'-triphosphate (ddNTP) was incorporated. Products were resolved on a denaturing polyacrylamide gel and detected by a laser (ABI 377 Automatic Sequencer). This apparatus can distinguish between the 4 ddNTPs because of different dye molecules which are covalently attached to each ddNTP. A modified version of the Amplitaq DNA polymerase (*Taq* based), Amplitaq FS, which incorporates dye terminators with a similar efficiency to that of normal dNTPs was used removing the need for an additional, phenol clean up step.

As Amplitaq FS is a *Taq* based enzyme, template extension is performed at 60°C and reactions can be cycled in a similar manner to that used for a PCR reaction. Cycle sequencing can provide better sequence data than normal dideoxysequencing using T7 DNA polymerase based enzymes because at the higher extension temperatures used template DNA has less secondary structures allowing the enzyme to proceed along the template unhindered.

Automated DNA sequencing required template DNA which was of a high quality (CsCl gradient-purified or Qiagen prepared DNA for plasmids

PCR Product Direct Sequencing



Cloned PCR Product Sequencing

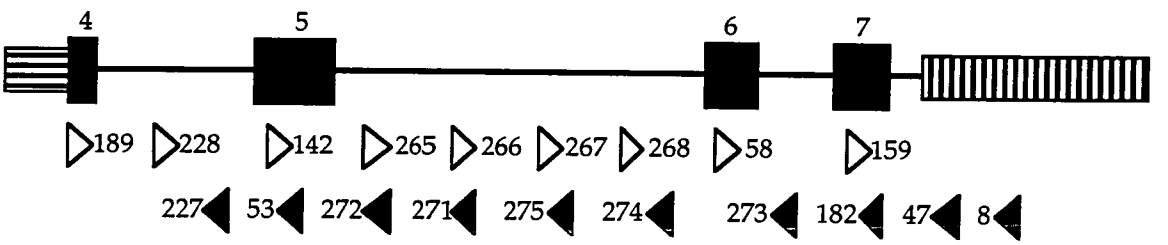


Figure 2.8: Location of primers used to sequence the 3' *Ren-1*^{d} homology arm: Schematic representation of the 3' end of the *Ren-1*^{d} gene (filled boxes are exons) either as PCR amplified from P1 template DNA or after cloning into the *Ren-1*^{d} targeting construct, pR1*neo*KO with location and orientation of PCR primers shown as arrow heads (forward primers are shown as arrowheads and reverse primers shown as filled arrowheads). Horizontally striped boxes represent the polyadenylation sequence of the PGK-*neo*pA selection cassette and vertically striped boxes represent pSP72 plasmid sequence.

or gel purification followed by Qiaex preparation or by using a Promega PCR Clean Up Column for PCR products). Sequencing reactions were performed and prepared for denaturing polyacrylamide gel electrophoresis according to the manufacturers instructions, except that 10.0µl, rather than 20µl, reactions were used in order to economise on materials.

To obtain the maximum sequence information, samples were loaded and run on a 1.0x TBE (90mM Tris-borate, 2.0mM EDTA; pH 8.0), 6.0M urea and 5.0% (29:1 acrylamide:bis-acrylamide) polyacrylamide gel. Gels were cast in gel frames supplied with the ABI 377 automatic sequencer (36cm run length from wells to laser) using ABI 0.2mm spacers and a 36 slot sharks tooth comb according to the manufacturers instructions. Polymerised gels were mounted in the apparatus and the top and bottom buffer reservoirs filled with 1.0x TBE. Samples (resuspended in 3.0µl Gel Loading buffer) were denatured at 100°C for 5 minutes and cooled rapidly on ice prior to

Lab ID	Primer Description	Sequence
JJM 189	Exon 4 Forward	GCGGTACCAG CTACATGGAG AACGGGTC
JJM 228	Intron D Forward	CAGTGTCTAA GCCTGTTCTG G
JJM 227	Intron D Reverse	GATGCCCTCG TCATAACCAG
JJM 142	Exon 5 Forward	CGGGATCCAG TTTGACGGGG TTCTAGG
JJM 53	Exon 5 Reverse	CCTGGGAGAG AATGTGG
JJM 265	Intron E Forward (E1)	CCCTCCATCC TCACAGAGCT
JJM 272	Intron E Reverse (E8)	CCCCTGCTGG GCAGTGAGCT
JJM 271	Intron E Reverse (E7)	TGACATCTAC TTCCACTCTT
JJM 266	Intron E Forward (E2)	GGGGACTCCG CAAAATGTGG
JJM 267	Intron E Forward (E3)	ACAAATGTGT GTTGTGAATT
JJM 275	Intron E Reverse (E61)	CTCCACGGGC ATGATGCTAC
JJM 268	Intron E Forward (E4)	AATGTCCTTG AGCTAGACAG
JJM 274	Intron E Reverse (E51)	ATCTTCCCTT GCTGGAGCCT
JJM 58	Exon 6 Forward	CCTGGCAGAT CACAATGAAG G
JJM 273	Intron F Reverse (F1)	AGGACAGAGC AAGCAGAGAG
JJM 159	Exon 7 Forward	TCATGCAAGC CCTGGGAGTC
JJM 182	Exon 7 Reverse	CTCCCAGGGC TTGCATG
JJM 47	T7	AATACGACTC ACTATAG
JJM 8	pSP72 *Bgl* II to *Xho* I	CAATACGCAA ACCGCCTC
JJM 84	Exon 8 Reverse	CGTAGTCCGT ACTGCTGAGT GTGTAGGCCC TGCCTCCC

Table 2.9: Sequence of primers used to sequence the 3' *Ren-1*^{d} homology arm: Table lists all sequencing primers used by lab ID number, gives a description of where each primer binds (and its orientation) and lists the nucleotide sequence.

loading. Gels were run overnight for 10 hours at 150W with a constant running temperature of 51°C. Other parameters were limited to 1680V and 50mA. The following day, gel computer images were scanned by eye to check for correct tracking of samples before extracting sequence information.

2.2.16 Mouse Tail DNA Preparations

This method is based on a Doug Hanrahan (Cold Spring Harbor cloning course) protocol originally adapted from various standard methods for preparing high molecular weight DNA (288). Following application of local anaesthetic (ethyl chloride BP; Syntex Pharmaceuticals Ltd,

Maidenhead, UK) the end 1.0cm of tail was removed from a 4-5 week old mouse and stored at -20°C until needed. Cut tails were sealed with Vet Seal tissue glue (B. Braun, Malsungen, Germany) and the mouse was eartagged for later identification. To digest the tissue, 600µl of tail buffer (50mM Tris-HCl; pH 8.0, 100mM EDTA, 100mM NaCl, 1.0% (v/v) SDS) and 35µl 10mg/ml Proteinase K (Boehringer Mannheim; Product code 1092 766) were added before incubating at 55°C overnight, inverting tubes to mix. Subsequently, RNA was degraded by incubation at 37°C for 1 hour after the addition of 20µl of 20µg/ml DNase-free RNase (RNase A (Sigma-Aldrich Company Ltd; Product code R-4875) made DNase-free by heating a 10mg/ml solution made in 10mM Tris-HCl; pH 7.5, 15mM NaCl at 100°C for 15 minutes).

To minimise shearing of high molecular weight DNA, "cut off" blue pipette tips were used for subsequent stages. Tail digests were extracted with 600µl phenol, 300µl buffered phenol/300µl chloroform; isoamyl alcohol (24:1) and finally 600µl chloroform; isoamyl alcohol (24:1). In each case samples were rotated on a vertical rotator for 15 minutes, phases separated by centrifugation for 2 minutes and the aqueous phase, along with any interphase, transferred to a fresh tube (in the final extraction stage the interphase was avoided). DNA was precipitated by adding 600µl isopropanol and pelleted by centrifugation for 2 minutes before resuspending in 200µl of TE (4°C, overnight). DNAs were reprecipitated with ammonium acetate and isopropanol (section 2.2.5) and pelleted by centrifugation for 2 minutes. Pellets were washed, air dried briefly, resuspended in 100µl of TE (4°C, overnight) and quantitated (section 2.2.2).

2.2.17 RNA Preparation

RNA was prepared by the guanidine isothiocyanate/phenol method (308) using the following freshly made stock solutions:- Stock Solution A (4M guanidinium thiocyanate, 25µM sodium citrate; pH 7.0, 1.0% (w/v) sarcosyl, 0.1M ß-mercaptoethanol, made up to 50ml using DEPC treated H_2O and adjusted to pH 7.0 with 1.0M NaOH) and RNAzol Stock Solution (1.0 volumes of Stock Solution A, 1.0 volumes of phenol and 0.1 volumes of 2.0M sodium acetate; pH 4.0).

Tissues were homogenised in 2.0ml RNAzol stock solution using a

Janke and Kunkel Ultra-turrex T25 homogeniser (supplied by Sartorius Ltd, Epsom, UK). Samples were placed on ice for 5-15 minutes before adding 200µl chloroform to each homogenate and mixing by shaking vigorously for 15 seconds. These were left on ice for 15 minutes before centrifugation in a Heraeus Varifuge 3.2RS at 3200rpm (2,200g) for 5 minutes to separate the phases. The top, aqueous layer was removed, avoiding the interface (contains DNA and protein) and transferred to a 2.0ml, screw top tube. RNA was precipitated overnight at -20°C by adding an equal volume of isopropanol. RNA was pelleted by centrifugation for 10 minutes, washed twice with 1.0ml of 70% (v/v) ethanol before air drying and resuspending in 100µl of 100% deionised formamide (protects against degradation of RNA samples by RNase) (309). Finally RNA recovery was quantified by spectrophotometry (section 2.2.2) and RNA quality was assessed by running 1.0µg of each sample on an agarose gel.

2.2.18 Primer Extension

Primer extensions were performed using a 38mer oligodeoxynucleotide complimentary to exon 8 which distinguishes between *Ren-1*^{d} and *Ren-2* as originally reported by Field and Gross (130). Briefly, primer was annealed to RNA and products extended using reverse transcriptase and a dATP, dGTP, dTTP and ddCTP mix resulting in a 5bp extended product from *Ren-1*^{d} and a 17bp extended product from *Ren-2* (Figure 2.10). These were then resolved on a denaturing polyacrylamide gel.

Primer was end labelled in a reaction containing 20ng (or 1.8pmol) oligodeoxynucleotide, 1.0x Kinase buffer (50mM Tris-HCl; pH 7.6, 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine and 0.1mM EDTA), 8.0µl γ^{32}P-ddATP (>5000 Ci/mmol) and 1.0µl (10 units) Polynucleotide Kinase made up to 15µl with DEPC H_2O. This was incubated at 37°C for 30 minutes before removing excess γ^{32}P-ddATP (and other dNTPs) by passing through a TE equilibrated NAP-5 column (Pharmacia Biotech). A 10µl aliquot of the labelled primer was counted and used to calculate cpm/µl. For each reaction, 2.0-4.0x10^5 cpm were co-precipitated with RNA, the quantity of which depended upon the abundance of the transcript from that tissue (*i.e.* for detection of renin 80µg kidney or 0.8µg DBA/2J submandibular gland total RNA was used). RNA and labelled primer were aliquoted before adding carrier RNA or glycogen and 0.1 volumes of 3M sodium acetate; pH 5.5. Samples were

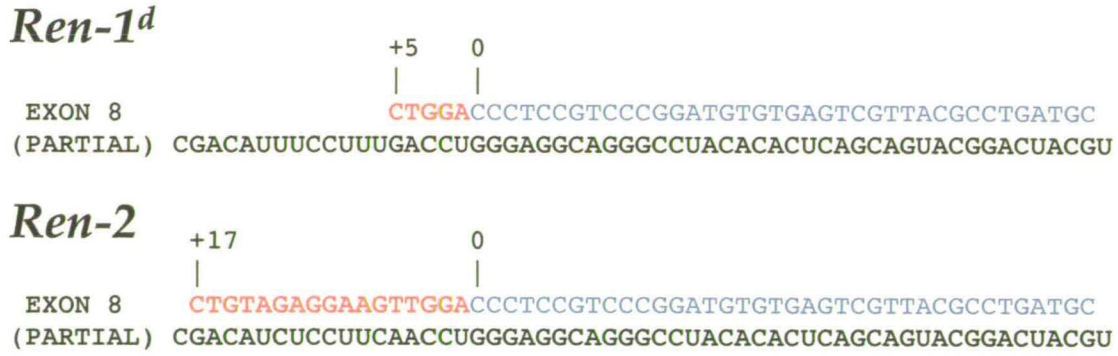


Figure 2.10: *Ren-1^d/Ren-2* diagnostic primer extension: The chosen extension primer binds to the same sequence in *Ren-1^d* or *Ren-2* and when extended, using reverse transcriptase and a ddCTP mix, results in differently sized products. Part of the mouse renin mRNAs coded for by exon 8 are shown (black), above which the extension primer (blue) and extended region (red) are depicted. The most 3' base of the primer is numbered 0 with extension products for *Ren-1^d* and *Ren-2* terminating at +5 or +17 bases respectively.

mixed thoroughly before the addition of 2.5 volumes of 100% ethanol and then mixed again.

After precipitation overnight at -20°C, samples were centrifuged for 30 minutes and the supernatant removed. Each pellet was dissolved in 4.0μl of DEPC H_2O and 32μl of Phosphate Formamide buffer (10mM NaH_2PO_4, 10mM Na_2HPO_4 in deionised formamide adjusted to pH 6.5). Pellets were completely resuspended before adding 4.0μl of Hybridisation buffer (4.5M NaCl, 0.1mM EDTA and 75mM Tris-HCl; pH 7.5). After mixing, samples were denatured at 80°C for 10-15 minutes before hybridising overnight at 42°C (determined empirically by Field and Gross (130)). Reactions were precipitated with 1.0 volumes 4.0M ammonium acetate and 2.0 volumes 100% ethanol, mixed thoroughly and left on dry ice for 1 hour.

Primer and RNA was spun down for 30 minutes and the pellets were washed, finally removing all ethanol traces with a P2 tip. Each pellet was completely resuspended in 50μl of Primer Extension buffer (10μl 5.0x Promega RT buffer, 2.0μl 5.0mM ddATP, 1.0μl 10mM dCTP, 1.0μl 10mM dGTP, 1.0μl 10mM dTTP, 34.5μl ROP dH_2O and 0.5μl Reverse Transcriptase) and incubated at 42°C for 90 minutes (tubes were flicked after 30 minutes to ensure complete resuspension).

RNA was specifically hydrolysed at 42°C for a further 2 hours by adding 50μl of 0.4M NaOH. This was neutralised by the addition of 50μl 1.0M Tris-HCl; pH 6.8. Carrier DNA (2.0μl of 1.0μg/μl pBluescript) was

added before precipitating reactions with 1.0ml 100% ethanol at -20°C overnight. DNAs were spun down for 1 hour and the pellets were washed twice, finally removing all traces of ethanol with a P2 tip and resuspending each pellet in 2.0µl DEPC H_2O and 4.0µl Gel Loading buffer (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF).

Samples were run on a 1.0x TBE, 8.3M urea and 12% polyacrylamide gel (19:1, acrylamide:bis-acrylamide) after prerunning the gel for 10-15 minutes. Gels were run at 35W (constant) for about 3-4 hours and fixed in 10% methanol, 10% acetic acid for 15 minutes before blotting onto prewetted Whatman 3MM paper, drying for 2 hours at 80°C under vacuum and exposing to film for 2-7 days. Expression was quantified by 7 day exposure in a phosphoimager cassette after adjusting for sample recovery by comparison of the signals to the primer band.

2.3 Tissue Culture Techniques and the Generation of Chimaeric Mice

2.3.1 ES Cell Culture

All cells were maintained in 6.0-7.5% CO_2, at 37°C in a humidified incubator (Heraeus; model B5060 EC/CO2) as described by Smith (294) with manipulations being performed inside a laminar flow sterile hood (Gelair ICN Flow Hood (Class 3), ICN Pharmaceuticals Ltd, Thame, UK) using culture grade plastic supplied by Corning (Bibby Sterilin, Stone, UK) or Nunc (Supplied by Gibco-BRL). All surfaces (including arms and hands) were sprayed with 70% industrial methylated spirits (BDH) before use to prevent bacterial or fungal contamination.

ES cells were cultured on 0.1% gelatine-coated (Sigma-Aldrich Company Ltd; Product code G-2500) tissue culture flasks in 1.0x GMEM (Gibco-BRL; Product code 12541-025) supplemented with 10% (v/v) foetal bovine calf serum (FCS; Globepharm, Surrey, UK), 0.1µM ß-mercaptoethanol, 2.0mM L-glutamine (Gibco-BRL; Product code 25030-024), 1.0mM sodium pyruvate (Gibco-BRL; Product code 11360-039), 1.0x MEM non-essential amino acids (Gibco-BRL; Product code 118110-017), and 0.23% (w/v) sodium bicarbonate (Gibco-BRL; Product code 11140-035).

Medium was made in 500ml aliquots and stored at 4°C for 2 to 4 weeks. DIA/LIF was also included at a 1:1000 dilution to maintain the ES cells in an undifferentiated state (292, 293). The term "ES cell medium" will now be used to refer to the medium described above including DIA/LIF. Cells were routinely washed in phosphate buffered saline (PBS) during many of the described protocols. PBS is 137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4 and 1.4mM KH_2PO_4; pH 7.3 and was made using one PBS tablet (Unipath, Basingstoke, UK; Product code BR14A) dissolved in 100ml of filter-sterilised UHP water before filter sterilising.

Cells were passaged by rinsing twice with PBS before adding enough TVP (0.025% trypsin (Gibco-BRL; Product code 25090-010), 1.0% chicken serum (Gibco-BRL; Product code 16110-033), 0.5mM EDTA dissolved in PBS) to just cover the cells. The flask was then incubated at 37°C for 2-3 minutes before neutralising the trypsin by adding ES cell medium. The medium in the cell suspension was pipetted several times to generate a single cell suspension. Cells were centrifuged for 5 minutes at 1,200rpm (approximately 200x*g* in a Denley BS400 Benchtop Centrifuge; Supplied by Life Sciences Intl. (UK) Ltd, Basingstoke, UK), counted using a hemacytometer and $1.0x10^6$ cells used to seed a $25cm^2$ flask containing pre-warmed medium (or scaled up for larger flasks).

2.3.2 Freezing and Thawing Cells

Cells were trypsinised and pipetted to obtain a single cell suspension and centrifuged at 1,200rpm for 5 minutes. The pellet was resuspended in freezing mix (ES cell medium + 10% (v/v) DMSO) and the number of cells were counted. After recentrifugation, cells were resuspended in 0.5ml freezing mix per $5.0x10^6$ cells and 0.5ml aliquots were transferred to cryotubes. These were frozen at -80°C overnight before transferring to a liquid nitrogen cell bank (Minnesota Valley Engineering Cryogenics; Product code XLC110; Supplied by Cryotecnics, Edinburgh, UK).

Frozen cells were thawed in a 37°C water bath, transferred to a 15ml Corning tube containing 15ml of ES cell medium and centrifuged. The cell pellet was resuspended in 10ml fresh ES cell medium and this was seeded into a $25cm^2$ flask. After 8 hours the medium was removed and fresh ES cell medium was added to remove final traces of DMSO.

2.3.3 Electroporation of ES Cells

Two confluent 150cm^2 flasks per electroporation (5.0x10^7 to 1.0x10^8 cells required per electroporation) were trypsinised in the normal manner and resuspended in PBS. The number of cells were calculated before recentrifuging in a 15ml Corning tube and resuspending to a final volume of 700µl with PBS. DNA for electroporation (resuspended in 100µl of sterile H_2O) was added to the cells and mixed. Electroporation was in a 0.4cm cuvette using the following settings on the Biorad Gene Pulser (Biorad Laboratories Ltd; Model no. 1652087) and Capacitance Extender (Biorad Laboratories Ltd; Model no. 1652078); Capacitance= 3.0µF and Voltage= 0.8kV, resulting in a typical time constant of 0.1 msec. The cells were transferred as quickly as possible to warm ES cell medium and plated out into twenty 90mm cell culture dishes, at two dilutions (10-fold difference). A plate of untransfected cells was seeded at the same time so that when G418 selection was applied it would be apparent when all sensitive cells had been killed. Twenty-four hours after electroporation, selection was started by including 175µg/ml G418 (Boehringer Mannheim; Product code 1464981) in the medium which was changed every 1-2 days depending on the number of cells and dead matter present in a dish. If a lot of dead cells were present the dish was washed twice with 5.0ml PBS before adding the fresh ES cell medium.

2.3.4 Expansion of G418 ES Cell Colonies

After selection in G418 for 7-8 days, clones were picked and expanded for freezing (storage) and DNA preparation (screening). ES cell medium (2ml) was aliquoted into each well of gelatinised 24-well plates and warm TVP (30µl) was aliquoted into 96-well plates before maintaining all plates at 37°C. A dish was washed twice in 10ml PBS and 7.0ml of PBS added to keep the cells hydrated. Individual clones were picked by scraping a colony with a P2 tip fitted to a P20 pipette lifting about 10µl of PBS with the cells and pipetting into the prewarmed TVP. Batches of 24 clones at a time were trypsinised before incubating at 37°C for 5 minutes and transferring to the gelatinised 24-well plates containing pre-warmed ES cell medium. Clones were grown to confluence, with feeding at least every 2 days. Clones at similar degrees of confluence were passaged together in an attempt to synchronise all clones on a plate. Cells were split 1:2 onto replica 24-well

plates (washed with 100µl PBS, trypsinised with 100µl of TVP then aliquoted, 30-40µl to a "freezing plate" and the rest to a "DNA plate"). After 24 hours, medium in the "freezing plates" was replaced with 250µl of Freezing Mix (ES cell medium + 10% (v/v) DMSO) and plates were frozen at -20°C before transferring them to -80°C for long term storage (310). "DNA plates" were left for a further 24-48 hours (until every well was confluent) to increase DNA yield.

2.3.5 ES Cell DNA Preps

DNA was prepared from ES cell colonies by a modification of the method by Laird *et al.* (311). Plates were aspirated and transferred to the molecular biology lab where 600µl of Lysis buffer (100mM Tris-HCl; pH 8.5, 5.0mM EDTA, 0.2% (w/v) SDS, 200mM NaCl and 100µg/ml Proteinase K (Boehringer Mannheim; Product code 745723)) was added to each well before incubating at 37°C overnight. Each Proteinase K digest was then transferred to a labelled tube and precipitated with 600µl isopropanol. Samples were mixed by rotating for 5-10 minutes and DNA was then pelleted by centrifugation for 15 minutes. Pellets were washed twice, air dried and resuspended in 100µl TE (aided by incubating at 65°C for 30 minutes and pipetting up and down if required).

2.3.6 Production of Chimaeric Mice and Breeding of Mutant Animals

Chimaeric mice were produced according to Bradley (312) as modified by Nichols (313). Blastocysts used for the production of chimaeras were flushed from C57BL/6J female mice on the fourth day of pregnancy with PB1 medium supplemented with 10% (v/v) FCS (100ml PB1 medium was made by dissolving the following in 100ml UHP H_2O resulting in a solution of pH 7.0-7.2; 822mg NaCl, 21mg KCl, 300mg Na_2HPO_4, 20mg KH_2PO_4, 104mg glucose, 4.5mg sodium pyruvate, 6.2mg penicillin 1.4mg $CaCl_2$, 1.0mg $MgCl_2$ and 1.0ml 1.0% phenol red). Unexpanded embryos were transferred to ES cell medium and placed in a humidified incubator at 37°C with 6.0% CO_2 to allow the blastocoel cavities to expand fully. For injection, blastocysts were transferred to small hanging drops of PB1 medium supplemented with 10% (v/v) FCS on a siliconised (Repelcote; BDH; Product code 63216 4J) coverslip suspended over a manipulation chamber (custom-made by Gary Robertson,

CGR, University of Edinburgh, Edinburgh, UK) flooded with liquid paraffin (Boots The Chemists). Trypsinised ES cells, generally singletons or pairs, were also placed in a hanging drop and the chamber refrigerated for at least 10 minutes.

Blastocyst injections were performed with a rounded holding pipette (internal diameter= 20μm) and a heat polished, flat ended injection pipette (internal diameter= 15μm), both pulled from glass capillary tubing (internal diameter= 100μm) using an electrode puller (Camden Instruments, Loughborough, UK), cut to size and heat polished with a microforge (DeFonbrune; Supplied by Micro Instruments Ltd, Witney, UK). Holding and injection pipettes were each attached to instrument holders (Ernst Leitz, Wetzlar, Germany) and operated by Leitz manipulators. The suction of both pipettes was controlled by specialised injectors (Narashige International Ltd, London, UK; holding pipette and DeFonbrune; injection pipette) by means of paraffin filled plastic tubing. An IMT2 image-corrected microscope (Olympus Optical Company (UK) Ltd, London, UK) was integrated into the micromanipulation assembly.

Between 10 and 20 cells were injected into each blastocyst. Operated embryos were allowed to recover for a few hours in ES cell medium in the incubator before being transferred to pseudopregnant recipients. Recipient MF1 females had been mated with vasectomised DBA/2J males 2.5 days previously. Embryos, typically 6-12, were transferred to one uterine horn of a recipient female. Contribution of ES cells to resulting pups was assessed by coat colour (sandy coloured hairs amongst the host black hairs indicated successful incorporation of the ES cells into the resulting individual). Germ-line transmission was assessed by crossing male chimaeras with female 129/Ola females. Generation of agouti pups indicated inheritance of a compliment of chromosomes from the host embryo. Generation of sandy coloured pups, however, indicated germ-line transmission and these animals were subsequently screened by Southern blot for inheritance of the disrupted allele. Heterozygote animals were intercrosseed to generate homozygote mutant animals.

2.4 Physiological Techniques

2.4.1 Blood Pressure Measurement

Mean blood pressure (40% of systolic + 60% of diastolic pressures) was measured via direct cannulation of the abdominal aorta in adult mice (8-15 weeks old) as previously described (286). Briefly, a cannula made from drawn polyethylene tubing (internal diameter= 11μm; Portex, Hythe, UK; Product code 800/100/100) or micro renathane tubing (internal diameter= 12μm; Braintree Laboratories Inc, Braintree, Massachusetts, USA; Product code MRE025) was inserted into the aorta (blood flow in the aorta and vena cava was occluded using a suture) and fixed in place using Vet Seal tissue glue. To prevent an animal biting through it's cannula, it was passed under the skin and brought out at the top of the neck, on the back of the head, roughly between the ears. Cannulas were filled with heparin-saline, flushed daily and prevented from leaking by the insertion of the end of a blunted, blocked (with paraffin wax) 26G needle. Blood pressure was measured 24 hours post operation by connecting the cannula to a pressure transducer (Viggo-Spectralab, Oxnard, California, USA) and printing the output on a chart recorder. Measurements were made over a 15 minute period in conscious, resting animals housed in restraining tubes. All animals had undergone training in restraining tubes for five consecutive days (beginning 7 days before the operation) for at least 30 minutes each day. Statistical significance was assessed using a Wilcoxon rank test and a Student t-test (unpaired). Results are given as mean blood pressure±SEM.

2.4.2 Prorenin and Renin Protein Assays

Animals were sacrificed by inhalation of 100% CO_2, and blood sampled immediately by cardiac puncture into 0.1 volumes of 125mM EDTA, 25mM Ω-phenanthroline. After centrifugation for 3-5 minutes plasma was snap-frozen in liquid nitrogen in 100μl aliquots. Plasma renin concentrations (PRC) and plasma prorenin concentrations (PPC) were calculated according to the method of Peters *et al.*(314).

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 5.0µl 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 (^{125}I-angiotensinogen), isolated from nephrectomised rat plasma (final concentration; 80mg/ml, 0.11% 2,3-dimercapto-1-propanol, 1.15mg/ml 8-hydroxychinolin 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 (315, 316). The 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±SEM.

2.4.3 Histological Analysis

To characterise any histological changes occurring in the *Ren-1^d* -/-, animals tissues from all three genotypes and both sexes were studied for abnormalities. The tissues studied were the submandibular glands, adrenal glands, kidneys and testes or ovaries.

2.4.3.1 General Histology

Animals were sacrificed by inhalation of 100% CO_2 and tissues were immersion-fixed in formal saline histological fixative (4.0% (w/v) formaldehyde in a 153mM NaCl solution; BDH Laboratory Supplies; Product code 36136 7L) for 24 hours before long-term storage in 70% (v/v) ethanol. Tissues were processed using a Citadel tissue processor (Shandon Southern Products Ltd, Cheshire, UK) on an 18 hour program passing through serial dehydration steps in graded ethanol concentrations (70%, 80%, 90% and 3x 100%), followed by dealcoholisation with a clearing agent, Histoclear (National Diagnostics, Atlanta, Florida, USA) x3 and paraffin wax immersion x2 at 60°C. Paraffin blocks containing tissues were then sectioned (2.0µm), dewaxed in xylol, stained with haematoxylin and eosin and mounted with

DPX Mountant. In addition kidney sections were also stained using a periodic acid-Schiff technique (PAS) to specifically stain the tissue carbohydrates of extra-cellular matrix. All sections were examined, in a blinded manner, by standard light microscopy (Leitz Diaplan or Leitz Laborolux S) and photographed with a Leica Wild MPS45 or MPS46 camera.

2.4.3.2 Immunohistochemistry

Kidneys were processed and sections were cut as described in the General Histology Method. These were immersed in TBS (tris-buffered saline) followed by blocking in 1.0% (v/v) H_2O_2 for 20 minutes before rinsing in TBS. Sections were trypsinised for 10-20 minutes and rinsed and washed in TBS. After covering the sections in normal rabbit serum (D4KO) which had been diluted 1:5 in TBS for 10 minutes they were drained and the primary polyclonal antiserum applied (provided by T. Inagami, Howard Hughes Medical Institute, Nashville Tennessee, USA). This was diluted 1:64,000 in 1:5 normal serum before rinsing twice in TBS (317). Secondary antibody was then applied (biotinylated sheep anti rabbit) and left for 30 minutes (kit supplied by Vector Laboratories Ltd., Peterborough, UK). Sections were rinsed twice in TBS before applying the avidin/biotin complex for 30 minutes. Finally sections were rinsed twice in TBS, washed well in TBS and mounted. All TBS washes were for 5 minutes each.

2.4.3.3 Electron Microscopy

Kidney sections from male wild-type, heterozygote and homozygous mutant animals were studied for any structural abnormalities by electron microscopy. Cubes of kidney cortex ($1.0mm^3$) were fixed in 3% gluteraldehyde solution (3.0% gluteraldehyde in 0.2M sodium cacodylate; pH 7.2) overnight and stored in 0.2M sodium cacodylate solution until processing (about 2-3 days). Sodium cacodylate solution (0.2M) is made by mixing 25ml Solution A (4.28g sodium cacodylate in 100ml dH_2O) with 2.1ml Solution B (1.7ml HCl made up to 100ml with dH_2O) and making up to 100ml with dH_2O. Tissues are then osmic acid treated (0.5g osmium tetroxide dissolved in 25ml 0.2M cacodylate buffer and 25ml dH_2O) for 2 hours before dehydrating in alcohol (3x 10 minutes in 10% (v/v) ethanol and 3x 30 minutes in 100% ethanol). Tissues were then prepared for embedding by immersing in epoxy propane twice (20 minutes each).

Embedding was in an araldite based resin (stored at 4°C) made from 38ml stock solution X (200ml of araldite resin CY212 and 200ml DDSA) and 2.0ml stock solution Y (20ml dibutylphtalate and 5.0ml BDMA accelerator). Araldite solution was removed from the fridge, equilibrated at room temperature and placed at 60°C for 15 minutes prior to use to make the resin less viscous and therefore easier to work with. An impregnation mould was then filled with 5.0ml resin using a 5.0ml syringe. Most of the epoxy propane was removed from the sample before transferring tissues to the impregnation mould with forceps (ensuring that the sample did not dry out). Blocks were left to impregnate overnight at room temperature. Finally tissues were embedded in resin by placing the desired number of embedding capsules in drilled wooden holders and filling each to the top with araldite. Tissues were transferred from the impregnation moulds using forceps. The holder, containing the capsules, was then incubated at 60°C for 3 days to allow polymerisation of the araldite to occur. Blocks were then cooled to room temperature, turned upsidedown and the individual araldite capsules were forced out using a press. Sections of 0.1μm were cut onto copper grids using a diamond knife before counterstaining with osmium tetroxide and lead citrate. Grids were viewed on a Philips CM12 transmission electron microscope with the "beam voltage" set at 80kV.

CHAPTER 3

PCR Amplification of Homology Arms

3.1 Introduction

The development of gene targeting, a methodology allowing the ablation or mutation of a specific gene, is proving to be a powerful tool in dissecting the function of a particular gene (318). The generation of a null mutation in the *Ren-1^d* gene of 129/Ola mice was chosen to assess directly the function of the Renin-1^d protein in the "two-renin gene" mouse.

As discussed in Chapter 1 the use of PCR to generate homology arms should reduce the time taken to build targeting vectors, allow more flexibility in positioning of homology arms and requires less information about the gene to be disrupted. In an attempt to develop this strategy it was decided to investigate the feasibility of PCR amplifying large DNA fragments from isogenic genomic DNA for use in targeting constructs, thus removing the need to screen genomic libraries.

Early attempts at amplifying homology arms from genomic DNA were not successful (Sharp *et al.*, unpublished data) therefore we chose an alternative, less complex template. P1 clones containing the renin genes (cloned from a 129/Ola ES cell line) were available in the laboratory and were utilised as templates instead. A potential problem with this strategy is the introduction of mutations during the PCR reaction. In order to minimise this risk a high fidelity PCR reaction was required. We aimed to achieve this using the thermostable DNA polymerase enzyme, *UlTma*, which included a 3' to 5' proof-reading exonuclease activity.

In this chapter the optimisation of PCR amplification of fragments of about 4.5kb is described. Sections on the optimisation of ligation conditions and the cloning of two *Ren-1^d* derived homology arms after restriction digestion of the PCR products are also included.

3.2 PCR Amplification of *Ren-1^d* Homology Arms For Gene Targeting

The gene targeting strategy chosen involved part of exons 3 and 4 of the *Ren-1^d* gene being replaced by the selectable marker PGK-*neo*pA (Figure 3.1). In order to do this, homology arms flanking the region to be deleted were produced from a strain 129/Ola-derived P1 clone by PCR amplification. The building of the targeting construct is outlined in Figure 3.1 (each step is

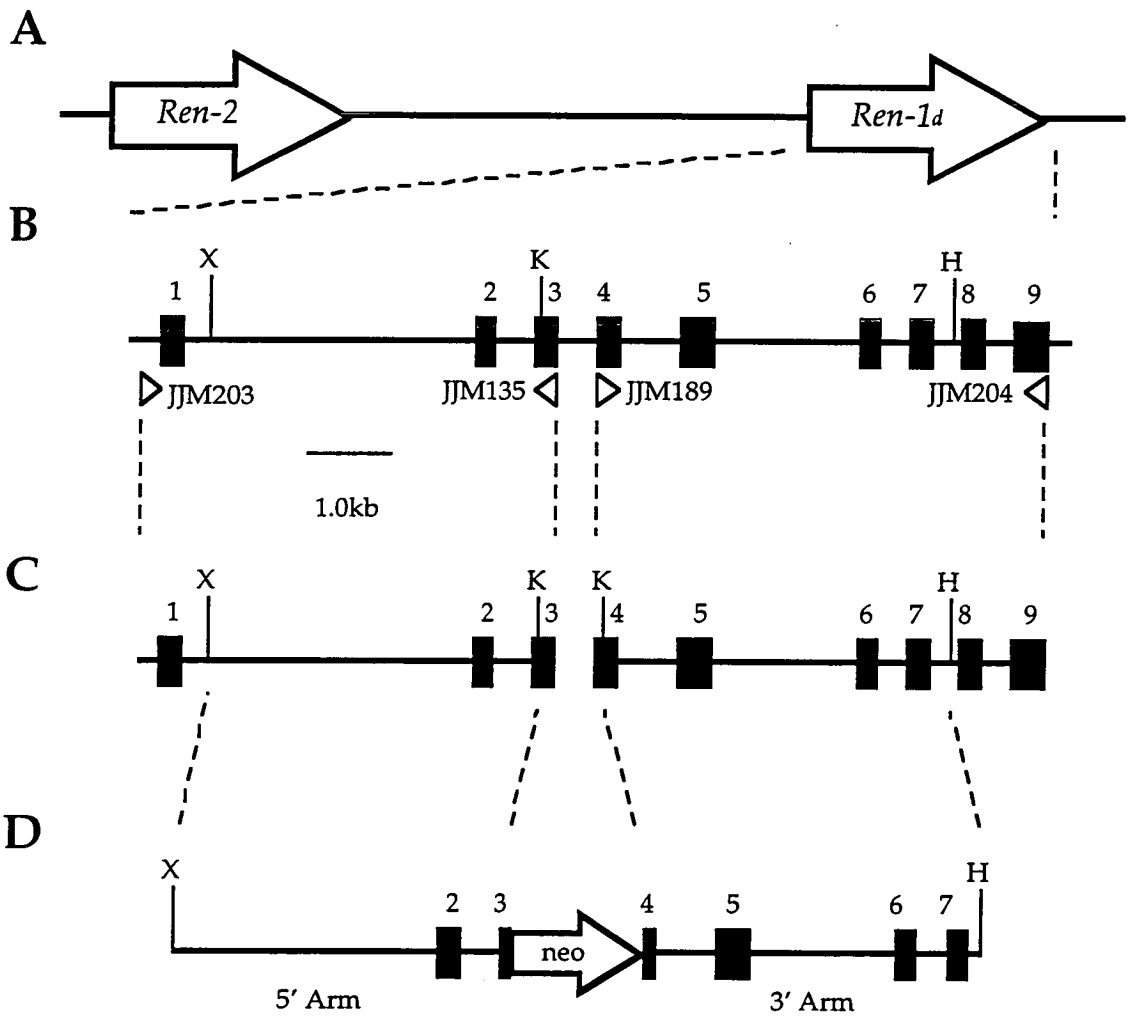


Figure 3.1: Gene targeting strategy for the disruption of the *Ren-1^d* gene using PCR to amplify the 5′ and 3′ arms of homology: A) The mouse 129/Ola *Ren* locus (arrows represent the direction of transcription). B) Enlarged view of the *Ren-1^d* locus with PCR primer pairs used (JJM203/135 and JJM189/204) indicated by open triangles (numbered black boxes represent exons). C) To facilitate cloning of the 5' and 3' PCR products, amplicons were digested internally, with *Xba* I (5' arm) or *Hin*d III (3' arm) and within the primers (*Kpn* I). D) The targeting construct deletes 92bp of exon 3, the third intron and 35bp of exon 4 and replaces them with a PGK-*neo* cassette in the same transcriptional orientation as the *Ren-1^d* gene. Only relevant restriction sites are shown:- H= *Hin*d III, K= *Kpn* I and X= *Xba* I.

discussed in more detail in this chapter and section 4.2). The resulting targeting construct contained a 3.7kb segment of *Ren-1^d* genomic sequence from intron A to exon 3, a PGK-*neo*pA selection cassette in the same transcriptional orientation as *Ren-1^d* and 3.6kb of *Ren-1^d* genomic sequence from exon 4 to intron G.

3.2.1 Size Range Amplification

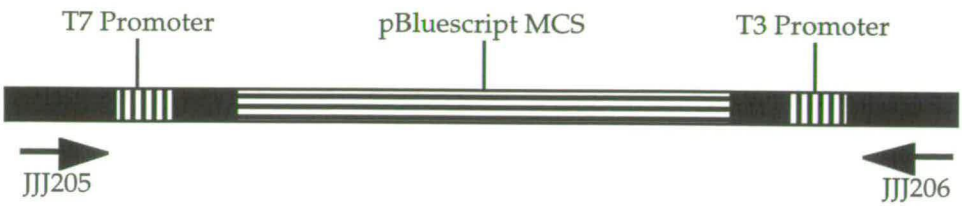
In order to test the ability of *UlTma* DNA polymerase to amplify large fragments, it was used to amplify a range of fragments inserted in pBluescript vectors (Figures 3.2). Template DNA (20pg) was amplified as described in section 2.2.6.2 for 40 cycles, annealing at 64°C for 6.5 minutes using the PCR primers JJM205 (CAGGGTTTTCCCAGTCACGA C) and JJM206 (CCAGTATCGACAAAGGACACAC).

Figure 3.2C shows the results of these amplifications in which the largest fragment detectable was 3.5kb (lane 5) and the largest attempted PCR fragment (predicted to be 4.9kb) was not detectable (lane 6). It was decided, therefore, to attempt to amplify the homology arms using *UlTma* DNA polymerase under these conditions. It may have been possible to amplify the larger fragment using this enzyme by altering the PCR conditions. Instead, however, it was decided to attempt to amplify the two *Ren-1^d* homology arms which were predicted to be 4.3 and 4.7kb. These are larger than the biggest fragment generated in this test experiment. However, the failure to detect the largest fragment in this test experiment could be due to many variables other than PCR conditions (*e.g.* template preparation, experimental error).

3.2.2 Amplification of 5′ and 3′ Arms

Amplification of the 5' and 3' homology arms was attempted from all four renin containing P1 clones available in the laboratory. Template DNA (10ng) was amplified using the 5′ PCR primers JJM203 and JJM135 and primers JJM189 and JJM204 for the 3' arm (see Figure 2.4 for primer binding sites and sequences).

A



B

Plasmid Name	JJM Number	Insert Size	Amplified Product Size
pSL1-16	JJM- 66	0.60kb	0.85kb
pLacZ5'	-	1.25kb	1.5 kb
p2335A1	-	2.3kb	2.55kb
pSLP BamA	JJM-116	3.2kb	3.45kb
prRP	JJM- 74	4.6kb	4.85kb

C

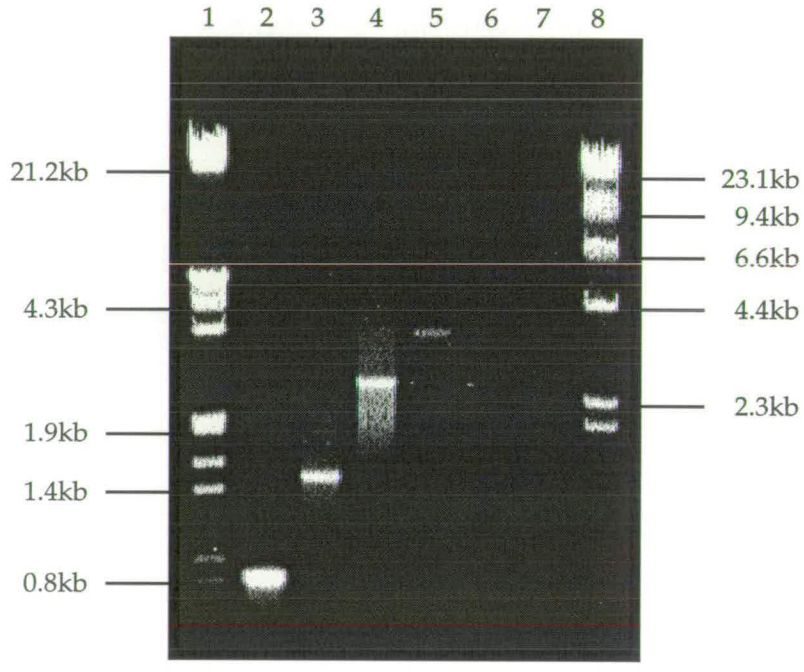


Figure 3.2: PCR Products Amplified from pBluescript Inserts: To determine the maximum size amplifyable with the *UlTma* enzyme, a range of product sizes were amplified. A) A schematic representation of the pBluescript Multiple Cloning Site (MCS) and the binding positions of the PCR primers used. B) Table showing the plasmids used as templates for PCR reactions, including the insert size and expected size of the PCR product. C) Amplification of a range of differently sized fragments inserted in the pBluescript polylinker. Lane 1= lambda *Hin*d III/*Eco*R I digested DNA, Lane 2= pSL1-16, Lane 3= pLacZ5', Lane 4= p2335A1, Lane 5= pSLP BamA, Lane 6= prRP, Lane 7= H_2O and Lane 8= lambda *Hin*d III digested DNA.

A fragment of the anticipated size (4.3kb) was obtained in amplification of the 5' arm from P1 clones P1-1249 (Figure 3.3A; lane 3) and P1-1251 (lane 5). No products were detectable from the other two P1 clones (P1-Ren and P1-1250; lanes 2 and 4 respectively). Amplification of the 4.7kb 3' arm fragment (Figure 3.3B) was observed from three P1 clones (P1-Ren, P1-1249 and P1-1251; lanes 2, 3 and 5 respectively) with no product being amplified from P1-1250 (lane 4).

3.2.3 Further Optimistion Of PCR Conditions

It has been shown that the quantity and specificity of PCR amplified product can be greatly increased by the use of a 2-step, instead of 3-step, PCR reaction, with the annealing/extension stages being combined at 65°C (319). Figure 3.4A shows a comparison made between amplification of the 5' homology arm using a 2-step (lanes 2-4) or the standard 3-step (lanes 5-7) amplification program from P1-1249 (lanes 2 and 5) and P1-1251 (lanes 3 and 6). In this experiment no clear differences can be seen between the two sets of conditions.

Several 'PCR enhancers' which increase the yield and specificity of

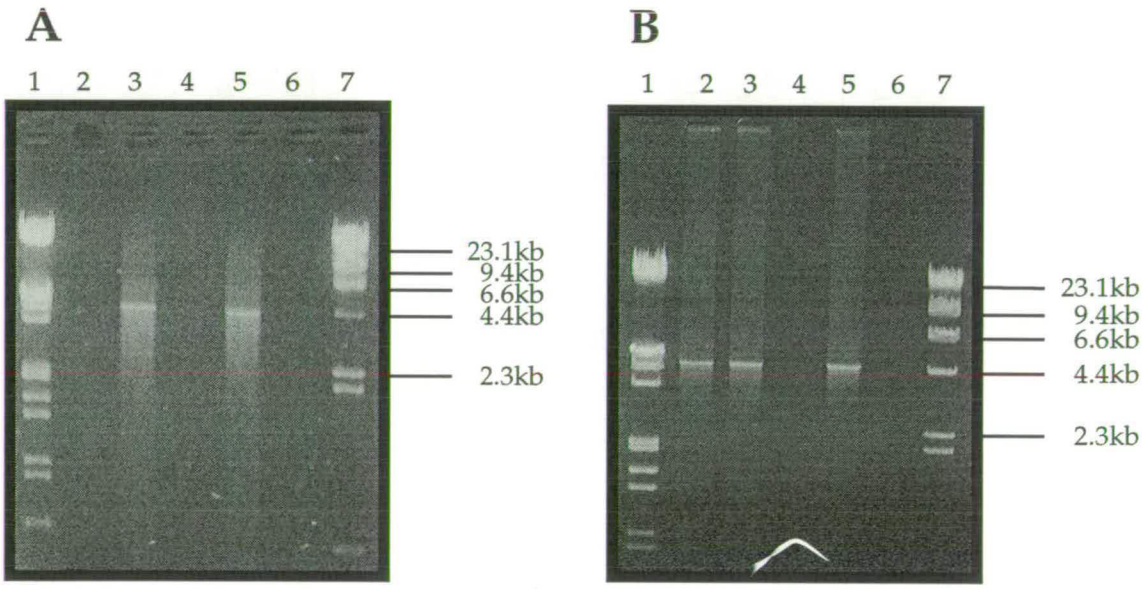


Figure 3.3: Amplification of the 5' and 3' homology regions: Amplification of the 5' (A) and 3' (B) homology arms from the four P1 clones. In panels A) and B) Lane 1= lambda *Hin*d III/*Eco*R I digested DNA, Lane 2= P1-Ren, Lane 3= P1-1249, Lane 4= P1-1250, Lane 5= P1-1251, Lane 6= H_2O and Lane 7= lambda *Hin*d III digested DNA.

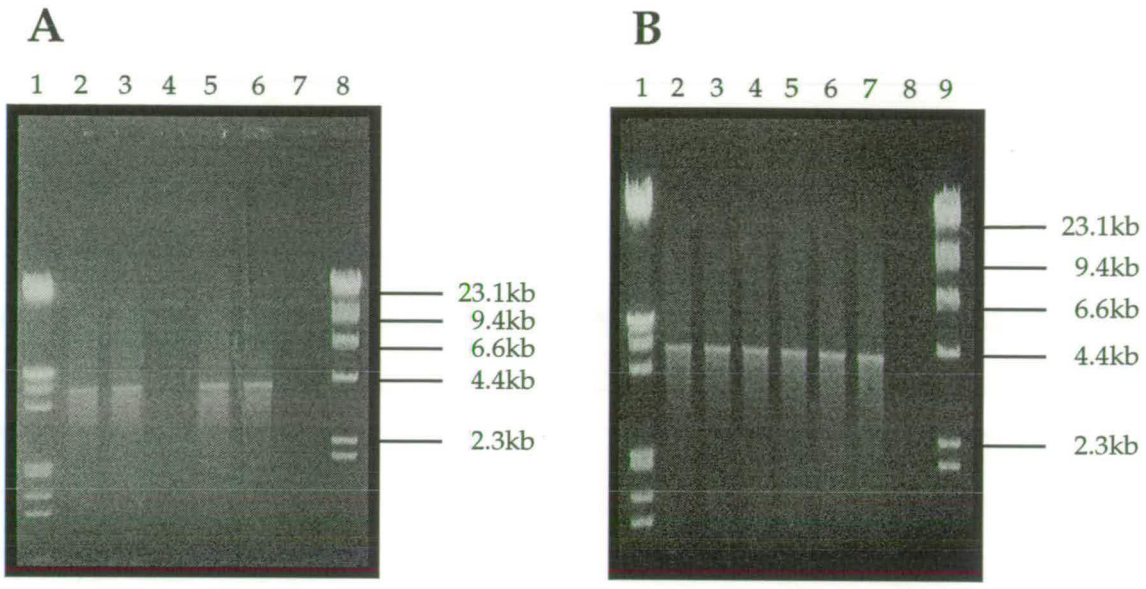


Figure 3.4: Attempted optimisation of the 5' PCR Reaction: A) Amplification of the 5' homology arm from two P1 clones (P1-1249 and P1-1251, 100pg template DNA) either in a standard 2-step reaction (Lanes 2-4) or in a 3-step reaction (Lanes 5-7). Lane 1= lambda *Hin*d III/*Eco*R I digested DNA, Lane 2 and 5= P1-1249, Lane 3 and 6= P1-1251, Lane 4 and 7= H_2O and Lane 8= lambda *Hin*d III digested DNA. B) Amplification of the 5' homology arm from two P1 clones (P1-1249 and P1-1251, 100pg template DNA) in the presence of 0.1 units Perfectmatch® DNA polymerase enhancer (Lanes 2 and 5), 1.0mM spermidine (Lanes 3 and 6) or in the absence of any 'PCR enhancer' (Lanes 4 and 7). Lane 1= lambda *Hin*d III/*Eco*R I digested DNA, Lane 2-4= P1-1249, Lane 5-7= P1-1251, Lane 8= H_2O and Lane 9= lambda *Hin*d III digested DNA.

amplification have been reported. Perfectmatch® DNA polymerase enhancer is a commercially available 'PCR enhancer' (Stratagene). Spermidine has also been shown to increase the yield and specificity of PCR amplification (320). It was shown that spermidine concentrations in the range of 0.2 to 2.0mM enhanced the amplification of target sequences. Perfectmatch® DNA polymerase enhancer (1.0 units/100ng template DNA; lanes 2 and 5) and 1.0mM spermidine (lanes 3 and 6) were compared to standard amplifications of the 5' arm (lanes 4 and 7) from P1-1249 (lanes 2-4) and P1-1251 (lanes 5-7) under the conditions given in section 2.2.6.2 and were found to have no effect on yield or specificity (Figure 3.4B).

3.3 Optimisation of Ligation Conditions

Initially, problems were experienced with the cloning of large PCR products, possibly due to suboptimal ligation conditions. To optimise this step a series of scaled-up test reactions were performed in order to make the

visualisation of ligation products on an agarose gel possible. Ligations were performed using a 3.7kb *Kpn* I/*Hin*d III DNA fragment (similar size to the arms of homology to be cloned) and *Kpn* I/*Hin*d III-digested pSP72poly4 (the vector preparation required for the cloning of the 3' homology arm). Both fragments were purified by running plasmid digests on a preparative agarose gel, excising the fragments and purifying the fragments by electroelution (section 2.2.8.1). The aim of this experiment was to determine optimal ligation conditions for the cloning of large PCR products, although this experiment would also determine the quality of the *Kpn* I/*Hin*d III digested pSP72poly4 vector preparation.

A total of sixteen ligations were performed aimed at testing the effects of molar ratios, ligation buffer used and DNA concentration. The normal reaction conditions suggested by Gibco-BRL were scaled up to 100ng of vector in a 200µl reaction, with the same amount of DNA in 20µl representing a "concentrated" reaction. Table 3.5 shows the composition of each ligation.

All 20µl reactions were made up to 200µl with 180µl sterile dH_2O. A 180µl sample of each was ammonium acetate/isopropanol precipitated in the presence of 25µg/ml glycogen (298) and resuspended in 7.5µl 1ng/µl proteinase K solution (to remove any remaining BSA from the Maniatis buffer). Samples were then run on an agarose gel to visualise the range of ligation products obtained from each reaction (Figure 3.6A).

The results show that, in the absence of DNA ligase and insert DNA, a single fragment representing linear vector was obtained (Lanes 2, 3, 11 and 12) whereas vector plus ligase (still with no insert) generated concatemers (Lanes 4, 5, 13 and 14), to an extent dependent on the DNA concentration and the buffer used.

When insert DNA was included in the reactions a more complex range of ligation products were produced (Lanes 6-9 and 15-18). Comparison of the two buffer types showed very little difference when insert DNA was included except for a slightly higher presence of lower molecular weight products with the Maniatis buffer (Lanes 6-9 compared to lanes 15-18; both buffers produced large concatemers which remained in the wells). The higher molar ratio tested (5:1 insert:vector) produced a slightly higher

Ligation → / Condition ↓	1	2	3	4	5	6	7	8
Ligation Buffer (M or G)	M	M	M	M	M	M	M	M
Reaction Volume (µl)	20	200	20	200	20	200	20	200
Molar Ratio (insert:vector)	no insert	no insert	no insert	no insert	2:1	2:1	5:1	5:1
Ligase (yes/no)	no	no	yes	yes	yes	yes	yes	yes

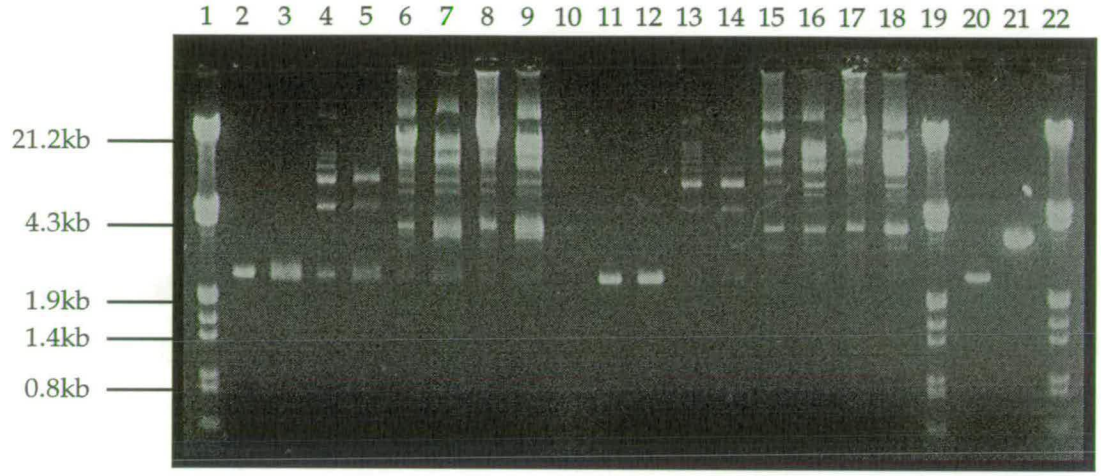
Ligation → / Condition ↓	9	10	11	12	13	14	15	16
Ligation Buffer (M or G)	G	G	G	G	G	G	G	G
Reaction Volume (µl)	20	200	20	200	20	200	20	200
Molar Ratio (insert:vector)	no insert	no insert	no insert	no insert	2:1	2:1	5:1	5:1
Ligase (yes/no)	no	no	yes	yes	yes	yes	yes	yes

Table 3.5: Composition of Test Ligation Reactions: To optimise ligation conditions three factors were tested. These were the use of different buffers (Maniatis or Gibco-BRL), the reaction volume (normal conditions in a 200µl reaction or a concentrated reaction containing the same quantity of DNA in 20µl) and the molar ratio of insert relative to vector (2:1 and 5:1).

proportion of concatemers regardless of the buffer used (Lanes 8, 9, 17 and 18). Increasing the relative DNA concentration generated ligation products of a generally higher molecular weight (Lanes 6, 8, 15 and 17).

Of the remaining 20µl samples, 10µl of each was used to transform competent cells, the results of which are shown in Figure 3.6B. These results are in accordance with the observations made from the agarose gel analysis of the ligations but allow more quantitative analysis. Taken together, the "vector only" ligations show that the vector preparation is virtually all cut to completion with both enzymes, with almost no background from vector minus ligase (*i.e.* all DNA is cut at least once) and only very low background from vector plus ligase (*i.e.* almost all the vector DNA is cut to completion with both enzymes).

A



B

Reaction No.	Colonies
1	1
2	2
3	33
4	32
5	1095
6	2584
7	1646
8	2384

Reaction No.	Colonies
9	2
10	1
11	10
12	21
13	1070
14	1836
15	871
16	1183

Figure 3.6: Test Ligation Products and Transformation Numbers: To determine the most efficient ligation conditions aliquots of each ligation were run on a gel and transformed into DH5 competent cells. A) Ligation products obtained from the test ligations detailed in Table 3.5. Lane 1= lambda *Hin*d III/*Eco*R I digested DNA, Lanes 2-9= Test Ligation Reactions 1-8 respectively, Lanes 11-18= Test Ligation Reactions 9-16 respectively, Lane 19= lambda *Hin*d III/*Eco*R I digested DNA, Lane 20= 100ng vector DNA (*Kpn* I/*Xba* I digested pSP72,poly4), Lane 21= 130ng insert DNA (*Kpn* I/*Xba* I digested) and Lane 22= lambda *Hin*d III/*Eco*R I digested DNA. B) Table showing the number of colonies obtained when 10µl (5.0ng vector DNA) of each test ligation was transformed into competent cells.

From the ligations which included insert DNA, it can be seen that Maniatis buffer generally gives higher colony numbers. When the two molar ratios are compared to each other little difference can be seen between them (excluding buffer differences). The DNA concentration has different effects according to the buffer used. In ligations performed using the Gibco-BRL buffer, the DNA concentration appears to have little effect. This is probably because this buffer includes PEG, a molecular crowding molecule which acts to increase the effective DNA concentration. In ligations performed using the Maniatis buffer, the best results were obtained using the more dilute

conditions (200µl reaction) which, when scaled down to 10ng vector in a 20µl reaction (as opposed to 100ng in a 200µl reaction), fall within Gibco-BRLs recommended conditions.

As a result of this experiment, subsequent ligations were set up using Maniatis buffer with insert ratios of 2:1 and 5:1 in a final volume of 20µl. DNA molar concentrations in the "dilute" reactions were also mimicked as closely as possible (*i.e.* 60fmol of vector and 120 or 300fmol insert in a 20µl reaction). Where recovery of vector or insert fragments was poor, scaled down 10µl ligation reactions were performed.

3.4 Cloning of the PCR Amplified Homology Arms

3.4.1 Fragment Preparation and Cloning of the 5' Arm

The first step in the construction of the *Ren-1^d* targeting vector was the cloning of the 5' PCR product (Figure 3.7). Restriction digestion of the PCR products with *Kpn* I to facilitate cloning was found to be a problem. However, this was not due to the enzymes ability to cut DNA or the ability to restriction digest the PCR product. It was concluded that the problems were caused by cutting at the *Kpn* I site present in the PCR primer.

To circumvent this problem two strategies were tested. The first was to biotinylate the exon 3 reverse primer (JJM213, Biotinylated *Ren-1^d* and *Ren-2* exon 3 primer; see Figure 2.4 for primer binding site and sequence). Using the same upstream primer (JJM203, -162bp *Ren-1^d* promoter primer; Figure 2.4), new PCR product was generated and digested as described in section 2.2.7.3. The second strategy was to design a new PCR primer in intron C, 390bp down stream of the *Kpn* I site in exon 3 (JJM212, *Ren-1^d* intron C primer; Figure 2.4). The result of a sequential *Kpn* I and *Xba* I digest would therefore produce four fragments, all of different sizes (140bp, 390bp, 480bp and 3700bp). Once again, new PCRs were performed and the PCR product was digested as described in section 2.2.7.1. Digested DNA was then electrophoresed on a preparative agarose gel, the desired 3.7kb fragment excised and the fragment gel purified (section 2.2.8.2).

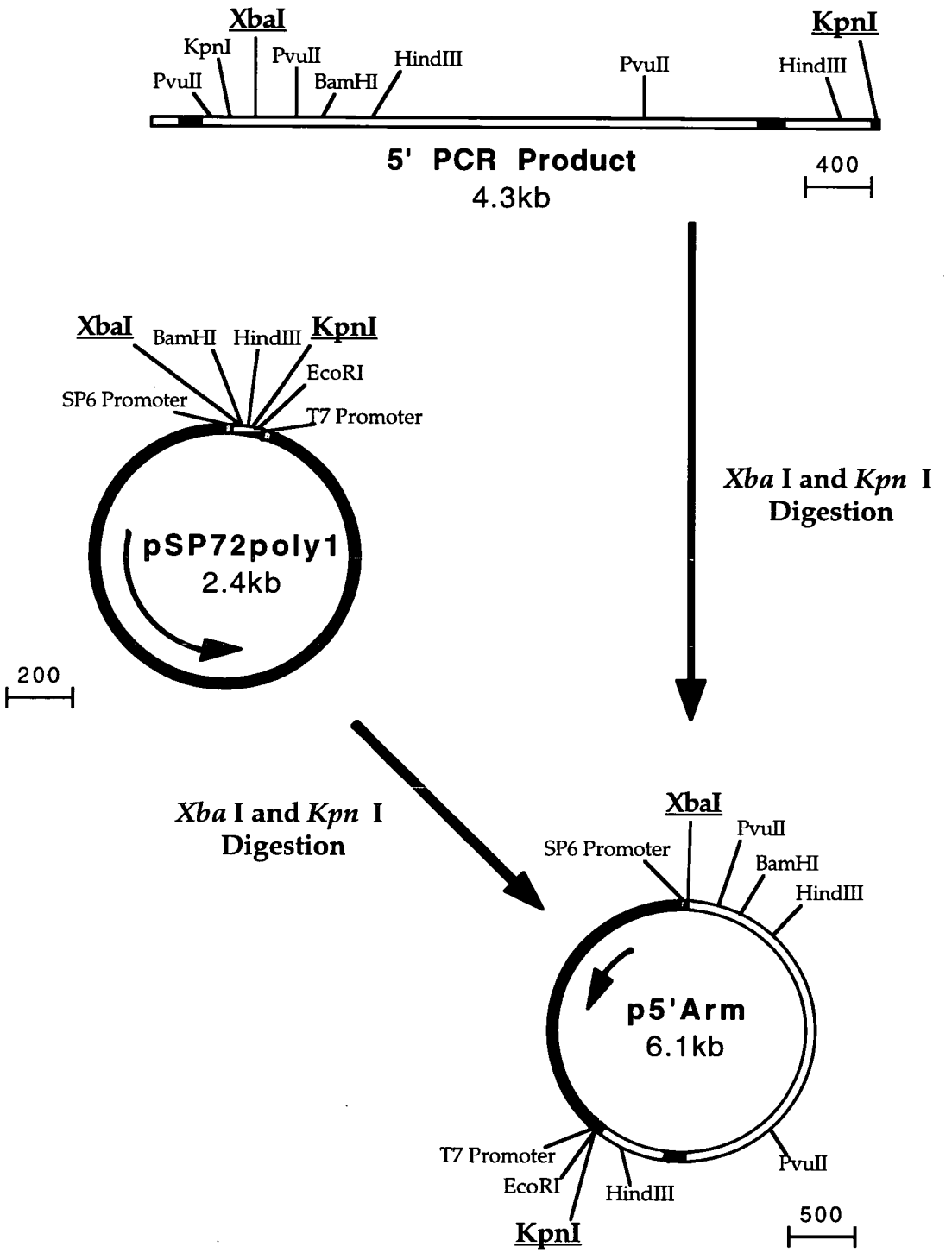


Figure 3.7: Cloning of the 5' homology Arm: The 5' PCR product was ligated into *Xba* I and *Kpn* I digested pSP72poly1 after restriction enzyme digestion at an internal *Xba* I site (present in intron A) and at a *Kpn* I recognition site present in the exon 3 reverse PCR primers (JJM135 or JJM213) to generate the plasmid p5'Arm. The *Kpn* I site in the reverse PCR primers corresponds to the endogenous *Kpn* I site present in exon 3.

Vector DNA (pSP72poly1) was prepared by digestion with *Xba* I followed by precipitation and digestion with *Kpn* I. Digested DNA was then run on a preparative agarose gel, and the desired 2.4kb vector fragment excised and Qiaex purified (section 2.2.8.2).

Ligations (10ng vector DNA in a 20µl reaction) were performed using fragments prepared by both methods and transformed into competent cells (Table 3.8A). Individual colonies (fifteen from each ligation containing insert DNA) were then used to prepare miniprep DNA, undigested DNA being analysed on an agarose gel to determine if plasmids contained any insert DNA, the results of which are shown in Table 3.8B.

Positive clones were then restriction mapped to characterise the insert (Figure 3.9A). Putative positive miniprep DNAs were double-digested with *Eco*R I and *Bam*H I, a digest which should result in fragments of 2.8 and 3.3kb after cleavage at an *Eco*R I site present in the 3' plasmid polylinker and a unique *Bam*H I site within intron A of the insert. *Bam*H I was chosen because it is possible to distinguish between *Ren-1*^{d} and *Ren-2* in this region using this

A

Biotinylated PCR Product		Intron C PCR Product	
Ligation Conditions	Colonies	Ligation Conditions	Colonies
Vector only -ligase	2	Vector only -ligase	1
Vector only +ligase	55	Vector only +ligase	70
Vector + Insert (2:1)	2160	Vector + Insert (2:1)	643
Vector + Insert (5:1)	2100	Vector + Insert (5:1)	346

B

Miniprep Origins	Colonies Containing Insert DNA
Biotinylated PCR Product (2:1)	3 (20.0%)
Biotinylated PCR Product (5:1)	6 (40.0%)
Total	9/30 (30.0%)

Intron C PCR Product (2:1)	2 (13.3%)
Intron C PCR Product (5:1)	0 (0.0%)
Total	2/30 (6.7%)

Table 3.8: Transformation results and positive clones recovered from ligations containing the 5' homology arm: A) Table showing the number of colonies observed when 50% of each ligation was transformed into DH5 competent cells. B) Fifteen minipreps were prepared from each plate containing vector plus insert. An aliquot of DNA from each was run on a gel, clones containing inserted DNA running at a higher molecular weight. The number of clones containing insert DNA is given (out of fifteen) and as a percentage in brackets.

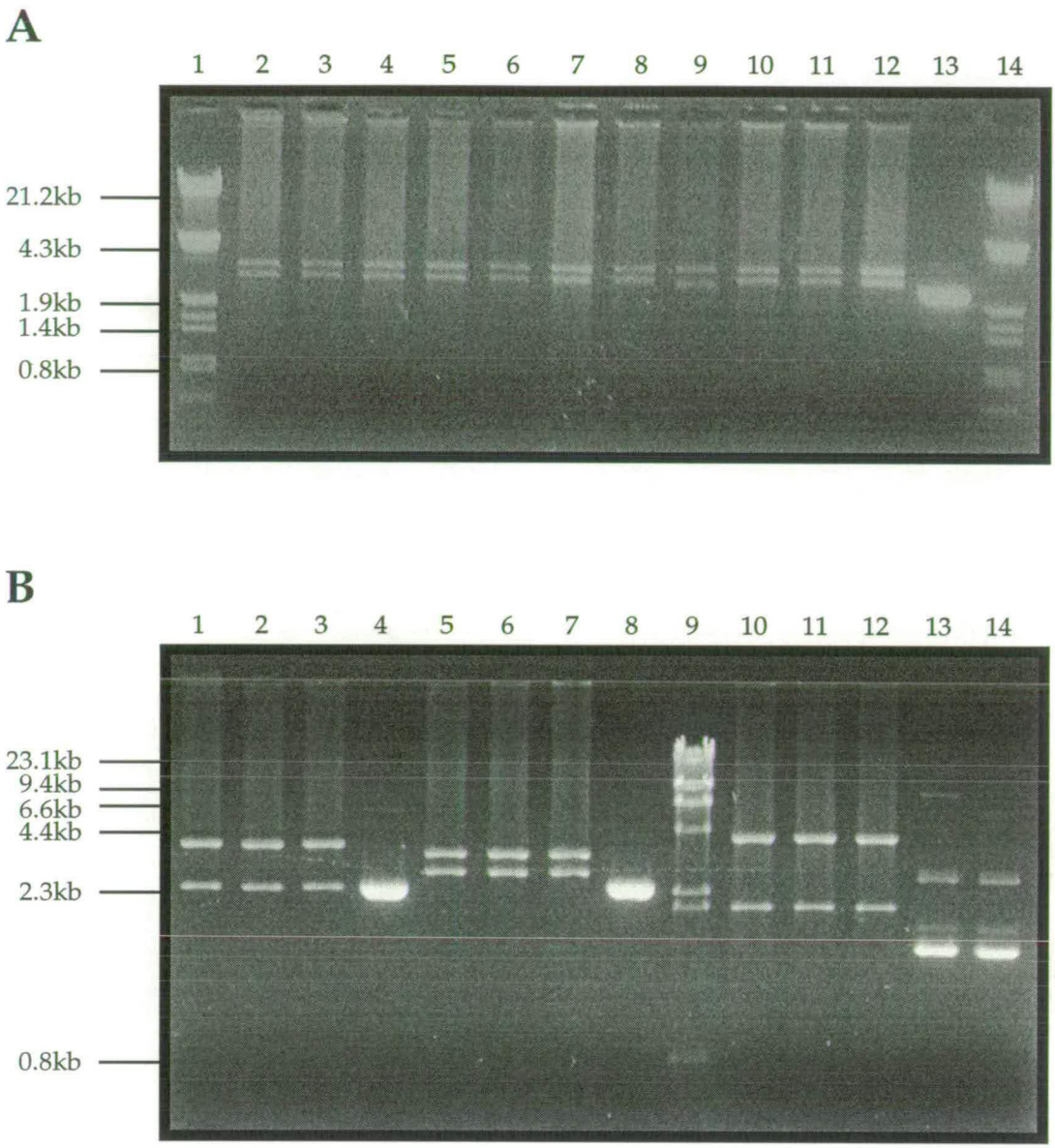


Figure 3.9: Mapping of putative p5'Arm clones: A) All eleven insert containing clones (and vector pSP72poly1) were restriction digested with *Bam*H I and *Eco*R I to confirm that the inserted DNA was *Ren-1*^{d} derived. Lane 1= lambda *Hin*d III/*Eco*R I digested DNA, Lane 2-12= insert-containing miniprep DNAs, Lane 13= pSP72poly1 and Lane 14= lambda *Hin*d III/*Eco*R I digested DNA. B) Three clones (and the vector pSP72poly1) were then further restriction mapped by digesting with *Xba* I plus *Kpn* I (Lanes 1-4), *Hin*d III (Lanes 5-8) and *Pvu* II (Lane 10-13). Lane 1, 5 and 10= miniprep DNA 3.1, Lane 2, 6 and 11= miniprep DNA 7.1, Lane 3, 7 and 12= miniprep DNA 12.1, Lane 4, 8 and 13= pSP72poly1 and Lane 14= undigested pSP72poly1.

enzyme, cutting once in *Ren-1*^{d} and four times (one common and three novel sites) in the equivalent *Ren-2* region. Digestion of the vector DNA (pSP72poly1) with the same enzymes should result in a single fragment of 2.4kb (lane 13). The results show that each DNA is only cut twice and is therefore *Ren-1*^{d} derived as expected.

Three clones were further restriction mapped (Figure 3.9B). A *Xba* I/*Kpn* I digest was used to excise the insert (expected fragments of 2.4 and 3.7kb corresponding to the vector and insert respectively) to confirm that neither site had been destroyed as a result of cloning the DNA fragment (this would suggest that an unusual insertion event had occurred and these clones would not be used in subsequent stages). *Hin*d III was used to confirm that the clones contained renin sequences, as both renin genes contain two restriction sites (in intron A and intron B) and therefore, digestion of plasmid DNA should result in two fragments of 2.8 and 3.3kb. To confirm that the insert DNA was *Ren-1^d*-derived a *Pvu* II digest was performed. A *Ren-1^d* clone should contain two *Pvu* II sites (both in intron A) whereas *Ren-2* contains only one (intron A). A *Ren-1^d* clone would therefore contain two fragments of 2.1 and 4.0kb. All clones showed the restriction pattern expected from a *Ren-1^d* insert and it was decided that clone 3, named p5'Arm 3.1, would be used for subsequent cloning stages. The cloning vector pSP72poly1 contains no *Pvu* II sites therefore the vector runs as undigested plasmid (compare lane 13 with lane 14). From preliminary mapping by restriction digestion all three clones gave the correct patterns and it was decided to continue with clone 3 which was named p5'Arm 3.1.

3.4.2 Fragment Preparation and Cloning of the 3' Arm

The 3' homology arm was cloned (Figure 3.10) into *Kpn* I and *Hin*d III-digested pSP72poly4. As reported above, the cloning of the 5' PCR product was problematical and the same was also true for the 3' homology arm. PCR-amplified material using primers JJM189 and JJM204 proved impossible to clone, therefore since biotinylation of one of the PCR primers proved most efficient in the cloning of the 5' homology arm, it was decided to try a similar approach for the cloning of the 3' arm.

A new biotinylated exon 4 forward primer (JJM210, biotinylated *Ren-1^d* exon 4 primer; Figure 2.4) was synthesised, new PCR material was amplified with this primer and the *Ren-1^d* and *Ren-2* exon 9 primer (JJM204; Figure 2.4), and attempts were made at digesting the DNA as described in section 2.2.7.3. This strategy proved unsuccessful because the desired fragment was never released from the magnetic beads, presumably because *Kpn* I was not cutting at the *Kpn* I recognition site introduced in the PCR primer. Here the *Kpn* I site was only two bases from the 5' end of the primer (as opposed to fourteen

in the 5' arm biotinylated exon 3 primer, JJM213) and it may have been this alone or in combination with steric hindrance caused by the biotin/streptavidin molecules that blocked *Kpn* I digestion.

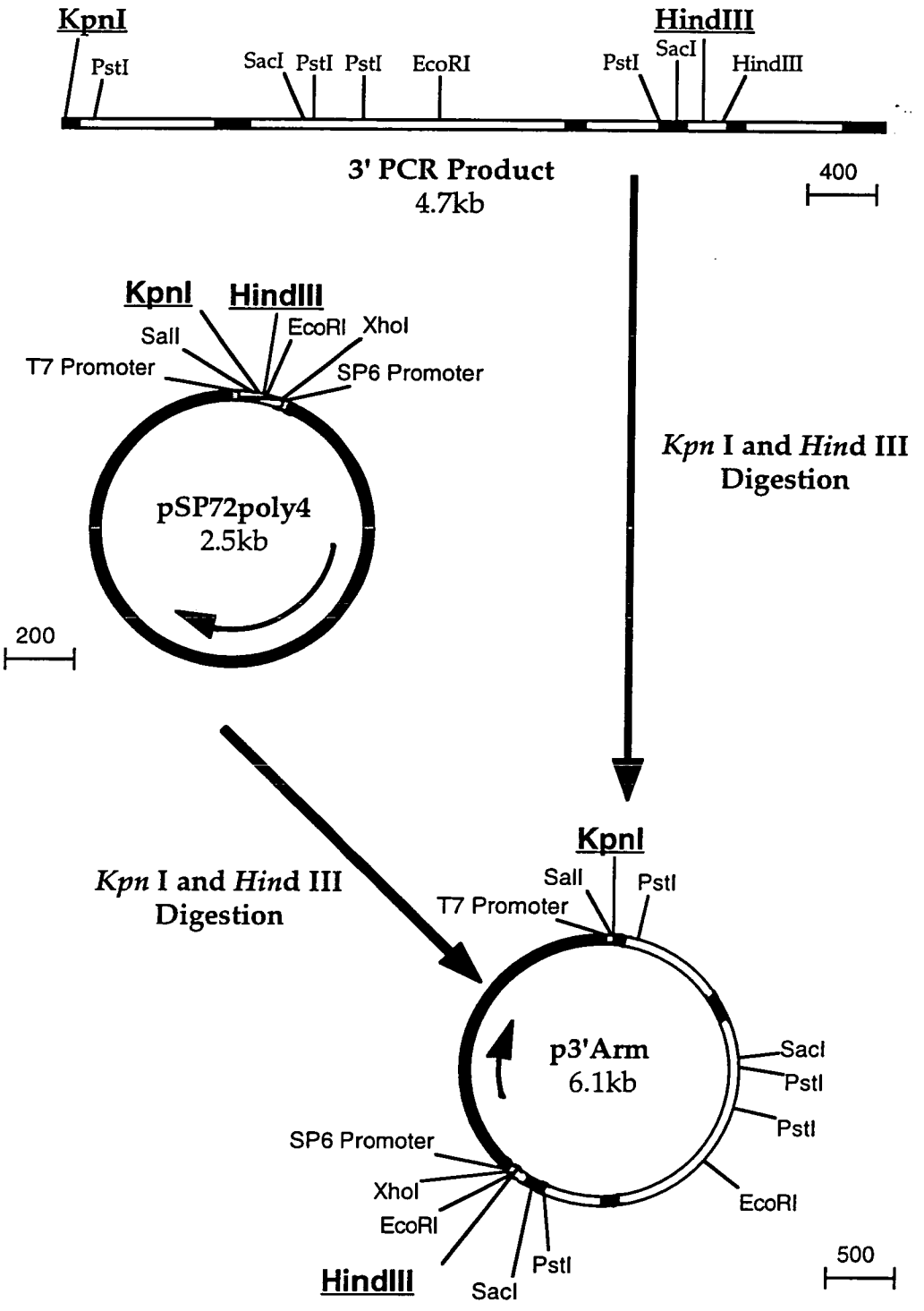


Figure 3.10: Cloning of the 3' homology Arm: The 3' PCR product was cloned into *Kpn* I and *Hind* III-digested pSP72poly4 after restriction digestion of the PCR product with *Kpn* I, a site being present within the forward PCR primers (JJM189, JJM210 or JJM224;) and at a *Hind* III site present within intron G to produce the plasmid p3'Arm.

A second primer was then synthesised spanning the same *Ren-1^d* homology but including nine bases upstream of the *Kpn* I site (JJM224, extended *Ren-1^d* exon 4 primer; Figure 2.4). PCR material generated with this primer and the *Ren-1^d* and *Ren-2* exon 9 primer (JJM204) was digested as described in section 2.2.7.2. Digested DNA was then run on a preparative agarose gel, the desired 3.6kb fragment excised and purified as described in the Amicom Spin Columns protocol (section 2.2.8.3).

Ligations were performed using 10ng (20µl reactions) of the previously tested, vector DNA (section 3.3). Figure 3.11A shows the resultant colony numbers, of which twenty-five were miniprepped, and the plasmid DNA restriction digested with *Sal* I and *Xho* I (each enzyme cuts once in the plasmid polylinker at either end of the homology arm, in effect excising the insert). pSP72poly4 DNA (vector DNA) was cut with each enzyme separately to confirm that they were active (lanes 15 and 16). Figure 3.11B shows twelve such digests with nine (75%) containing an insert (3.6kb) of the correct size (vector fragment is 2.5kb). In total eighteen (72%) out of the 25 clones screened contained insert DNA.

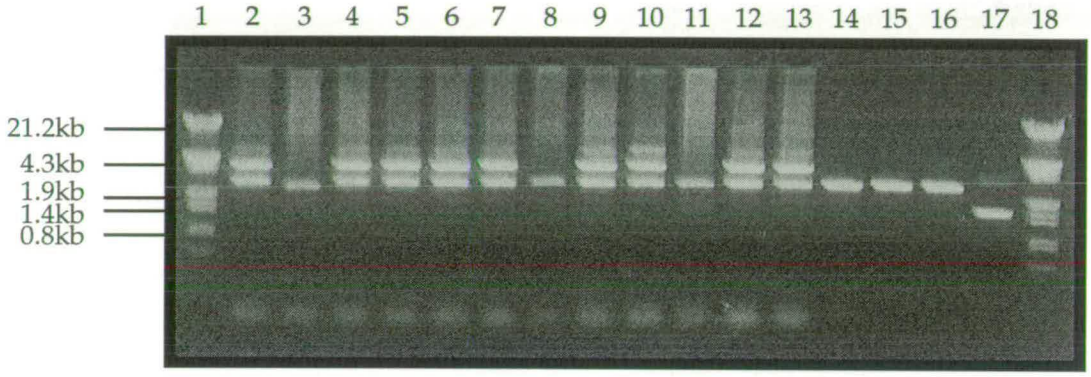
Three clones were further restriction mapped (Figure 3.11C). Putative positive miniprep DNAs were mapped by restriction digestion with the enzymes *Eco*R I, *Sac* I and *Pst* I. *Eco*R I cleaves once in the plasmid polylinker at the 3' end of the insert and once in intron E in the centre of the insert resulting in two fragments of 1.5kb and 4.6kb. Restriction digestion with *Sac* I should result in two fragments of 2.1kb and 4.0kb as a result of cutting at the *Sac* I sites present in the insert (intron E and exon 7). The 3' homology arm contains four *Pst* I sites (intron D, two in intron E and intron F) with none being present in the plasmid vector. Such a digest would therefore result in four fragments of 0.3, 1.3, 1.7 and 2.8kb. The cloning vector pSP72poly1 contains no *Sac* I or *Pst* I sites therefore the vector runs as undigested plasmid (compare lanes 8 and 13 with lane 14).

From preliminary mapping by restriction digestion all three clones gave the correct patterns and it was decided to continue with clone 4 which was named p3'Arm 4.1. Since this region of the gene contains few polymorphic restriction sites, it was not possible to prove that the cloned fragment was derived from *Ren-1^d*, however this was later confirmed by sequencing of the 3' arm (section 4.3).

A

Ligation Conditions	Colonies
Vector only -ligase	1
Vector only +ligase	20
Vector + Insert (2:1)	811
Vector + Insert (5:1)	1083

B



C

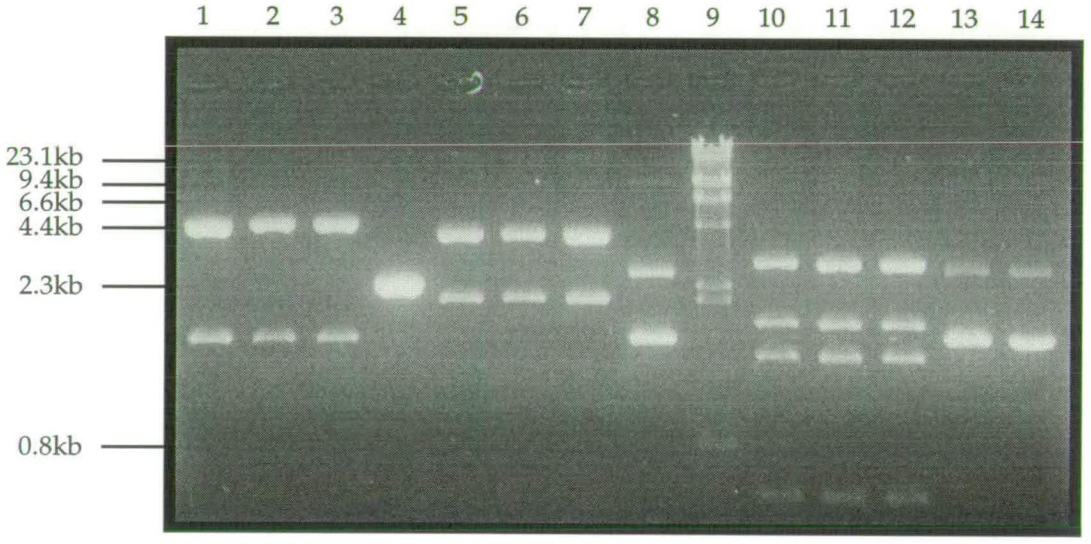


Figure 3.11: Transformation results and mapping of putative p3'Arm clones: A) Table showing the number of colonies observed when 50% of each ligation was transformed into DH5 competent cells. B) Twelve of the twenty-five miniprepped DNAs restriction digested with *Sal* I and *Xho* I to excise the inserted DNA, if present. Lane 1= lambda *Hin*d III/*Eco*R I digested DNA, Lane 2-13= miniprep DNAs 1-12, Lane 14= *Sal* I and *Xho* I digested pSP72poly4, Lane 15= *Sal* I digested pSP72poly4, Lane 16= *Xho* I digested pSP72poly4, Lane 17= undigested pSP72poly4 and Lane 18= lambda *Hin*d III/*Eco*R I digested DNA. C) Three clones (and the vector pSP72poly4) were then further restriction mapped by digesting with *Eco*R I (Lanes 1-4), *Sac* I (Lanes 5-8) and *Pst* I (Lane 10-13) Lane 1, 5 and 10= miniprep DNA 3.1, Lane 2, 6 and 11= miniprep DNA 4.1, Lane 3, 7 and 12= miniprep DNA 5.1, Lane 4, 8 and 13= pSP72poly4 and Lane 14= undigested pSP72poly4.

3.5 Discussion

Throughout this chapter the amplification and subsequent cloning of homology arms generated by PCR has been described. The two regions of 4.3 and 4.7kb, were amplified from P1 template DNA using the proof-reading enzyme *UlTma*. Amplicons were then subjected to restriction digestion to facilitate the cloning of two fragments, each of about 3.7kb, for use as the 5' and 3' homology arms.

The successful amplification (from P1 clones) and cloning of PCR products several kilobases in length, is a step towards an alternative strategy to that of cloning homology arms from genomic clones. Since these PCR reactions were performed, several papers have been published in which the further optimisation of PCR has been reported (321-323). One paper reports the amplification of up to 15kb from genomic DNA or 40kb from a lambda clone using a mixture of *Taq* and a proof-reading enzyme, *Pfu* (321). It is believed that this is more efficient because *Taq* is responsible for the majority of the DNA synthesis whereas the proof-reading enzyme excises any misincorporated residues. In other words, the high processivity of *Taq* is combined with the mismatch editing of a proof-reading enzyme. Experiments in our laboratory using the Expand™ PCR kit (Boehringer Mannheim) which utilises a mixture of *Taq* and the proof-reading enzyme *Pwo*, have lead to the successful amplification of large fragments (up to 9kb) from genomic DNA (M. Sharp *et al.*, personal communication). This would allow the building of targeting constructs without the need to identify any genomic clones.

This system offers several advantages over the more commonly used strategy including; 1) the ability to introduce restriction sites for cloning of material and screening of ES cell DNAs, 2) the ability to use highly isogenic DNA, as homology can be amplified directly from ES cell DNA and 3) the requirement for minimal mapping data, *e.g.* a cDNA sequence and preliminary genomic map for the design of primers, prediction of amplicon sizes and the development of a screening strategy.

Although not straightforward, it has been possible to clone PCR generated homology arms. The next chapter describes the construction of a *Ren-1^d* directed targeting construct using the two homology arms described in this chapter.

CHAPTER 4

Construction of a *Ren-1^d* Targeting Construct

4.1 Introduction

In this chapter the construction of a *Ren-1^d* directed gene targeting vector is described. This was built using the two homology arms described in Chapter 3 and a PGK-*neo*pA selection cassette. As the homology arms were generated by PCR, the introduction of mutations during the reaction was possible. This would reduce the homology thereby affecting the targeting efficiency and therefore an estimation of the error rate in the 3' Arm PCR reaction was determined.

4.2 Construction of a *Ren-1^d* Knock-Out Vector

Each cloning step in the assembly of pR1*neo*KO, a *Ren-1^d* directed targeting construct, is detailed in a figure in each of the corresponding results sections (4.2.1-4.2.3). Briefly, a new polylinker was cloned into p5'Arm 3.1 before sequential addition of the 3' arm and PGK-*neo*pA selection cassette.

4.2.1 Insertion of a Modified Polylinker into p5'Arm

In order to introduce the 3' arm into the targeting construct it was necessary to introduce a new polylinker fragment into p5'Arm (Figure 4.1).

Vector DNA (p5'Arm 3.1) was either digested with *Kpn* I followed by precipitation and *Not* I digestion or *Not* I digested followed by precipitation and *Kpn* I digestion. This was because the two sites are close to each other in the polylinker and it was not known how efficiently either enzyme would cut in a second digestion step as the site would be close to the end of the linear molecule. Vector DNA was then run on a preparative agarose gel, the desired 6.1kb fragment excised and purified as described in the Amicom Spin Columns protocol (section 2.2.8.3).

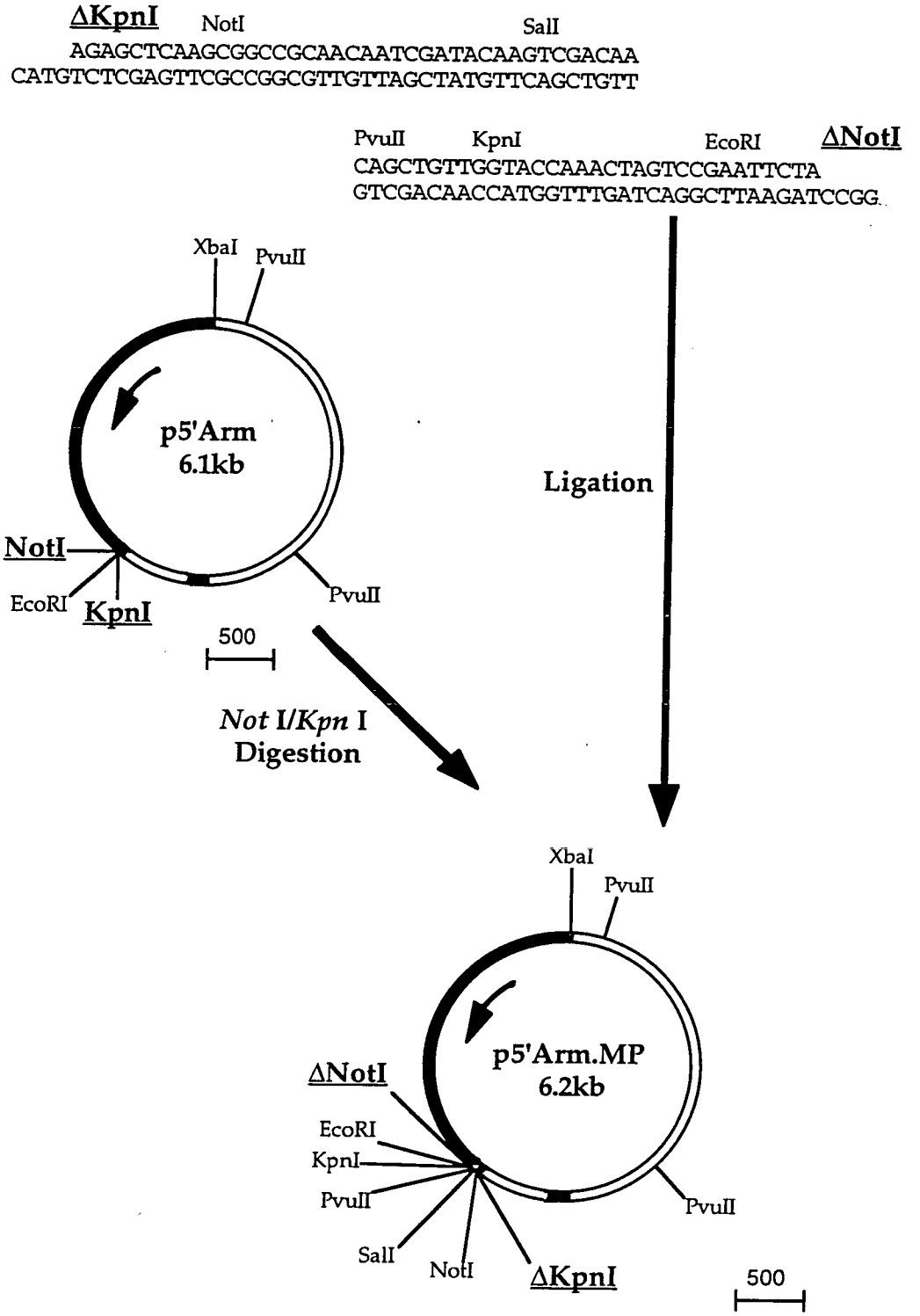


Figure 4.1: Insertion of a new polylinker into p5'Arm: A pair of self complementary oligodeoxynucleotides (see Figure 2.6 for oligodeoxynucleotide sequences and the restriction sites that they contain) were annealed and cloned into *Kpn* I and *Not* I digested p5'Arm 3.1 as described in section 2.2.9.2. The sequences of the oligonucleotides were designed in such a way that both cloning sites were destroyed (indicated by Δ symbol) when inserted into the plasmid.

Ligations were set up using 10ng (20µl reactions) of each prepared vector DNA using molar ratios of 5:1, 50:1 or 500:1 (insert:vector). Part of each ligation (10µl) was then transformed into competent cells and plated out. Both methods of vector preparation showed a low background, with the *Not* I followed by *Kpn* I digestion being optimal. Significantly more colonies were seen on plates with vector plus oligodeoxynucleotide insert at 50:1 and 500:1 ratios (both preparations).

Miniprep DNA was prepared from eighteen colonies and then restriction digested with *Pvu* II. p5'Arm, the vector DNA, contains two *Pvu* II sites (in intron A) whilst the inserted oligodeoxynucleotides introduce a third site which should therefore produce three fragments (1.4, 2.1 and 2.7kb) when run on an agarose gel. Figure 4.2A shows the results of these digests alongside a *Pvu* II digest of the parental plasmid p5'Arm 3.1 (lane 20; fragments are 2.1 and 4.0kb). Of the eighteen clones screened, seven (39%) showed the same restriction pattern as the parental plasmid while the other eleven (61%) clones had the three fragment pattern expected from clones containing the oligodeoxynucleotide insert.

Three clones were then subjected to further restriction mapping (Figure 4.2B) to confirm the presence of other restriction sites required for subsequent cloning steps, and run in parallel with similarly digested parental plasmid (p5'Arm 3.1). This was done using a series of double digests using *Xba* I and a second enzyme which cut in the new polylinker (*Sal* I, *Not* I, *Kpn* I and *Eco*R I). *Xba* I cleaves this plasmid once in the plasmid polylinker at the 5' end of the homology arm. *Sal* I, *Not* I, *Kpn* I and *Eco*R I should all be unique, cutting at the 3' end of the homology arm in the newly inserted polylinker fragment. Therefore, digestion with *Xba* I and either *Sal* I, *Not* I, *Kpn* I or *Eco*R I should result in two fragments corresponding to the plasmid (2.5kb) and the homology arm (3.7kb).

All plasmids showed the correct patterns and it was decided to use clone p5'Arm.MP 1.1 in subsequent cloning steps. Note that *Xba* I/*Not* I, *Xba* I/*Kpn* I and *Xba* I/*Eco*R I digested plasmids do not differ from the parental plasmid because these new sites (*Not* I, *Kpn* I and *Eco*R I) were also present in the old polylinker removed from the parental plasmid (p5'Arm 3.1). Therefore the confirmation that the new polylinker was present was only determined by the presence of newly introduced *Pvu* II and *Sal* I sites.

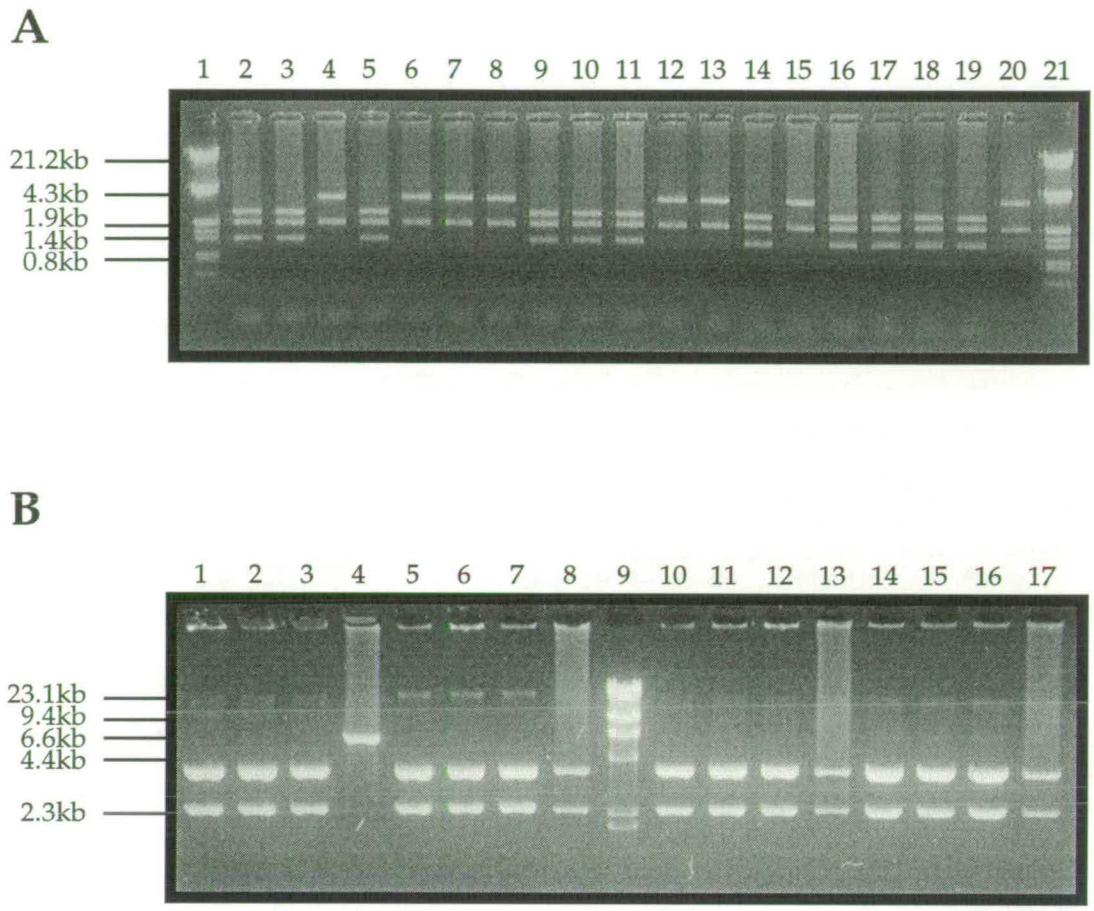


Figure 4.2: Mapping of putative p5'Arm.MP clones: A) All eighteen clones (and the vector p5'Arm 3.1) were restriction digested with *Pvu* II to confirm that the inserted DNA was renin derived and contained an extra *Pvu* II site, present in the new polylinker. Lane 1= lambda *Hin*d III/*Eco*R I digested DNA, Lane 2-19= miniprep DNAs 1-18, Lane 20= p5'Arm 3.1 and Lane 21= lambda *Hin*d III/*Eco*R I digested DNA. B) Three clones (and the vector p5'Arm 3.1) were then further restriction mapped by digesting with *Xba* I plus *Sal* I (Lanes 1-4), *Xba* I plus *Not* I (Lanes 5-8),*Xba* I plus *Kpn* I (Lanes 10-13) and Xba I plus *Eco*R I (Lanes 14-17). Lane 1, 5, 10 and 14= miniprep DNA 1.1, Lane 2, 6, 11 and 15= miniprep DNA 2.1, Lane 3, 7, 12 and 16= miniprep DNA 4.1, Lane 4, 8, 13 and 17= p5'Arm 3.1 and Lane 9= lambda *Hin*d III digested DNA.

4.2.2 Insertion of 3′ Arm into p5′Arm.MP

To create a plasmid containing both the *Ren-1*^{d} homology arms, the 3' arm was cloned into the plasmid p5'Arm.MP (Figure 4.3). As problems had been encountered with *Kpn* I digestion at sites near the ends of linear DNA molecules, it was decided that vector DNA (p5'Arm.MP1.1) would be digested firstly with *Kpn* I followed by *Eco*R I digestion. Digested DNA was run on a preparative agarose gel, the desired 6.2kb fragment excised and the fragment Qiaex purified (section 2.2.8.2).

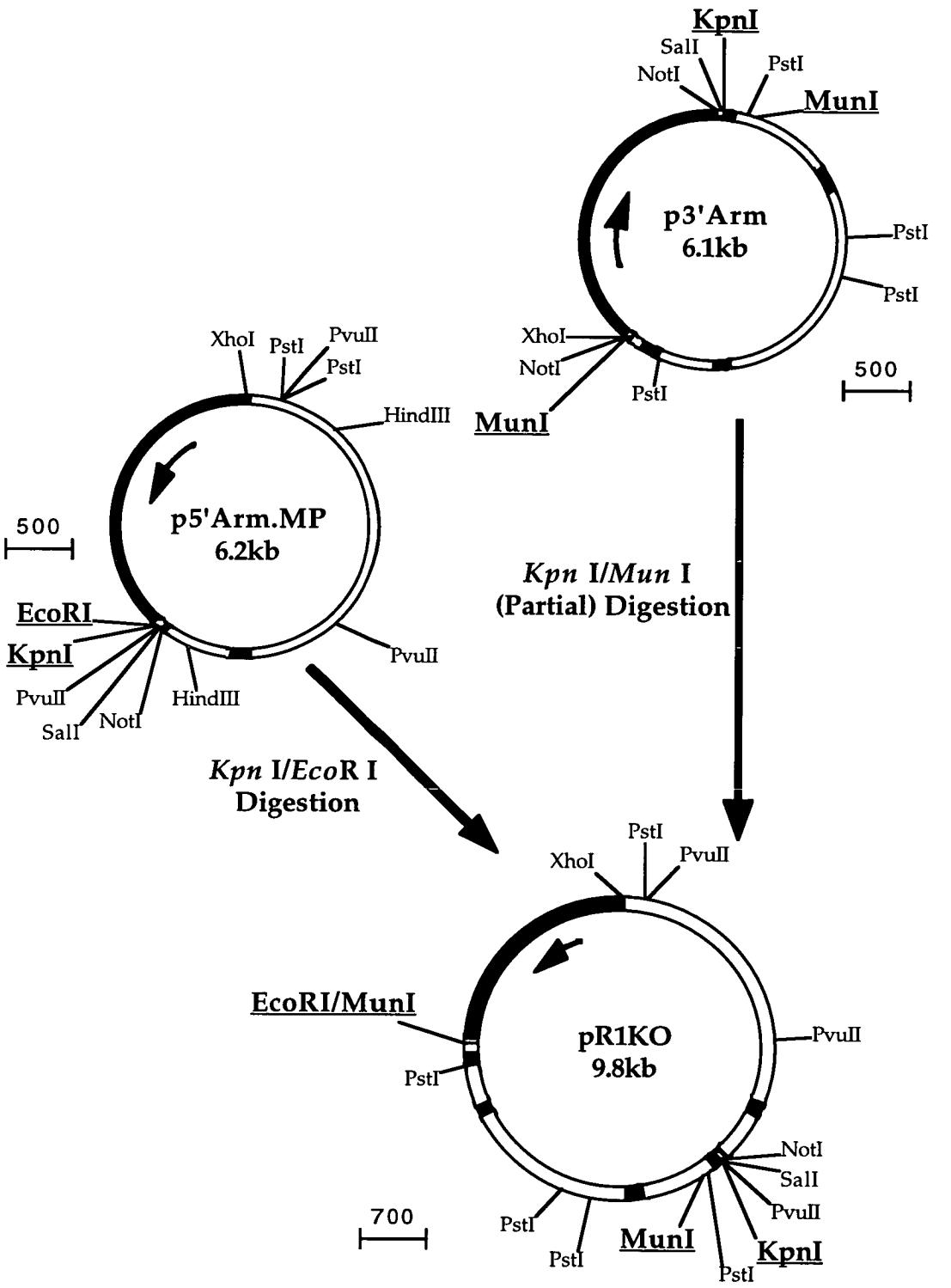


Figure 4.3: Subcloning of the 3' homology arm into p5'Arm.MP: Vector DNA was prepared by digesting p5'Arm.MP 1.1 with *Eco*R I and *Kpn* I. The 3' arm was prepared for cloning by restriction digesting the plasmid p3'Arm 4.1 with *Kpn* I before partially digesting with *Mun* I.

The insert DNA (3' arm) needed to be prepared by *Mun* I partial digestion of *Kpn* I digested p3'Arm 4.1. This was done by *Kpn* I-digesting 40µg of p3'Arm 4.1 which was then precipitated and digested in a total volume of 240µl with 40 units of *Mun* I. Aliquots (30µl containing 5µg DNA) were taken at various time points (0, 4, 8, 12, 16, 20, 30, 40 and 60 minutes) and the digestion stopped by pipetting into a tube containing EDTA. An aliquot (0.5µg) of each time point was run on an agarose gel (Figure 4.4). As can be seen, the digest was complete after 12 minutes (lane 4) with the majority of partially digested fragments being visible at 4 minutes (lane 2). The remaining DNA from the "4 minute" time point was run on a preparative agarose gel, the desired 3.6kb fragment was excised, and the DNA prepared using Amicom Spin Columns (section 2.2.8.3).

Ligations were set up using vector DNA (10ng in 10µl reactions) and the purified *Kpn* I/*Mun* I (partial) 3'Arm fragment. These ligations yielded a

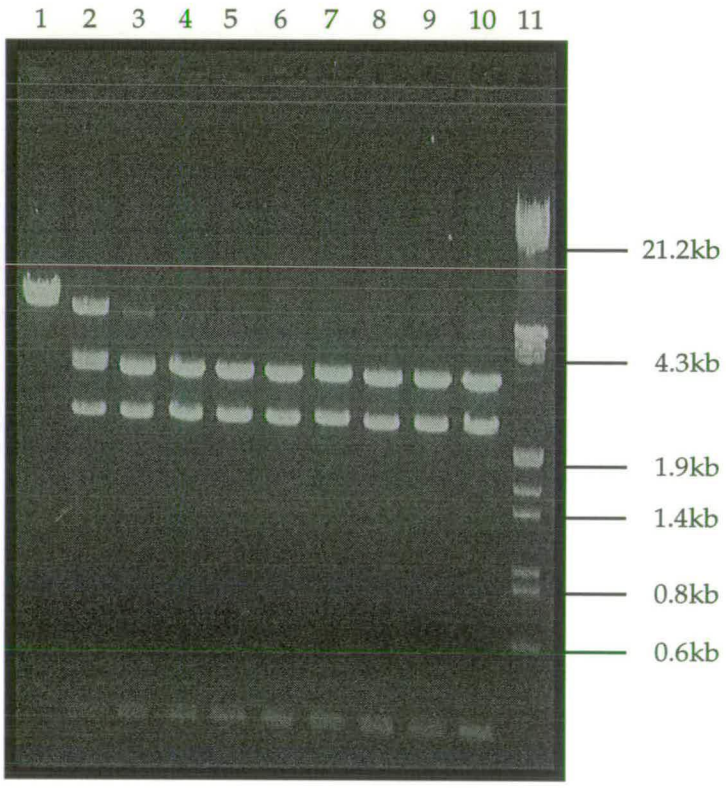


Figure 4.4: Partial *Mun* I digestion of *Kpn* I-digested p3'Arm 4.1: Aliquots of a *Mun* I digest of *Kpn* I-digested p3'Arm 4.1 were removed at 0, 4, 8, 12, 16, 20, 30, 40 and 60 minutes and an aliquot of each time-point was run on an agarose gel. Lanes 1-9= a 0.5µg aliquot from time points 0, 4, 8, 12, 16, 20, 30, 40 and 60 minutes respectively, Lane 10= p3'Arm 4.1 cut to completion with *Kpn* I plus *Mun* I and Lane 11= lambda *Hin*d III/*Eco*R I digested DNA.

3-fold increase in colonies compared to the background observed in "vector only plus ligase" reactions. Miniprep DNA was prepared from eighteen colonies and then restriction digested with *Pst* I. The parental plasmid (p5'Arm.MP 1.1) contained only one *Pst* I site (intron A) and the insert contained four *Pst* I sites (intron D, two in intron E and intron F) which would result in a single fragment of 6.2kb from parental plasmid (lane 20) and five fragments of 0.3, 1.2, 1.7, 2.9 and 3.7kb from the new construct. Figure 4.5A shows the restriction pattern observed for each clone. Of the eighteen clones screened, sixteen (89%) contained insert DNA.

Three clones were further mapped (Figure 4.5B) by restriction digestion (*Sal* I/*Xho* I, *Not* I/*Xho* I and *Pvu* II,). The two double digests were performed to confirm that the *Not* I and *Sal* I sites required for the final cloning step were still present. *Xho* I is a unique site in the 5' polylinker upstream of the 5' homology arm and when used in combination with *Not* I or *Sal* I, it should produce two fragments, the 5' homology arm (3.7kb) and a larger one containing the plasmid vector and 3' homology arm (6.1kb). These sites should also be unique in the parental plasmid (p5'Arm.MP 1.1; lanes 4 and 8) resulting in fragments of 3.7kb (5' homology arm) and 2.5kb (plasmid vector). *Pvu* II cuts at three sites in p5'Arm.MP (once in the polylinker fragment which will separate the 5' and 3' homology arms and twice in intron A) and does not cut in the 3' arm. Therefore, upon correct insertion of the 3' arm, the 2.7kb fragment observed in a digest of the parental plasmid should increase to 6.3kb with the other two remaining the same (1.4 and 2.1kb). All three clones show the correct patterns for all three sets of digests and clone 2, named pR1KO 2.1, was chosen for use in the final cloning step.

4.2.3 Insertion of PGK-*neo* into pR1KO

The final step in the construction of the *Ren-1*^{d} targeting construct was the insertion of a selectable marker, PGK-*neo*pA into pR1KO (Figure 4.6).

Parental plasmid DNA (pR1KO 2.1) was either digested with *Not* I followed by *Sal* I or digested with *Sal* I followed by *Not* I because of the problems associated with digestion close to the end of the linear molecule. Both vector DNAs were then run on a preparative agarose gel, the desired 9.8kb fragments excised and Qiaex purified (section 2.2.8.2).

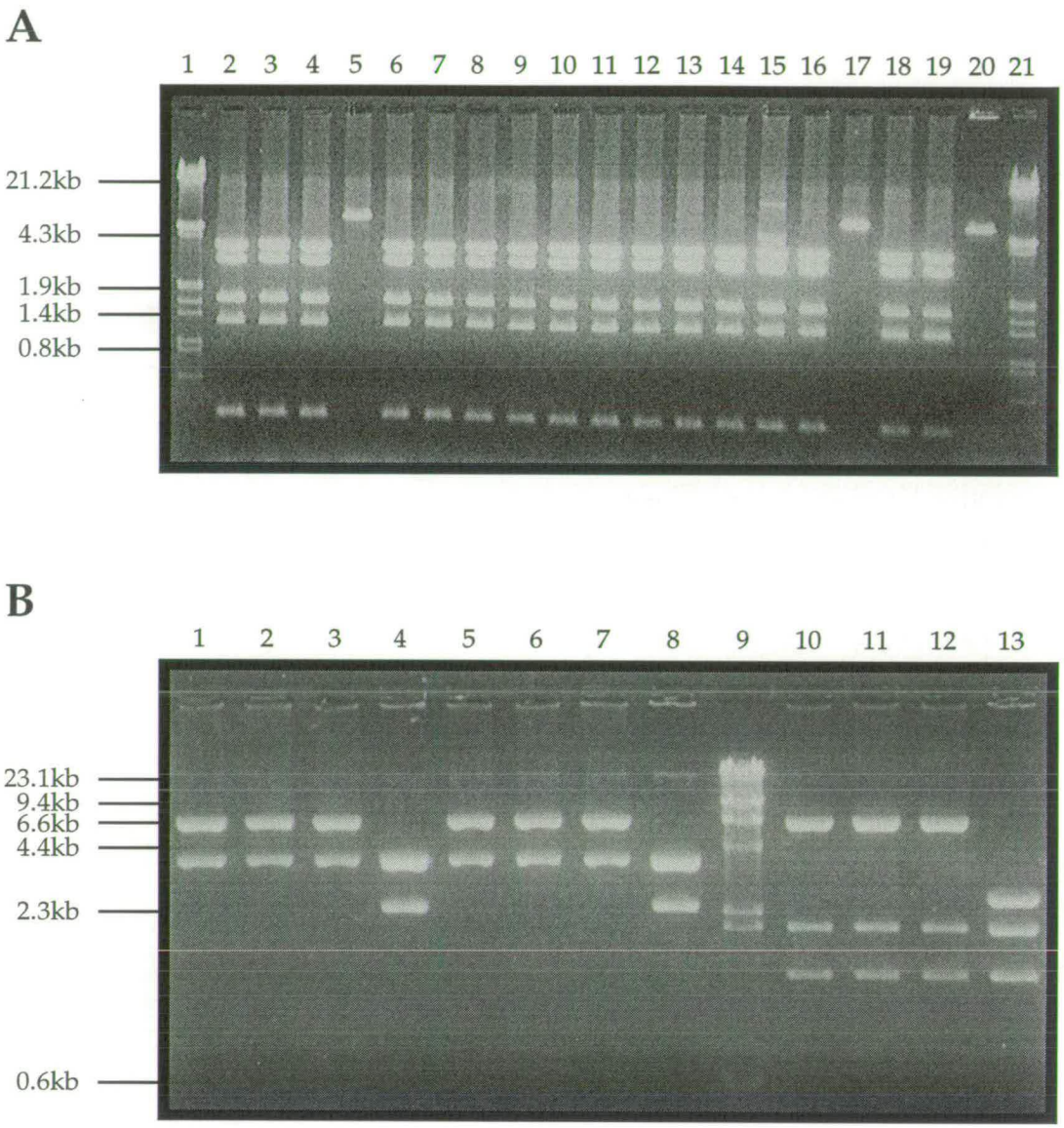


Figure 4.5: Mapping of putative pR1KO clones: A) All eighteen clones (and the vector p5'Arm.MP 1.1) were restriction digested with *Pst* I to confirm that the new plasmids contained both the 5' and 3' renin derived homology arms. Lane 1= lambda *Hin*d III/*Eco*R I digested DNA, Lane 2-19= miniprep DNAs 1-18, Lane 20= p5'Arm.MP 1.1 and Lane 21= lambda *Hin*d III/*Eco*R I digested DNA. B) Three clones (and the vector p5'Arm.MP 1.1) were then further restriction mapped by digesting with *Sal* I plus *Xho* I (Lanes 1-4), *Not* I plus *Xho* I (Lanes 5-8) and *Pvu* II (Lanes 10-13). Lane 1, 5 and 10= miniprep DNA 1.1, Lane 2, 6 and 11= miniprep DNA 2.1, Lane 3, 7 and 12= miniprep DNA 3.1, Lane 4, 8, 13 and 17= p5'Arm.MP 1.1 and Lane 9= lambda *Hin*d III digested DNA.

Ligations were set up using both vector preparations (40ng in 20µl reactions) with insert DNA and a fraction of each was transformed into DH5 competent cells and plated out. Both vector preparations gave low backgrounds and both also gave a 7-fold enrichment when insert DNA was included. Sixteen colonies were miniprepped and the DNA double-digested

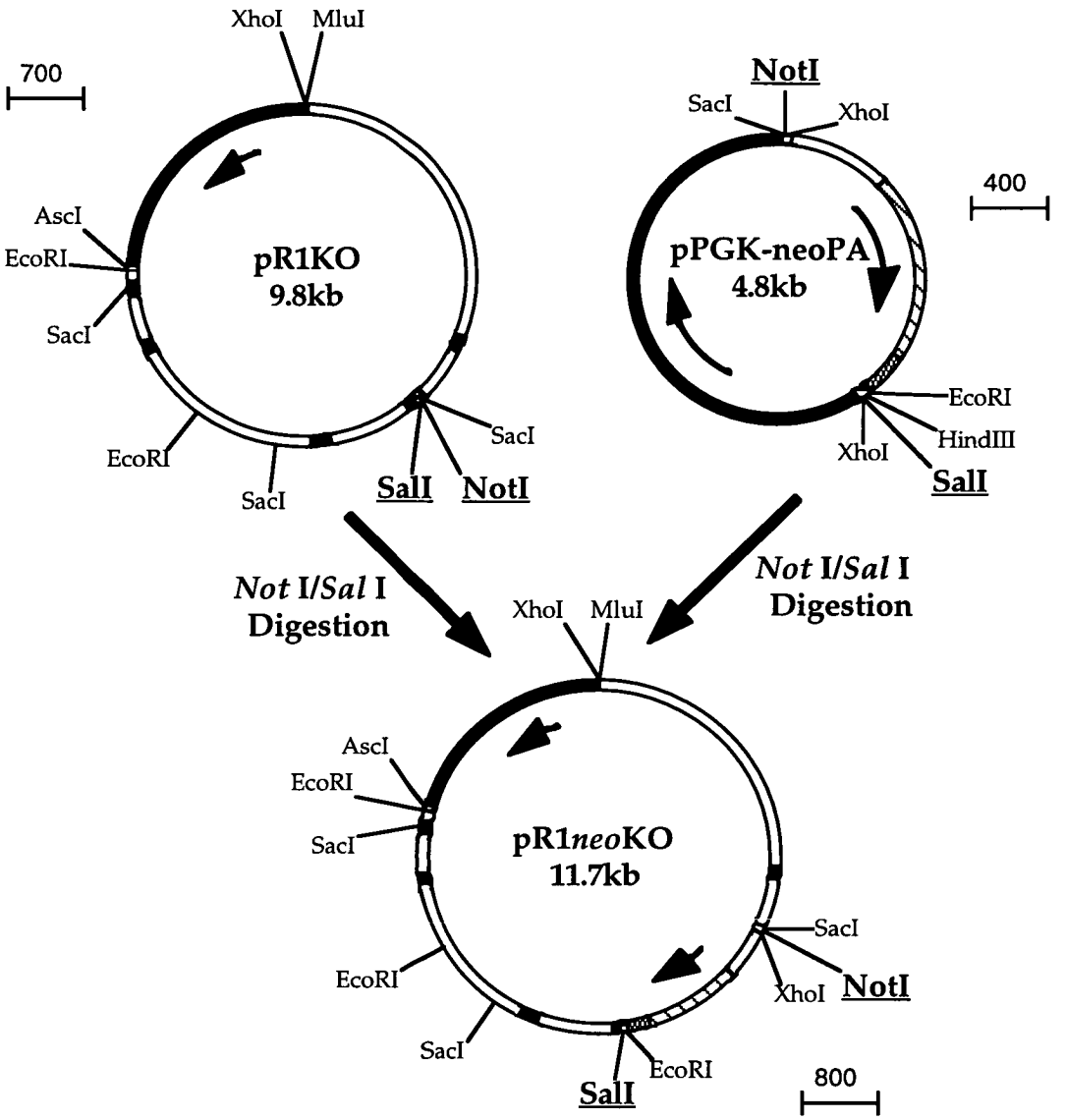


Figure 4.6: Insertion of the PGK-*neo*pA selection cassette into pR1KO: The 1.9kb PGK-*neo*pA *Not* I/*Sal* I fragment preparation used in the *Ren-2* targeting construct pR2*neo*KO (JJM-156) (286) was also used to construct the *Ren-1*^{d} targeting construct. This was ligated into *Not* I and *Sal* I-digested pR1KO 2.1.

with *Not* I and *Sal* I, a combination which should excise the 1.9kb PGK-*neo*pA fragment (vector plus 5' and 3' homology arms is 9.8kb). Figure 4.7A shows the result where 13 (81%) of the 16 colonies tested were positive for the insert DNA (note that the positive clones have two fragments corresponding to the parental plasmid (lane 18) and the insert DNA (lane 19), which were also run on the gel).

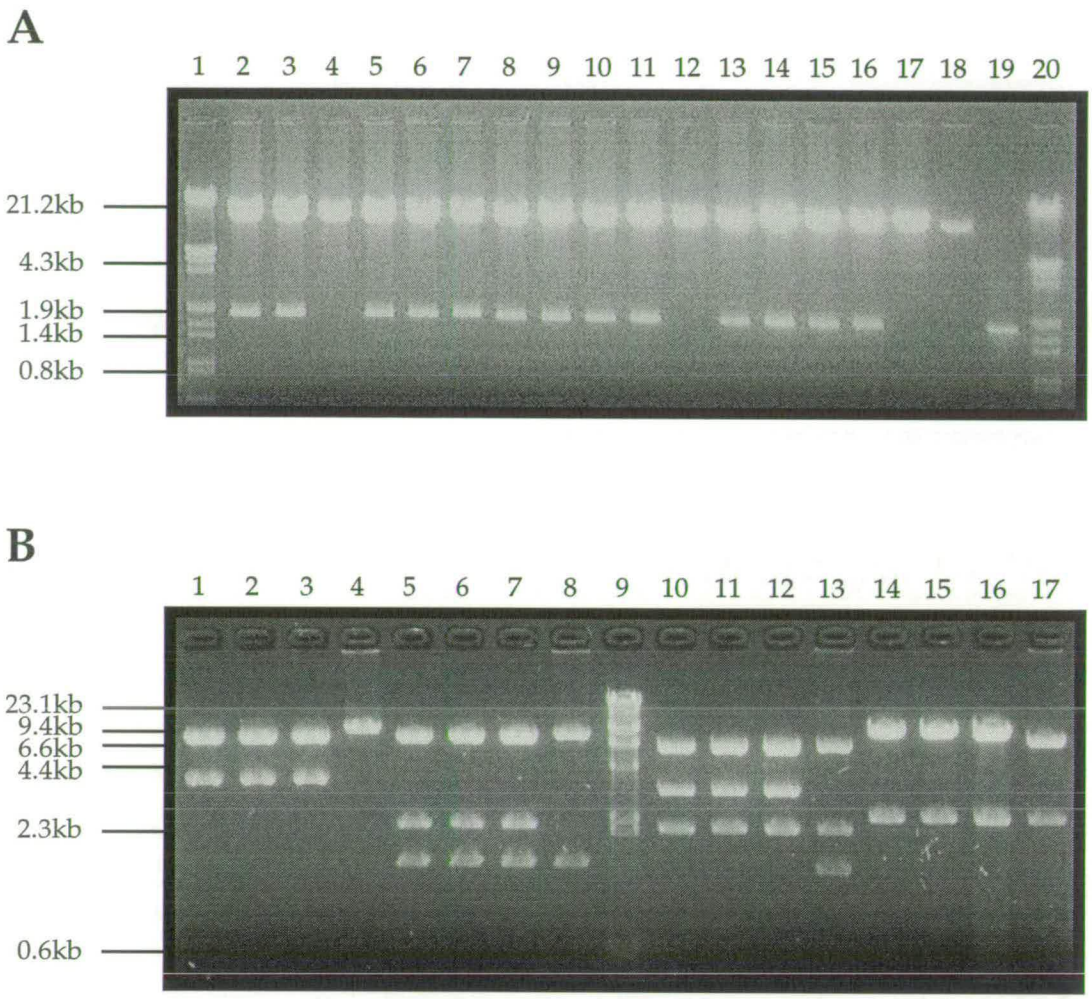


Figure 4.7: Mapping of putative pR1*neo*KO clones: A) All sixteen clones (and the vector pR1KO 2.1) were restriction digested with *Not* I plus *Sal* I to excise the inserted DNA, if present. Lane 1= lambda *Hin*d III/*Eco*R I digested DNA, Lane 2-17= miniprep DNAs 1-16, Lane 18= pR1KO 2.1, Lane 19= 200ng *Not* I/*Sal* I PGK-neopA fragment (insert DNA) and Lane 20= lambda *Hin*d III/*Eco*R I digested DNA. B) Three clones (and the vector pR1KO 2.1) were then further restriction mapped by digesting with *Xho* I (Lanes 1-4), *Eco*R I (Lanes 5-8), *Sac* I (Lanes 10-13) and *Asc* I plus *Mlu* I (Lanes 14-17). Lane 1, 5, 10 and 14= miniprep DNA 5.1, Lane 2, 6, 11 and 15= miniprep DNA 6.1, Lane 3, 7, 12 and 16= miniprep DNA 7.1, Lane 4, 8, 13 and 17= pR1KO 2.1 and Lane 9= lambda *Hin*d III digested DNA.

Three clones were further mapped (Figure 4.7B) by restriction digestion (*Xho* I, *Eco*R I, *Sac* I and *Asc* I/*Mlu* I). Insertion of the selectable marker introduces a second *Xho* I site present at the 5' end of the PGK-*neo*pA fragment. Therefore, upon *Xho* I digestion, parental plasmids will only have one fragment of 9.8kb while the new plasmid will have two fragments of 3.7 and 8.0kb. The parental plasmid contains two *Eco*R I sites (in the 3' polylinker and in intron E of the 3' arm) and the final construct should contain an additional site at the 3' end of the PGK-*neo*pA selection cassette.

Therefore, parental plasmids should contain two fragments of 1.5 and 8.3kb while the new construct should contain three fragments of 1.5, 2.2 and 8.0kb. Both parental and new plasmids contain three *Sac* I sites (central polylinker fragment which ends up between the 5' homology arm and the 5' end of the PGK-*neo*pA selection cassette, intron E and exon 7). However, after digestion the parental fragment of 1.4kb in size should increase by 1.9kb to 3.3kb with the other two fragments remaining the same (2.1 and 6.3kb). An *Asc* I/*Mlu* I digest was also performed to check that these sites were unique in the plasmid. Each enzyme should cut once resulting in two fragments corresponding to the plasmid vector DNA (2.4kb) and the targeting construct fragment to be electroporated into ES cells (9.3kb; lanes 14 to 16) or 7.4kb from the parental plasmid (lane 17).

All clones gave the correct patterns for all four digests and clone 5, named pR1*neo*KO 5.1, was alkaline lysis maxiprepped and caesium chloride double banded (sections 2.2.11 and 12) ready for electroporation into ES cells.

4.3 Sequence Analysis of the 3' Arm

As the proof-reading DNA polymerase *UlTma* was used in preference to *Taq* in order to keep the number of errors introduced in the PCR step to a minimum (or none), the entire 3' homology arm was sequenced so that it could be compared with the corresponding sequence from strain 129/Ola mice. Unfortunately, the 129/Ola sequence has not been published and was therefore also required for this comparison. This sequence was obtained by direct sequencing of PCR product generated by amplification from P1-1249, the P1 clone used as a template for the generation of all cloned material. All sequencing was performed as described in section 2.2.15 using the ABI377 automatic sequencing apparatus (Applied Biosystems), ABI sequencing kits (Applied Biosystems) and the sequencing primers shown and listed in Figure 2.8 and Table 2.9.

The aim in this sequencing project was, wherever possible, to produce a "contig" for each region with at least 2-fold coverage on each strand. A summary of the two sequencing strategies is shown in Table 4.8. This was achieved in the case of the cloned 3' arm in the targeting construct but was not possible when PCR product was sequenced directly due to problems obtaining sequence at the ends of the PCR product and difficulties

	Sequencing of Cloned 3' Arm	Direct Sequencing of 3' Arm PCR Product
Total No. of Runs	66	64
Average Run Length	412	460
Cover Above Threshold	99%	87%
Cover Below Threshold	0%	1%
Only on One Strand	1%	12%
Only Sequenced Once	0%	0%

Table 4.8: Summary of 3' homology arm sequencing strategies: Targeting construct, pR1*neo*KO 5.1 and PCR product were sequenced as described in section 2.2.15. The number of reactions performed and the average sequence length is given for each project. During sequencing of the cloned 3' arm in pR1*neo*KO 5.1 almost all of the sequence was covered above the threshold set (twice on each strand). Only 55 bases at the extreme 5' end of the homology arm were not sequenced on both strands (due to the lack of an appropriate sequencing primer). The direct sequencing of the PCR product was more problematical as only 87% of the sequence was covered above the threshold set. Of the remaining sequence, 12% was sequenced on one strand only and 1%, although sequenced on both strands fell, below the thresholds set.

sequencing a polyG tract in intron E (DBA/2J sequence contains 12 G residues).

As mentioned above, problems were encountered with the sequencing of a polyG tract and this is thought to be due to errors in the PCR reaction rather than an artifact of the sequencing reaction. Figure 4.9A shows a sequencing trace going through the polyG tract using the targeting construct as a template. Here, fifteen G residues can be seen clearly, with the sequence after these residues being easy to read as well. Figure 4.9B shows a sequencing trace using the same primer with the PCR product. In this case, no sequence can be read after the G residues and many shadow peaks can be seen at -1 and +1 positions, and often -2 and +2 as well. This suggests that a heterogeneous population of PCR products is generated in the PCR reaction since sequencing of the cloned material posed no problems. By looking at the sequence of the PCR product, at least twelve real peaks can be seen and the possibility of a thirteenth cannot be ruled out. However, from this plot (and others) it does not appear that there are more than thirteen, the extra peaks, in actual fact, being false, shadow peaks.

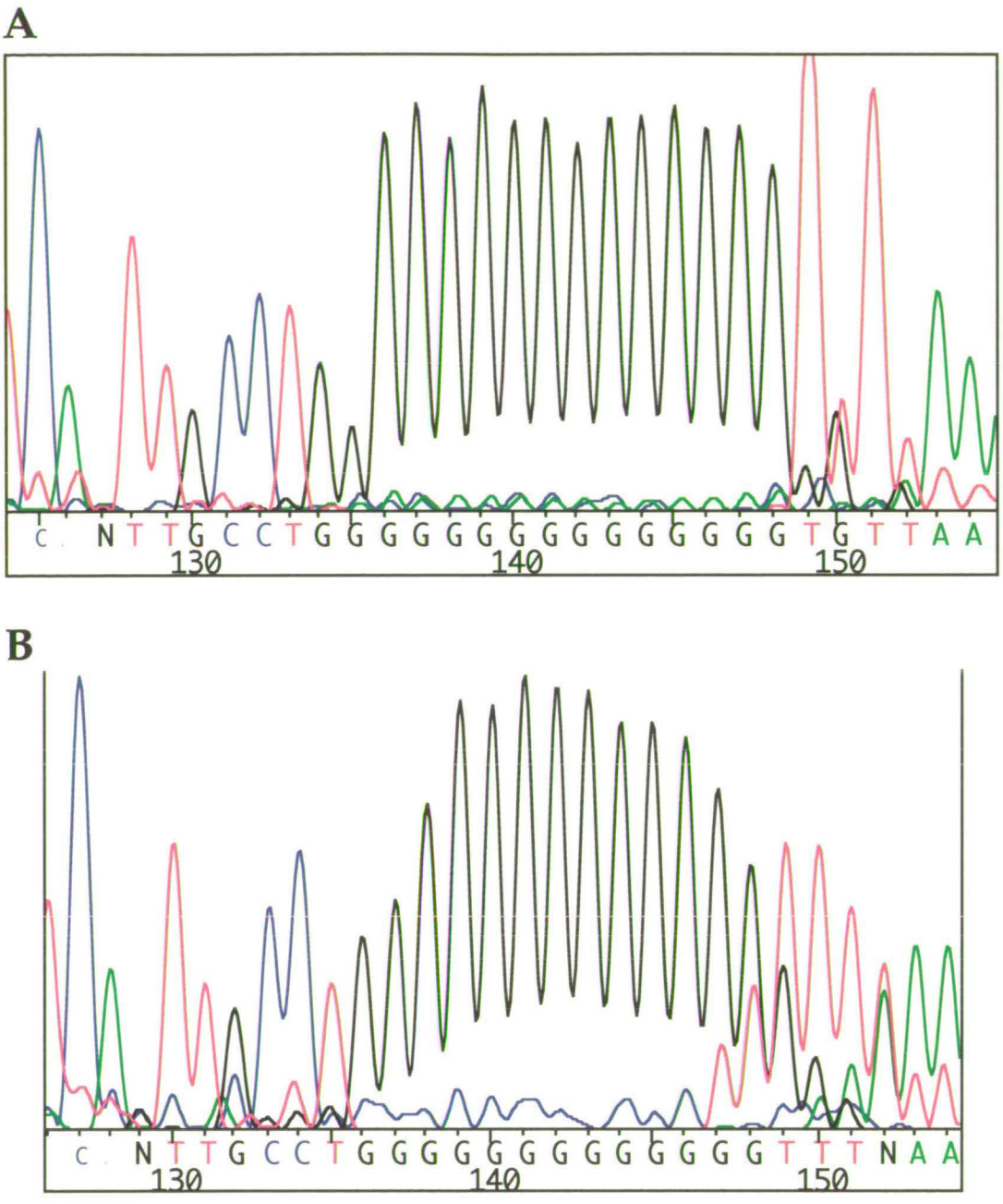


Figure 4.9: Sequencing of the polyG tract in intron E using sequencing primer JJM 267: A) Extract of the sequence trace obtained when sequencing the targeting construct, pR1*neo*KO 5.1. Fifteen G residues can be seen with following sequence also easily read. B) Extract of the sequence trace obtained when sequencing the PCR product directly. An uncertain number of G residues (probably 12 or possibly 13) can be seen with following sequence being impossible to read.

To prove that the PCR product was in fact a heterogeneous population, the two extra 3'Arm clones which were mapped in section 3.4.2 were sequenced using the same primer p3'Arm 3.1 was found to contain twelve

residues and p3'Arm 5.1 contained eight G residues thus supporting this hypothesis. In an attempt to solve these problems, new PCR product was generated using *Taq* DNA polymerase. When this was sequenced, the same staggering of bands was observed suggesting that this is a problem common to different thermostable DNA polymerases.

The two consensus sequences were aligned to identify any mismatches, *i.e.* errors, which had arisen during the PCR reactions, and a summary is given in Table 4.10 (The actual alignment is shown in Figure 4.11). In total there are 11 differences between the two sequences, a sequence similarity of 99.7%, with the longest uninterrupted region of homology being 1.2kb, situated in the centre of the homology arm.

A comparison was also made between the published DBA/2J sequence (89) and the newly determined 129/Ola sequence (Figure 4.11). Here, 28 differences (99.2% identity) were identified (Table 4.10). This figure may be even lower, as many of the differences occur in GC-rich regions which can be difficult to sequence since they result in compressions. As many as fifteen of these differences reside in GC-rich regions (at least two G or C residues together), and if all of these are the results of sequencing compressions a new estimation of the identity is 99.6%. This means that rather than being isogenic, the 3' homology arm used, actually displays a similar degree of sequence identity to that of a closely related strain. Targeting results obtained using constructs built with non-isogenic DNA have been published (275) and generally show a lower targeting efficiency although clones undergoing homologous recombination can still be identified.

Type of Mutation	129/Ola Cloned	DBA/2 Published
Base-Pair Substitution	7	10
Base-Pair Deletion	1	13
Base-Pair Insertion	3	5
Total	11	28
% Similarity	99.7	99.2

Table 4.10: Summary of differences between the cloned 3' homology arm and the corresponding DBA/2 region compared to the 129/Ola PCR sequence: Differences within the 129/Ola Cloned and DBA/2 Published sequences are divided into base-pair substitutions, deletions and insertions and their corresponding numbers are given.

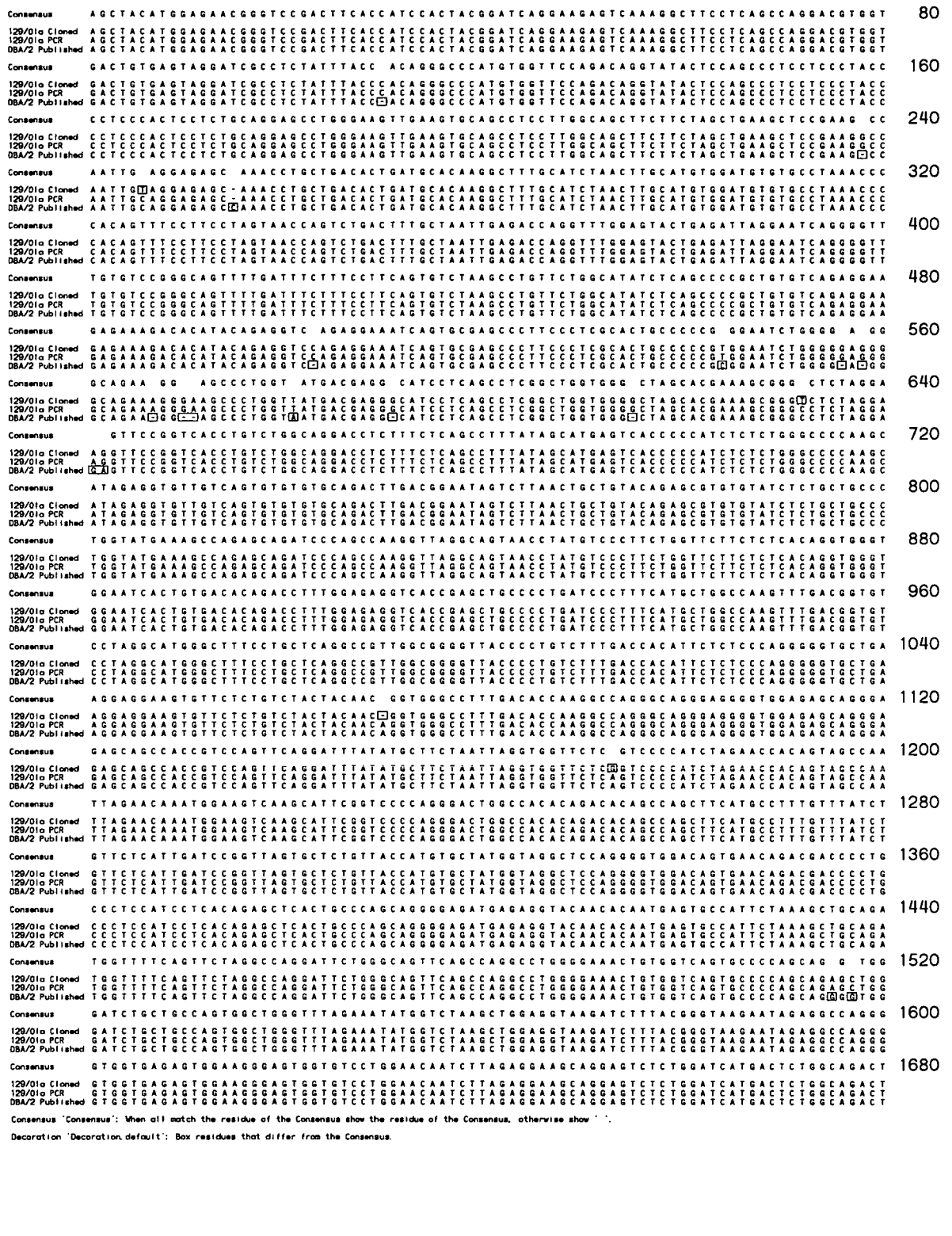
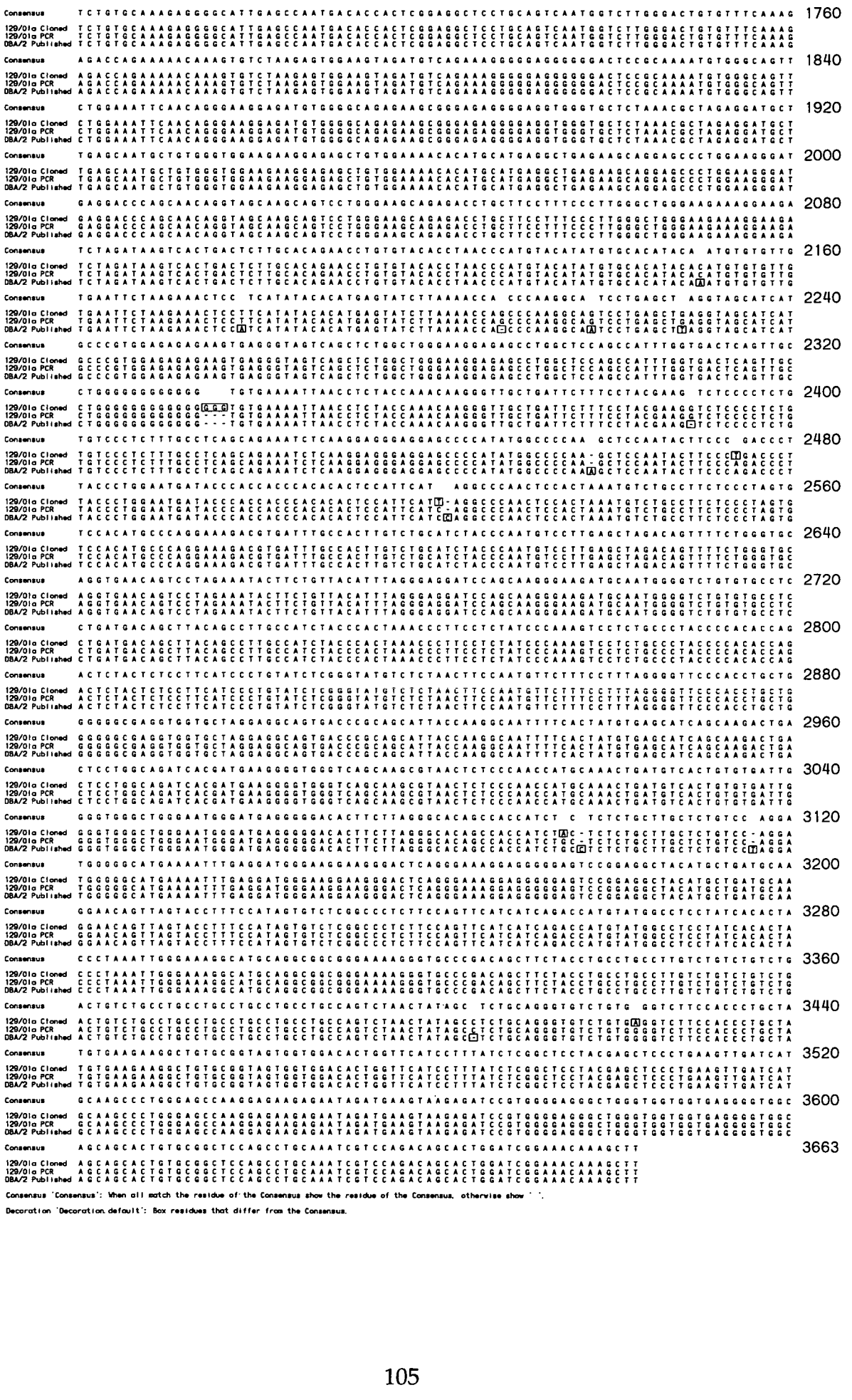


Figure 4.11: Three way sequence comparison between the 129/Ola cloned sequence, the 129/Ola PCR sequence and the DBA/2 Published sequence: The three sequences are shown above together with the consensus sequence. Any bases which do not match the 129/Ola PCR sequence are boxed and no consensus base is given at this position. Continued on the next page.



Consensus 'Consensus': When all match the residue of the Consensus show the residue of the Consensus, otherwise show ' '.

Decoration 'Decoration default': Box residues that differ from the Consensus.

4.4 Discussion

In this chapter the construction of a *Ren-1^d* directed gene targeting vector is reported. This construct, pR1*neo*KO, contains a total of 7.3kb of homology and a PGK-*neo*pA cassette for selection of G418-resistant ES cell colonies.

The discovery that the cloned 3' homology arm contains eleven mismatches was unexpected. In a similar experiment within the laboratory, the 3' homology arm of a *Ren-2* directed targeting construct which was amplified using the proof-reading enzyme *Pfu* (Stratagene) contained no errors compared with the 129/Ola sequence for that region (M. Sharp *et al.*, unpublished data). Even if *Taq* had been used, calculations using published error rates and template doubling estimations (324) suggest that only 3-5 errors could be expected. An explanation for this has been published recently. Here the author determined the error rates for many thermostable DNA polymerases, both proof-reading and *Taq* (324). In general, these results show that proof-reading enzymes have a lower mutation rate than *Taq*. However the highest mutation rate of all (5.0×10^{-5}) was found for *UlTma* (compared to 8.0×10^{-6} for *Taq*). Cline suggests that this may be related to the structure of *UlTma*. Most other proof-reading enzymes are unique proteins isolated from different thermophillic bacteria whereas *UlTma* is a genetically modified version of a *Taq* like enzyme (*Tma*) created by removal of the N-terminal portion of the protein. He suggested that although this enzyme now possesses a 5' to 3' proof-reading activity (at significantly lower levels than other proof-reading enzymes) it may have a much reduced specificity for the incorporation of the correct nucleotides in the 3' to 5' direction.

Although not straightforward, it has been possible to build a targeting construct from PCR generated homology arms. Sequencing of one of these arms reveals that the PCR reaction using *UlTma* has resulted in the introduction of errors reducing the isogenicity of the homologous DNA. These mutations may lead to a reduced targeting efficiency but previous studies performed using non-isogenic targeting constructs still resulted in targeted clones (275). Confirmation of this would come from the next step of the project which was to generate targeted ES cells.

CHAPTER 5

Generation and Transmission of a *Ren-1^d* Null Mutation

5.1 Introduction

Having built a targeting vector, the next step in this project was to use the construct to disrupt the *Ren-1^d* gene in ES cells and subsequently to derive new mouse lines from these cells. After targeting construct DNA had been electroporated into ES cells and the drug-resistant colonies selected, it became necessary to distinguish between clones in which the targeting construct had inserted randomly and the desired clones in which homologous recombination had occurred. The standard procedure for screening for targeted events is Southern blot hybridisation (although PCR based strategies can also be used). Screening by Southern blot hybridisation involves identifying the predicted homologous event in both the 3' and 5' arms of homology using probes external to the homology present in the targeting construct.

In this chapter the screening of G418-resistant ES cell clones and transgenic mice by Southern blot hybridisation is described. Firstly, ES cell DNAs were screened allowing the identification of clones in which the *Ren-1^d* gene was disrupted. These cells were then used to generate chimaeric mice which were bred to produce pups showing coat colour transmission. Pups were then screened by Southern blot hybridisation for inheritance of the *Ren-1^d* null allele and animals containing this engineered gene were used to produce homozygous and more heterozygous offspring. After extensive breeding and screening enough animals were generated and genotyped for subsequent analysis.

Since the *Ren-1^d* and *Ren-2* genes display such strong sequence similarity it may be possible to get aberrant gene targeting, (*i.e.* the disruption of the *Ren-2* gene with the *Ren-1^d* targeting construct) and also means that most genomic probes will detect both the *Ren-1^d* and *Ren-2* genes.

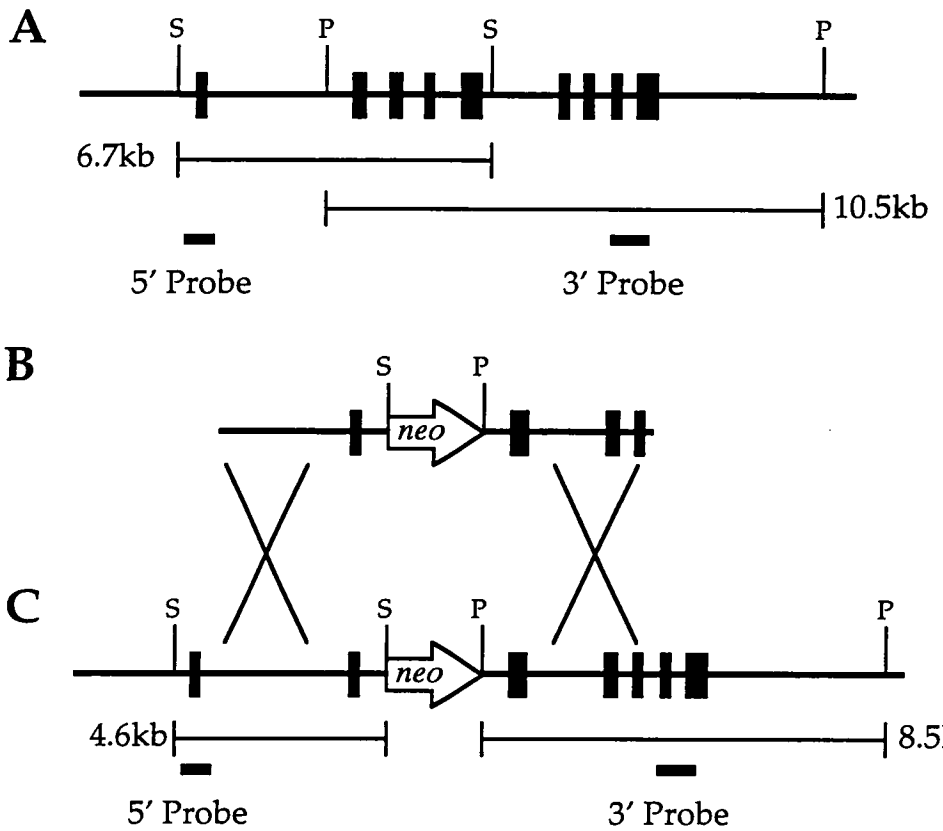
A previous paper by Miller *et al.* (287) reported successful targeting of the *Ren-1*^{d} locus without observing any aberrant targeting within the *Ren-2* gene. Sharp *et al.* (286) reported similar observations concerning the targeting of the *Ren-2* gene. This suggests that the mechanism which differentiates between truly homologous sequences and similar sequences is highly specific and can differentiate between the two genes. The screening strategy is common to both renin gene targeting experiments and must distinguish between all four possible results, *i.e.* the endogenous *Ren-1*^{d} and *Ren-2* genes as well as the corresponding targeted alleles. Figure 5.1 shows a simplified graphic representation of the screening strategy showing only the predicted fragment sizes for the endogenous and targeted *Ren-1*^{d} gene. These do, however, differ from the expected *Ren-2* endogenous and targeted fragment sizes, all of which are summarised in Table 5.1D. Figure 5.1 shows the 5' screening using *Sac* I, however *Eco*R I should give similar results.

5.2 Identification of *Ren-1*^{d} +/- ES Cells

Passage 24 E14Tg2a ES cells (291) were electroporated with 150µg of *Asc* I/*Mlu* I digested pR1*neo*KO DNA. After eight days selection in ES cell medium containing G418, well separated colonies were picked and expanded in 24 well plates, where possible splitting for freezing and DNA preparations. Not all colonies were suitable, due to contamination, failure to grow or growth at a slow rate (probably due to seeding at too low a density). This left 313 clones (passage 26) suitable for subsequent analysis (Table 5.2 summarises the ES cell culture results).

5.2.1 Test Southern

During the initial screening experiment it was intended to load as many samples onto a gel as possible to speed up the procedure. A test Southern blot hybridisation was performed to determine if both, or either, of the digests (*Sac* I and *Eco*R I) to be used in conjunction with the 5' "external" probe allowed identification of targeted ES cell clones when digests were only run a short distance into the gel. This was done by blotting wild-type 129/Ola DNAs and DNAs from ES cells and mice in which the *Ren-2* gene is known to have been disrupted (286). In each digest the two endogenous fragments are of similar sizes and migrated as a doublet. Therefore, to show



D

Digest (Probe)	Endogenous *Ren-1d*	Targeted *Ren-1d*	Endogenous *Ren-2*	Targeted *Ren-2*
Sac I (5' Probe)	6.7kb	4.6kb	7.0kb	4.9kb
*Eco*R I (5' Probe)	8.7kb	7.6kb	9.3kb	8.2kb
Pvu II (3' Probe)	10.5kb	8.5kb	7.8kb	5.6kb

Figure 5.1: Screening strategy for the identification of *Ren-1*^{d} targeting events: A) The *Ren-1*^{d} locus is shown along with the position and sizes of the endogenous *Sac* I and *Pvu* II fragments detected using the 5' and 3' "external" probes respectively. B) The *Ren-1*^{d} targeting vector fragment (*Asc* I and *Mlu* I digested pR1*neo*KO) which was electroporated into ES cells. C) The *Ren-1*^{d} -/- allele expected after homologous recombination in both homology arms is shown. The position and sizes of the novel *Sac* I and *Pvu* II fragments detected using the 5' and 3' "external" probes respectively are also shown. Restriction sites are P= *Pvu* II and S= *Sac* I (not all restriction sites are shown). D) Predicted fragment sizes from targeted and endogenous *Ren-1*^{d} and *Ren-2* genes when the listed enzymes are used to digest DNA before hybridising with the 5' or 3' "external" probes.

that the chosen 5' probe (a *Ren-2* genomic fragment) hybridised satisfactorily to *Ren-1*^{d} sequences, negative 129/Ola liver DNA was spiked with 1 or 10 copy number equivalent of *Ren-1*^{d} (pRn34) or *Ren-2* (pRen2-5'XK) containing plasmids (each of which migrated at a different size to the endogenous fragments).

Stage	Number of Colonies
Total Colonies Picked	432
- Lost due to Contamination	- 8
- Lost due to Failure to Grow	- 84
- Lost due to Very Slow Growth Rate	- 27
Total Colonies for Screening	313
- DNA Preparations Lost	- 9
Total Number of Clones Screened	304 (100.0%)
Clones Positive by 5' Screen	4 (1.3%)
Clones Positive by 3' Screen	3 (1.0%)
Clones Injected Into Blastocysts	2

Table 5.2: Summary of ES cell culture and screening: A total of 432 colonies were picked for subsequent screening. After losses due to contamination, failure to grow or loss of DNA pellets, 304 colonies were screened with the 5' "external" probe. Four of these were positive, three of which were also targeted in the 3' arm. After blastocyst injection of two of these clones, one transmitted the disrupted gene through the germ-line.

This test blot (Figure 5.3) shows that, as expected, the *Ren-2*-derived 5' probe hybridises satisfactorily to both *Ren-1*^{d} and *Ren-2* sequences as expected (lanes 1-4 and 12-15). The plasmid pRen2-5'XK contains unique *Eco*R I and *Sac* I sites resulting in a hybridising fragment of 12.3kb in each digest. The *Ren-1*^{d} derived plasmid (pRn34), however, contains one *Sac* I site and two *Eco*R I sites, therefore the probe detects a full sized, 9.4kb fragment in the *Sac* I digests and a smaller, 5.2kb fragment in the *Eco*R I digested DNAs.

The *Eco*R I digests do not seem to be informative as no additional fragment can be seen in the lanes containing *Ren-2* targeted ES cell DNAs (lanes 9-10). The *Sac* I digests, however, do show the presence of an extra fragment (lanes 19-21). For this reason it was decided to do the routine screening of the ES cell clones by probing blotted *Sac* I-digested DNA with the external 5' probe. As the targeted fragment is well separated from the endogenous fragments (which appear as a doublet) it was decided to run samples double loaded, *i.e.* run with two sets of samples loaded 10cm apart. It should be noted that the high molecular weight fragments hybridising in lanes 6 and 8 are due to the fact that the tail DNAs were not digested.

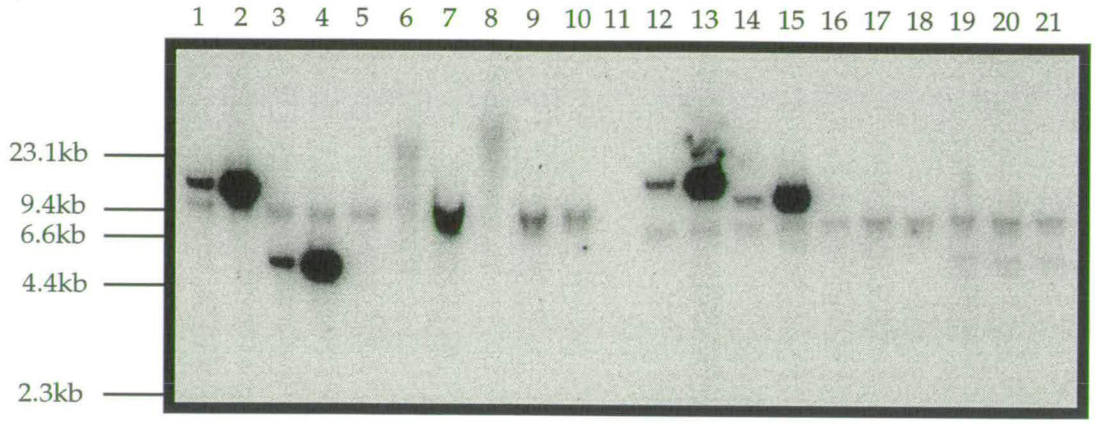


Figure 5.3: Test Southern blot hybridisation: *Eco*R I- (Lanes 1-10) and *Sac* I-digested (Lanes 12-21) and Southern blotted DNAs from a wild-type 129/Ola mouse liver either, on its own, or spiked with a *Ren-1*^{d} (pRn34) or *Ren-2* (pRen2-5'XK) containing plasmid. Also included were a nontargeted ES cell DNA (P5A6), ES cell DNAs in which *Ren-2* had been targeted (P2A1 and P5A5 (286)) and tail DNAs from a wild-type 129/Ola and *Ren-2* targeted mouse (ear tags 524 and 526 respectively (286)). Lane 1 and 12= wild-type 129/Ola liver DNA spiked with 1 copy of pRen2-5'XK, Lane 2 and 13= wild-type 129/Ola liver DNA spiked with 10 copies of pRen2-5'XK, Lane 3 and 14= wild-type 129/Ola liver DNA spiked with 1 copy of pRn34, Lane 4 and 15= wild-type 129/Ola liver DNA spiked with 10 copies of pRn34, Lane 5 and 16= wild-type 129/Ola liver DNA, Lane 6 and 17= tail DNA 524, Lane 7 and 18= ES cell DNA P5A6, Lane 8 and 19= tail DNA 526, Lane 9 and 20= ES cell DNA P2A1, Lane 10 and 21= ES cell DNA P5A5 and Lane 11= lambda *Hin*d III digested DNA. These samples were run in conjunction with lambda *Hin*d III, the positions of the fragments being shown on the left hand side of this figure.

5.2.2 Southern Blot Analysis of ES Cell DNAs

ES cell DNAs were digested with the restriction enzyme *Sac* I, blotted after agarose gel electrophoresis and hybridised with the 5' probe. An example of such an autoradiograph is shown in Figure 5.4. Clone 1D6 (lane 9) has an extra fragment of approximately the right size to be indicative of a *Ren-1*^{d} targeting event. Three other such clones (5' targeting efficiency of 1.3%) were identified, and were then screened for a homologous recombination event in the 3' arm by Southern blot hybridisation of *Pvu* II-digested and blotted DNA (Figure 5.5). The results show that three of the four clones identified had also undergone homologous recombination in the 3' arm (an overall targeting efficiency of 1.0%) and were therefore suitable for injection into blastocysts to generate chimaeric mice (Table 5.2).

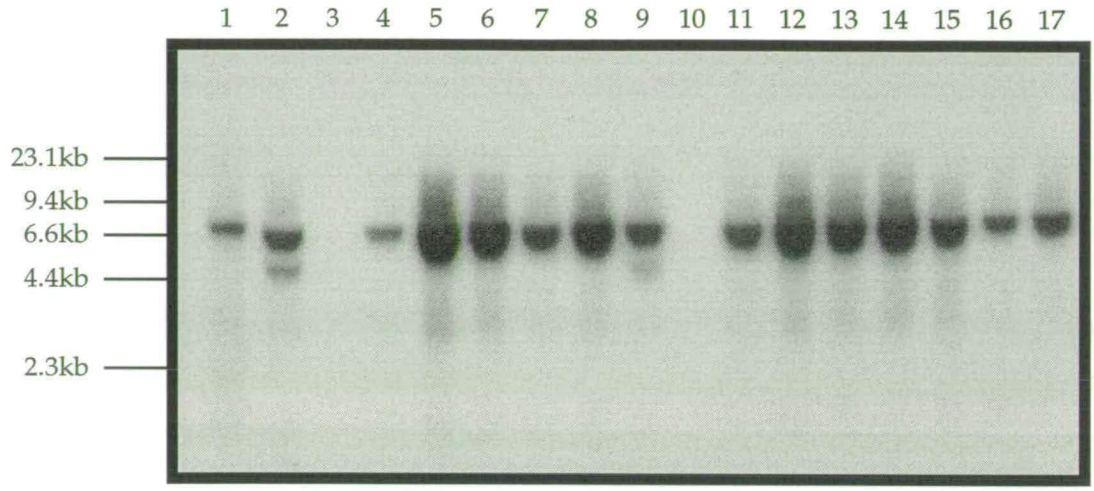


Figure 5.4: 5' Screening by Southern blot hybridisation: *Sac* I-digested and Southern blotted DNAs from G418 resistant ES cells clones elecroporated with the pR1*neo*KO targeting construct. Wild-type 129/Ola liver DNA was included as a negative control with DNA from ES cell clone P2A1 being a positive control (286), possessing a targeted disruption of the *Ren-2* gene. Clone 1D6 appears to have undergone homologous recombination in the 5' homology arm. DNA from ES cell clone 2A1 was lost during the preparation of the DNA therefore no fragments hybridise in this lane. Lane 1= wild-type 129/Ola liver DNA, Lane 2= P2A1, Lane 3= lambda *Hin*d III marker DNA, Lane 4-9= 1D1-1D6, Lane 10-16= 2A1-2A6 and Lane 17= 2B1. These samples were run in conjunction with lambda *Hin*d III marker DNA, the positions of the fragments being shown on the left hand side of this figure.

At this stage it was possible to say that the three most promising clones had under gone homologous recombination in the 3' arm of *Ren-1*^{d}, but at the 5' end the situation was not definitive. It was possible to say that the construct had inserted into a renin gene. However, because the expected sizes of the targeted fragments are so similar in size and further, because the DNA preparations contain salt causing fragments of the same size to migrate varying distances it was impossible to size the fragments accurately at this stage. A more accurate and diagnostic *Sac* I Southern was performed later using mouse tail DNA preparations and will be discussed in section 5.3.

5.3 Generation of Chimaeric Mice and Analysis of Progeny

Two of the three correctly targeted ES cell clones were successfully defrosted and expanded, and cells from both clones were injected independently into mouse blastocysts (Table 5.2). Eighteen chimaeric animals were born showing the expected distortion of the sex ratio in favour of males (ES cells used were XY karyotype) as shown in Table 5.6. Male mice

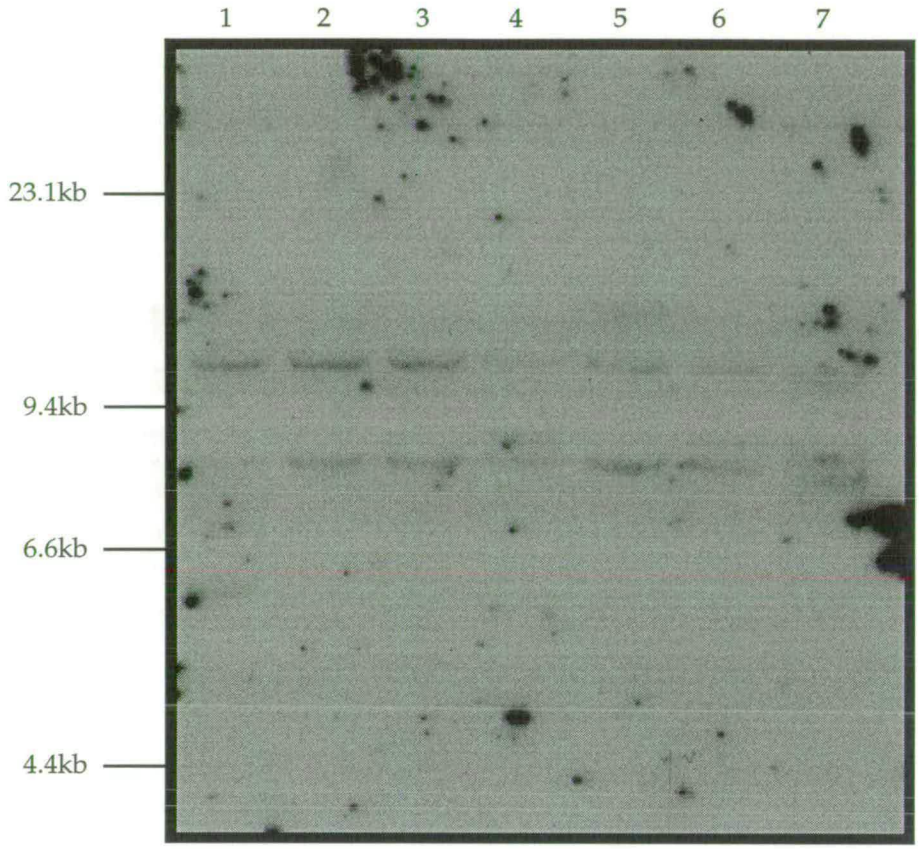


Figure 5.5: 3' Screening by Southern blot hybridisation: *Pvu* II-digested and Southern blotted DNAs from wild-type ES cells (ES cell clones 2D5 and 4C6), an ES cell clone (P2A1) known to contain a disruption of the *Ren-2* gene (286) and the four ES cell clones which are believed to have undergone homologous recombination in the 5' homology arm (1D6, 2D4, 4C5 and 9D5). Clones 1D6, 4C5 and 9D5 have undergone homologous recombination between the 3' homology arm and the *Ren-1*^{d} gene. ES cell clone 2D4 shows no band indicative of homologous recombination and has a larger fragment of 13.0kb hybridising to the probe, this fragment is of unknown origin and no further experiments were performed with these cells. Lane 1= P2A1, Lane 2= 2D5, Lane 3= 4C6, Lane 4= 1D6, Lane 5= 2D4, Lane 6= 4C5 and Lane 7= 9D5. These samples were run in conjunction with lambda *Hind* III marker DNA, the positions of the fragments being shown on the left hand side of this figure.

were bred with 129/Ola females in an attempt to get germ-line transmission of the targeted gene. Female chimaeras were not bred as they are often infertile (325). Of the fourteen male chimaeras, only two (2168 and 2175) produced pups showing coat colour transmission (both from clone 9D5). A photograph of male chimaera 2168 is shown in Figure 5.7.

Clone	Male	Female	Total
1D6	2	2	7
9D5	5 (2)	2	11
Total	14	4	18

Table 5.6: Summary of chimaeric mice: Eighteen chimaeric mice derived from two independent ES cell lines were born. These showed the expected distortion of the sex ratio (14 males:4 females). Two animals showed germ-line transmission of coat colour, with one of these passing the disrupted allele to it's pups.

Pups fathered by a C57BL/6J-derived chimaeric mouse (and 129/Ola mother) are either agouti or beige, the latter being the result of germ-line transmission of a complement of 129/Ola chromosomes. The first three coat colour transmission pups born were shown, by way of Southern blot hybridisation, not to have inherited the disrupted *Ren-1*^{d} gene from their respective fathers (Figure 5.8). The next coat colour transmission pups were a litter of four and a single female pup. These were also screened by *Sac* I digesting tail DNA, blotting and hybridising with the 5' probe (Figure 5.8) and were found to have inherited the disrupted allele.

Figure 5.7: Chimaeric animal 2168: This animal was the transmitting animal used to start the breeding colony when it passed the disrupted allele to five pups (2896-99 and 3193).

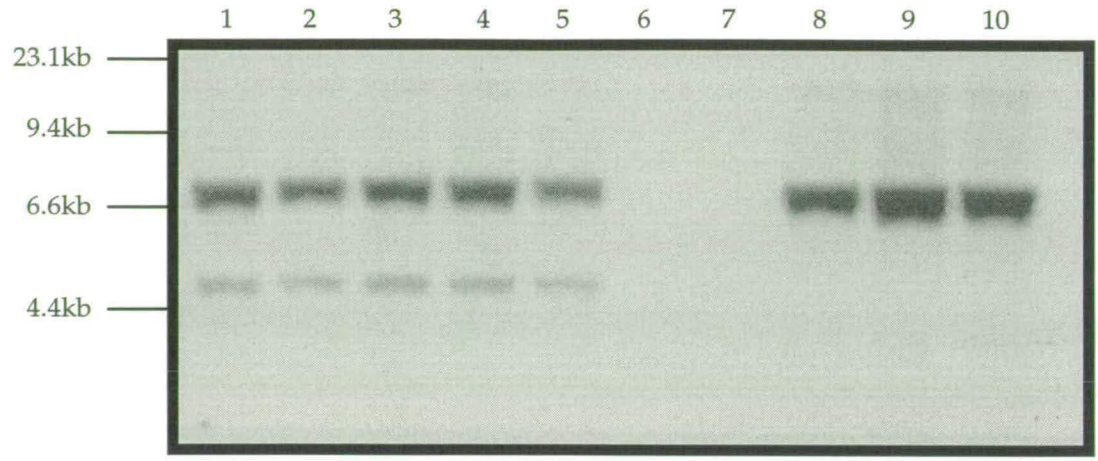


Figure 5.8: 5' Screening of coat colour transmission animals: *Sac* I-digested and Southern blotted DNAs from pups showing coat colour transmission. The animals screened were 2552-3, 2896-9 and 3193 (fathered by chimaera 2168) and animal 2563 (fathered by chimaera 2175). Animals 2896-99 and 3193 had inherited a disrupted allele whereas animals 2552-3 and 2563 had not. Heterozygous animals were then used to set up a breeding colony. Lane 1= 2896, Lane 2= 2897, Lane 3= 2898, Lane 4= 2899, Lane 5= 3193, Lane 6= lambda *Hin*d III/*Eco*R I digested DNA, Lane 7= lambda *Hin*d III digested DNA, Lane 8= 2552, Lane 9= 2553 and Lane 10= 2563. These samples were run in conjunction with lambda *Hin*d III and lambda *Hin*d III/*Eco*R I marker DNA, the positions of the lambda *Hin*d III fragments being shown on the left hand side of this figure.

5.4 Confirmation of *Ren-1^d* 5' Arm Homologous Recombination

The recombination event in the 5' homology arm in section 5.2.2 could, theoretically, be the result of an aberrant targeting event in the *Ren-2* gene. To prove that homologous recombination had occurred between *Ren-1^d* and the 5' arm of the *Ren-1^d* targeting construct, a Southern blot (Figure 5.9) was performed using *Sac* I and *Eco*R I digested tail DNAs (5.0µg). Tail DNAs from both a *Ren-2* and *Ren-1^d* targeted heterozygote mouse were run in parallel lanes after restriction enzyme digestion. As an additional control, these two DNAs were also mixed with each other prior to digestion and electrophoresis. This was in case the two targeted fragments had migrated similar distances in which case it would be possible to see a doublet in the lanes containing both DNAs.

The results of the *Sac* I digests proved conclusively that *Ren-1^d* had been disrupted by homologous recombination. In all four digests (lanes 1-4) the two endogenous fragments (6.8 and 7.1kb) have been resolved and migrate

to a point above both the targeted *Ren-2* fragment (lane 1) and the targeted *Ren-1^d* fragment (lane 2). The two targeted fragments also migrate at different sizes proving that in the animals derived from the 9D5 cell line the *Ren-1^d* gene had been targeted. This is confirmed in lanes three (10µg total) and four (5.0µg total) where two distinct fragments can be seen at 4.7kb and 5.0kb, corresponding to a *Ren-1^d* and *Ren-2* targeted fragment respectively.

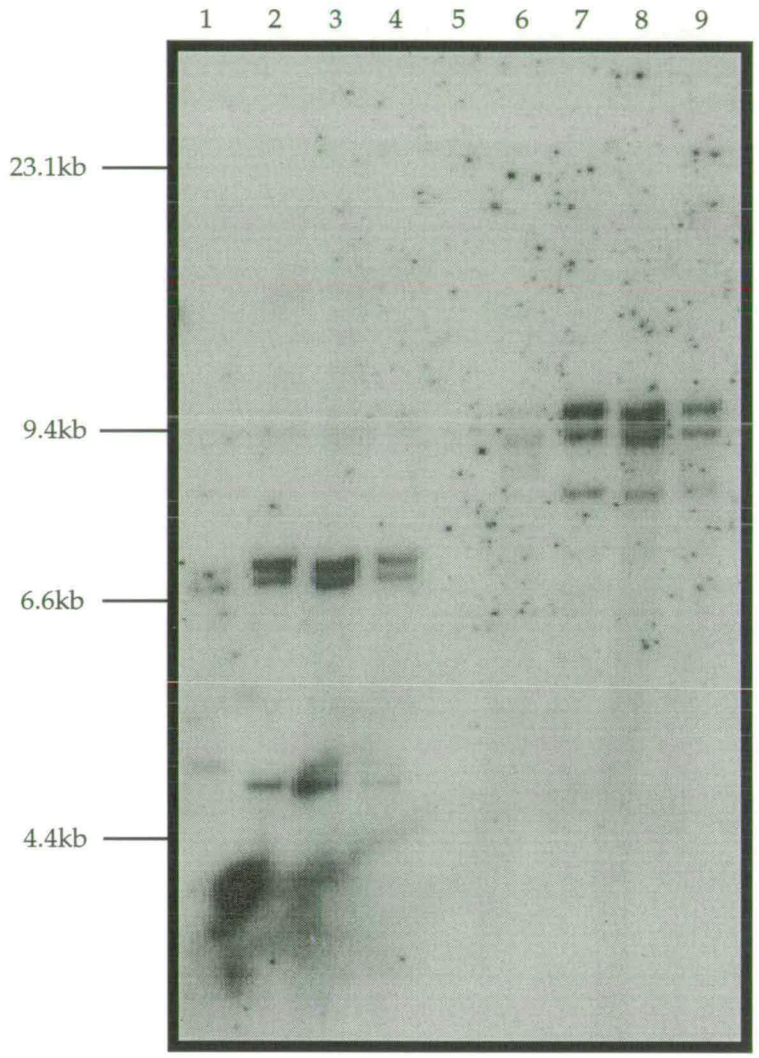


Figure 5.9: Confirmation of homologous recombination between the *Ren-1^d* gene and *Ren-1^d* targeting construct: *Sac* I- (Lanes 1-4) and *Eco*R I-digested (Lanes 6-9) and Southern blotted DNAs from *Ren-1^d* (animal 3553) or *Ren-2* (animal 2416) heterozygous animals (286) run either alone, or together, in the same lane. Both *Sac* I and *Eco*R I digestion results in differently sized "targeted" fragments from *Ren-1^d* or *Ren-2* heterozygous animals proving that the 9D5 *Ren-1^d* targeted ES cell line underwent homologous recombination between the 3' homology arm and the corresponding region of *Ren-1^d*. Lane 1 and 6= 5.0µg 2416 DNA, Lane 2 and 7= 5.0µg 3553 DNA, Lane 3 and 8= 5µg 2416 DNA plus 5µg 3553 DNA, Lane 4 and 9= 2.5µg 2416 DNA plus 2.5µg 3553 DNA and Lane 5= lambda *Hin*d III digested DNA. These samples were run in conjunction with lambda *Hin*d III and lambda *Hin*d III/*Eco*R I marker DNA, the positions of the lambda *Hin*d III fragments being shown on the left hand side of this figure.

The results of the *Eco*R I digests, although not as clear cut, also support this finding. The endogenous fragments which migrated as a doublet on the Test Southern blot (section 5.2.1) have been resolved with two fragments of 9.1 and 9.8kb representing the wild-type *Ren-1*^{d} and *Ren-2* fragments, respectively. The smaller, *Ren-1*^{d} targeted fragment (8.1kb) can be seen clearly in all lanes containing this DNA (lanes 7-9), whereas the larger, *Ren-2* targeted fragment (8.6kb) can be seen clearly only in the digest containing the *Ren-2* targeted DNA alone (lane 6). The *Ren-2* targeted fragment can be seen in the digests containing both DNAs but it is very faint.

5.5 Maintenance of Transgenic Lines

Having achieved germ-line transmission of the mutated *Ren-1*^{d} allele, a series of matings were performed in order to generate enough wild-type, heterozygous and homozygous animals for subsequent analysis. All animals in the growing colony were analysed by Southern blot hybridisation as described previously (*Sac* I or *Pvu* II digested tail DNAs). A preference for *Pvu* II-digested DNAs in conjunction with the 3' probe was adopted because, using this strategy, it was easy to distinguish all three genotypes. In wild-type animals the 3' probe hybridises to the two endogenous fragments (7.8 and 10.5kb); in heterozygous animals three fragments hybridise, the two endogenous fragments and a 8.5kb *Ren-1*^{d} targeted fragment (the endogenous *Ren-1*^{d} fragment should be reduced in intensity by 50% because there is now only one wild-type allele present); animals homozygous for the *Ren-1*^{d} mutation displayed two fragments hybridising to the 3' probe, these being the endogenous *Ren-2* fragment (7.8kb) and the *Ren-1*^{d} targeted fragment (8.5kb); no wild-type *Ren-1*^{d} fragments hybridised since they were replaced in these animals by two mutated copies of the gene.

The Southern blot hybridisation analysis of DNA isolated from a litter of pups produced from a heterozygous intercross is shown in Figure 5.10. Lanes 1-4 show the fragments detected upon hybridisation of *Pvu* II-digested tail DNAs from the litter of four pups and lane 5 shows the genotype of the father as heterozygous. Here, all three genotypes have been generated in a ratio which does not differ significantly from the expected 1:2:1 ratio predicted for a heterozygous intercross (lane 1 is a wild-type, lanes 2 and 4 are heterozygotes and lane 3 shows a homozygous pattern).

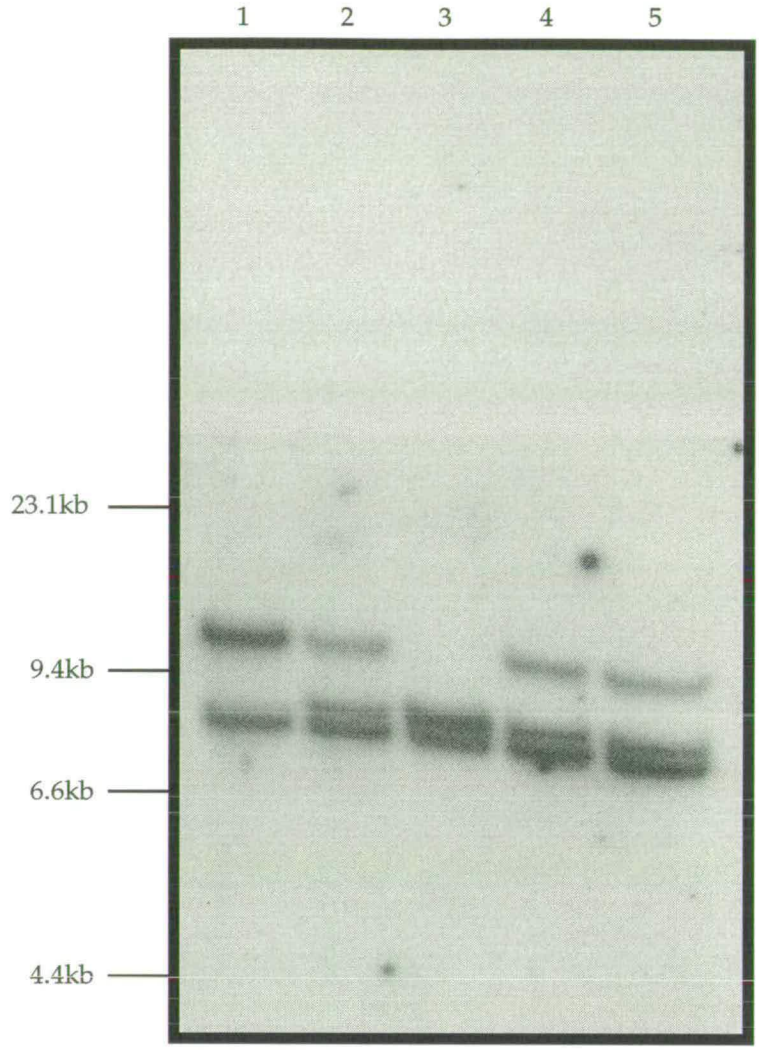


Figure 5.10: Genotyping by Southern blot hybridisation of four pups from a heterozygous intercross: *Pvu* II-digested and Southern blotted DNAs from pups resulting from a heterozygous intercross. The animals screened were 3827-30 and their father 2896. Animal 3829 had inherited two disrupted alleles, whereas animals 3828 and 3830 inherited only one copy of the disrupted gene and animal 3827 inherited two wild-type copies of the *Ren-1*^{d} gene. Lane 1= 3827, Lane 2= 3828, Lane 3= 3829, Lane 4= 3830 and Lane 5= 2896. These samples were run in conjunction with lambda *Hin*d III and lambda *Hin*d III/*Eco*R I marker DNA, the positions of the lambda *Hin*d III fragments being shown on the left hand side of this figure.

At first, the colony was expanded and homozygous animals generated by crossing heterozygous brothers and sisters. An extract from the family tree of Line 2168 is illustrated in Figure 5.11 and shows how a chimaeric mouse (male 2168) was first used to generate heterozygous mice (male 2896 and females 2897-2899) which were, in turn, cross-bred to generate a second generation (3399-3404) containing homozygous animals (3402 and 3407). Subsequently, homozygous males were often crossed with heterozygous or homozygous females in order to generate the numbers of homozygote

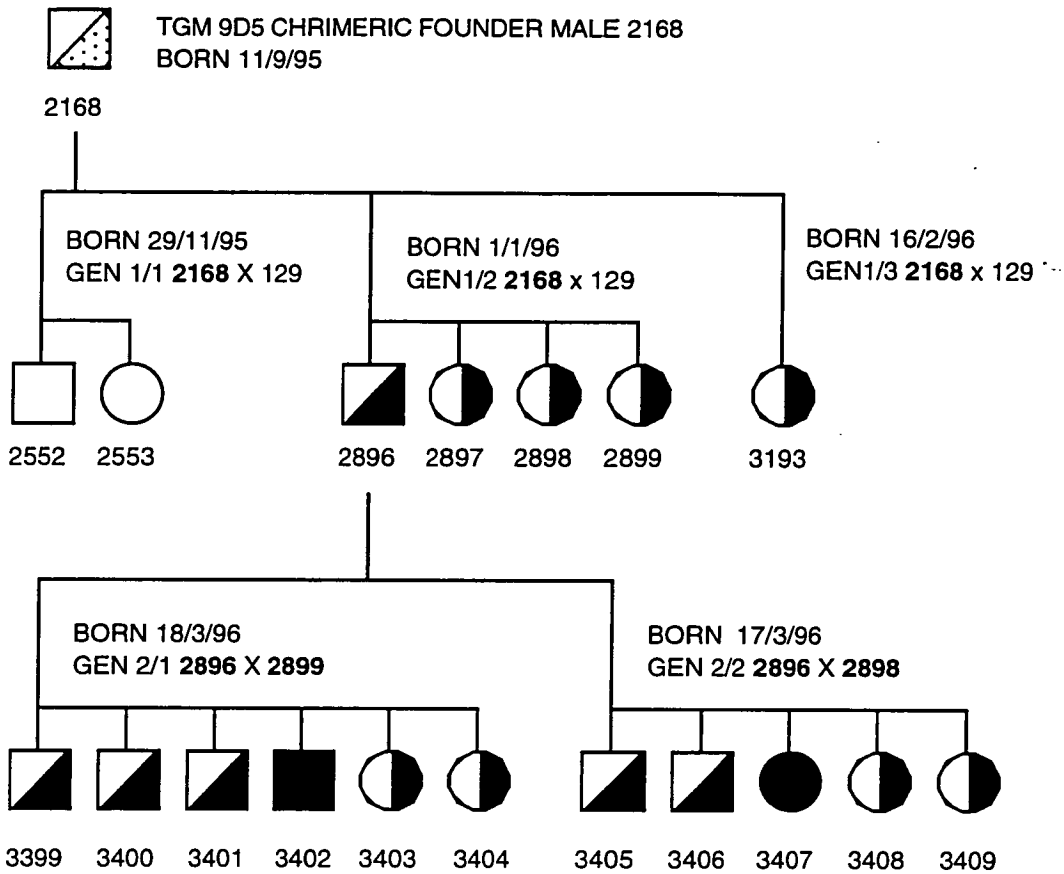


Figure 5.11: Extract from the family tree of line 2168: Initially chimaeric male 2168 initially had three litters containing pups showing coat colour transmission which were analysed by Southern blot hybridisation. The first two animals were negative and the remaining five were found to be heterozygous. The second generation of pups were generated by crossing heterozygous male 2896 with females 2898 and 2899. Of the resultant litters, two homozygous animals were identified (3402 and 3407) with the remaining animals being heterozygotes.

animals required for the phenotypical characterisation. Wild-type animals used for phenotypical analysis were not always littermates since they were often taken from the laboratory stocks of strain 129/Ola mice. This, however, should not affect the results as all three genotypes are on an inbred background and vary only at the *Ren-1*^{d} locus.

5.6 Discussion

This chapter described the screening, by Southern blot hybridisation of ES cells for homologous recombination in the *Ren-1*^{d} gene. The screening of all animals derived from chimaeric animals was also described.

ES cells were screened first by hybridising *Sac* I digested DNA blotted

onto nylon membranes. This was in order to screen all 307 clones as quickly as possible to identify any colonies with a targeting event. At this stage, it was not known whether the recombination event had occurred in the 5' region of *Ren-1^d* or *Ren-2*. Having identified four clones in this manner, they were then rescreened using the 3' probe and *Pvu* II-digested DNA. This confirmed that three of the four clones had undergone homologous recombination between the 3' arm of the *Ren-1^d*-directed gene targeting construct and the corresponding region of the *Ren-1^d* gene. As the limited supply of ES cell DNAs was now exhausted, the confirmation of *Ren-1^d* targeting in the 5' homology arm could not be performed until mouse tail DNAs were available.

Two of the correctly targeted clones were used to generate chimaeric mice, of which, two transmitted coat colour through the germ-line. Pups were screened by Southern blot hybridisation and the first five heterozygous animals were identified. All animals were screened by Southern blot hybridisation using either the 5' probe and *Sac* I digested DNA (for the differentiation between heterozygotes and wild-type animals) or the 3' probe with *Pvu* II-digested DNA for litters likely to contain animals homozygous for the mutation. The latter method was preferred as homozygous mutant animals clearly lose the *Ren-1^d*-hybridising fragment, whereas when using a *Sac* I digest it is difficult to distinguish *Ren-1^d* and *Ren-2* endogenous fragments which migrate as a doublet.

Confirmation of recombination between the 5' homology arm and the *Ren-1^d* gene was obtained by Southern blot hybridisation in conjunction with the 5' probe using *Sac* I-and *Eco*R I-digested DNA. By sizing the fragments accurately, using careful electrophoretic conditions, it was possible to show that the line derived from male 2168 (9D5 ES cells) are correctly targeted in the 5' arm. This mouse line, therefore, contains a disrupted *Ren-1^d*, having undergone homologous recombination in both the 5' and 3' homology arms.

In Chapter 3 a higher than expected number of mutations were found when the 3' homology arm was compared to the 129/Ola sequence. Although this reduces the isogeny, and may therefore affect the targeting efficiency, it was still possible to identify clones which had undergone homologous recombination. These screening strategies were used to screen all new born pups in order to generate enough animals for phenotypical analysis, the topic covered in the next chapter.

CHAPTER 6

Characterisation of the *Ren-1^d* -/- Phenotype

6.1 Introduction

The generation of mice carrying a mutation in the *Ren-1^d* gene allowed the characterisation of the "null phenotype", the absence of Renin-1^d protein. Disruption of the *Ren-1^d* gene permitted the study of the Renin-2 protein in isolation from the Renin-1^d protein and permitted the investigation of the ability of Renin-2 protein to perform all the known functions of renin *in vivo*.

Other constituents of the RAS have been disrupted by gene targeting. Many of these publications report common findings, these being hypotension (276-279, 281, 282) and altered kidney morphology (276, 278-280, 283). In these publications, hypertrophy of vessel walls, and in particular the interlobular arteries, was observed, as well as areas of cortical thinning and severe atrophy.

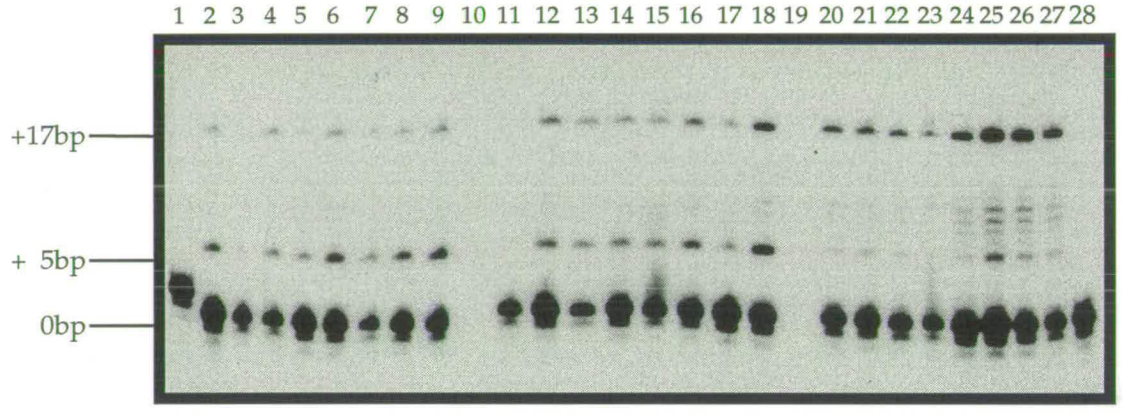
The targeted disruption of the *Ren-2* gene in mice has also been reported (286). This resulted in *Ren-1^d* being the only active renin gene present. The *Ren-2* -/- mice develop normally, display no histological abnormalities in adult kidneys, adrenals or submandibular glands and have blood pressures indistinguishable from wild-type littermates. However, they do display elevated circulating renin concentrations and reduced circulating prorenin concentrations compared to wild-type and heterozygous littermates. So far, no function for *Ren-2* has been found which cannot be compensated for in adult mice, presumably by a factor such as Renin-1^d.

In this chapter homozygous mice were first characterised and compared to heterozygous and wild-type animals, by looking at mRNA expression and renin concentrations in plasma. Once this basic characterisation had been completed animals were then studied for blood pressure homeostasis and histological abnormalities.

6.2 Renin Gene Expression Analysis

The dideoxynucleotide primer extension assay using the exon 8 primer (section 2.2.18) which distinguishes between *Ren-1*^{d} and *Ren-2* was used to investigate the expression levels of both genes in all three genotypes (130). Expression of the *Ren-1*^{d} gene in heterozygous animals did not differ from wild-type mice, suggesting a compensatory upregulation of the wild-type copy of the *Ren-1*^{d} gene (Figure 6.1A). No *Ren-1*^{d} transcript was expected from *Ren-1*^{d} -/- animals, however, a faint *Ren-1*^{d} mRNA-derived signal was

A



B

	+/+		+/-		-/-	
Sex	*Ren-2* Expression	Expression	*Ren-2* Expression	Expression	*Ren-2* Expression	Expression
Male	317±145	100±46%	449±182	142±41%	900±44	284±5%
Female	483±63	100±13%	569±161	118±29%	1867±428	386±23%

Figure 6.1: Kidney RNA primer extension assays in *Ren-1*^{d} knock-out mice: A) A ddCTP primer extension assay designed to distinguish between *Ren-1*^{d} and *Ren-2* mRNAs was performed as described in section 2.2.18. Animals from each genotype and sex were analysed, the above phosphoimage being obtained after 7 days exposure to a phosphoimage screen. An upregulation of *Ren-2* can be seen in the male and female *Ren-1*^{d} -/- animals and a faint *Ren-1*^{d} signal is detectable in male and female homozygous animals. Lane 1= HepG2 RNA (negative control), Lane 2-5= Male +/+ RNAs, Lane 6-9= Female +/+ RNAs, Lane 10-13= Male +/- RNAs, Lane 14-17= Female +/- RNAs, Lane 18-22= Male -/- RNAs, Lane 23-25= Female -/- RNAs and Lane 26= SMG RNA minus RT (negative control). B) Quantitation of *Ren-2* expression (extract from appendix 1). *Ren-2* expression is given in arbitary units, these were then converted to percentages of wild-type activity. Both male and female *Ren-1*^{d} -/- animals display an upregulation of *Ren-2* tra nscription in the kidney.

present. This may be caused by aberrant splicing of the mRNA around the disrupted exons 3 and 4, resulting in a truncated mRNA. Negative controls showed no signal, other than the primer, as expected.

Ren-2 gene expression in the kidneys of wild-type and heterozygous mice did not differ, whereas a slight upregulation was seen in male and female homozygous mutant animals, compared to wild-type mice (Figure 6.1A). Signal strengths were quantified after normalising for sample recovery (Table 6.1B). This showed that *Ren-2* expression was upregulated by 2.84±0.05-fold in males, and 3.86±0.23-fold in females compared to sex-matched wild-type mice. All raw data and subsequent analysis is shown in Appendix 1.

To prove that no wild-type *Ren-1^d* message was being produced, an RT-PCR experiment was performed including a primer which hybridised to a part of exon 4 which was deleted by the *Ren-1^d* targeting construct (section 2.2.6.3). When used in conjunction with an exon 9 primer, a product of 1.0kb would be generated, which was then restriction digested with the enzyme *Ear* I to generate three fragments from a *Ren-1^d* transcript or two fragments from *Ren-2* (Figure 6.2). In this manner, it was possible to prove that no wild-type *Ren-1^d* message was synthesised in *Ren-1^d* -/- animals (lane 1). In lane 1 a larger fragment is also present, this is thought to be full-

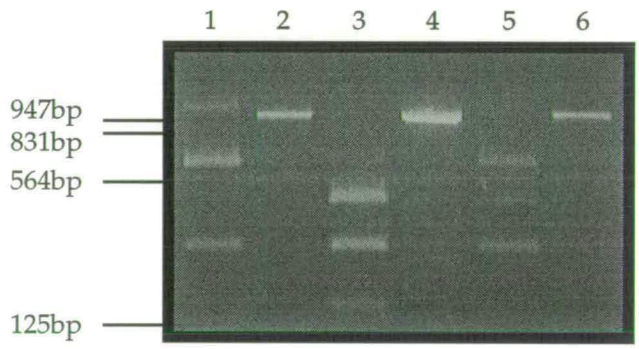


Figure 6.2: Renin mRNA analysis in targeted mice: Amplification of renin cDNA from mouse kidney-derived RNA results in a 999bp product (Lanes 2, 4 and 6). The *Ren-1^d* and *Ren-2* cDNAs can be distinguished by digestion with *Ear* I (Lanes 1, 3 and 5), to produce fragments of 652bp and 347bp from a *Ren-2* cDNA (*Ren-1^d* -/- kidney; Lane 1) or 484bp, 347bp and 168bp from a *Ren-1^d* cDNA (*Ren-2* -/- kidney (286); Lane 3). Lane 5 corresponds to *Ear* I digested cDNA prepared from a *Ren-1^d* +/- kidney, which demonstrates fragments diagnostic of both *Ren-1^d* and *Ren-2* gene expression (not all fragments are visible). These samples were run in conjunction with lambda *Hind* III/*Eco*R I marker DNA, the positions of the fragments being shown on the left hand side of this figure.

length PCR product due to incomplete digestion and appears to run at a diiferent size because of the salt present in the restriction buffer.

6.3 Plasma Enzyme Concentrations

Plasma active renin concentration (PRC) and plasma prorenin concentration (PPC) were determined for plasmas from all three genotypes (section 2.4.2). Table 6.3 summarises all the "raw data" measurements which were used for statistical analysis and graphical representation (Figure 6.4). The nature of this assay means that, occasionally, some clearly erroneous measurements are recorded and are routinely excluded from subsequent calculations. With this in mind, some data points were excluded from subsequent calculations where it was believed figures were not correct. This was done by calculating the means and standard deviations for each sex and genotype and removing any points which lay outside the mean±2 standard deviations according to the directions of Dr. Jörg Peters. Even where only one of the parameters measured lay outside these limits, both measurements were removed from subsequent calculations. An example of this is female animal 3837, where the PRC value is 3.6x the standard deviation higher than the group mean. The PPC value lies within the limits set, but both points were removed from subsequent analyses.

These results show that in males (Figure 6.4A) there is no difference in PRC between the three genotypes (+/+= 240±58, +/-= 170±34 and -/-= 244±63ng Ang I/ml/hour; P>0.05), whereas an increase in PPC is observed in homozygous mutant animals (1341±116ng Ang I/ml/hour) compared to both wild-type (717±64ng Ang I/ml/hour; P=0.0003) and heterozygous males (566±33ng Ang I/ml/hour; P=0.0003). In females (Figure 6.4B), PRC was significantly reduced in homozygous mutant animals (123±28ng Ang I/ml/hour) compared to wild-type controls (229±32ng Ang I/ml/hour; P=0.027), while heterozygous females had an intermediate level (164±34ng Ang I/ml/hour; P>0.05). PPC in females showed the same trend observed in males with female homozygotes showing significantly higher levels (1632±238ng Ang I/ml/hour) compared to wild-type (557±56ng Ang I/ml/hour; P=0.0003) and heterozygous females (528±42ng Ang I/ml/hour; P=0.0003).

+/+		
Animal +Sex	PRC	PPC
0129 M	95	1268
1293 M	620	330
3549 M	514	485
3550 M	49	575
3720 M	1947*	2399*
3723 M	121	801
3728 M	242	542
3831 M	449	989
3838 M	239	712
4613 M	129	618
4614 M	84	754
4617 M	61	527
4628 M	251	1199
4629 M	78	648
4631 M	89	732
4632 M	57	731
4633 M	113	934
5184 M	894	332

+/-		
Animal +Sex	PRC	PPC
3399 M	389	764
3401 M	75	520
3548 M	93	624
3721 M	497	680
3839 M	140	397
3840 M	88	496
3841 M	97	423
4177 M	162	708
4187 M	251	1166*
4188 M	887*	525
4615 M	82	501
4616 M	91	608
4623 M	260	657
4630 M	81	339
5183 M	138	647
5188 M	189	560

-/-		
Animal +Sex	PRC	PPC
3402 M	53	1151
4148 M	55	903
4179 M	300	1316
4186 M	520	1324
4624 M	341	1576
4626 M	165	1250
5189 M	273	1866

+/+		
Animal +Sex	PRC	PPC
1291 F	329	509
1292 F	322	383
3551 F	89	470
3730 F	209	742
3772 F	420	428
3842 F	3881*	922
4618 F	212	950
4619 F	256	522
4620 F	158	654
4635 F	136	430
4638 F	157	483

+/-		
Animal +Sex	PRC	PPC
3553 F	48	448
3555 F	104	622
3727 F	378	654
3826 F	126	741
3835 F	103	295
3837 F	7196*	480
4180 F	454	756
4181 F	277	685
4191 F	238	591
4611 F	174	540
4621 F	89	251
4622 F	53	404
4634 F	155	434
4636 F	55	540
4637 F	41	431

-/-		
Animal +Sex	PRC	PPC
3829 F	197	2591
4189 F	42	964
4190 F	157	1247
4192 F	54	1046
5186 F	77	1455
5187 F	258	1545
5190 F	141	2744
5191 F	56	1463

Table 6.3: Circulating renin levels obtained from individual *Ren-1^d* targeted mice: Experimental mice numbers are listed by genotype and sex (M=male, F=female) and the corresponding plasma active renin concentration (PRC) and plasma prorenin concentrations (PPC) are given in ng Ang I/ml/hour. Measurements marked with a * lie more than 2x the standard deviation above or bellow the mean for that group and were therefore removed before calculating new means and assessing statistical significances. The second parameter measured in plasma from these animals was also excluded from subsequent calculations.

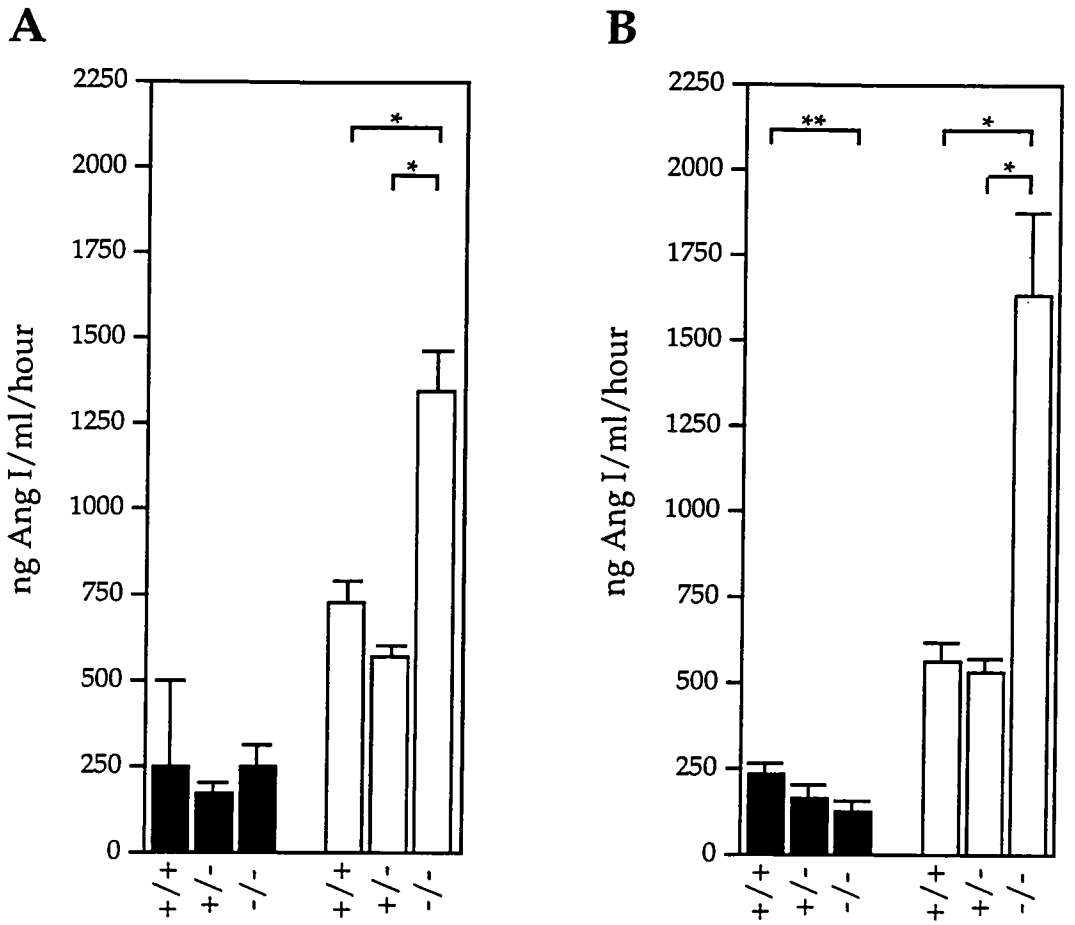


Figure 6.4: Histograms showing circulating renin levels: A) Plasma active renin concentrations (PRC; solid bars) and plasma prorenin concentrations (PPC; open bars) in male *Ren-1^d*-targeted mice. PPC in *Ren-1^d* -/- male mice (n=7) is significantly higher than Ren-*1^d* +/- (n=14) and wild-type mice (n=17). B) Plasma active renin concentrations (PRC; solid bars) and plasma prorenin concentrations (PPC; open bars) in female *Ren-1^d*-targeted mice. PRC in the female *Ren-1^d* -/- mice (n=8) are significantly decreased compared to wild-type (n=10) animals but not the intermediate *Ren-1^d* +/- females (n=14). PPC in the female *Ren-1^d* -/- mice are significantly increased compared to wild-type and *Ren-1^d* +/- animals. * denotes a P-value of 0.003 by Wilcoxon rank test and ** denotes a P-value of 0.027 by Wilcoxon rank test.

6.4 Blood Pressure Homeostasis

Mean arterial blood pressures were calculated from systolic and diastolic readings 1 day after the cannulation operation as described in section 2.4.1 (Table 6.5) and are shown in Figure 6.6. In males, no statistically significant (P>0.05) blood pressure differences are observed between wild-type (93.6±5.2mm Hg), *Ren-1^d* +/- heterozygous (93.0±4.9mm Hg) and *Ren-1^d* -/- homozygous (92.3±3.8mm Hg) male mice. However, a significantly lower blood pressure is seen in *Ren-1^d* -/- females (80.9±3.4mm Hg)

+/+	
Animal (Sex)	Blood Pressure
1291 M	118
3549 M	104
3550 M	89
3720 M	67
3723 M	93
3728 M	100
3831 M	89
5184 M	89

+/-	
Animal (Sex)	Blood Pressure
3399 M	110
3401 M	100
3547 M	119
3548 M	104
3721 M	76
4177 M	60
4187 M	85
4188 M	88
4623 M	92
5183 M	96
5188 M	93

-/-	
Animal (Sex)	Blood Pressure
3402 M	110
4147 M	94
4148 M	92
4179 M	84
4186 M	82
4624 M	105
4626 M	91
5189 M	80

+/+	
Animal (Sex)	Blood Pressure
1292 F	87
1293 F	103
1294 F	86
1295 F	88
1297 F	94
3552 F	102
3730 F	100
3772 F	89

+/-	
Animal (Sex)	Blood Pressure
3553 F	95
3555 F	83
3727 F	96
3835 F	86
4180 F	79
4181 F	77
4191 F	83

-/-	
Animal (Sex)	Blood Pressure
3829 F	65
4149 F	74
4189 F	71
4190 F	89
4192 F	88
5186 F	84
5187 F	92
5191 F	84

Table 6.5: Blood pressures (mean arterial blood pressures) from individual *Ren-1^d* targeted mice: Experimental mice numbers are listed by genotype and sex (M=male, F=female) and the corresponding mean arterial blood pressures are given in mm Hg.

compared with wild-type females (93.6±2.5mm Hg; P=0.012 by Wilcoxon rank test and P<0.01 using an unpaired Student t-test). In female heterozygotes, an intermediate blood pressure can be seen (85.6±2.8mm Hg), but this is not statistically different from either wild-type or *Ren-1^d* -/- mice (P>0.05).

6.5 Histomorphological Analysis

To ascertain the effects of disruption of the *Ren-1^d* gene on organ morphology, tissues known to express renin were sectioned, stained and examined for differences between genotypes. Kidneys, adrenal glands, submandibular glands (males only) and testes or ovaries from *Ren-1^d* -/- (n=4), *Ren-1^d* +/- (n=4) and wild-type animals (n=2) were analysed. No

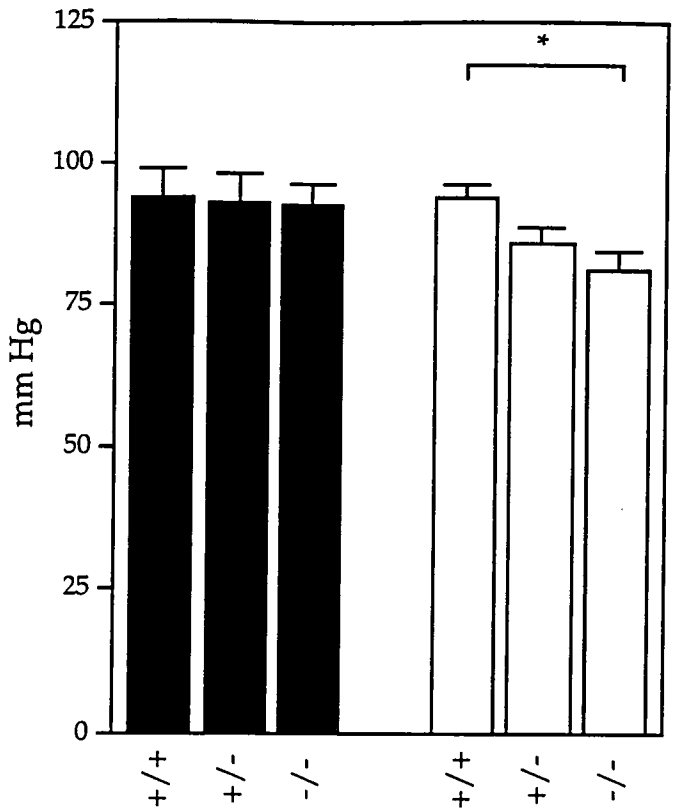


Figure 6.6: Mean blood pressures in *Ren-1^d* targeted mice: Mean blood pressures in *Ren-1^d* knock-out mice are shown in males (solid bars) and females (open bars). Female homozygotes have significantly lower mean blood pressure than wild-type females. Sample sizes are male *Ren-1^d* +/+ (n=8), male *Ren-1^d* +/- (n=11), male *Ren-1^d* -/- (n=8), female *Ren-1^d* +/+ (n=8), female *Ren-1^d* +/- (n=7) and female *Ren-1^d* -/- (n=8). * denotes a P-value of 0.012 by Wilcoxon rank test or P<0.01 using an unpaired Student t-test.

differences were observed in adrenal glands, submandibular glands, testes or ovaries from all 3 genotypes in both sexes (Figure 6.7).

Kidney sections showed subtle abnormalities in *Ren-1^d* -/- animals of both sexes (Figure 6.8), although no vasculature differences were observed as reported for other RAS knock-outs (276, 278-280, 283). When stained with haematoxylin and eosin, sections from the kidneys of *Ren-1^d* -/- animals invariably showed hypercellularity of the macula densa, with these cells also displaying an altered morphology. This manifested itself as a columnar appearance of the tubular epithelial cells which contrasted with the cuboidal morphology of the wild-type controls.

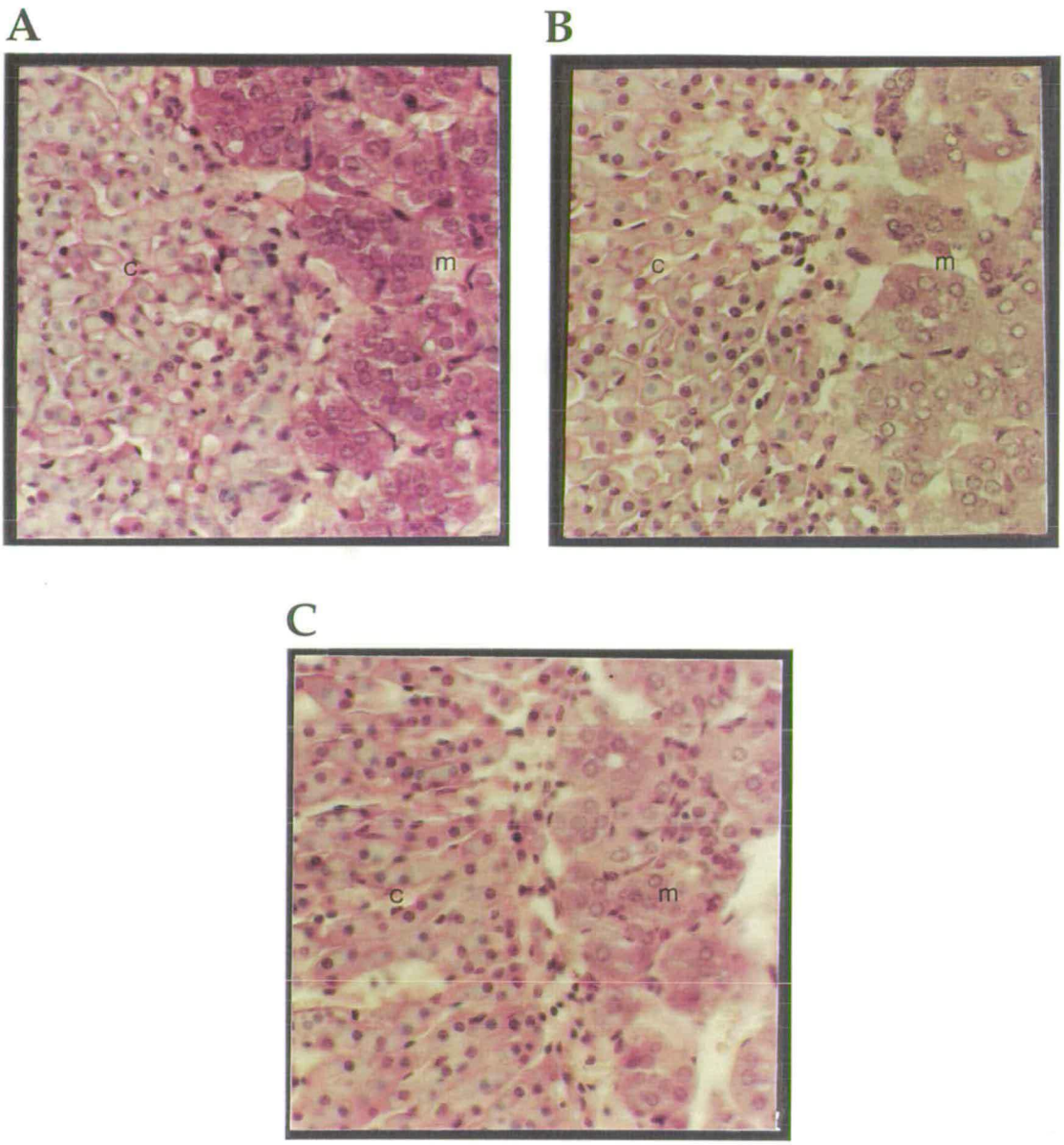


Figure 6.7: Adrenal, submandibular gland, testis and ovary histology: Tissues were sectioned and stained before visualisation using light microscopy. Adrenal glands sections from wild-type (A and D), *Ren-1*^{d} +/- (B and E) and *Ren-1*^{d} -/- (C and F) male (A to C) or female (D to F) mice were studied for histological abnormalities. No differences in adrenal gland morphology were observed. c= adrenal cortex and m= adrenal medula.

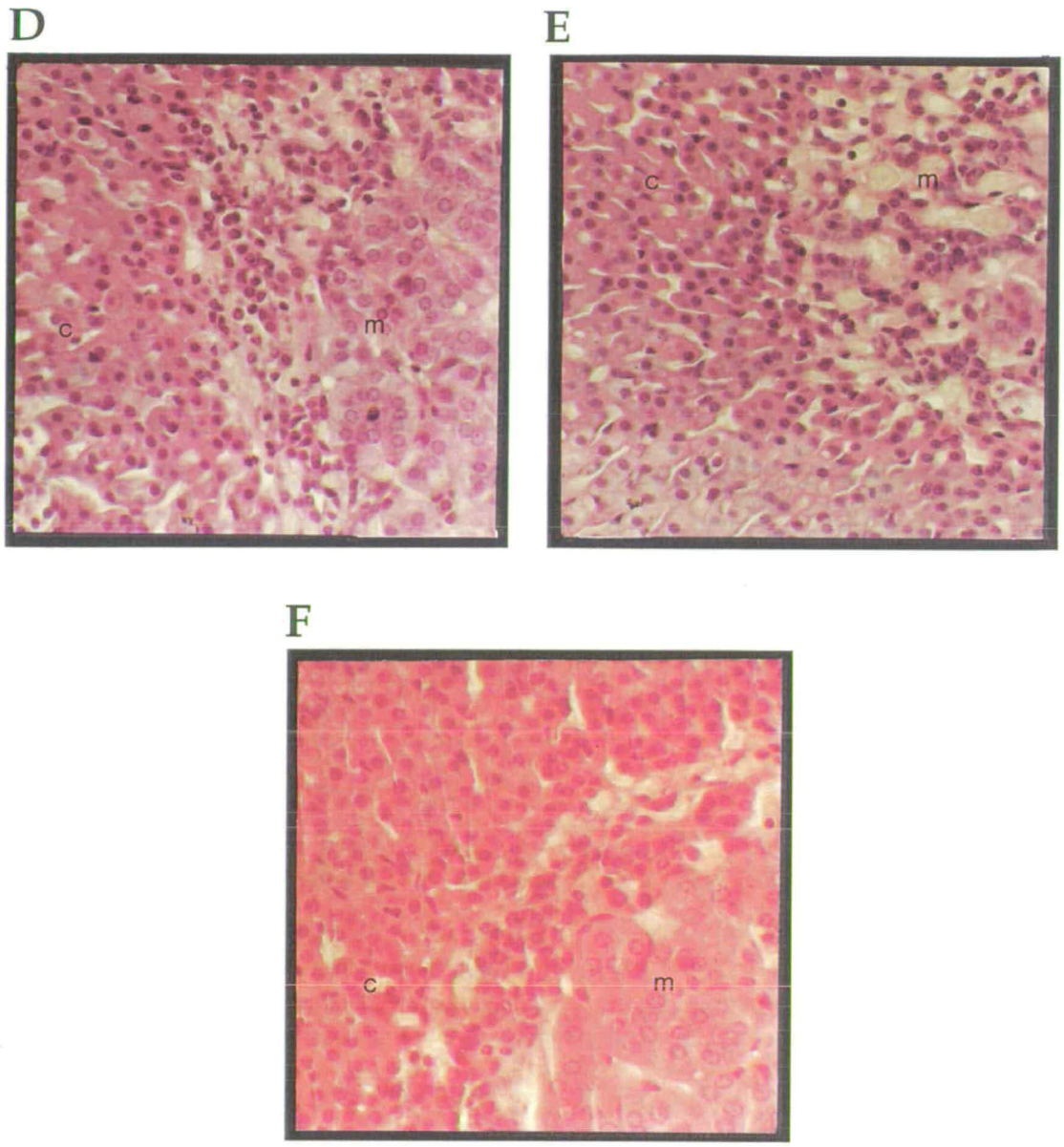


Figure 6.7 (continued): Adrenal, submandibular gland, testis and ovary histology: Tissues were sectioned and stained before visualisation using light microscopy. Adrenal glands sections from wild-type (A and D), *Ren-1*^{d} +/- (B and E) and *Ren-1*^{d} -/- (C and F) male (A to C) or female (D to F) mice were studied for histological abnormalities. No differences in adrenal gland morphology were observed. c= adrenal cortex and m= adrenal medula.

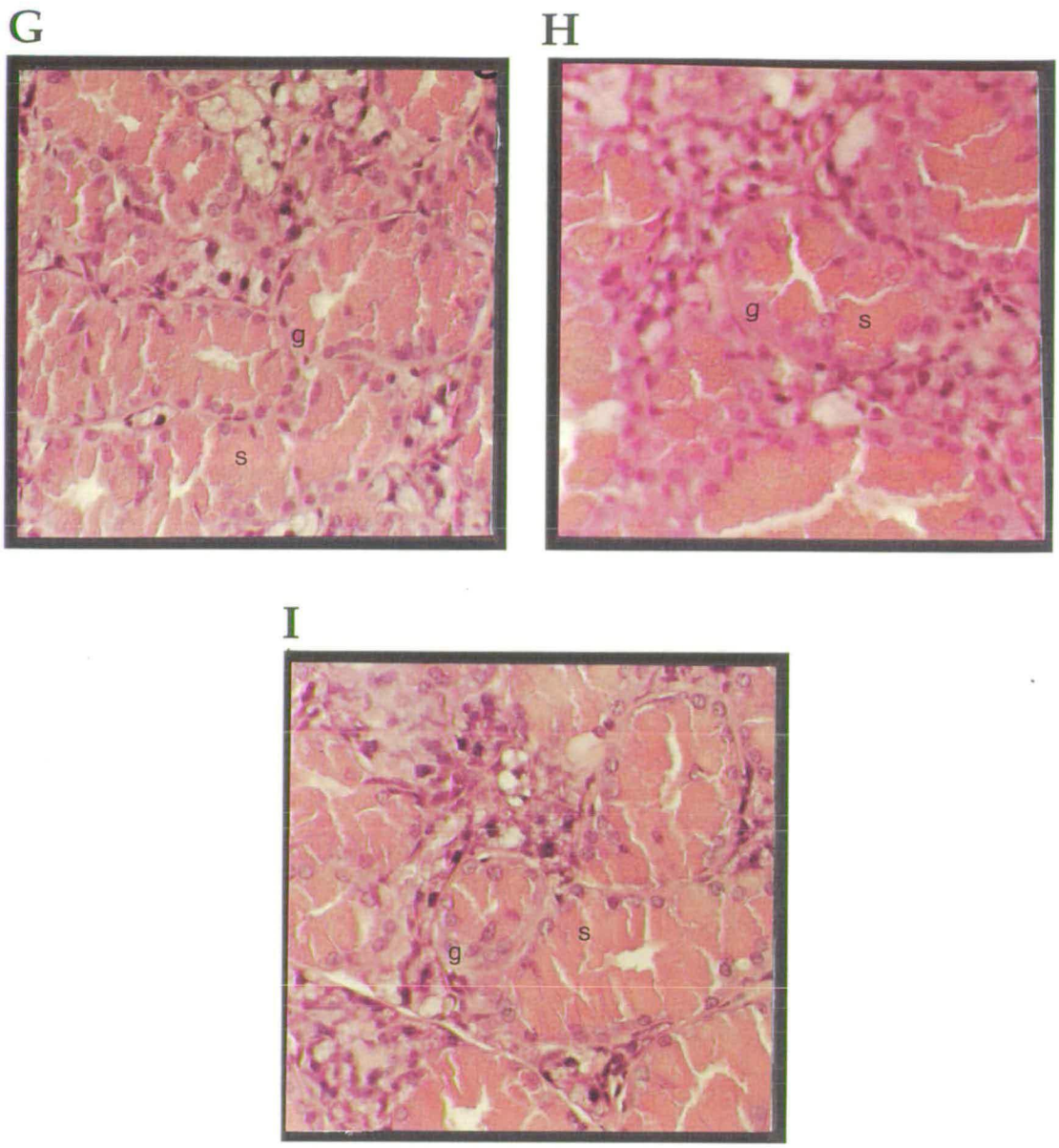


Figure 6.7 (continued): Adrenal, submandibular gland, testis and ovary histology: Tissues were sectioned and stained before visualisation using light microscopy. Submandibular glands sections from wild-type (G), *Ren-1*^{d} +/- (H) and *Ren-1*^{d} -/- (I) male mice were studied for histological abnormalities. No differences in submandibular gland morphology were observed. g=glandular epithelium and s= secreted proteinaceous material.

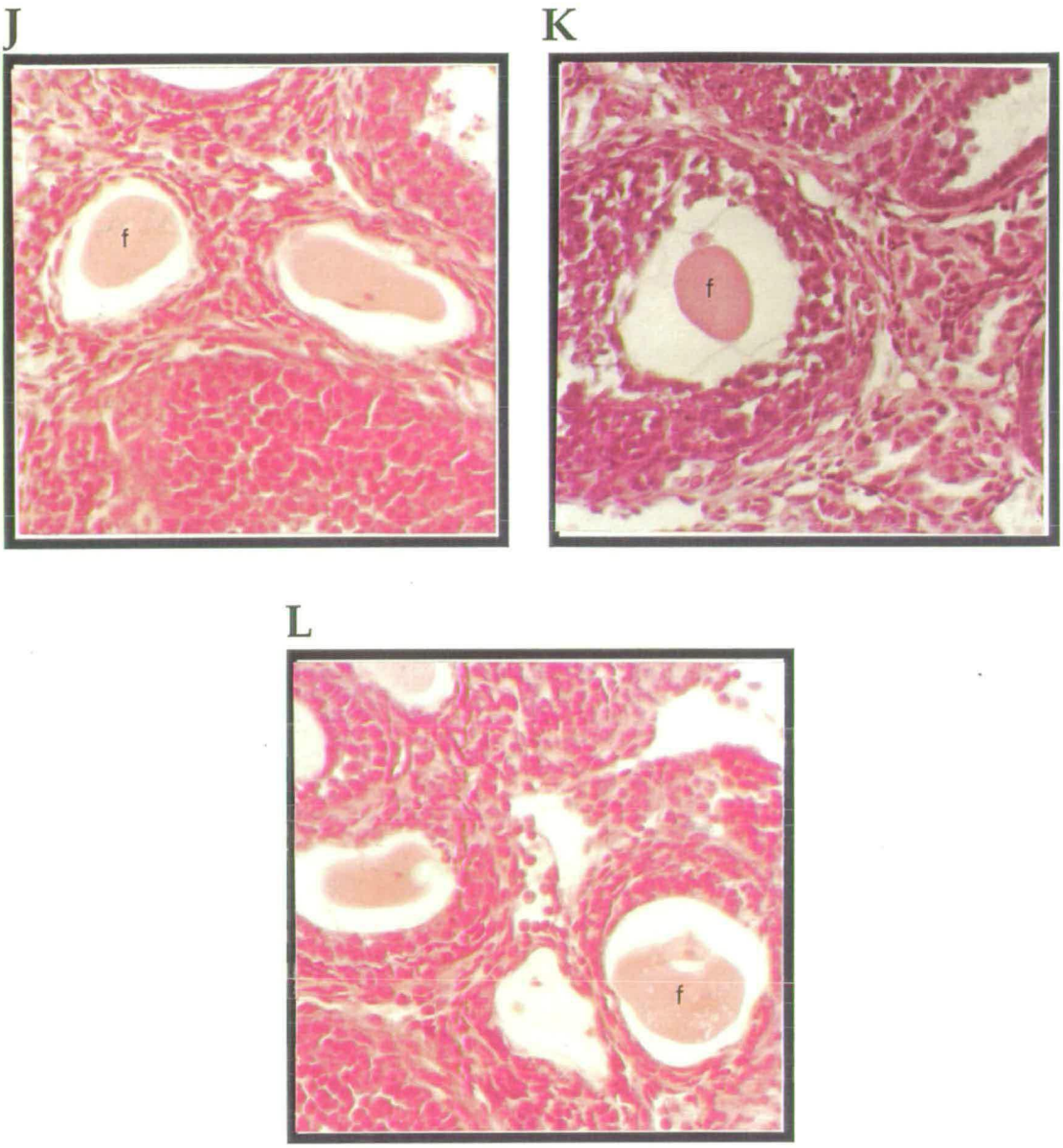


Figure 6.7 (continued): Adrenal, submandibular gland, testis and ovary histology: Tissues were sectioned and stained before visualisation using light microscopy. Sections of ovary tissue from wild-type (J), *Ren-1*^{d} +/- (K) and *Ren-1*^{d} -/- (L) female mice were studied for histological abnormalities. No differences in ovary morphology were observed. f= developing folicle.

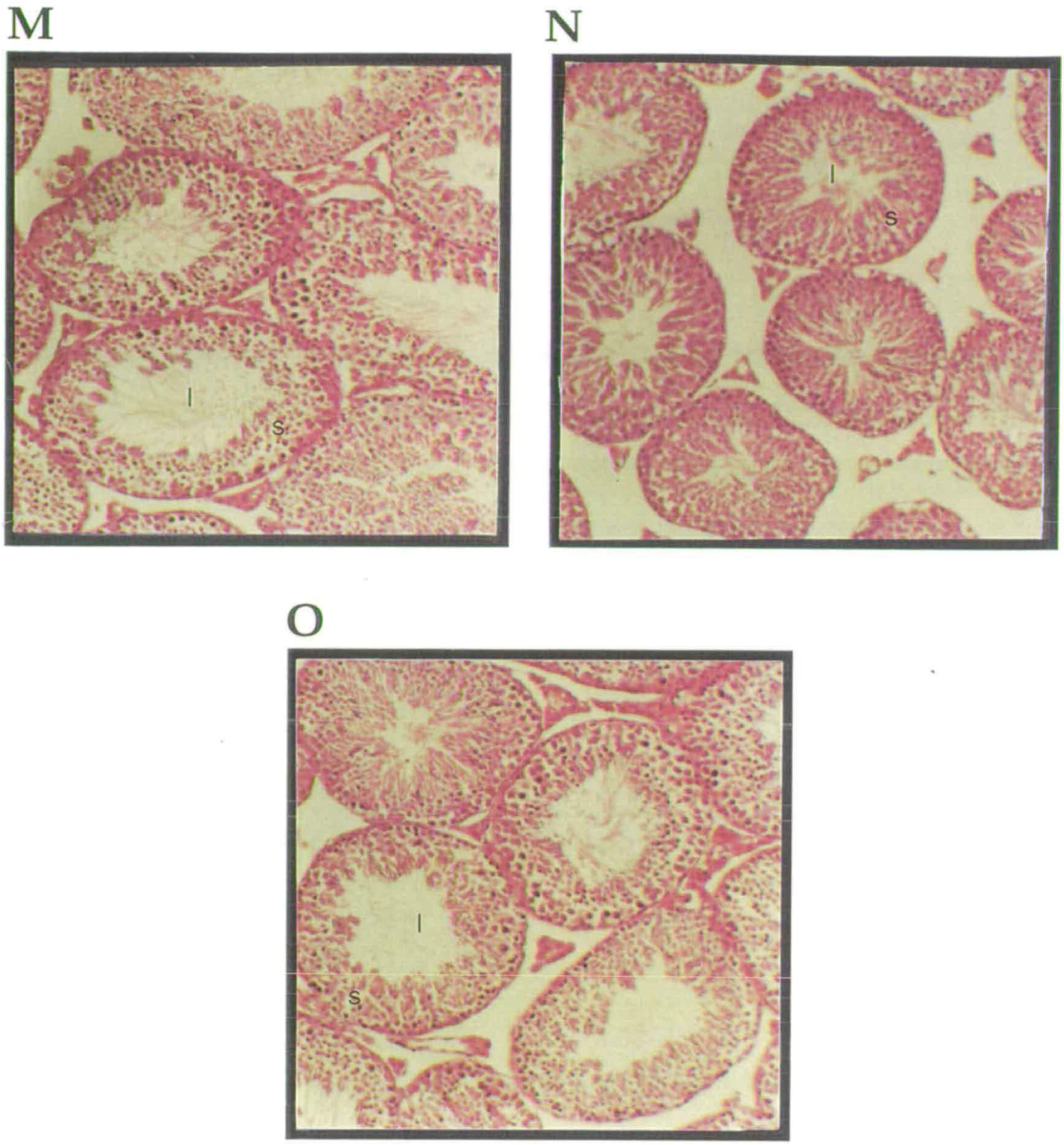


Figure 6.7 (continued): Adrenal, submandibular gland, testis and ovary histology: Tissues were sectioned and stained before visualisation using light microscopy. Sections of testis tissue from wild-type (M), *Ren-1*^{d} +/- (N) and *Ren-1*^{d} -/- (O) male mice were studied for histological abnormalities. No differences in testis morphology were observed. s= seminiferous tubule and l= seminiferous tubule lumen.

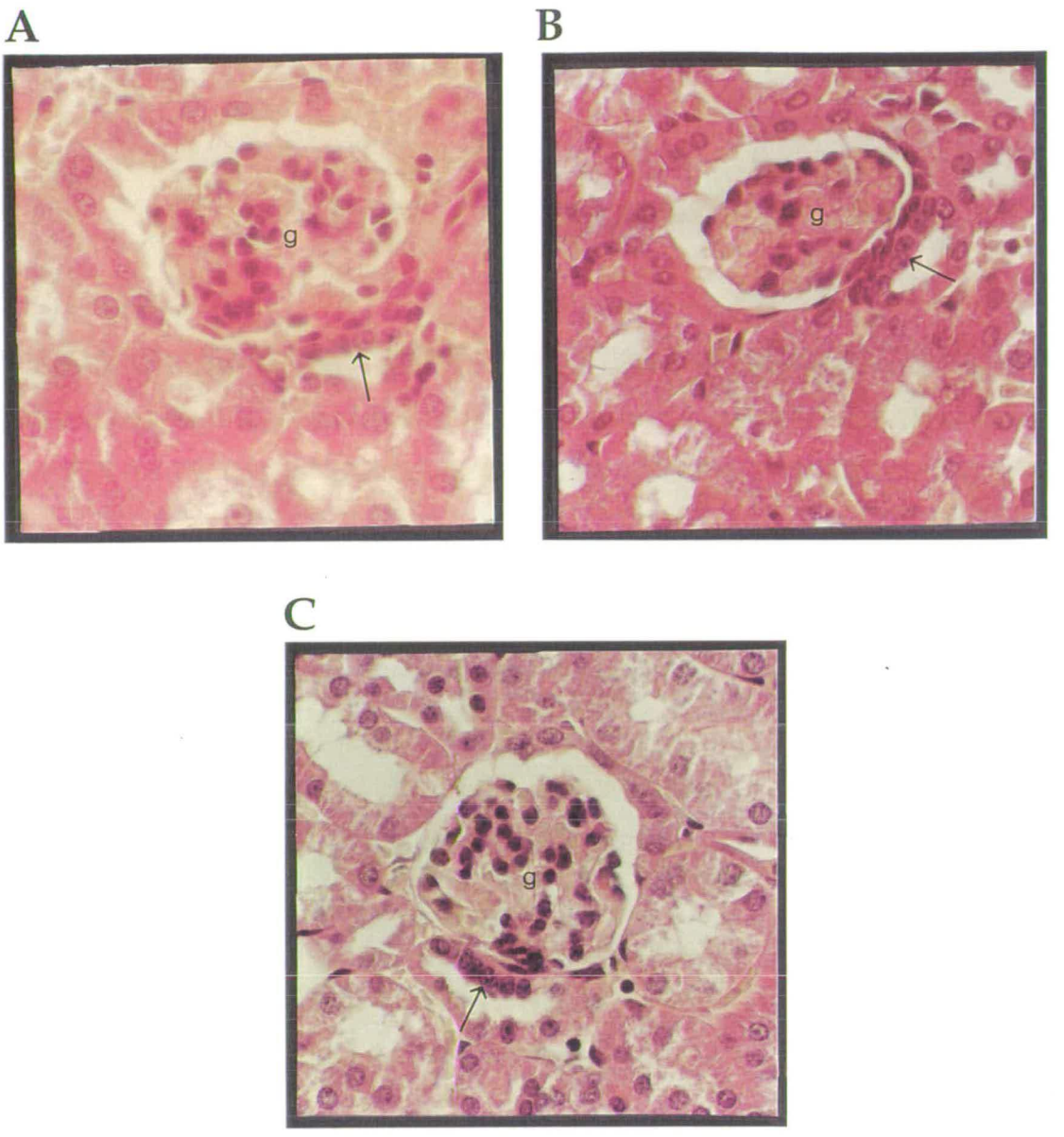


Figure 6.8: Kidney histology: Kidney sections from wild-type (A and D), *Ren-1^d* +/- (B and E) and *Ren-1^d* -/- (C and F) from male (A to C) or female (D to F) mice were studied for histological abnormalities. Differences were noted in macula densa cell morphology in male and female *Ren-1^d* -/- animals compared to wild-type and heterozygous controls. This manifested itself as an increase in macula densa cell height (arrow; panels C and F), giving the cells a more columnar appearance compared to the more cuboidal shape in control animals (arrow; panels A, B, D and E). g= glomerulus.

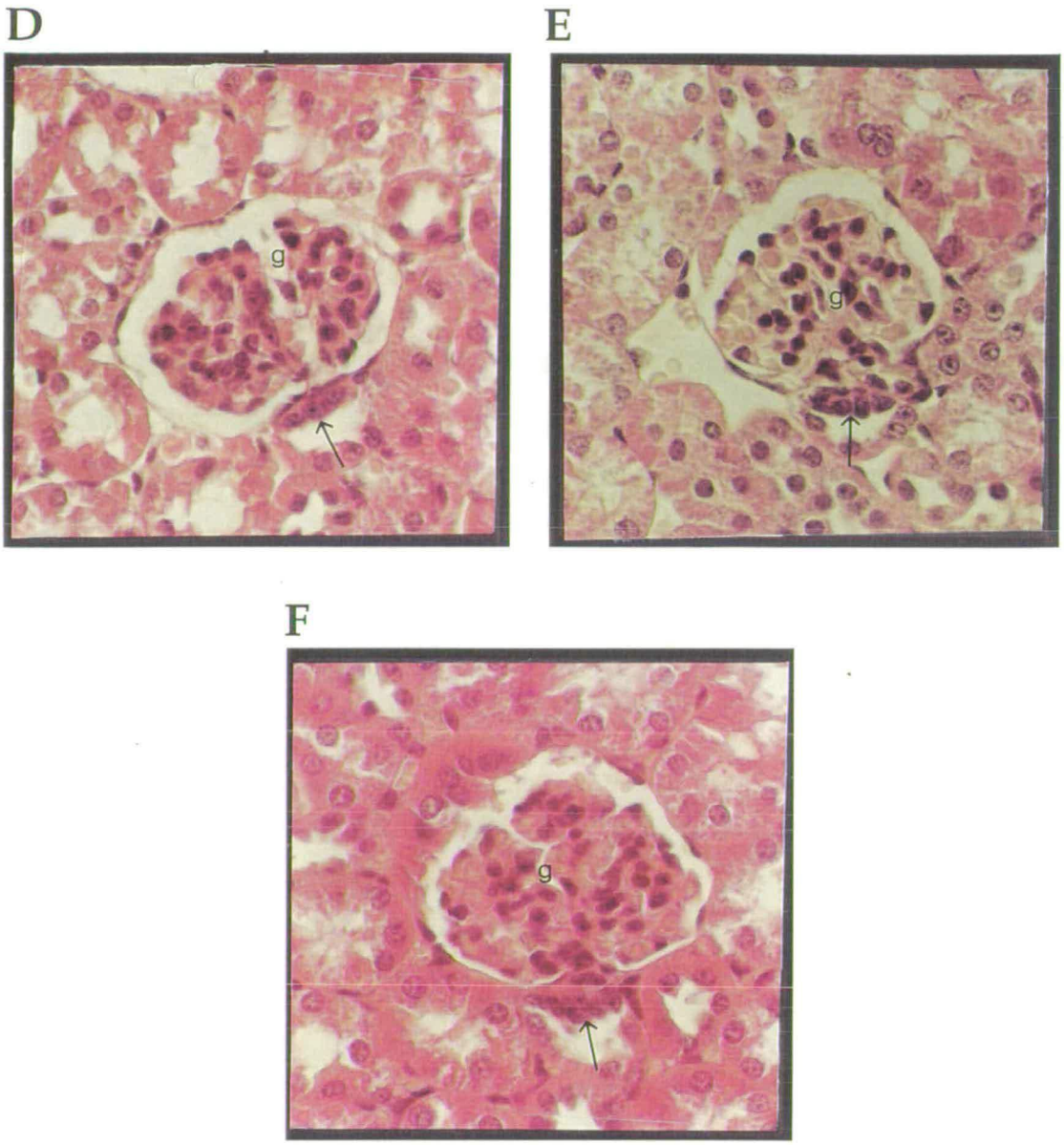


Figure 6.8 (continued): Kidney histology: Kidney sections from wild-type (A and D), *Ren-1^d* +/- (B and E) and *Ren-1^d* -/- (C and F) male (A to C) or female (D to F) mice were studied for histological abnormalities. Differences were noted in macula densa cell morphology in male and female *Ren-1^d* -/- animals compared to wild-type and heterozygous controls. This manifested itself as an increase in macula densa cell height (arrow; panels C and F), giving the cells a more columnar appearance compared to the more cuboidal shape in control animals (arrow; panels A, B, D and E). g= glomerulus.

In an attempt to quantify this observation the central three macula densa cells were measured in five kidney regions from each of four individual mice (n=20) of each genotype. Wild-type and *Ren-1^d* +/- had macula densa cells 6.1μm (range 5.6-6.3μm) and 6.0μm (range 5.8-6.2μm) in height respectively (Table 6.9). *Ren-1^d* -/- mice had macula densa cells 7.9μm (range 7.6-8.1μm) in height, a 30% increase in cell height. This change in cell shape causes a "doming" of the macula densa cells toward the lumen of the distal tubule, in turn, causing the lumen to adopt a more triangular shape in cross-section, compared to the normal, oval/circular pattern, observed in wild-type mice.

To investigate the expression levels and cellular localisation of the Renin-2 protein in these animals, immunohistochemistry was performed (Figure 6.10). A polyclonal antibody which can detect both Renin-1^d and Renin-2 proteins was used to show a strong, granular renin positive staining in the juxtaglomerular cells of kidneys from heterozygote and wild-type animals of both sexes, as expected. However, in *Ren-1^d* -/- kidneys an

Genotype	Measurement 1	Measurement 2	Measurement 3	Measurement 4	Measurement 5
+/+ 1	6.3	5.9	5.7	5.9	6.0
+/+ 2	6.0	6.2	5.9	6.1	6.1
+/+ 3	5.6	5.9	5.8	6.0	6.0
+/+ 4	5.9	6.2	6.3	6.2	6.3

Genotype	Measurement 1	Measurement 2	Measurement 3	Measurement 4	Measurement 5
+/- 1	6.1	6.1	6.0	5.8	6.0
+/- 2	5.9	5.9	6.1	6.2	6.2
+/- 3	5.9	5.8	5.8	6.0	5.9
+/- 4	6.1	6.1	6.2	6.0	6.0

Genotype	Measurement 1	Measurement 2	Measurement 3	Measurement 4	Measurement 5
-/- 1	7.7	7.7	7.9	8.0	7.9
-/- 2	8.0	8.0	8.1	7.9	8.0
-/- 3	7.6	7.7	7.6	7.8	7.7
-/- 4	8.1	8.0	7.9	7.9	7.9

Table 6.9: Macula densa measurements: The central three macula densa cells in five JGA (per animal) were measured in four animals from each genotype. Results are given in μm.

absence of granular renin-staining in the smooth muscle layer of the afferent arteriole was observed. The only detectable signal seen in these sections was a low-level, uniform and diffuse staining (approximately 10% of the controls) around the afferent arteriole. This was consistent with cytoplasmic staining, as would be expected for a constitutively secreted protein. These results also show that Renin-2 protein is not stored in detectable quantities in juxtaglomerular cells of these mice.

Having observed a lack of granular immunoreactivity in the *Ren-1^d* -/- animals, transmission electron microscopy was used to determine the subcellular structure of the juxtaglomerular cells (Figure 6.11). This revealed an absence of secretory granules, the subcellular structures in which renin is normally stored. These cells, however, have an abundance of rough endoplasmic reticulum (a distinguishing feature of JG cells). The lack of granulation observed correlates well with the absence of granular renin immunostaining in *Ren-1^d* -/- animals.

6.6 Discussion

In this, the final results chapter, an initial characterisation of the *Ren-1^d* -/- phenotype was described. Initially, animals were analysed at the mRNA and protein level. This was to confirm firstly that the *Ren-1^d* gene had been successfully disrupted and secondly, to determine the effects of disruption of the *Ren-1^d* gene on the expression of the closely related and highly conserved *Ren-2* gene and the resultant protein, Renin-2. Mean arterial blood pressures were measured in animals of all three genotypes and both sexes. Tissues known to express renin were then taken and examined for histological and subcellular structural differences/abnormalities.

RT-PCR, followed by diagnostic *Ear* I digestion was used to confirm the absence of any wild-type *Ren-1^d* mRNA. Using primer extension, an upregulation of *Ren-2* expression was seen in *Ren-1^d* -/- mice. Analysis of circulating renin protein showed no significant differences in plasma active renin concentration (PRC) in male *Ren-1^d* -/- mice but significantly lower PRC in female *Ren-1^d* -/- mice. Circulating plasma prorenin concentrations (PPC) in *Ren-1^d* -/- mice were significantly higher in both sexes. This may correspond to the upregulation of *Ren-2* mRNA expression observed by primer extension. These results are the reverse of observations made in the

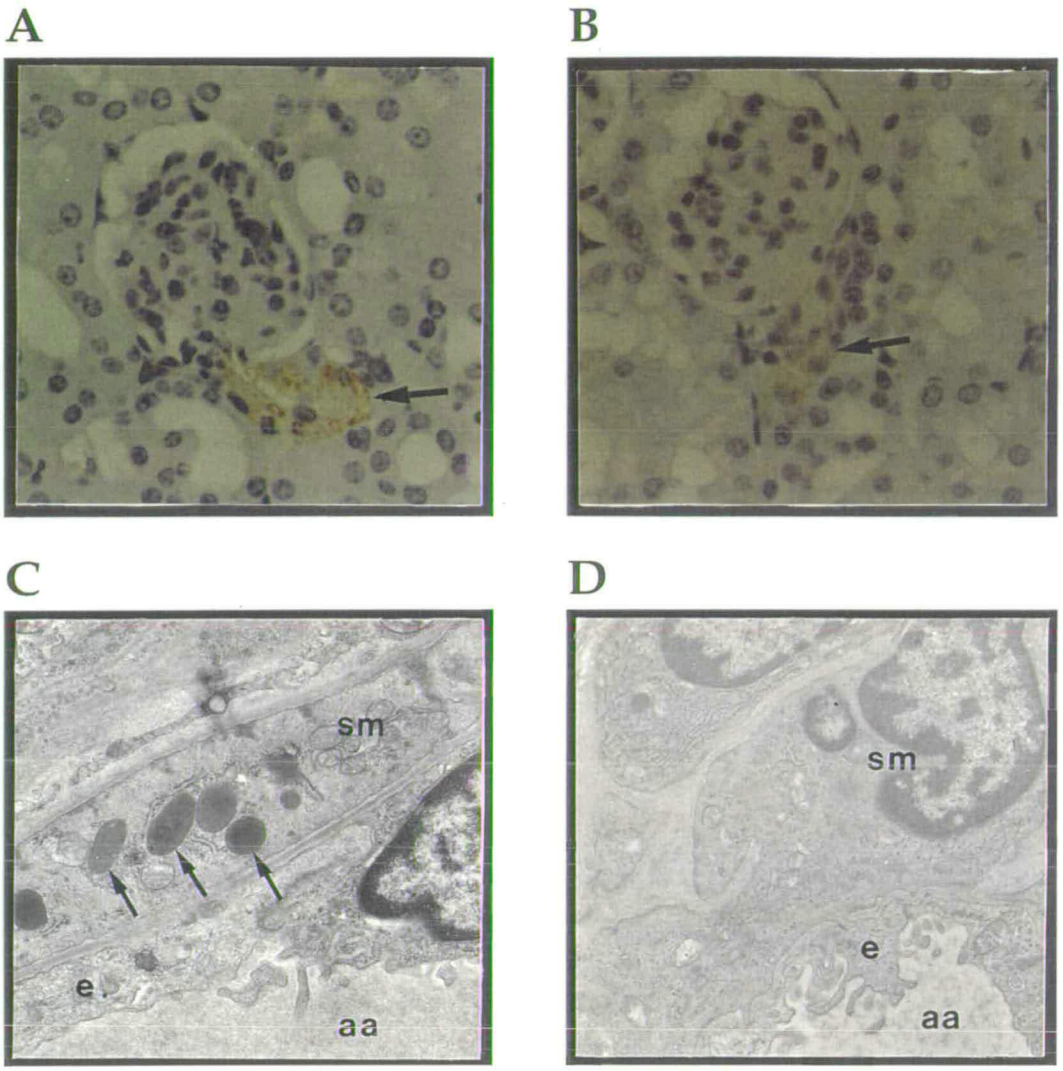


Figure 6.10: Kidney immunohistochemistry and electron microscopy: Wild-type (A and C) and *Ren-1*^{d} -/- (B and D) kidney sections after immunostaining for renin (A and B) or electron microscopy (C and D). A) Strong immunostaining for renin is visible in the medial layer of a wild-type afferent arteriole (arrow;). B) In contrast, sections from a *Ren-1*^{d} -/- mouse kidney show only faint cytoplasmic immunoperoxidase staining for renin in a few cells near the vascular pole (arrow). C) and D) Electron microscopy of Wild-type (C) and *Ren-1*^{d} -/- (D) kidney sections. C) Prominent dense cytoplasmic granules (arrows) can be seen in the afferent arteriolar smooth muscle cells (sm) of wild-type animals. aa= afferent arteriole lumen, e= endothelial cell. D) In contrast no cytoplasmic granules can be seen in the smooth muscle cells of the afferent arterioles of *Ren-1*^{d} -/- animals. Afferent arterioles were defined by tracing there origins from the interlobular artery in semi-thin sections.

Ren-2 gene targeted mice (286) which showed increased levels of active renin and reduced prorenin levels in the plasma of *Ren-2* -/- animals. Taken together, these results suggest that Renin-2 protein is a major component of circulating prorenin.

Blood pressures in males showed no differences between the three

genotypes, whereas female homozygous mutant animals had a significantly lower blood pressure, possibly related to the lower levels of PRC. This sexual dimorphism could be due to increased synthesis and/or activation of renin in an extrarenal tissue of male mice.

Histological analysis of kidneys from all *Ren-1^d* -/- animals show subtle abnormalities in the macula densa cells of the distal tubule. These morphological changes are remarkable in that they are cause by the lack of expression of the *Ren-1^d* gene, in the presence of a functional *Ren-2* gene. Immunohistochemistry revealed a significant reduction in renin-positive staining in the juxtaglomerular cells of the kidney, from punctate staining consistent with renin storage granules to a more diffuse form, consistent with cytoplasmic staining. The absence of these storage granules in the juxtaglomerular cells was subsequently confirmed by transmission electron microscopy. A functional *Ren-1^d* gene is, therefore, required for granule formation and lack of this enzyme alone is enough to block the formation of these structures. This function cannot be supplied by the product of the *Ren-2* gene. This supports the hypothesis that in the kidney, *Ren-2* is expressed and the gene product is constitutively released into the plasma in the prorenin form, whereas the *Ren-1^d* gene product is sequestered by the regulated pathway, processed and released as active renin in a regulated manner.

All *Ren-1^d* -/- animals displayed kidney abnormalities, including hypercellularity of the macula densa and a lack of renin storage granules in JG cells. In addition, female *Ren-1^d* -/- mice displayed significantly reduced blood pressures compared to wild-type controls. The reduction in blood pressure in females and the kidney abnormalities observed in both sexes prove that the *Ren-1^d* and *Ren-2* gene products are not wholly functionally identical, that is *Ren-2* cannot fully substitute the functions of *Ren-1^d*. Many of the points raised here are discussed in greater detail in Chapter 7.

CHAPTER 7

Discussion

7.1 Introduction

The aim of this project was to ablate Renin-1^d function by gene disruption using a replacement-type targeting vector. This was to allow the role of *Ren-2* function in blood pressure regulation and development in 'two-renin gene' mice to be studied (in isolation from other renin genes) and to address the question of functional redundancy between the two genes.

Disruption of the *Ren-1^d* gene in mice, confirmed by RT-PCR, resulted in hypotension in female *Ren-1^d -/-* animals, the first evidence that disruption of a renin gene affects blood pressure. In addition to this, the phenotype of the *Ren-1^d -/-* animals alludes to other important functions of the *Ren-1^d* gene. *Ren-1^d* was found to be essential for normal macula densa morphology where absence of the Renin-1^d protein resulted a 30% increase in cell height. More striking however are the changes observed in juxtaglomerular cell morphology where a complete absence of renin secretory/storage granules was observed. These results prove for the first time that *Ren-1^d* and *Ren-2* are not functionally equivalent, with *Ren-1^d* being required for blood pressure homeostasis and normal macula densa and juxtaglomerular cell morphology.

Sharp *et al.* (286) have reported the disruption of the *Ren-2* gene in which no kidney morphology or blood pressure differences were observed. This suggests that although *Ren-1^d* is essential and cannot be substituted for by *Ren-2*, the role of *Ren-2* may however be replaced by *Ren-1^d*. This would be consistent with the *Ren-1^d* gene fulfilling all renin functions after the gene duplication event allowing *Ren-2* to undergo mutation with no constraints on its functionality.

7.2 Gene Targeting

7.2.1 PCR Mediated Gene Targeting at the *Ren-1^d* Locus

Here, the disruption of the *Ren-1^d* gene has been described. This was achieved using a targeting construct in which both homology arms were amplified by PCR from a 129/Ola template. Cloning of PCR fragments was found to be problematical, but this problem was solved by cutting once at an internal site within the amplicon and by ensuring enough buffer sequence was included between a restriction site and the end of an oligodeoxynucleotide.

This system offers several advantages over the more commonly used strategy including; 1) the ability to introduce restriction sites for cloning of material and screening of ES cell DNAs, 2) the ability to use highly isogenic DNA, as homology can be amplified directly from ES cell DNA and 3) the requirement for minimal mapping data, *e.g.* a cDNA sequence and preliminary genomic map for the design of primers, prediction of amplicon sizes and the development of a screening strategy. The ability to amplify fragments of 10kb and above from genomic DNA using a combination of enzymes (321-323) allows the PCR amplification of homology arms directly from ES cell DNA. This then is a fourth advantage, the need to identify genomic clones being removed.

A major consideration in this strategy is the enzyme (or enzymes) used to amplify the homology arms. In order to maximise the targeting efficiency amplicons should contain no errors and therefore be truly isogenic. One way to achieve this is to use proof-reading DNA polymerases. For the amplification of the *Ren-1^d* homology arms the proof-reading DNA polymerase, *UlTma* was used. To determine the fidelity of the PCR reaction the 3' homology arm in the targeting construct was compared to the 129/Ola sequence (generated by direct sequencing of PCR product). This resulted in the identification of eleven differences between the two sequences, 2- to 3-fold higher than would be expected in amplifications using the *Taq* DNA polymerase (324). Cline (324) proposed that *UlTma* has a low fidelity because it is a genetically modified version of a *Taq* like enzyme (*Tma*) created by removal of the N-terminal portion of the protein. It is postulated

that although this enzyme now possesses a 5' to 3' proof-reading activity it may have a much reduced specificity for the incorporation of the correct nucleotides in the 3' to 5' direction. This is not a problem with all proof-reading enzymes. In a similar experiment within the laboratory, the 3' homology arm of a *Ren-2* directed targeting construct which was amplified using the proof-reading enzyme *Pfu* (Stratagene) contained no errors compared with the 129/Ola sequence for that region (M. Sharp *et al.*, unpublished data).

In Chapter 5 the successful disruption of the *Ren-1^d* gene was described. This proves that PCR amplified homology arms can successfully mediate homologous recombination, even when one of the arms is known to contain errors which disrupt the overall isogeny. Two of the correctly targeted ES cell clones were used to generate chimaeric mice, of which, two transmitted coat colour through the germ-line. These animals were then characterised for abnormalities arising from the introduced mutation.

7.2.2 Genetical Background Considerations

Care must be taken in interpreting the results of gene knockout experiments. Ideally chimaeric mice should be crossed with inbred mice from which the ES cells were derived to maintain genetic homogeneity. This is not always feasible as 129/Ola mice are well known for their low fertility and reduced fecundity. This problem can be partly circumvented by crossing chimaeric mice with another inbred line.

The *Agt* modified chimaeric mice (260, 276) were crossed with the inbred strain C57BL/6J. The resulting F_1 mice are genetically identical, possessing a complement of chromosomes from each parent. Thus heterozygous mutant animals and control littermates can be directly compared, the only genetic difference being at the mutated locus. However when these heterozygotes are intercrossed to generate *Agt* -/- and *Agt* 2/2 animals, the original 129/Ola and C57BL/6J chromosomes will segregate randomly at meiosis. In a large F_2 population, any unlinked gene affecting the phenotype will occur on a purely random basis.

In most analyses it is assumed that any phenotype observed is due to the disruption of the gene, however, the influence of linked genes cannot be

ruled out. Kim *et al.* (276) addressed this question by comparing their F_2 *Agt* -/1 and *Agt* 2/1 animals. These animals can be compared because the wild-type, "1" gene and the neighbouring linked genes are all derived from the C57BL/6J strain, whereas the disrupted or duplicated genes ("-" or "2") and all linked genes are derived from the 129/Ola strain, *i.e.* with regard to linked genes these animals only vary at the *Agt* locus. Kim *et al.* (276) then compared the blood pressure results obtained with these animals to the results obtained with the complete set of animals, and found no significant difference implying that no linked genes were affecting blood pressure.

The best way to circumvent such problems would be to breed onto a single genetic background, *i.e.* the strain from which the ES cells were derived. This is difficult with the 129/Ola strain because of the small litter sizes but would theoretically rule out the effects of linked genes since, by definition, the chromosomes of an inbred mouse will be identical except for the modified gene. Alternatively ES cells could be derived from other inbred mouse strains which have a higher fertility and fecundity.

In the case of the *Ren-1^d* gene targeting experiment all animals were kept on a 129/Ola genetic background. As discussed above the effects of linked genes can affect the phenotype observed and, as it is well known that the *Ren-1^d* and *Ren-2* genes are tightly linked, it was deemed pertinent not to cross onto another background. Maintaining the *Ren-1^d* mutation on a pure 129/Ola background prevents the possible introduction of variation at the *Ren-2* locus. By keeping the *Ren-1^d* -/- phenotype on a pure 129/Ola background it also allows direct comparisons with the *Ren-2* -/- animals to be made, as they were also generated and bred in a similar manner.

Another potential problem with gene targeting experiments is the amount of time ES cells spend in cell culture. During this time random, recessive mutations accumulate in the chromosomes and have no effect on the viability of the cells. This means that 129/Ola chromosomes and 129/Ola ES cell chromosomes are not the same. F_1 animals (fathered by a chimaera) are assumed only to vary at the targeted locus but will in actual fact vary at several loci due to the accumulation of mutations in the ES cell derived complement of chromosomes. At this stage F1 animals are typically brother/sister mated, a procedure which will produce animals homozygous at the targeted locus. Unfortunately, all random mutations accumulated in

the ES cells during cell culture which are linked to the targeted locus will also become homozygous. Therefore any observed phenotype may be due to these random mutations and not the gene disrupted by gene targeting. To prevent this occurring the F1 animals described above should be back-crossed to 129/Ola mice for several generations, a procedure which reduces the presence of ES cell derived chromosome regions. This however takes several generation and therefore a long time. In the case of the *Ren-1^d* targeting experiment backcrossing to 129/Ola mice has only been performed a few timesmeaning that there is still a significant contribution of ES cell derived chromosomal segments. Although we cannot say that the *Ren-1^d* -/- phenotype is due purely to this introduced mutation it is possible to say that this is very likely as the *Ren*-2 gene targeting experiment was performed using the same ES cells and none of the *Ren-2* -/- animals display this phenotype.

7.3 *Ren-1^d* -/- Phenotype

7.3.1 Renin Secretion

Much of our current understanding of the regulation of renin processing and secretion is based on tissue culture experiments in which cell lines were transfected with preprorenin cDNA expression vectors. Several cell lines have been used for these studies, including Chinese hamster ovary cells, which lack any granular secretion machinery and secrete prorenin exclusively (326-330) and AtT20 cells, a mouse pituitary cell line which display both a constitutive and regulated secretion capable of producing both active and inactive renin, secretion of the former being further stimulated by cAMP (328, 331-335). Similar observations have also been made in cultured tumoural cells (110, 336, 337) and human kidney slices (112).

Transfection of AtT20 cells with mouse *Ren-1^d* and *Ren-2* cDNA constructs has been reported (329, 338-340). In these publications both Renin-1^d and Renin-2 can be processed by both the constitutive and regulated secretory pathways. Whether this occurs in mouse juxtaglomerular cells *in vivo* is not known. Evidence exists to suggest that AtT20 cells are not a true model for secretion of renin from JG cells, for

example the prorenin processing enzymes in the two cell types are known to be different (339) and although active renin is produced by AtT20 cells it is of the one-chain and not the two-chain form produced by the mouse kidney or submandibular gland (107-109).

Upon studying kidney sections by electron microscopy it was found that JG cells from *Ren-1^d -/-* animals lack dense renin storage granules. This finding was also confirmed by immunohistochemical staining of kidney sections where the punctate, granular staining in wild-type animals was reduced by 95% and gave a more diffuse pattern consistent with cytoplasmic staining in *Ren-1^d -/-* animals with no large granular staining being present.

These results suggest that *Ren-1^d* is required for the formation of storage granules as in the absence of Renin-1^d these vesicles fail to form. As no granules form, even when *Ren-2* is present, granule formation cannot be catalysed by *Ren-2*. The signal that directs renin to the secretory granules cannot be clearly defined in this experiment, for example it may be that only *Ren-1^d* can direct assembly of the storage granules whereas both proteins could have the necessary signal to direct the gene products to these granules. Therefore in this experiment the direction of *Ren-2* to secretory granules would be masked because of the absence of storage/secretory vesicles in *Ren-1^d -/-* animals.

Ren-1^d -/- animals were found to have significantly higher levels of circulating prorenin in both males and females. This could be an attempt to compensate for the lack of Renin-1^d release through the regulated secretory pathway by increasing the release of inactive Renin-2 through the constitutive secretory pathway. This is supported by the finding that *Ren-2* transcription is upregulated by 3- to 4-fold in *Ren-1^d -/-* animals. The mechanism leading to the upregulation of *Ren-2* derived prorenin is unknown, but may be signaled by the lack of JG storage/secretory granules or a direct absence of Renin-1^d protein.

Female *Ren-1^d -/-* animals, but not males, had a statistically significantly lower level of circulating active renin. Assuming the maturation of renin only occurs in storage granules in JG cells and not in the cytoplasm, then only prorenin should be released from the kidney into the plasma by the constitutive pathway. If the JG cells are the only

physiologically significant source of active renin, then these animals would be expected to have no active renin present in the plasma This is not the case and prorenin must therefore be activated upon uptake by another organ, released in the active form from an extrarenal tissue which expresses *Ren-2*, or activated in the cytoplasm of the JG cells or in the plasma.

These results prove that a functional *Ren-1^d* gene is, therefore, required for granule formation in the JG cells of the kidney and that lack of this enzyme alone is enough to block the formation of these structures. These results also support the hypothesis that in the kidney *Ren-2* is expressed and the gene product constitutively released into the plasma in the prorenin form. Assuming that renin is activated elsewhere in these animals, these results suggest that the *Ren-1^d* gene product alone, is sequestered by the regulated pathway, processed and released as active renin in a regulated manner.

7.3.2 Reduced Blood Pressure in Female *Ren-1^d* -/- Mice

Disruption of other constituents of the RAS have been reported, several of which document reductions in blood pressure (276-279, 281, 282). In the case of the *Ren-1^d* gene a sexual dimorphism was observed with respect to blood pressure. The ablation of *Ren-1^d* in males had no effect on blood pressure, whereas in females a statistically significant reduction of 12.7mmHg was seen in *Ren-1^d* -/- animals, with *Ren-1^d* +/- females showing an intermediate blood pressure, which at present does not reach statistical significance.

The finding that blood pressure is only reduced in the *Ren-1^d* -/- females may be related to the statistically significant reductions in plasma active renin concentrations in these animals. The first step in the RAS is the rate limiting step in humans, rats and mice (5, 6). However renin is rate limiting in humans and rats (5) and angiotensinogen is rate limiting in mice (6). Therefore it may be necessary to reduce renin concentrations considerably before this enzyme is no longer in excess. In *Ren-1^d* -/- females active renin concentrations are reduced by 50% compared to wild-type animals, yet a reduction in blood pressure is seen. It may be that this has reduced renin levels below the threshold at which it is in excess over angiotensinogen, or alternatively another mechanism may be involved.

These animals contain active renin as determined by cleavage of the rat angiotensinogen molecule in a plasma assay. As these animals are *Ren-1^d* -/- any active renin detected must be derived from the *Ren-2* gene. The mouse Renin-2 protein is known to be able to cleave the rat angiotensinogen molecule since transgenic rats containing a *Ren-2* transgene develop hypertension (249). However, it is not known whether the mouse Renin-2 protein can cleave the mouse angiotensinogen molecule efficiently *in vivo*. Therefore the actual renin activity in these mouse plasmas may be much lower than determined in the protein assays described here. This could explain the reduced blood pressure in females associated with what appears to be only a modest reduction (relative to angiotensinogen concentration) in active renin concentrations.

Blood pressure is only reduced in the female *Ren-1^d* -/- animals. Possible explanations for the sexual dimorphism could be; 1) the high level expression of the *Ren-2* gene in the submandibular gland of males compared to females; 2) the oestrus cycle, and the effect that this has on blood pressure, for example in the adrenal gland, a known extrarenal site of renin synthesis.

This experiment has provided the first proof that renin is required for maintenance of normal blood pressure, at least in females and that in mice with regards to blood pressure homeostasis *Ren-1^d* and *Ren-2* are not functionally equivalent. The lack of*Ren-2* having no effect on blood pressure in male or female *Ren-2* -/- animals.

7.3.3 Altered Macula Densa Cell Morphology

The macula densa cells of the proximal tubule in the kidney lie in close proximity to the JG cells. It is thought that the macula densa cells detect the sodium chloride load in the distal tubule and then signal the regulation of active renin release from the JG cells accordingly (166, 184, 185). Signaling by the macula densa cells is thought to be by nitric oxide (NO) (193-195). Low sodium chloride levels in the distal tubule would cause an increased production of nitric oxide which in turn would trigger the release of renin from the JG cells.

Ren-1^d -/- animals of both sexes display abnormalities of the macula densa cells. An increase in cell number and height is observed with macula

densa cells from *Ren-1^d -/-* animals being 30% longer than wild-type controls. This may reflect perturbations in the RAS in these animals and suggests an involvement of thetubuloglomerular feedback loop due to altered chloride and fluid balances.

It is striking to find that disruption of expression of the *Ren-1^d* gene in neighbouring cells has such a strong effect on the macula densa cells where renin is not expressed. At present however, the sequence of events leading to this macula densa phenotype is not understood although this line of investigation will be continued.

7.3.4 An Alternative Theory

These results could be explained by an alternative hypothesis in which a lack of *Ren-1^d* primarily causes a reduction in blood pressure. To compensate for this all granular renin in the JG cells of the kidney is released, in males this is enough to normalise blood pressure and in females it is not. This model differs from that previously described in that here *Ren-2* can also be sequestered into storage granules and processed to active renin, making the presence of active renin in the *Ren-1^d -/-* animals much easier to explain. If the primary effect of removing *Ren-1^d* is a reduction in blood pressure which would affect sodium balance causing the macula densa cells to continually stimulate the JG cells to release renin. The macula densa phenotype may there fore be due to chronic production of the messenger molecule (NO). If this were what is happening in the JGA of these animals one might expect to see metaplastic transformation of the afferent arteriole resulting in an increase in the length of renin immunoreactivity in the afferent arteriole. This was not observed suggesting that this model does not reflect what is happening *in vivo*. However, this hypothesis should not be written off as differences between *Ren-1^d* and *Ren-2* may account for the lack of increased immunoreactivity in the afferent arteriole.

7.4 Future Experiments

The finding that *Ren-1^d -/-* animals lack storage granules suggests that these animals and the *Ren-2 -/-* animals may make good *in vivo* models for the studying of renin secretion. Before this can be confirmed the JG cells of the *Ren-2 -/-* animals need to be characterised by immunohistochemistry and

electron microscopy to study the granulation of these cells. Even without this extra information *Ren-1^d* -/- animals can be used to assess factors required for granule formation. To do this transgenic animals could be generated using various mutated versions of the *Ren-1^d* gene and tested for their ability to rescue granulation in the JG cells. Mutated *Ren-2* transgenes could also be used to try and rescue the lack of granulation phenotype.

Glycosylation is known not to be required for the direction of prorenin to storage granules in AtT20 cells (333, 340, 341), but this has never been tested in JG cells *in vivo*. Glycosylation could therefore be tested as a signal for granule formation by making transgenic animals (on a *Ren-1^d* -/- background) which express a glycosylated form of *Ren-2* or a deglycosylated *Ren-1^d* and looking for storage granules in the JG cells of these animals. Another area of interest may be the pro segment where some groups (330, 335), but not others (333, 337) have found signals required for the direction of human prorenin to storage/secretory granules *in vitro*.

In order to further understand the mechanisms leading to hypotension in *Ren-1^d* -/- females attempts could be made at restoring normal blood pressure in these animals by inducing submandibular gland expression of *Ren-2* in females by administering testosterone. The reciprocal experiment, the removal of the submandibular glands form male *Ren-1^d* -/- mice could be performed in a bid to try and lower there blood pressures to similar levels to those seen in *Ren-1^d* -/- females.

In a bid to further understand the phenotype observed in the macula densa cells experiments will be performed which are designed to challenge the tubuloglomerular feedback loop. For example blood pressure and kidney morphology will be analysed in animals after subjection to different diets containing varying levels of sodium. By further challenging theses animals, *e.g.* with a low sodium diet, it may be possible to induce larger decreases in blood pressure in female animals or to induce a decrease in blood pressure in male animals. If the alternative theory, in which storage granules are absent due to chronic stimulation to release renin is postulated, maintaining these animals on a high salt diet, a regime which normally inhibits renin secretion, may result in the presence of storage granules, good evidence for this theory, as well as proving that *Ren-1^d* is not required for granule formation.

7.5 Concluding Remarks

These results prove that the mouse *Ren-1^d* and *Ren-2* genes are not functionally identical. The *Ren-1^d* gene product being essential for granule formation in JG cells of the kidney, as well as macula densa morphology and blood pressure homeostasis. At present no function of *Ren-2* has been identified that cannot be completely substituted for by *Ren-1^d* suggesting that *Ren-1^d* fulfills all renin functions in the mouse, leaving *Ren-2* to undergo random mutations with no constraints on the proteins function. Using the *Ren-1^d* -/- mice and the *Ren-2* -/- mice it should be possible to dissect further renin gene function and the underlying mechanisms in storage, maturation and release of renin. *Ren-1^d* -/- animals will also provide an opportunity for investigation of the role of macula densa cells in the tubuloglomerular feedback loop, especially in response to perturbations in the RAS.

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Appendix A:- RNA Quantitation Calculations

AREA	X-POS	Y-POS	OBJECT	Adj. Factor								
6004	131	427	1-84	1.20811878								
2266	179	435	2-84	0.45596222								
2165	234	437	3-84	0.4356391								
5207	286	439	4-84	1.04774725								
4296	337	441	5-84	0.86443676								
1688	400	443	6-84	0.33965765								
3988	454	443	7-84	0.80246131								
3985	515	439	8-84	0.80185765								
3057	638	411	9-84	0.61512644								
6288	700	413	10-84	1.26526498								
2174	760	409	11-84	0.43745007								
6775	826	409	12-84	1.36325862								
3823	888	405	13-84	0.76926018								
4694	947	405	14-84	0.94452191								
8595	1013	413	15-84	1.72947717								
7066	1071	415	16-84	1.42181335								
4552	1193	419	17-84	0.91594882								
5014	1251	419	18-84	1.00891199								
3652	1302	423	19-84	0.73485173								
2661	1354	423	20-84	0.53544372								
8814	1417	425	21-84	1.77354413								
12838	1464	423	22-84	2.58324932								
6213	1507	421	23-84	1.25017355								
3458	1563	415	24-84	0.69581525								
				Adjusted Ren1								
635	127	327	1+5	525.61								
93.19	174	331	2+5	204.38	Male WT Ren1							
405.9	233	333	3+5	931.73	Mean	±SEM	% of WT	±SEM				
361.4	279	335	4+5	344.93	501.66	157.71	100.00	31.44				
879.4	334	337	5+5	1017.31					M:F ratio	0.46		
356.4	399	337	6+5	1049.29	Female WT Ren1							
702.9	452	331	7+5	875.93	Mean	±SEM	% of WT	±SEM				
1125	517	327	8+5	1402.99	1086.38	112.06	100.00	10.31				
98.95	637	309	9+5	160.86								
826.9	698	311	10+5	653.54	Male HET Ren1							
515.1	758	307	11+5	1177.51	Mean	±SEM	% of WT	±SEM				
672.7	824	305	12+5	493.45	621.34	211.89	123.88	34.10				
731.8	883	305	13+5	951.30					M:F ratio	0.75		
909.8	945	301	14+5	963.24	Female HET Ren1							
427	1011	305	15+5	246.90	Mean	±SEM	% of WT	±SEM				
1640	1068	311	16+5	1153.46	828.72	199.39	76.28	24.06				
219.7	1189	315	17+5	239.86								
277.9	1248	319	18+5	275.45								
186.3	1298	323	19+5	253.52	Male HOM Ren1							
246.2	1349	323	20+5	459.81	Mean	±SEM	% of WT	±SEM				
325.8	1410	323	21+5	183.70	282.47	46.85	56.31	16.59				
834.9	1468	325	22+5	323.20	Female HOM Ren1				M:F ratio	0.73		
457.3	1499	321	23+5	365.79	Mean	±SEM	% of WT	±SEM				
331.3	1559	317	24+5	476.13	388.37	45.57	35.75	11.73				
				Adjusted Ren2								
325	123	133	1+17	269.01								
78.45	169	135	2+17	172.05	Male WT Ren2							
321	229	133	3+17	736.85	Mean	±SEM	% of WT	±SEM				
93.78	279	131	4+17	89.51	316.88	144.72	100.00	45.67				
403.1	332	129	5+17	466.32					M:F ratio	0.66		
155.4	399	131	6+17	457.52	Female WT Ren2							
284.7	448	129	7+17	354.78	Mean	±SEM	% of WT	±SEM				
525.3	514	125	8+17	655.10	483.43	62.57	100.00	12.94				
37.33	637	111	9+17	60.69								
663.5	701	107	10+17	524.40	Male HET Ren2							
400.4	767	107	11+17	915.30	Mean	±SEM	% of WT	±SEM				
401.3	835	105	12+17	294.37	448.69	182.08	141.61	40.58				
355.5	882	103	13+17	462.13					M:F ratio	0.79		
731.6	947	103	14+17	774.57	Female HET Ren2							
283.3	1008	103	15+17	163.81	Mean	±SEM	% of WT	±SEM				
1247	1070	105	16+17	877.05	569.39	161.44	117.78	28.35				
829.5	1184	105	17+17	905.62								
867.5	1240	105	18+17	859.84								
756.8	1291	109	19+17	1029.87	Male HOM Ren2							
501.7	1344	109	20+17	936.98	Mean	±SEM	% of WT	±SEM				
1358	1406	111	21+17	765.70	899.60	43.54	283.92	4.84				
2619	1441	111	22+17	1013.84	Female HOM Ren2				M:F ratio	0.48		
2796	1495	111	23+17	2236.49	Mean	±SEM	% of WT	±SEM				
1636	1548	107	24+17	2351.20	1867.18	427.95	386.23	22.92				
Raw Data					Adjusted Data							
Mean:	±SEM	Band		Male WT Ren1		100		Male WT Ren2		100.00		
4969.71	525.82	JJM84		Male HET Ren1		124		Male HET Ren2		141.61		
				Male HOM Ren1		56		Male HOM Ren2		283.92		
552.54	74.09	*Ren1*		Female WT Ren1			100	Female WT Ren2			100	
				Female HET Ren1			76	Female HET Ren2			118	
738.30	149.14	*Ren2*		Female HOM Ren1			36	Female HOM Ren2			386	

Appendix B:- Publications

1. A. F. Clark, M. G. F. Sharp and J. J. Mullins (1996): "Gene targeting and its application to basic hypertension research" *Clin Sci* **90;** 435-46.

2. M. G. F. Sharp, D. Fettes, G. Brooker, A. F. Clark, J. Peters, S. Fleming and J. J. Mullins (1996): "Targeted inactivation of the *Ren-2* gene in mice" *Hypertension* **28;** 1126-31.

3. A. F. Clark, M. G. F. Sharp, S. D. Morley, S. Fleming, J. Peters and J. J. Mullins (1997): "Renin-1 is essential for normal renal juxtaglomerular cell granulation and macula densa morphology" *J Biol Chem* **(in press).**

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Editorial Review

Gene targeting and its application to basic hypertension research

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GENE TARGETING

The development of gene targeting, a methodology allowing the ablation or mutation of a specific gene, is proving to be a powerful tool in the dissection of many single gene and, more recently, multigenic disorders. This technology involves the modification *in vitro* of totipotent embryonic stem (ES) cells derived from pre-implantation mouse embryos [1]. ES cells can be grown in culture, modified by gene targeting and returned to a mouse embryo where they can contribute to the germ cell population in the resulting chimaeric mouse [2]. Subsequent breeding of the chimaeric mice can result in the establishment of new mouse lines derived entirely from the modified ES cells (Fig. 1).

Modification of these cells involves disrupting the gene of interest with a targeting construct, consisting of two arms of homology and a 'selectable marker' placed between them. The two arms of homology are identical to DNA sequences flanking the region to be ablated and it is these regions of homology which mediate the specificity in the recombination event.

Types of targeting vector

There are two distinct types of vector used for gene targeting experiments [3], termed replacement vectors and insertion vectors (Fig. 2). Replacement vectors contain two stretches of homologous sequence interrupted by a selectable marker. After linearization at one end of the homology arms and transfection into ES cells, recombination in both homologous regions results in the replacement of part of the endogenous gene with the selectable marker. The complete removal of the plasmid vector may give increased efficiency and removes the possibility of plasmid sequences inserting in the genome, the effects of which are unpredictable. In contrast, insertion vectors result in the entire construct being integrated into the region of homology. Such vectors include a single region of homology and a gene encoding a selectable marker. Before the transfection of ES cells, the construct is linearized within the homologous region, generating two arms of homology, with the plasmid backbone and selectable marker between them. By a slight modification of the insertion vector strategy it is also possible to duplicate a region of the targeted locus. One potential problem with the use of insertion vectors for gene knockout is the fact that no part of the endogenous gene is deleted, and it may be possible to generate a wild-type mRNA via intron splicing around the inserted DNA (exon skipping). This was shown to occur when an insertion-type vector was used to target the *CFTR* gene (cystic fibrosis transmembrane conductance regulator) [4]. In this instance, residual activity is thought to be critical for the viability of the mice since in two other CFTR mouse models created using replacement vectors, the homozygous mice die at an early age [5–7].

When a targeting construct is introduced into ES cells it can integrate into the genome either randomly or into its homologous site. As the frequency of homologous recombination is generally much lower than random integration, it is best to develop stringent screening strategies for detecting targeted events (homologous recombination at the targeted locus). Targeted events are commonly detected by Southern blot hybridization [8], in which genomic DNA is digested with one or more restriction enzymes and diagnostic differences between the targeted and non-targeted gene are visualized using radioactive hybridization probes. Such differences are created by the introduction of restriction sites within the selectable marker (Fig. 3), or by virtue of

Key words: blood pressure, embryonic stem cell, gene targeting, homologous recombination, mouse.

Abbreviations: ACE, angiotensin I-converting enzyme; ANG I, angiotensin I; ANG II, angiotensin II, ANP, atrial natriuretic peptide; EDRF, endothelium-derived relaxing factor; ES cells, embryonic stem cells; GC-A, guanylyl cyclase-A receptor; HSV-*tk*, herpes simplex virus thymidine kinase gene; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PGK, phosphoglycerate kinase.

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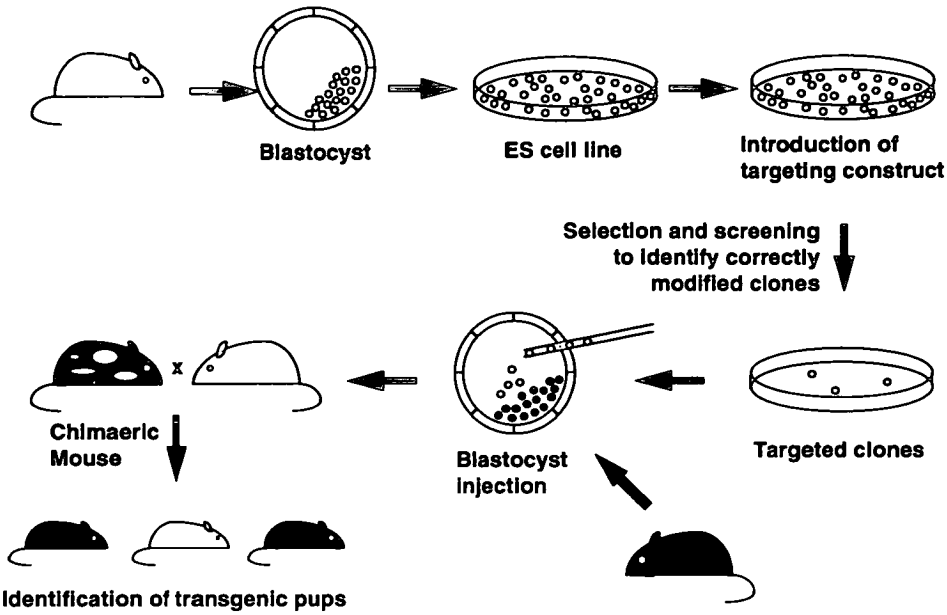

Fig. 1. Generation of novel mouse lines from gene targeted ES cells. ES cell clones are derived from pre-implantation mouse embryos and maintained in cell culture. Targeting construct DNA is introduced into the cells and clones containing the construct selected. These cells can then be reintroduced into mouse blastocysts, resulting in chimaeric mice which can be bred to generate new mouse lines.

the removal of endogenous sites during the recombination events.

Methods of selection

Central to any targeting strategy is the selection of ES cells into which the targeting construct has inserted. This is achieved using a selectable marker, such as the *Escherichia coli* neomycin phosphotransferase II gene (*neo*), which renders transfected cells expressing the gene resistant to the mammalian antibiotic G418. Such cells can then be selectively maintained in the presence of the appropriate antibiotic. Using this approach the targeting efficiency (relative frequency of homologous recombination events to total drug resistant colonies) will be low since the expression of drug resistance is independent of the integration site; however, it is a perfectly adequate and commonly used method.

In order to reduce the number of colonies which need to be screened, other selection procedures have been developed. A powerful method for selection of targeted events is the use of vectors lacking a promoter [9] or a polyadenylation signal [10]. Here, in order for the selectable marker to be expressed, the targeting construct must insert appropriately into the transcribed region of a gene. In the case of random insertions this will be relatively rare and will therefore increase the relative number of targeted events isolated. A limitation of this system is that it relies on transcription of the targeted gene in ES cells.

Positive/negative selection can also be used to enrich for targeted events [11]. This method relies on the fact that random integrations tend to insert via their ends, whereas homologous recombination events occur within the region of homology, such that any non-homologous sequences flanking these regions are lost. Cells can be selected positively for the expression of an antibiotic resistance gene, and selected negatively using the herpes simplex virus thymidine kinase gene (HSV-*tk*), the expression of which renders cells sensitive to the base analogue gancyclovir. If an HSV-*tk* gene is placed outside the region of homology, only random integrants should contain HSV-*tk* and will therefore be selected against in the presence of gancyclovir.

Regions of homology

The length of the homologous sequence present in the targeting vector has a direct effect on the targeting efficiency, and typically a total length of 5–10 kb is used. The term 'isogenic' refers to DNA derived from the same strain, and although historically non-isogenic DNA has been used in targeting experiments, it has recently been shown that when isogenic DNA is used the frequency of targeted events is greatly increased. te Riele et al. [12] compared isogenic and non-isogenic DNA by targeting the *Rb-1* gene in 129 ES cells using parallel vectors in which the homology arms were derived from either the 129 mouse (isogenic) or the BALB/c mouse (non-isogenic). By using the same selectable marker for both constructs, differences in efficiency could be directly attributed to the source of the

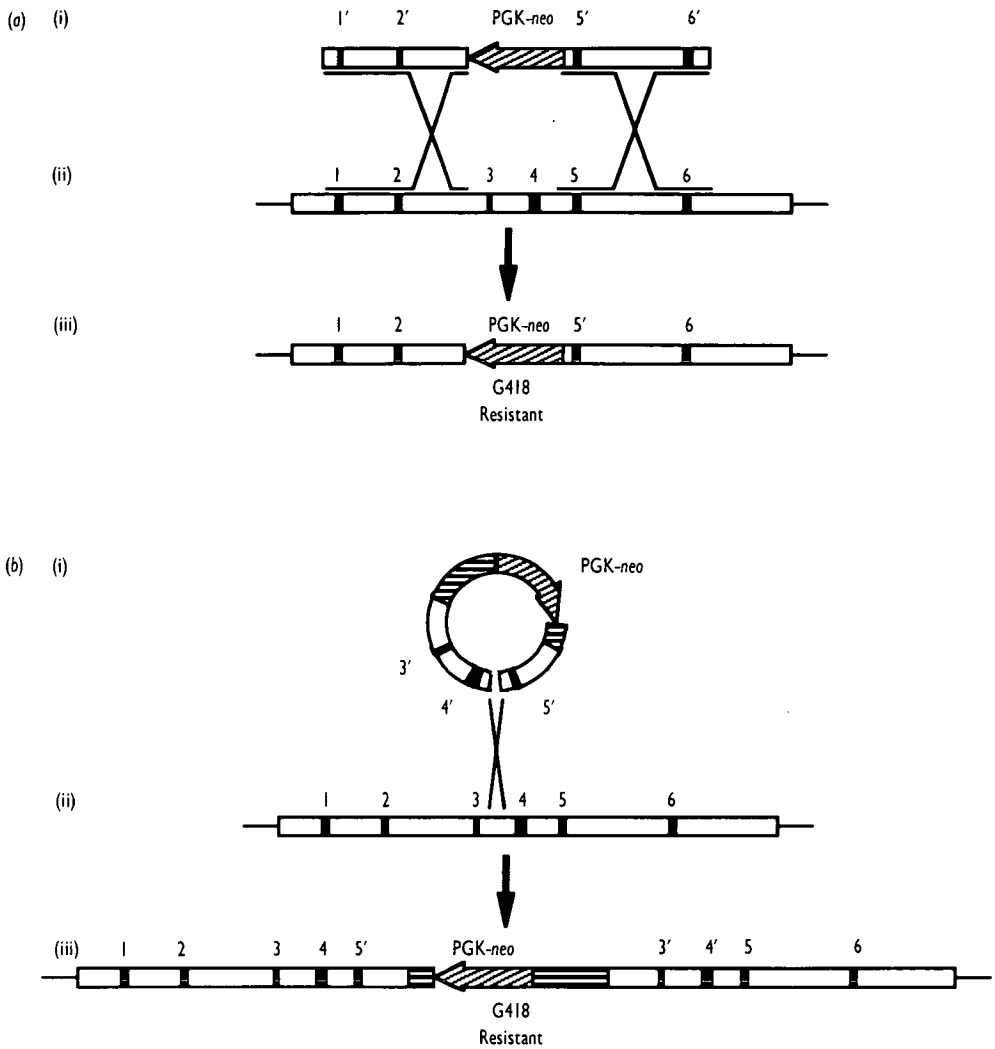

Fig. 2. Two methods for the disruption of a gene by gene targeting. (*a*): (i) shows a sequence replacement targeting vector including a phosphoglycerate kinase (PGK)-*neo* selectable marker (diagonal stripes, arrow indicates the direction of transcription) flanked by two homology arms (black bars = exons, white box = introns, ′ = exon derived from targeting construct); (ii) shows the genomic locus against which the targeting construct has been designed; (iii) shows the targeted locus after homologous recombination has occurred. Crosses indicate the sites at which recombination has occurred; this is only an example and recombination could have occurred anywhere in the two homology arms. (*b*): (i) shows a sequence insertion targeting vector including a PGK-*neo* selectable marker linearized within the homology region (horizontal stripes = plasmid vector sequences); (ii) shows the genomic locus against which the targeting construct has been designed; (iii) shows the targeted locus after homologous recombination has occurred.

homologous DNA. Using 129 DNA, a targeting frequency of 35% (33/94 G418 resistant colonies) was obtained, while the frequency when BALB/c DNA was used was only 1 in 144 (0.7%) G418 resistant colonies, representing a 50-fold increase in targeted events when isogenic DNA was used. When the degree of homology between the two inbred strains of mice was studied it was found that the longest stretch of perfect homology was only 278 bp within the region tested (1.68 kb) and the authors suggest that the decrease in targeting efficiency may reflect a lack of sufficiently long regions of perfectly matched homology. These data only apply to the *Rb-1* locus and care must be taken in extrapolating these results to other loci. However, in general it is believed that using isogenic DNA significantly increases targeting efficiency.

Deng and Capecchi [13] addressed the question of how much homology is required for efficient targeting and found that replacement and insertion vectors had a strong dependence on the length of homology between the targeting vector and the targeted locus. An exponential relationship was observed between targeting efficiency and length of homology, reaching a plateau when the total length of homology reached 14 kb. These authors also

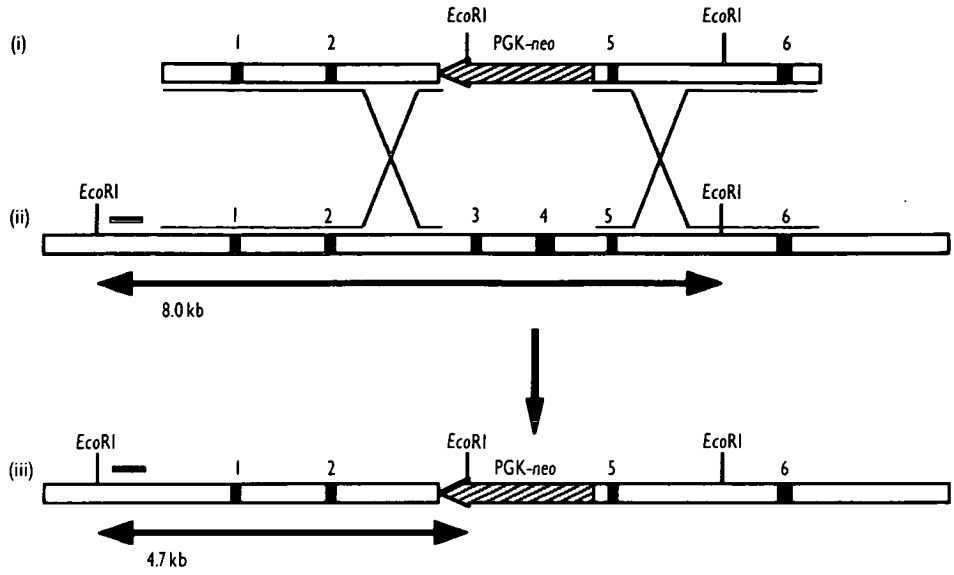

Fig. 3. Example of a screening strategy using an *Eco*RI digest and external probe to identify targeted events after electroporation of a replacement type vector. (i) shows a sequence replacement targeting vector including a PGK-*neo* selectable marker (diagonal stripes, arrow indicates the direction of transcription) flanked by two homology arms (black bars = exons, white box = introns, ′ = exon derived from targeting construct); (ii) shows the genomic locus against which the targeting construct has been designed, the predicted size of the endogenous EcoRI fragment after Southern blotting and screening with the external probe (horizontal black bar); (iii) shows the targeted locus after homologous recombination has occurred and the predicted size of the targeted EcoRI fragment after Southern blotting and screening with the external probe.

reported an improvement in the targeting efficiency through the use of isogenic DNA.

Gene targeting has had a large impact on our understanding of single gene disorders such as cystic fibrosis, but the technology can also be applied to more complex traits such as blood pressure.

RENIN-ANGIOTENSIN SYSTEM

The renin–angiotensin system is one of the most studied pathways involved in blood pressure regulation (Fig. 4). The first step in this pathway is the conversion of angiotensinogen to angiotensin I (ANG I) by renin, the activity of which is rate limiting in humans. In contrast, it is the substrate, angiotensinogen, which is the limiting factor in the mouse [14, 15]. ANG I is re-cleaved by angiotensin I-converting enzyme (ACE) to produce the active octapeptide, angiotensin II (ANG II), a peptide hormone which exerts its effects in a receptor-mediated manner.

Two pharmacologically distinct types of ANG II receptors have been identified, AT_1 and AT_2. The AT_1 receptor has two known subtypes, AT_{1A} and AT_{1B}, which have a wide tissue distribution and are expressed from distinct, but highly homologous genes [16–21]. It is believed that ANG II performs its range of physiological functions via the AT_1 receptors, both of which have been implicated in blood pressure regulation. The function and significance of the AT_2 receptor is as yet unknown [22–24]. ANG II functions include vasoconstriction,

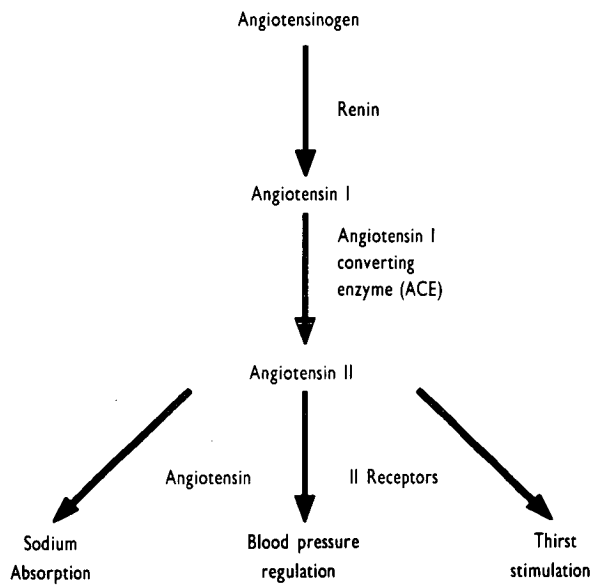

Fig. 4. Key elements of the renin–angiotensin system. Angiotensinogen is converted to ANG I by renin followed by cleavage to produce the active octapeptide ANG II by the enzyme ACE. ANG II then exerts its range of physiological effects via the ANG II receptors.

increasing renal sodium reabsorption and stimulating the thirst centre of the central nervous system, and it has been hypothesized that the renin–angiotensin system may be involved in the pathogenesis of hypertension (reviewed in Skott and Jensen [25]). Furthermore, polymorphisms in the genes

encoding renin [26–28], angiotensinogen [29, 30], ACE [31, 32] and angiotensin receptors [33–35] have all been reported to be associated with hypertension in humans or in animal models of hypertension. Although there are numerous transgenic studies of the renin–angiotensin system and other blood pressure regulatory systems, for the purpose of this review we will focus on the use of homologous recombination.

Angiotensinogen gene (*Agt*)

An elegant series of studies involved the duplication of the entire angiotensinogen locus [36], and the complementary disruption of the gene by conventional targeting [37]. The duplication of the angiotensinogen gene was achieved by using an insertion-type targeting vector. Sequences from upstream and downstream that are believed to encompass all control sequences of the gene were used as the homology in the targeting construct. The 8 kb 5′ homology arm included 3 kb of promoter and exon 1, and the 1.8 kb 3′ homology arm included exons 4 and 5 and extended 200 bp beyond the polyadenylation site. The 8 kb gap between 5′ and 3′ homology arms, spanning exons 2 and 3, was repaired (filled in) by cellular mechanisms during the recombination event, resulting in duplication of the targeted locus [36].

By breeding animals possessing either the duplicated or disrupted locus it was possible to generate mice containing 0 to 4 copies of the angiotensinogen gene [36, 37]. This resulted in mice expressing 0 to 145% of normal plasma angiotensinogen levels, increasing in a non-linear but gene copy number-dependent manner. This relationship between gene copy number and angiotensinogen levels was also extended to include blood pressure, where mean arterial pressure was found to be proportional to gene copy number. Linear increases in blood pressure of 8.3 ± 2.3 mmHg (mean arterial pressure) were reported for each additional copy of the *Agt* gene [37].

Of particular interest in the experiments of Kim et al. [37] is the null phenotype; the complete absence of angiotensinogen. No data have been reported for the blood pressure of homozygous mutant mice, probably because the viability of these mice is severely reduced. Surviving homozygous mutant (*Agt* −/−) animals were found to have no obvious defects at birth although adult mice displayed pathological changes in the kidney. *Agt* −/− animals were found to have thickening of medial layers of vessel walls, caused by an increase in cell number and loss of structural organization, being most noticeable in the interlobular arteries. The mechanism underlying the wall thickening is unknown but may reveal a novel response to the low blood pressure or to the complete absence of angiotensinogen. General cortical thinning with foci of severe atrophy was also observed in the kidneys of *Agt* −/− animals. The areas of atrophy consisted of shrinkage and loss of tubules, interstitial fibrosis and interstitial infiltration of chronic inflammatory cells, and were postulated to be caused by ischaemic damage as a result of reduced blood flow through the arteries. The kidneys of all other animals (1 to 4 copies of *Agt*) appeared to be normal.

The relationship between copy number and plasma angiotensinogen levels was not linear. The values one would have expected are 50% of wild-type activity for every copy of *Agt* present, as opposed to the 0, 35, 100, 124 and 145% observed for mice containing 0, 1, 2, 3 or 4 copies of the *Agt* gene respectively. There are two explanations for these results. The lower level of angiotensinogen present in the *Agt* −/1 animals is postulated by Kim et al. [37] to be related to the increased expression of renin in these animals. As the level of renin is much higher, a greater percentage of angiotensinogen would be expected to be converted to ANG I and ultimately to ANG II, therefore resulting in an increase in blood pressure. However, the expected increase in blood pressure is not observed. The lower than expected levels of angiotensinogen observed in the 3 (*Agt* 2/1) and 4 (*Agt* 2/2) copy animals can be explained by negative feedback on *Agt* expression when elevated levels of angiotensinogen protein are present, or it could be related to the structure of the *Agt* locus. Although all known *cis*-acting elements required for normal *Agt* levels were duplicated, additional, more distant sequences may be essential for recapitulating expression *in vivo*. This can be investigated by studying the *Agt* 1/1 and *Agt* 2/− animals, both of which have two angiotensinogen genes. When levels in these groups of mice were compared, the *Agt* 2/− were found to have only 55% of the *Agt* 1/1 (wild-type) levels, suggesting that not all the regulatory elements were duplicated in this experiment, or that the close proximity of the two gene copies is inhibitory. Interestingly, this difference in angiotensinogen level had no statistical effect on blood pressure in the two groups of animals.

In an independent experiment in which the angiotensinogen gene was disrupted [38], no difference in blood pressure was observed in heterozygous (*Agt* −/+) animals, but a significant reduction in systolic blood pressure in the homozygous mutant (*Agt* −/−) animals was reported (66.9 ± 4.1 mmHg compared with 100.4 ± 4.4 mmHg in wild-type animals). Similarly, diastolic and mean blood pressures were also reduced. When renin levels in both homozygous mutant and heterozygous animals were studied, renin levels in heterozygotes did not differ from wild-type values whereas null animals showed a 600 to 800% increase in renin expression levels.

Angiotensin-converting enzyme gene (*Ace*)

In addition to the full-length transcript widely expressed from the *Ace* gene, in post-meiotic sper-

matogenic cells a truncated transcript encoding a testis-specific form of ACE is also expressed. The precise function of this testis-specific isoform is unknown; however, the gene targeting strategy used by Krege et al. [39] to inactivate the *Ace* gene resulted in the disruption of both transcripts, permitting the role of ACE in blood pressure regulation and fertility to be studied.

Male heterozygotes showed a significant reduction in blood pressure of 15–20 mmHg, with female heterozygotes being indistinguishable from their wild-type litter mates. The reason for this sexual dimorphism is unclear, since in both sexes serum ACE activity was reduced. When homozygous mutant animals were studied, it was found that both males and females were hypotensive with a reduction of 35 mmHg in mean arterial pressures. Homozygous mutant mice also displayed histological changes in the kidney similar to those of the angiotensinogen mutant mice described earlier, with thickening of artery walls caused by an increase in the number of disorganized cells, as well as cortical thinning with focal areas of atrophy.

When the fertility of homozygous mutant animals was assessed, females were found to be fertile whereas males had severely reduced fertility with one out of five males tested being fertile. A more detailed study of the males showed that they were still capable of mating and that testis pathology, sperm count and sperm morphology were all normal, suggesting that homozygous mutant males may have a reduced ability to fertilize ova.

ANG II type 1A receptor gene (*Agtr1a*)

ANG II exerts its vasopressive effects via the AT_1 receptors. Ito et al. [40] disrupted the AT_{1A} receptor gene (*Agtr1a*) by gene targeting using a replacement vector. The resulting homozygous mutant mice were viable and displayed no outwardly visible abnormalities. Binding of ANG II was studied in homozygous mutant mice (*Agtr1a* $-/-$) using radiolabelled ANG II and in general was found to be reduced. Using the receptor antagonists losartan (DuP 753) and PD123319, which block binding to type 1 and type 2 receptors respectively, it was possible to show that the ANG II binding observed in the kidneys of homozygous mutant mice was mediated through the AT_2 receptor. The response to ANG II injection was also studied with the direct infusion of ANG II into wild-type mice, resulting in an increase in blood pressure which lasted 20 s followed by a delayed depressor effect lasting longer than 500 s. Infusion into heterozygous mice resulted in a similar short-lived rise in blood pressure, but the depressor effect was shortened to about 400 s while ANG II infusion into homozygous mutant mice had no effect on blood pressure.

Systolic blood pressure decreased by 12 mmHg in the heterozygotes and 24 mmHg in the homozygotes when measured by the tail-cuff method, and by 17 and 43 mmHg respectively when measured by cannulation of the carotid artery. Contrary to the observations in the *Agt* and *Ace* targeting experiments already discussed, the homozygous mutant animals displayed no abnormal histopathology in the kidney.

Taken together, these results tell us that the AT_{1A} receptor is not essential for normal development and survival, or for normal kidney development. However, the AT_{1A} receptor is essential for the pressor and depressor effects observed on infusion of ANG II. The AT_{1A} receptor is also responsible for almost all of the ANG II binding occurring in the kidney, and is involved in the regulation of blood pressure under normal conditions.

An independent report of the disruption of the AT_{1A} receptor [41] also found systolic blood pressure decreases, with 10 and 22 mmHg reductions being observed in hetero- and homozygotes respectively. Similar changes were observed in diastolic blood pressures and expression of renin was found to be upregulated in the kidneys of homozygous mutant mice, resulting in a 7–8-fold increase in plasma renin. The study used a modification of the gene targeting strategy to express *lacZ* under the control of the *Agtr1a* promoter. *lacZ* is a gene which can be used to mark cells because of the ability of its encoded protein, β-galactosidase, to convert colourless chromogenic substrates into blue precipitates that can be easily visualized. After incubation with a β-galactosidase substrate (Bluo-Gal), blue staining was observed in the glomerulus and juxtaglomerular apparatus. To confirm that this expression was equivalent to that of the endogenous gene, antisense probes for the AT_{1A} receptor mRNA were also used and shown to mirror the expression pattern of *lacZ*. Using an AT_{1B}-specific probe it was shown that no AT_{1B} receptor expression was present in the kidney.

The results of the AT_{1A} receptor knockout experiments help to dissect the phenotypes observed in the earlier renin–angiotensin system knockouts. Since a decrease in blood pressure was observed in the absence of histopathological alterations in the kidney, the changes reported for the *Ace* and *Agt* knockouts may not be mediated through the AT_{1A} receptor, although intercross experiments would confirm whether this was the case.

ANG II type 2 receptor gene (*Agtr2*)

Hein et al. [42] reported the disruption of *Agtr2* using a replacement-type targeting construct. Since the *Agtr2* gene is located on the X chromosome, in the first generation only females were capable of inheriting the disrupted *Agtr2* gene from the chimaeric father. After three generations (F3) it was possible to study homozygous females and hemizygous males, both of which developed normally. These animals showed no abnormal organ or skeleton development and produced litters of comparable

sizes to wild-type animals. To confirm the absence of AT_2 receptors, 18.5-day *post coitum* embryos were examined for RNA expression and ligand binding. RNA blot analysis revealed no expression in hemizygous mutant males or homozygous mutant females, and using the radioligand CGP42112 it was possible to show the absence of AT_2 receptors in the membranes of E18.5 embryos. This removal of AT_2 receptors did not affect the expression of the AT_{1A} receptor. When blood pressure was studied in these animals no difference was observed between wild-type mice and hemizygous mutant males. Similarly, injection of ANG II in both groups of mutant mice resulted in the expected pressor effects previously shown to be mediated, at least in part, by the AT_{1A} receptor.

As ANG II is also important in the central control of many physiological responses, including thirst, the drinking habits of the mutant mice were also studied. When wild-type and mutant mice were given drinking water *ad libitum* no differences were observed; however, if water was withdrawn for 40 h and then returned, the two groups of animals responded differently. In the subsequent 3 h period the water intake of the mutant mice was significantly lower than the wild-type control animals. The AT_2 receptor is highly expressed in the locus corneleus, a part of the brain involved in integration of sensory information and arousal. Since the intracerebroventricular injection of ANG II stimulates exploratory behaviour, the locomotive activity of AT_2 receptor-deficient mice was evaluated. Activity was measured on two consecutive days, in a light or dark environment. In the light periods, activity was not significantly different; however, in the dark the mutant mice displayed a lower activity compared with their wild-type controls. The physiological significance of the reduced activity is not known and it should be noted that this may be directly responsible for the alteration in water intake in the mutant mice, rather than a direct effect of ablation of *Agtr2* expression.

A second report describing the disruption of the AT_2 receptor used a replacement-type vector [43]. In this study, third generation (F_3) hemizygous mutant males or homozygous mutant females also developed normally, showed no abnormal organ or skeleton development and produced litters of comparable sizes to wild-type animals. Mutant mice were found to display lower levels of exploratory behaviour (reduced ambulation); however, contrary to the findings of Hein et al. [42], basal blood pressure was found to be elevated in the mutant animals with systolic blood pressure being 118.2 ± 5.0 mmHg in hemizygous mutant males compared with 94.2 ± 1.7 mmHg in control males. Administration of captopril, an ACE inhibitor, reduced the blood pressure of both groups of animals to similar values. These animals were then subjected to increasing doses of ANG II and at all doses tested the mutant mice exhibited elevated blood pressures compared with the wild-type controls. Finally, the administration of losartan, an AT_1 receptor antagonist, reduced blood pressure to the same levels as the original captopril treatment in both groups of mice. Ichiki et al. [43] suggest that since basal blood pressure remains higher even when the AT_1 receptors are blocked, the AT_2 receptor may act to limit the response of the AT_1 receptors to ANG II. The theory is further supported by the fact that infusion of ANG II into captopril-treated mutant mice resulted in a larger increase in blood pressure compared with controls. In light of these results and the reduction in blood pressure observed in other renin–angiotensin system gene targeting experiments, Ichiki et al. [43] postulate that the regulation of blood pressure *in vivo* is dependent on a balance between AT_1 and AT_2 receptor activation.

Hein et al. [42] also report a similar altered response to infusion of low doses of ANG II into mice pretreated with captopril. Unlike the findings of Ichiki et al. [43] no significant differences were observed in baseline blood pressure between wild-type and mutant mice. These apparent discrepancies may be due to differences between the background strains onto which the mutations have been crossed (FVB/N as opposed to C57BL/6J).

Renin genes (*Ren1^c^*, *Ren1^d^*, *Ren2^d^*)

Mice differ from other animals inasmuch as many of the inbred strains and all of the wild strains studied contain two closely linked genes, termed *Ren1^d^* and *Ren2^d^*, encoding distinct renin isozymes. The genes in strains containing two copies (e.g. 129) are termed *Ren1^d^* and *Ren2^d^*, and in single-renin-gene mice (e.g. C57BL/6J) the gene is termed *Ren1^c^*. The ablation of renin expression must therefore take this into consideration. Three possible strategies exist for the ablation of renin production: disruption of the *Ren1^c^* gene in ES cells derived from 'one-renin-gene' mice, disruption of each gene consecutively in ES cells derived from 'two-renin-gene' mice, or the use of a targeting construct to simultaneously disrupt both genes by deleting part of each gene and all the sequence between them.

The availability of mice totally lacking a renin structural gene will complement the existing gene knockout studies and determine whether angiotensins can be produced *in vivo* by an alternative enzymic pathway. The use of ES cells from two-renin-gene mice (129) will enable the individual roles of Ren1 and Ren2 during development, and in the adult, to be defined, and by intercrossing with other transgenic strains the individual contributions of various sites of renin expression can be further investigated. Studies on the renin genes in our laboratory have shown that *Ren2^d^*(−/−) animals are viable, fertile and have no histological abnormalities in the kidney, adrenal gland or submandibular gland (M. G. F. Sharp et al. unpublished work). In combination with the *Ren2^d^* knockout, additional

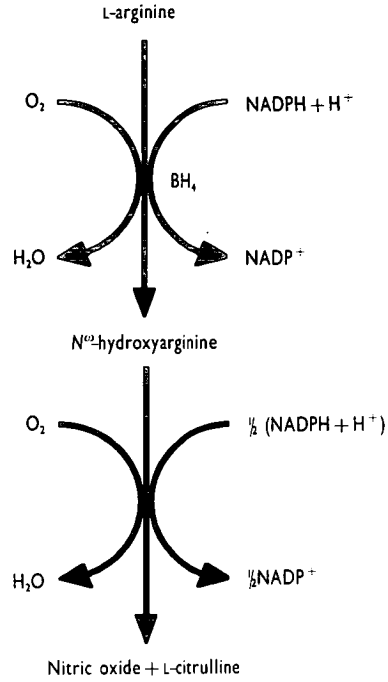

Fig. 5. Summary of the reaction catalysed by NOSs. L-arginine is converted to NO and L-citrulline by the NOS group of enzymes. This two-step reaction yields a short lived intermediate, N^{ω}-hydroxyarginine. BH_4 = tetrahydrobiopterin.

targeting studies at the Ren locus will further elucidate some of the remaining questions.

NITRIC OXIDE SYNTHASE (NOS)

Nitric oxide (NO), a highly diffusible free radical gas, was first reported in the late 1980s as a signal transduction molecule. It is now well-documented that NO plays a role in several biological systems, including the regulation of blood pressure [44]. Reviews by Knowles and Moncada [45] and Bredt and Snyder [46] cover aspects of gene structure and expression, the reaction catalysed and physiological roles for NO.

NO is produced from L-arginine by a group of enzymes termed nitric oxide synthases (NOSs) as shown in Fig. 5. To date, three isoforms have been identified with homology of between 50 and 60%. However, when equivalent genes are compared between species, a high degree of similarity is observed (e.g. 93% between rat and human neuronal NOS). Although within a species the three genes (and proteins) are not highly conserved, isoform-specific inhibitors are not available, making it difficult to assign the effects of inhibitors to a particular NOS isoform. In light of this the use of gene targeting provides an extremely useful tool in the dissection of the roles of the NOS genes.

Inducible NOS or iNOS (also called NOS II) is expressed by many cell types in response to cytokines or bacterial products. Mice lacking the iNOS protein, as a result of gene targeting [47, 48], display a reduced resistance to micro-organism infection [47] and are less effective at protecting against the proliferation of lymphoma tumour cells [48], confirming a role for NO in inflammation responses. These mice are also resistant to carrageenan inflammation [47] and hypotension elicited by endotoxin [47, 48].

Neuronal NOS or nNOS (also called NOS I) is expressed at high levels in the brain, especially in the cerebellum, as well as other sites in the central and peripheral nervous systems. The nNOS gene has also been successfully disrupted by gene targeting [49] and the resulting homozygous mutant mice showed no immediately obvious phenotype. However, further studies revealed that the mutant mice had dilated stomachs associated with constricted pyloric sphincters, mimicking a condition observed in humans called infantile hypertrophic pyloric stenosis, which is believed to involve a lack of NO production [49]. These mice also displayed resistance to brain damage caused by vascular stroke [50], highly aggressive behaviour and exhibited an altered and excessive sexual behaviour [51].

Endothelial NOS or eNOS (NOS III) is expressed in endothelial cells where the production of NO has been shown to have a potent vasodilatory action [44]. Endothelium-derived relaxing factor (EDRF) is a molecule released by endothelial cells in response to acetylcholine, bradykinin and substance P, resulting in vascular relaxation [52–55]. It has long been postulated that endothelial NO, produced from arginine in a reaction catalysed by eNOS, and EDRF were one and the same [54, 56, 57]. Although the use of NOS blockers results in an increase in blood pressure [55, 58, 59] this had not formally been shown to be caused by blockade of eNOS activity.

Mice containing a mutant eNOS gene were generated using a replacement-type targeting construct [60]. Homozygous mutant mice were generated and found to be viable, fertile and appeared normal with respect to outward appearance and behaviour. Western blotting showed the absence of the eNOS protein in the mutant mice in a panel of tissues normally found to express the eNOS isoform (brain, heart, lung and aorta). NOS enzyme activity was also measured in the aorta of mutant mice and was found to be reduced to trace levels, which may be due to nNOS activity within neurons present in the tissue preparation. EDRF activity was measured in eNOS mutant mice and was found to be absent, as would be expected if EDRF was endothelial NO. Aortic rings were precontracted in the presence of noradrenaline and then subjected to increasing concentrations of acetylcholine, which was found to relax the aortic rings from wild-type mice (EDRF activity) but had no effect in the eNOS homozygous mutant mice.

Although endothelium-derived NO is known to affect blood pressure it is difficult to predict the

effect of disrupting the gene *in vivo*, since several other systems may be modulated to compensate. However, when mean blood pressure was measured, either under anaesthetic or in the conscious state, the homozygous mutant animals were found to be significantly hypertensive (conscious mean blood pressure readings in homozygotes were 117 ± 10 mmHg compared with 97 ± 8 mmHg in wild-type animals), proving that endothelial NO plays a major role in the regulation of blood pressure.

The role of NO in blood pressure regulation is not straightforward, as demonstrated by the administration of non-specific NOS inhibitors to the mutant animals. Treatment of wild-type animals with L-nitroarginine results in an increase in mean arterial blood pressure (measured by femoral artery catheterization), consistent with the blocking of a potent vasodilator. However, treatment of the eNOS homozygous mutant mice with L-nitroarginine resulted in a decrease in mean arterial blood pressure. This treatment would have been expected to have no effect if the eNOS protein is the only NOS enzyme contributing to blood pressure control. This indicates that one of the other NOS proteins is also involved in the maintenance of blood pressure, and Huang et al. [60] suggest that nNOS may also play a role based on the occurrence of anaesthesia-induced hypotension in nNOS mutant mice.

More evidence for the role of NOS in blood pressure control comes from studying its expression in the kidney. The macula densa cells of the kidney form part of the juxtaglomerular apparatus, an important structure in the regulation of blood pressure not least because of its critical role in the renin–angiotensin system. The macula densa cells are believed to sense the pressure in the proximal tubule and secrete a signal molecule (possibly NO) which is detected by the juxtaglomerular cells, which then respond by increasing or decreasing renin release. Ramipril, an ACE inhibitor, and losartan, an AT_1 receptor antagonist, cause an increase in renin concentration [61]. NOS inhibitors cause a decrease in renal renin mRNA levels and in plasma renin activity and in combination with ramipril or losartan completely blunt the expected rise in renin concentration [61], suggesting that NO is directly involved as a signalling molecule. The decrease in blood pressure of the eNOS mutant mice caused by L-nitroarginine could therefore be due to a decrease in renin release caused by the lack of stimulation of juxtaglomerular cells by NO from the macula densa cells. It will be very interesting to measure the expression of renin in these animals, both untreated and treated with NOS inhibitors. As the three NOS genes are all present on different chromosomes it should be possible, by cross-breeding mutant lines, to generate double and triple mutants, the phenotypes of which should be highly informative.

One may have expected other blood pressure regulating systems to compensate for disruption of the eNOS gene but this was not found to be the case. Huang et al. [60] postulate that other mechanisms, e.g. the renin–angiotensin system, may have evolved to protect against hypotension and are therefore ill-equipped to cause a reduction in blood pressure. Alternatively, NO may be involved in creating the baroreceptor set point, a mechanism known to be affected by NOS inhibitors.

ENDOTHELIN AND ATRIAL NATRIURETIC PEPTIDE (ANP) PATHWAYS

Endothelin-1 is a potent vasoconstrictor and has been found to induce the release of EDRF/NO and inhibit the release of renin. The gene coding for endothelin-1 has been disrupted by gene targeting [62], resulting in an increase in blood pressure in the heterozygous animals and lethality in homozygous mutant animals. The removal of a vasoconstrictive molecule was expected to cause a decrease in blood pressure, but this was not observed. Possible explanations are that the lack of endothelin-1 throughout development has led to problems in the integration of blood pressure regulating systems or that endothelin-1 may have vasodilatory activities *in vivo*, as suggested by the stimulation of NO and inhibition of renin production by endothelin-1.

ANP is synthesized primarily by the atria of the heart and released in response to atrial distension. ANP is believed to mediate a vasodilatory action via receptors present in the vasculature, kidney and adrenal gland. ANP [63] and the guanylyl cyclase-A receptor, GC-A [64], through which ANP is believed to act, have both been disrupted by gene targeting. *proANP* $-/+$ animals did not differ from wild-type litter mates, although homozygous mutant animals (*proANP* $-/-$) were found to be hypertensive when fed on standard chow (0.5% NaCl). When the same animals were fed on chow containing 2.0% NaCl the *proANP* $-/-$ displayed even higher blood pressures, confirming the salt-sensitive nature of the phenotype. The disruption of the *GC-A* gene also resulted in a hypertensive phenotype; however, in this instance the hypertension was independent of salt concentration (salt-resistant hypertension). This contradiction in results can best be explained by the presence of another natriuretic activity acting through a receptor other than GC-A [64].

GENETIC BACKGROUND CONSIDERATIONS

Care must be taken in interpreting the results of gene knockout experiments. Ideally, chimaeric mice should be crossed with inbred mice from which the ES cells were derived to maintain genetic homogeneity. This is not always feasible as 129 mice are well known for their low fertility and reduced fecundity. This problem can be partly circumvented by crossing chimaeric mice with another inbred line.

The *Agt* modified chimaeric mice [36, 37] were

Table 1. Summary of published gene targeting papers in which an effect on blood pressure is observed

Gene	Gene product	Phenotype
Agt	Angiotensinogen	Hypotension [38].
		Hypotension, reduced viability. Thickening of intrarenal arteries, cortical atrophy [37].
Ace	ACE	Hypotension, male fertility reduced. Thickening of intrarenal arteries, cortical atrophy [39].
Agtr1a	ANG II type 1a receptor	Hypotension, no response to ANG II infusion. Normal kidney histology [40].
		Hypotension [41].
Agtr2	ANG II type 2 receptor	Normotensive, normal response to ANG II [42].
		Hypertensive, altered responses to ANG II and captopril [43].
eNOS	Endothelium-derived NO	Hypertension, decrease in blood pressure with NOS inhibitors [60].
ET-1	Endothelin-1	Hypertension and craniofacial abnormalities [62].
proANP	ANP	Salt-sensitive hypertension [63].
GC-A	GC-A	Salt-resistant hypertension [64].

crossed with the inbred strain C57BL/6J. The resulting F_1 mice are genetically identical, possessing a complement of chromosomes from each parent. Thus, heterozygous mutant animals and control litter mates can be directly compared, the only genetic difference being at the mutated locus. However, when these heterozygotes are intercrossed to generate *Agt* $-/-$ and *Agt* 2/2 animals, the original 129 and C57BL/6J chromosomes will segregate randomly at meiosis. In a large F_2 population, any unlinked gene affecting the phenotype will occur on a purely random basis.

While in most analyses it is assumed that any phenotype observed is due to the disruption of the gene, the influence of linked genes cannot be ruled out. Kim et al. [37] addressed this question by comparing their F_2 *Agt* $-/1$ and *Agt* 2/1 animals. These animals can be compared because the wild-type '1' gene and the neighbouring linked genes are all derived from the C57BL/6J strain, whereas the disrupted or duplicated genes ('$-$' or '2') and all linked genes are derived from the 129 strain, i.e. with regard to linked genes these animals only vary at the *Agt* locus. Kim et al. [37] then compared the blood pressure results obtained with these animals to the results obtained with the complete set of animals and found no significant difference, implying that no linked genes were affecting blood pressure.

The best way to circumvent such problems would be to breed onto a single genetic background, i.e. the strain from which the ES cells were derived. This is difficult with the 129 strain because of the small litter sizes but would rule out the effects of linked genes since, by definition, the chromosomes of an inbred mouse will be identical except for the modified gene. Alternatively, ES cells could be derived from other inbred mouse strains which have a higher fertility and fecundity.

DISCUSSION AND CONCLUSIONS

Gene targeting experiments have begun to elucidate further the mechanisms of blood pressure regulation and to complement both pharmacological and physiological studies. In addition to the use of genes known to be directly involved in blood pressure regulation, many other gene targeting experiments have had effects on blood pressure (see Table 1) and will yield valuable information.

A problem commonly associated with gene targeting experiments, but not examined in detail here, is that of redundancy between genes. In this case, one observes no differences between the targeted and wild-type mice because a gene other than the one disrupted is compensating for the loss of the targeted gene. This is common when targeting a member of a highly related gene family, but may still provide valuable information regarding the regulation and functioning of the gene family being investigated. Animals lacking functionally overlapping genes can be readily intercrossed to explore the relationship between such genes.

At present, most gene targeting experiments result in the ablation of a gene product by disruption of the gene locus. Techniques are available for the introduction of more subtle mutations, for example introducing mutations observed in human disorders. Gene targeting has been used to recreate the common human cystic fibrosis mutation, ΔF508, in mice [65–67], and such techniques allow further refinement of animal models for human diseases to be performed. An additional development of gene targeting is the use of the Cre/*loxP* recombination system [68, 69]. Cre is a protein which mediates homologous recombination between two specific *loxP* sites. Introduction of *loxP* sites in a gene targeting experiment and the subsequent removal of intervening sequences by Cre recombinase can recreate a functional gene. As this only occurs in the presence of Cre protein this can be regulated both tissue-specifically and/or temporally. Traditional transgenic animals expressing Cre either tissue-specifically or under the control of an inducible promoter can be crossed with gene knockout mice, resulting in selective re-activation of the gene.

Gu et al. [70] used Cre/*loxP* recombination to knock out the DNA polymerase β gene specifically in T-cells. The disruption of this gene in all cells resulted in a lethal phenotype. As this gene was believed to be important in the development of

lymphocytes, Cre recombinase (expressed in transgenic animals under the control of the T-cell-specific *lck* gene promoter) was used to knock out the gene specifically in T-cells. In this way the role of a gene in individual tissues or at specific times in development can be dissected from the global effect of disrupting a gene.

At present, ES cell lines are only available from a few inbred mouse lines. As some physiological questions are either difficult or impossible to address in the mouse, the generation of ES cells from other species would be highly advantageous. At present, pluripotential rat ES cells have been used to generate chimaeric rats but germ-line transmission was not achieved [71]. For gene targeting experiments to be viable in species other than the mouse, germ-line transmission must be achieved, and much work is currently underway with this objective in mind following both traditional mouse protocols and other novel strategies.

Gene targeting experiments in the mouse are permitting the systematic disruption and mutagenesis of genes involved in blood pressure regulation. Although such experiments have already been valuable, the continuing development of the technology is likely to provide further possibilities for refining the questions which can be addressed and will undoubtedly deepen our understanding of the complex genetics of blood pressure homoeostasis.

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Targeted Inactivation of the *Ren-2* Gene in Mice

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Abstract Several recent studies have demonstrated that ablation of genes of the renin-angiotensin system can have wide-ranging and sometimes unexpected effects. Renin is directly involved in blood pressure regulation and is encoded by a single gene in most mammals. Wild mouse strains and some inbred laboratory strains have a duplicated renin gene (*Ren-2*), the physiological significance of which is unclear. Significant differences exist in the structure and expression of these renin genes, but as yet, no distinct biological function that distinguishes these genes has been defined. We have used gene targeting to discover the effects of inactivating the duplicated (*Ren-2*) gene in strain 129 mice, and we show that mice lacking the *Ren-2* gene are viable and healthy. There appear to be no histopathological differences in renin-expressing tissues between *Ren-2*–null mice and their controls. Studies of our *Ren-2*–null mice allow, for the first time, a direct evaluation of the ability of the *Ren-1*^{d} gene to regulate blood pressure in the absence of expression of the *Ren-2* enzyme. We observed no alteration to blood pressure in adult mice homozygous for the mutated *Ren-2* gene, even though the concentration of active renin is increased and of prorenin is decreased in plasma of these mice. *Ren-1*^{d} is therefore capable of regulating normal blood pressure and despite a different tissue expression profile, is functionally equivalent to *Ren-1*^{c}. (***Hypertension. 1996;28:1126-1131.***)

Key Words • mice, transgenic • genes • molecular biology • mutation • recombination, genetic • renin

The renin-angiotensin system (RAS) is involved in the regulation of blood pressure (BP) and electrolyte balance as well as in the pathogenesis of several diseases, including hypertension. The aspartyl protease renin catalyses the cleavage of the plasma glycoprotein angiotensinogen, its only known substrate, thereby initiating the first and rate-limiting step in the generation of the potent vasoactive octapeptide hormone angiotensin II (Ang II). Renin is secreted primarily from modified smooth muscle cells of the afferent arteriole of the kidney, which constitute a major element of the juxtaglomerular apparatus.

Molecular cloning has demonstrated that renin is encoded by a single gene in humans, rats, and some strains of laboratory mice. However, wild strains and some inbred laboratory mouse strains have a duplication of approximately 30 kb of DNA on chromosome 1, encompassing the whole of the original renin locus (designated *Ren-1*^{d}) and including a second functional renin gene designated *Ren-2*. The single gene present in some inbred strains is designated *Ren-1*^{c}. The rat and mouse renin genes all span approximately 13 kb and are split into nine exons. The *Ren-1*^{c} and *Ren-1*^{d} genes are 99% identical, and the *Ren-1*^{d} and *Ren-2* genes have 97% sequence identity in their respective coding regions; thus, all three murine genes give rise to highly conserved proteins, which are approximately 97% similar at the amino acid level.[1] The gene duplication event is thought to have occurred 3 to 10 million years ago, after the speciation of the mouse.[2] The most notable differences in the renin-2 protein are the changes at three potential asparagine-linked glycosylation sites present in the renin-1 protein and reduced thermostability compared with renin-1.[3]

The *Ren-1*^{d} and *Ren-2* genes are expressed at approximately equal levels in the juxtaglomerular cells of the kidney, but these genes are differentially expressed in a number of tissues. The *Ren-1*^{c} gene is expressed in the developing adrenal gland, but it is developmentally restricted and the mRNA becomes undetectable near birth. The *Ren-1*^{d} and *Ren-2* genes are both expressed at similar levels in the adult adrenal gland of DBA/2 mice, and the expression level varies through the estrus cycle.[4] The granular convoluted tubule cells of the submandibular gland show a profound differential expression of renin genes, such that *Ren-2* is expressed in 100-fold excess over *Ren-1*^{c} and is regulated by androgens[5] and *Ren-1*^{d} expression is detectable by ribonuclease protection only.[6] On a per-cell basis, the relative amount of mRNA from the *Ren-2* gene in the submandibular gland is approximately equal to the level in the kidney. The *Ren-1*^{d} and *Ren-2* genes are also differentially expressed in the interstitial Leydig cells of the testis and a population of subcutaneous fibroblasts in the developing fetus. The renin-2 protein has biochemical properties similar to those of the renin-1 protein,[7] but it has not yet been possible to attribute a specific role to the product of the *Ren-2* gene. It is not known whether this gene is involved in any aspect of cardiovascular homeostasis or indeed whether it is functionally equivalent to the *Ren-1*^{d} gene. The duplicated mouse renin genes have long been used as a model system for the analysis of differential gene expression and of the evolutionary development of gene families.

The introduction of the mouse *Ren-2* gene into transgenic rats illustrates that in these circumstances, the renin gene can profoundly affect BP regulation, as this genetic modification results in severe hypertension.[8] It is thought that the rate of Ang II generation is limited by renin ac-

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tivity in all species except the mouse, in which the reaction is substrate limited. In support of this, expression of the rat angiotensinogen gene in transgenic mice causes hypertension,[9] whereas introduction of extra mouse renin genes does not alter BP.[4] The powerful studies of Kim and coworkers[10] have shown that titration of gene copy number for the mouse angiotensinogen gene not only increases plasma angiotensinogen levels in line with gene copy number but also results in a near-linear increase in BP.

The physiological role of individual genes can be investigated by specifically removing or altering the function of the gene in the absence of any other genetic changes. This is possible by use of cellular mechanisms of homologous recombination in embryonic stem (ES) cells, which can then be used to derive a genetically modified mouse strain.[11,12] This approach has been applied recently to some of the genes of the RAS and has demonstrated unequivocally that the genes encoding angiotensinogen,[10,13,14] angiotensin-converting enzyme,[15] and angiotensin type 1A receptor[16,17] are all important in the maintenance of normal BP. Common findings of the studies on angiotensinogen and angiotensin-converting enzyme gene knockouts are histopathological changes in the adult kidney, including medial hyperplasia of the interlobular arteries and afferent arterioles, interstitial fibrosis, and cortical thinning.[10,13-15] Because of the precise nature of the genetic change introduced in targeting experiments, particularly when performed in an inbred mouse strain such that normal littermates are genetically identical except for the introduced mutation, the data generated provide the proof of causation of a particular phenotype by a defined mutation in a candidate gene.[18] As part of a strategy to define pathological effects of ablating all renin gene expression, we attempt here to define separately the physiological functions of the *Ren-1*^{d} and *Ren-2* genes.

Methods

Materials

Plasmid pPGK*neo*, ES cell line E14Tg2a, and differentiation inhibiting activity were gifts from Austin Smith.

Amplification of Genomic Regions and Building of Targeting Construct

Two regions of the *Ren-2* gene, extending from exon 1 to exon 3 (4.32 kb) and from exon 5 to exon 9 (3.76 kb), were amplified from a partially characterized 129/Ola genomic clone with the use of the following primers: region 1 (4.32 kb): forward: 5′-GGACAGGAGGAGGATGCCTC-3′; reverse: 5′-AAG GTCTGGGGTGGGGTACC-3′; and region 2 (3.76 kb): forward: 5′-CGGGATCCAGTTTGACGGGGTTCTAGG-3′; reverse: 5′-CGGGATCCGGCGCGCCTTGCGGATGAAGGTGGCAC-3′. DNA was amplified for 40 cycles with *Pfu* DNA polymerase (Stratagene). The amplification conditions were denaturation for 1 minute at 95°C; annealing for 1 minute at 70°C (region 1) or 64°C (region 2) for 6.5 minutes at 74°C; and a single final extension period of 10 minutes at 74°C. Amplifications were performed in 20 mmol/L Tris-HCl (pH 8.75), 10 mmol/L KCl, 10 mmol/L $(NH_4)_2SO_4$, 100 μg/mL bovine serum albumin, 0.1% Triton X-100, 2 mmol/L $MgCl_2$, 0.2 mmol/L (each) dNTP, 0.5 μmol/L (each) primer, and 50 to 100 ng template DNA. Two factors contribute to the high number of cycles required to produce sufficient material for cloning: (1) the relative inefficiency of *Pfu* polymerase compared with *Taq* DNA polymerase, and (2) the fact that the large polymerase chain reaction products shown here are at the limit of amplification for pure proofreading enzymes using genomic clone template DNA.

These amplified products were digested with the restriction endonucleases *Xba* I and *Kpn* I (5′ arm of homology) or *Kpn* I and *Hin*dIII (3′ arm of homology) to yield larger fragments that were cloned into a pSP72-based plasmid and smaller fragments that were used as genomic probes flanking the targeting vector sequences during the screening of recombinant ES cell clones. The selectable marker cassette PGK*neo*, which has the neomycin phosphotransferase gene driven by the phosphoglycerokinase promoter, was inserted between the regions of genomic DNA to yield the plasmid pR2 *neo*KO (Fig 1). This plasmid was linearized before transfection into ES cells. The entire 3′ homology arm was sequenced, and these data were compared with the 3′ arm polymerase chain reaction product sequenced directly with the use of an automated sequencer (ABI Prism 377, Perkin-Elmer). No differences were found between the targeting construct and the genomic sequence, and so no mutations were introduced during polymerase chain reaction amplification.

Gene Targeting

The ES cell line E14Tg2a[19] was grown on gelatin-coated plastic in Glasgow Minimal Essential Medium supplemented with 1× nonessential amino acids, 0.25% (wt/vol) sodium bicarbonate, 0.4 mmol/L β-mercaptoethanol, 2 mmol/L glutamine, 1 mmol/L pyruvate, 10% fetal calf serum, and mouse DIA/LIF. Electroporation of 1×10^8 cells with 150 μg linearized DNA was followed 24 hours later by 9 days of selection in G418 (175 μg/mL). Individual clones were picked and expanded and then screened by Southern blot hybridization of *Pvu*II-digested DNA to the 3′ hybridization probe (Fig 1). DNAs from clones that appeared to be targeted at the *Ren-2* locus were then digested with *Sac* I, and the filters were hybridized with the 5′ flanking probe to confirm that homologous recombination had occurred in both arms of the targeting construct (unpublished observations, 1995). These combinations of restriction enzymes and probes were chosen because they distinguish between the two closely linked renin genes and also between the predicted results of homologous recombination at either gene.

Animal Handling and Breeding

Correctly targeted ES cells were thawed, expanded, and injected into blastocysts derived from C57/Bl6 mice and offspring containing a good proportion of ES cell–derived tissue identified by coat color chimerism. These chimeric mice were bred with 129/Ola female mice; thus, transmission of the disrupted *Ren-2* gene from ES cells to 129/Ola mice maintains a pure, inbred genetic background. All mice were bred in-house, fed standard chow and tap water ad libitum, and maintained in accordance with the Animals (Scientific Procedures) Act, 1986.

Gene Expression Analysis

RNA was prepared with the guanidinium isothiocyanate/phenol method,[20] quantified by UV spectroscopy, and visually assessed by agarose gel electrophoresis. Primer extension with a 38-mer oligonucleotide specific for exon 8 of all mouse renin genes was by the method of Field and Gross.[5] RNA expression was quantified by a PhosphorImager (Molecular Dynamics) and was adjusted for sample recovery by comparison of the signal from the primer. Polyacrylamide gels (12%) were exposed for 7 days to Kodak X-Omat film and then for a further 7 days for PhosphorImager analysis.

BP Measurements

Mean BP was measured by direct cannulation of the abdominal aorta in adult (12- to 20-week-old) mice. A fine cannula made from drawn polyethylene tubing (Portex) was inserted into the vessel, while the aorta and vena cava were supported under tension with a suture to prevent blood flow and hence blood loss. The aortic flow was stopped for a maximum of 30 seconds, which is sufficient time to insert the catheter into the aorta and fix it in place with tissue glue. After at least 24 hours of recovery, the

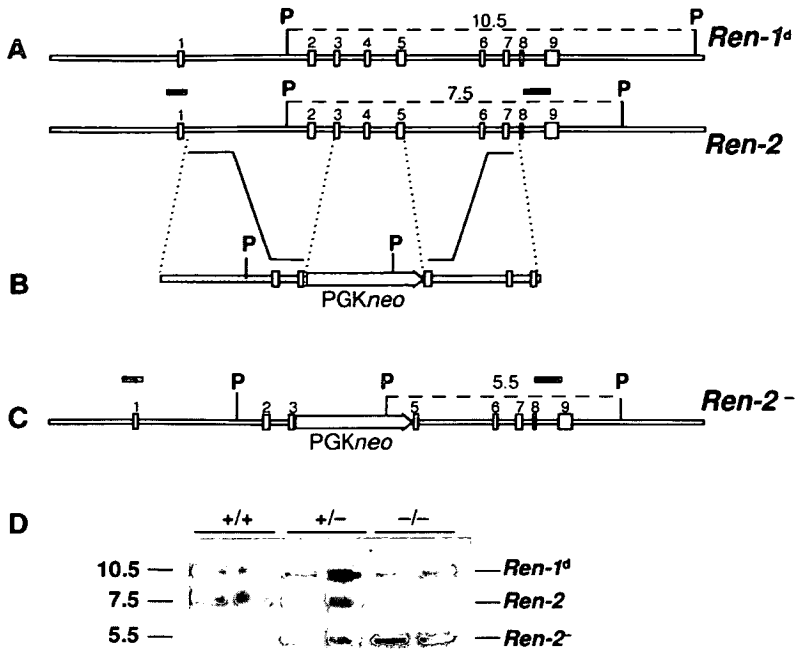


FIG 1. Gene targeting at the *Ren-2* locus. A, Mouse *Ren-1*^{d} and *Ren-2* genes are aligned to show the high similarity between these loci. Positions of the probes used to screen embryonic stem (ES) cell clones (horizontal black bars) are external to the genomic DNA used in the targeting construction. Exons (open boxes) are numbered. B, Targeting construction pR2 *neo*KO showing genomic DNA (narrow bars) and selectable marker cassette PGK*neo* (neomycin phosphotransferase gene driven by the phosphoglycerokinase promoter) (arrow). Recombination events within each homology arm result in the replacement of exon 4 and parts of exons 3 and 5 with the PGK*neo* gene, resulting in the null *Ren-2*$^-$ allele as shown in C. Restriction endonuclease recognition sites (P, for *Pvu*II) used in screening ES cell clones and sizes of fragments detected by the 3′ probe of the *Ren-2* gene are shown. D, Southern blot shows expected band sizes for *Ren-1*^{d} (10.5 kb), *Ren-2* (7.5 kb), and *Ren-2*$^-$ (5.5 kb) genes in wild-type (+/+), heterozygous (+/−), and homozygous null (−/−) littermates with the 3′ probe of the *Ren-2* gene.

catheter was connected to a pressure transducer (Viggo-Spectralab), and BP was measured on a chart recorder. The catheter was prefilled with heparin-saline and flushed daily. Measurements were made in conscious mice in restraining tubes for at least 15 minutes for each mouse. Mean BP was calculated as 60% of mean diastolic pressure plus 40% of mean systolic pressure. Each mouse had undergone 10 days of training over the preceding 2 weeks involving at least 10 minutes of restraint per day. All data were collected between 10 AM and 3 PM, and the data gathered from mice measured on more than 1 day were averaged.

Plasma Renin and Prorenin Measurements

Blood was collected by cardiac puncture immediately after death into fresh Ωphenanthroline (0.05 mol/L) and EDTA (0.1 mol/L) on ice in a ratio of 10 μL per 100 μL whole blood. Blood was spun immediately for 6 minutes at 4000*g*; plasma was snap-frozen in liquid nitrogen and stored at −70°C until assayed. Plasma renin and prorenin concentrations were determined as previously described.[21] Briefly, inactive renin in 20 μL plasma was activated with 40 μL trypsin (400 U/mL, dissolved in TES buffer: 0.1 mol/L *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid [pH 7.2], 0.01% neomycin, 10 mmol/L EDTA). Samples were incubated on ice for 10 minutes, and the reactions were stopped by the addition of 40 μL soybean trypsin inhibitor (600 U/mL, in TES buffer). Active renin was measured after addition of 80 μL TES buffer without trypsin, as described below.

Pretreated samples were incubated with lyophilized renin substrate isolated from nephrectomized rat plasma (final concentration: 80 mg/mL; 0.11% 2,3-dimercapto-1-propanol, 1.15 mg/mL 8-hydroxychinolin in TES buffer). The reaction was stopped with radioimmunoassay buffer (0.1 mol/L Tris-acetate, pH 7.4) (1) immediately before the incubation and (2) 1 to 3 hours after incubation at 37°C. Generated Ang I was measured by radioimmunoassay.[22,23]

Histology

Mice were killed by exposure to 100% CO_2, and tissues were fixed in 10% phosphate-buffered formal saline for 24 hours before dehydration and embedding in paraffin wax. Sections of 6 μm were mounted on slides and stained with hematoxylin and eosin. Multiple sections from kidneys, adrenal, and submandibular glands were examined in a blinded fashion by two experimenters using standard light microscopy.

Results

Targeting Efficiency

Screening of G418-resistant ES cells transfected with pR2 *neo*KO with the 3′ flanking probe (Fig 1) identified 15 of 228 clones that possessed a restriction fragment size diagnostic of homologous recombination in the *Ren-2* gene. The 5′ flanking probe was used to show that 13 of these clones were correctly targeted also in the 5′ arm of homology, which is an overall targeting frequency of 5.7%. We used two of these ES cell clones to make chimeric mice that transmitted the disrupted *Ren-2* gene to their offspring. None of the targeted cells screened in this way had evidence of the recombination at the highly homologous *Ren-1*^{d} locus.

Analysis of Renin Gene Expression

The transcripts of the *Ren-1*^{d} and *Ren-2* genes can be distinguished by a dideoxynucleotide primer extension assay[5] as well as by ribonuclease protection.[6] As shown by primer extension (Fig 2), *Ren-2*–derived transcripts were not detectable in adult *Ren-2*–null mice in either kidney or submandibular gland RNA at this level of sensitivity. The relative level of *Ren-2*–derived RNA, which has been studied in a limited number of mice and in heterozygous mouse kidney, is 55% (range, 54.7% to 55.4%; n=2) compared with wild-type littermates and is 63.3% (range, 44.5% to 79.3%; n=3) in the male heterozygote submandibular gland. The level of *Ren-1*^{d} mRNA detected in the kidney is slightly increased to 107.6% (range, 101.1% to 114.1%; n=2) and 117.3% (range, 107.2% to 127.3%; n=2) in heterozygous and *Ren-2*–null mice, respectively.

Analysis of *Ren-2* Knockout Mice

Homozygous *Ren-2*–null mice are healthy and viable. Intercrossing of F_1 heterozygotes produced the expected numbers of wild-type, heterozygous, and homozygous mice. The adult *Ren-2*–null mice had no gross abnormalities postmortem and had normal histomorphology of the kidney, adrenal gland, and submandibular gland compared

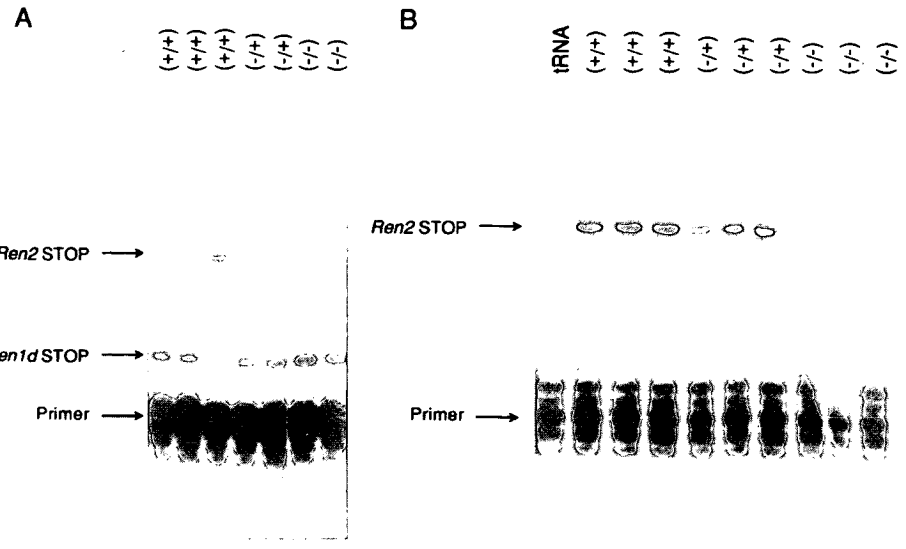
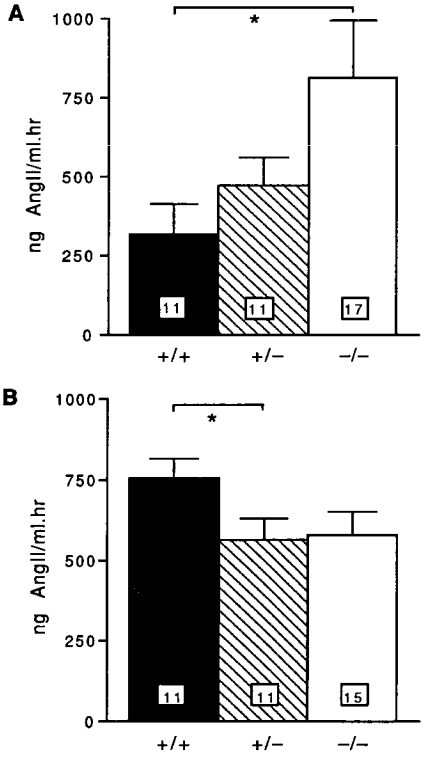


FIG 2. Gene-specific primer extension analysis of 80 μg of kidney (A) and 2 μg of submandibular gland (B) total RNA. The 38-mer oligodeoxynucleotide primer used produces extension products of +5 nucleotides from *Ren-1*^{d} mRNA (*Ren1d* STOP) and +12 nucleotides from *Ren-2* (*Ren2* STOP) mRNA in the presence of ddCTP and dGTP, dTTP, and dATP. Adult male wild-type (+/+), heterozygous (+/−), and homozygous (−/−) littermates were used. tRNA indicates 80 μg of yeast tRNA negative control RNA.

ith wild-type littermates (n=2 for males and females, npublished observations, 1996).

lasma Renin and Prorenin Concentrations

The prorenin level in the plasma of heterozygous mice as lower than that of wild-type mice ($P<.05$, t test), as nown in Fig 3. Homozygous *Ren-2*–null mice also had decreased plasma prorenin, but this difference did not reach the 5% level of significance ($.05<P<.10$). Interestingly, plasma renin concentration was higher in *Ren-2*–null mice than in wild-type mice ($P<.05$, t test) and was at an intermediate level in heterozygous mice (P=NS). This increase in plasma renin concentration in the absence of two copies of the *Ren-2* gene may be due to increased synthesis and release of renin-1 by a feedback mechanism linked either to the lower levels of circulating prorenin or to expression of renin-2 itself.



G 3. Plasma renin concentration (A) and prorenin concentraon (B) in male and female wild-type (solid bars), heterozygous atched bars), and homozygous (open bars) mice expressed as ean+SE. Numbers inside bars indicate sample size. *$P<.05$, tudent's t test. Wild-type (7 males, 4 females), heterozygote (4 ales, 7 females), and homozygote (4 males, 12 [A] or 10 [B] males) mice show no difference between sexes within each enotype, so pooled data are presented. Angl indicates angiosin I.

Does Loss of Renin-2 Affect Resting BP?

To determine whether the lack of a functional *Ren-2* gene has any effect on the resting BP of 129/Ola mice, we measured mean BP by direct cannulation of the abdominal aorta (Fig 4). Measured in this way, BP values in wild-type, heterozygous, and *Ren-2*–null mice did not differ ($P>.1$, t test). Mixed genetic backgrounds could affect BP, irrespective of the presence or absence of an intact *Ren-2* gene. For this reason, we chose to breed purely within the 129/Ola inbred mouse strain. Because we did not backcross to a different strain, there was no segregation of un-

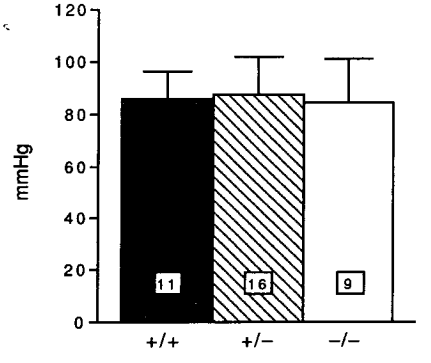


FIG 4. Mean blood pressure as measured by aortic cannulation. Values for male and female mice were pooled and are expressed as mean+SE. Mean blood pressure values did not differ significantly between pooled and sex-matched groups (unpublished observations, 1996). Numbers inside bars indicate sample size. Wild-type (solid bar; 6 males and 5 females), heterozygous (hatched bar; 7 males and 9 females), and homozygous (open bar; 2 males and 7 females) mice are shown.

linked polymorphic genes in subsequent generations and no false association of a phenotype with the introduced mutation due to selection for 129 alleles linked to the targeted locus.[18] All offspring were genetically identical except for the mutated gene.

Discussion

It is presently unclear what the exact role of the duplicated *Ren-2* gene is in the regulation of BP and electrolyte and fluid balance in the mouse strains that carry this gene. The aim of this study was to inactivate the *Ren-2* gene by homologous recombination and thus (1) determine whether the remaining renin gene, *Ren-1*^{d}, is sufficient to maintain normal BP, and (2) reveal unrecognized functions of this gene. The *Ren-2* renin gene was inactivated, and mice carrying the null allele were analyzed for changes in resting BP, expression of the *Ren-1*^{d} gene, levels of active and inactive renin in the circulation, and histopathological changes in renin-expressing organs.

The *Ren-1*^{d} gene is the orthologue of the *Ren-1*^{c} gene of "single-gene" mouse strains, and it may be reasonable to expect, on the basis of the high degree of similarity, that the *Ren-1*^{d} gene product is capable of performing all of the functions of the renin proteins from strains and species that contain only a single renin gene. Previously, studies comparing the *Ren-1*^{c} and *Ren-1*^{d} genes have had to take into account the presence of the *Ren-2* gene in "two-gene" mice. Inactivation of the *Ren-2* gene has generated a new mouse strain that is better suited to the direct comparison of the orthologous *Ren-1*^{c} and *Ren-1*^{d} genes. The physiological functions of *Ren-1*^{d} gene expression can now be assessed in the absence of the *Ren-2* gene and so without the confounding effects of the overlapping expression of this similar activity. The effect of genetic background in these comparisons could be taken into account by parallel studies on the wild-type mice of each strain (ie, C57/Bl6 and 129) and by sampling a large number of F_2 mice derived from an intercross between *Ren-2*–null (129) and C57/Bl6 mice after the offspring are typed and sorted according to which *Ren-1* alleles (*Ren-1*^{c} or *Ren-1*^{d}) are present.

In this study, we aimed to show whether the *Ren-1*^{d} gene product alone is sufficient for normal BP regulation and also hoped to uncover any critical function of the renin-2 protein not previously recognized. The major sites of expression of the *Ren-2* gene are the juxtaglomerular cells of the kidney, the X-zone of the adrenal gland, and the granular convoluted tubule cells of the submandibular gland; however, the specific physiological properties of this protein are not defined. The high level of expression in the secretory epithelial cells of the submandibular gland may be the incidental result of the disruption of a negative regulatory DNA element, located in the 5′ region of the *Ren-2* gene, by the insertion of a repetitive sequence termed M2.[24] Renin-2 is also expressed and secreted from the juxtaglomerular cells of the kidney and so may play a role in Ang II generation in the classic circulating RAS. We have used a generally applicable, rapid strategy for the construction of vectors for use in gene targeting experiments based on amplification and cloning of long regions of chromosomal homology by the polymerase chain reaction. The result of the homologous recombination reported here is predicted to completely remove enzyme activity. The absence of renin-2 has no effect on the resting BP of young adult mice. However, the normal function of the renin-2 protein may become manifest only after an environmental or physiological stimulus or insult. Studies are ongoing in this respect. The mutation at *Ren-2* does affect the level of prorenin in the circulation, and it could be that circulating prorenin is predominantly derived from the *Ren-2* gene in normal "two-gene" mice. There is a concomitant increase in the plasma renin concentration in animals with depressed levels of prorenin, suggestive of a compensatory response. The signals that might mediate a feedback response may involve the activation of prorenin in tissues (or in the circulation) or some direct signal passed through the prorenin molecule itself.

Overall, we have shown that the mouse *Ren-2* gene is not essential and that mutation of this gene does not affect kidney, submandibular gland, or adrenal gland histomorphology. The levels of the inactive zymogen prorenin are reduced in homozygous *Ren-2*–null mice, and yet resting BP does not change in these mice. An activity other than that encoded by the *Ren-2* gene is sufficient for all the functions of renin that have been described to date, and that activity is probably the product of the *Ren-1*^{d} gene. More detailed analysis of these mice, under different physiological conditions, is being used to distinguish any functions that may be ascribed to the *Ren-2* gene only.

Genetic ablation of the renin substrate angiotensinogen illustrates profound alterations in BP and kidney vascular morphology.[10,13] The question of whether the *Ren-2* gene is active in the regulation of the classic circulating RAS and is able to participate in the normal regulation of BP will come from the targeted inactivation of the *Ren-1*^{d} gene in mice that retain the normal *Ren-2* gene. The *Ren-1*^{d} gene has been targeted in mouse ES cells,[25] but mice derived from these targeted cells have not been reported to date. We have generated mice that carry a mutation of the *Ren-1*^{d} gene, and homozygous null mice are healthy and viable (unpublished observations, 1996). Comparison of these strains, which are isogenic except for the renin gene defects, will allow various functions of hemodynamic regulation and physiology of the RAS to be associated with one or both of the forms (nonglycosylated and potentially glycosylated) of renin present in "two-gene" mice. The mice reported here will be used for further study of the role of the renin-2 protein in mouse physiology and to characterize which functions of renin are performed by the products of each gene. This may aid in the elucidation of pathologically important sites or forms of renin expression in human populations.

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Renin-1 is essential for normal renal juxtaglomerular cell granulation and macula densa morphology

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Running Title: Morphological Changes in Mice Lacking *Ren-1^d*

The secretion of renin from granules stored in renal juxtaglomerular cells plays a key role in blood pressure homeostasis. The synthesis and release of renin and the extent of granulation is regulated by several mechanisms including signalling from the macula densa, neuronal input and blood pressure. Through the use of a gene targeting vector containing homology arms generated using the polymerase chain reaction, we have inactivated the *Ren-1^{d}* gene, one of two mouse genes encoding renin, and report that lack of renin-1^{d} results in altered morphology of the macula densa of the kidney distal tubule, and complete absence of juxtaglomerular cell granulation. Further, *Ren-1$^{d-/-}$* mice exhibit sexually dimorphic hypotension. The altered growth morphology of the macula densa in *Ren-1^{d}*-null mice should provide a tool for the investigation of the JG cell–macula densa signalling. Further, the current data indicate that expression of the *Ren-1^{d}* gene is a prerequisite for the formation of storage granules, even though the related protein renin-2 is present in these mice, suggesting that renin-1^{d} and renin-2 are secreted by distinct pathways *in vivo*.

INTRODUCTION

Renin (EC 3.4.23.15) is an aspartyl protease which catalyses the first step in the renin angiotensin system, the end-product of which is the potent vasopressor peptide hormone, angiotensin II (AngII[1]). This octapeptide acts to increase peripheral vascular resistance, and promote salt and fluid retention in concert with the hormone aldosterone. Renin is synthesised principally in the kidney juxtaglomerular (JG) cells, a group of modified smooth muscle cells located at the distal end of the renal afferent arteriole of the glomerulus (1). JG cells are in close contact with the macula densa, a specialised plaque of epithelial cells of the kidney distal tubule, which signal to the renal arterioles to regulate glomerular filtration rate and the secretion of renin, in response to ionic concentration and flow rate in the distal tubule (2, 3), the so-called tubuloglomerular feedback loop. Except for the submandibular gland (SMG) of the mouse the JG cells are the only site where prorenin, the inactive zymogen, is known to be converted to the active form of renin. SMG renin does not, however, make its way into the plasma in large quantities, and is thought not to play a significant role in blood pressure regulation under normal circumstances [reviewed in Bing *et al.* (4)]. The release of renin from JG cells is mediated by two pathways; regulated release of the mature, active renin from modified lysosomal storage granules, and constitutive release of the inactive zymogen. While the regulated pathway of renin secretion is responsive to baroreceptor, neurogenic and macula densa signals (5), the physiological significance of the constitutive secretion of prorenin is not understood, nor are the molecular pathways which link secretory signals to renin maturation and release. Clarification of the mechanisms underlying these processes will be crucial to our understanding of the control of renin activity locally in the renal glomerulus, and in the plasma, and the regulation of fluid and ion homeostasis.

Human and rat genomes contain a single gene for renin but mice display two alternative genotypes at the *Ren* locus. Thus some inbred mouse strains (*e.g.* strain

C57BL/6) have only a single renin structural gene, termed *Ren-1^c*, while others (e.g. DBA/2 and 129/Ola) possess two renin genes, termed *Ren-1^d* and *Ren-2*. This probably results from the recent duplication of 21kb of DNA containing a *Ren-1^c*-like ancestral gene (6, 7). All three mouse renin genes share the same overall genomic organisation, and encode highly homologous but distinct proteins, with approximately 97% similarity at the amino acid level, but having different glycosylation potentials [reviewed in (8)]. This arises because the renin-2 enzyme lacks putative consensus sites for asparagine-linked glycosylation, whereas renin-1^c and renin-1^d proteins can be glycosylated at three asparagine residues. The mouse renin genes are expressed in distinct, though overlapping, tissue-specific and developmental patterns (8). It has therefore been difficult to dissect the individual roles of each gene to date, or to determine if renin-1^d and renin-2 play functionally equivalent roles *in vivo*. For example, *Ren-1^d* and *Ren-2* are expressed at equivalent levels in JG cells, but *Ren-2* is expressed at high levels (2% of total SMG protein) in the submandibular gland and is under the control of various hormones, including testosterone, whereas *Ren-1^d* is only detectable at trace levels in this organ (9). The evolution of non-identical, tightly regulated developmental expression profiles, and the biochemical differences between renin-1 and renin-2 proteins suggest that the two genes may indeed possess functionally distinct properties.

The presence of two genetically distinct forms of renin, susceptible to manipulation by gene targeting, offers a unique opportunity to determine the importance of renin glycosylation for its role *in vivo* and to dissect the functions of renin which in other animals are subserved by a single gene product. We have recently reported the targeted disruption of the *Ren-2* gene in mice (10), which results in *Ren-1^d* being the only active renin gene present. *Ren-2*-null mice display elevated circulating active renin concentrations, and reduced circulating (inactive) prorenin, however no abnormalities in the histomorphology of adult kidneys, adrenals or submandibular glands, nor in resting blood pressures, have been found in adult animals to date (10). Here, we describe the generation of mice in which the *Ren-1^d* gene has been inactivated by homologous recombination. As part of a generalised construction strategy, the regions of DNA providing *Ren-1^d* gene homology in the targeting vector have been generated by long-range PCR amplification of isogenic substrate DNA, using a proof-reading DNA polymerase. The phenotype of *Ren-1$^{d-/-}$* mice shows that the *Ren-1^d* and *Ren-2* genes are not functionally equivalent. First, female mice show a significant reduction in resting blood pressure. Further, the level of plasma active renin is decreased while inactive prorenin is increased. Finally, the deletion of the glycosylated renin-1 results in the complete absence of dense secretory/storage granule formation in JG cells and altered morphology of the macula densa cells of the kidney distal tubule.

EXPERIMENTAL PROCEDURES

Construction of a Ren-1^d *Gene Targeting Vector*–Regions of *Ren-1^d* gene homology for incorporation into the targeting vector were generated by long-range PCR amplification using, as template, DNA from a bacteriophage P1 clone containing the entire mouse 129/Ola *Ren-1^d* gene (P1-1249[2]) and the primer pairs, for the 5' arm, JJM 203 (5'-CCGCTCGAGTCTGGACAGCCTACATGAC-3') and JJM 135 (5'-AAGG-TCTGGGGTGGGGTACC-3') and for the 3' arm, JJM 224 (5'-GCCGCTCGAGGTACCA-

GCTACATGGAGAACGGGTC-3') and JJM 204 (5'-GCAAGCTTGACAAAATGGCC-CCCAGGAC-3'). Natural (5' arm) and artificially introduced (3' arm) *Kpn* I sites used in cloning are underlined. Reactions (100µls) in 10mM Tris-HCl; pH 8.8, 10mM KCl, 0.002% (v/v) Tween 20®, 1.0mM $MgCl_2$, 40µM each dATP, dCTP, dGTP, dTTP, 5µM each primer, 10-100ng template DNA and 5 units of *ULTma* DNA polymerase (Perkin Elmer ABI, Warrington, UK) were 40 cycles of 95°C for 1 minute, 66°C (5' arm) or 68°C (3' arm) for 1 minute and 72°C for 6.5 minutes, followed by one period of 10 minutes at 72°C. Each PCR product was cloned into a plasmid vector, and then manipulated to flank the PGKneo selectable marker gene. The final targeting vector, pR1*neo*KO, contained 3.5kb and 3.7kb segments of the *Ren-1^d* gene flanking the selectable marker gene.

Gene Targeting and Generation of Mutant Mice–ES cells were grown in GMEM + 10% foetal calf serum supplemented with mouse or human DIA/LIF on gelatin-coated plastic, as described (11). Targeting vector DNA (150µg; pR1*neo*KO), was linearised by digestion with *Asc* I and *Mlu* I and electroporated into 5×10^9 E14Tg2a cells Hooper, 1987 #512 , a strain 129-derived embryonal stem cell line, with a discharge of 0.8kV at 3µF on a BioRad Gene Pulser. Following G418 selection (175µg/ml), drug-resistant colonies were expanded and genomic DNA prepared (12). Homologous recombination events were detected by Southern blotting of DNA digested with *Sac* I and hybridised with an external 5' probe (a 297bp *Pvu* II/*Bam* HI fragment, containing exon 1of the *Ren-1^d* gene). Clones selected in this way were also hybridised with an external 3' probe after digestion with *Pvu* II (a 746bp *Hind* III/*Nco* I fragment, containing *Ren-1^d* exon 8 and part of exon 9; see Figure 1D). One targeted clone was used to generate male chimaeras, which were crossed with 129/Ola females to generate inbred 129/Ola heterozygote offspring. These mice were intercrossed to produce an F2 generation with wild-type, heterozygous and homozygous inbred, *Ren-1$^{d-/-}$* mice.

Gene expression–Kidney RNA was amplified by reverse transcription-coupled PCR, using the Expand RT kit (Boehringer Mannheim), according to the manufacturer's instructions. cDNA was randomly primed with hexanucleotides, and renin sequences amplified using primers JJM 56 (5'-CCAGCCCAGACCTTCAAAGTC-3') and JJM 141 (5'-CCAGACAAATGGCCCCCAAG-3'), specific for exons three and nine of the mouse renin genes, respectively. Amplification was for 10 cycles of 94°C for 10 seconds, 62°C for 20 seconds and 68°C for 45 seconds, plus 25 cycles with a 5 second increment in each 68°C extension phase. The resulting renin cDNA (999bp) was digested with *Ear* I, an enzyme which digests *Ren-1^d* cDNA twice, and *Ren-2* cDNA once.

Histological Analysis–Following sacrifice of animals by CO_2 anesthesia, tissues were immersion-fixed in 4% (w/v) formaldehyde, 0.9% (w/v) NaCl for 24 hours and embedded in paraffin wax. Subsequently, 2µm sections from kidneys, submandibular glands, adrenal glands and testes were stained with haematoxylin and eosin and examined, in a blinded manner, by standard light microscopy.

Measurement of Blood Pressure–Mean blood pressure (40% of systolic + 60% of diastolic pressures) was measured via direct cannulation of the abdominal aorta in adult mice (8-15 weeks old). Measurements were made over a 15 minute period in conscious, resting animals housed in restraining tubes. All animals had undergone training (30 minutes/day) in restraining tubes for 5 consecutive days, beginning 7 days

prior to the operation. Operations were performed as described (10). Briefly, a cannula made from drawn polyethylene tubing (Portex, Hythe, UK; Product Code 800/100/100) was inserted into the aorta (while the blood flow in the aorta and vena cava was occluded using a suture) and fixed in place using tissue glue. Cannulae were filled with heparin-saline, and were flushed daily. Blood pressure was measured 24 hours post operation by connecting the cannula to a pressure transducer (Viggo-Spectralab, Oxnard, Ca, USA) and printing on a chart recorder. Statistical significance was assessed using a two-tailed Student's *t*-test.

Measurement of Renin Concentration–Animals were sacrificed as above, and blood sampled immediately by cardiac puncture into 0.1 volumes of 125mM EDTA; 25mM Ω-phenanthroline. Plasma was snap-frozen in liquid nitrogen in 100µl aliquots. Plasma renin concentrations (PRC) and plasma prorenin concentrations (PPC) were calculated according to the method of Peters *et al.* (14). Total renin concentration was measured by activating 20µl of plasma with 40µl of trypsin (400 units/ml, dissolved in TES buffer; 0.1M N-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid, pH7.2, 0.01% neomycin, 10mM EDTA). Samples were incubated on ice for 10 minutes and trypsin-activation stopped by the addition of 40µl of soybean trypsin inhibitor (600units/ml, in TES buffer). Plasma active renin was measured by the addition of 80µl of TES buffer (without trypsin) to 20µl of plasma. Pre-treated samples were incubated with lyophilised renin substrate, isolated from nephrectomized rat plasma (final concentration; 80mg/ml, 0.11% 2,3-dimercapto-1-propanol, 1.15mg/ml 8-hydroxyquinoline in TES buffer). Reactions proceeded for 1-3 hours at 37°C, and were stopped with RIA buffer (0.1M tris-acetate, pH7.4). The AngI generated by the plasma renin was measured by radioimmunoassay (15, 16). Plasma prorenin concentration is determined as the difference between total renin concentration and plasma active renin concentration. Statistical significance was assessed using the Wilcoxon rank test.

RESULTS

Generation of Ren-1^d *Deficient Mice*–Initally, conditions were established for the efficient PCR amplification of DNA fragments in the size range 3-4kb, using the thermostable proof-reading *ULTma* DNA polymerase (described in Experimental Procedures). Subsequently, two regions of the *Ren-1^d* gene, extending from exon 1 to exon 3 and exon 4 to exon 9 (Fig. 1A and 1B) were successfully amplified using *Ren-1^d*-specific primers, from bacteriophage P1 clone P1-1249, which contains the entire mouse 129/Ola *Ren-1^d* gene. PCR products were then digested and used to assemble the final targeting vector, pR1*neo*KO, in which 3.5kb and 3.7kb segments of *Ren-1^d* gene homology flank the selectable marker (Fig. 1C). Following electroporation of the targeting construct into ES cells, Southern analysis using 5' and 3' external probes (Fig. 1D and not shown) identified 3 from 313 (1%) drug-resistant colonies that were correctly recombined in both the 5'and 3' arms. One clone was used to generate male chimeras, which were crossed with 129/Ola females to generate inbred 129/Ola heterozygote offspring. These mice were intercrossed to produce an F2 generation with wild type, heterzygote and homozygote inbred *Ren-1$^{d-/-}$* mice (Fig. 1E). The absence of recombination within the *Ren-2* gene demonstrates the previously reported (17) highly specific recombination achievable, even when targeting closely related genes.

Gene Expression Analysis– Amplification of total kidney RNA from *Ren-1*$^{d-/-}$ mice by RT-PCR, followed by restriction digestion of the product with *Ear* I, confirmed that the disrupted *Ren-1*^{d} gene is unable to produce functional *Ren-1*^{d} mRNA and that *Ren-2* mRNA is the only gene product present in these mice (Fig. 2). Primer extension analysis[3], showed that*Ren-2*-derived mRNA is 2.8±0.05- and 3.9±0.23-fold more abundant in the kidneys of *Ren-1*$^{d-/-}$ males and females, respectively, compared to wild-type mice.

Renin Measurements–Plasma renin concentration (PRC) and plasma prorenin concentration (PPC) were determined from mouse plasma samples (Fig. 3). PRC levels did not differ significantly between males of all three genotypes (+/+=240±58, +/−=170±34, −/−=243±63 ng AngI/ml/hr; $P>0.05$) (Fig. 3A, solid bars). However, PPC was significantly higher in *Ren-1*$^{d-/-}$ male mice (1341±116 ng AngI/ml/hr), as compared to both wild-type (717±64 ng AngI/ml/hr; $P<0.0003$) and heterozygous males (566±33 ng AngI/ml/hr; $P<0.0003$) (Fig. 3A, open bars). In females, PRC was reduced in *Ren-1*$^{d-/-}$ mice (123±28 ng AngI/ml/hr) compared to controls (229±32 ng AngI/ml/hr; $P<0.027$), while heterozygous females had an intermediate level (164±34 ng AngI/ml/hr) (Fig. 3B, solid bars). Similar to male mice, PPC measurements revealed a significant increase in circulating prorenin in female *Ren-1*$^{d-/-}$ homozygotes (1632±238 ng AngI/ml/hr) compared to *Ren-1*$^{d+/-}$ mice (528±42 ng AngI/ml/hr; $P<0.0003$) and wild-type females (557±56 ng AngI/ml/hr; $P<0.0003$) (Fig. 3B, open bars).

Blood Pressure Homeostasis–Measurement of mean arterial blood pressures in males showed no significant ($P>0.05$) difference between *Ren-1*^{d} genotypes (Table 1). However, a significant decrease in blood pressure of 12.7 mmHg was seen in *Ren-1*$^{d-/-}$ females compared with wild-type controls ($P<0.01$).

Histomorphological Appearance–Kidneys, adrenal glands, submandibular gland and testes or ovaries from *Ren-1*$^{d-/-}$ (n=4), *Ren-1*$^{d+/-}$ (n=4) and wild-type animals (n=2) from both sexes were studied. No differences were observed in adrenal glands, submandibular glands, testes or ovaries from all three genotypes in both sexes. However, kidney sections showed two significant abnormalities in both *Ren-1*$^{d-/-}$ males and females (Fig. 4A and 4B). The macula densa of *Ren-1*$^{d-/-}$ mice exhibited hypercellularity, and an altered epithelial morphology in which the cells showed a columnar appearance, which contrasts with the cuboidal morphology of the wild-type controls. The central three macula densa cells were measured in five JG regions from each of four individual mice (n=20) of each genotype. Wild-type and *Ren-1*$^{d+/-}$ mice had macula densa cells of 6.1μm (range 5.6-6.3μm) and 6.0μm (range 5.8-6.2μm) in height (basolateral to apical dimension), repectively, whereas the height of macula densa cells in *Ren-1*$^{d-/-}$ mice was 7.9μm (range 7.6-8.1μm). This represents a 30% increase in cell height in the *Ren-1*^{d}-deficient mice. Immunostaining of kidney sections with an antibody specific for renin showed that, in contrast to the granular appearance of the controls, *Ren-1*$^{d-/-}$ mice exhibited diffuse, uniform, low-level cytoplasmic renin staining (approximately 5% of controls) consistent with constitutive secretion, and indicating that renin-2 is not stored in large quantities in the JG cells of these mice (Fig. 4C and 4D). Kidney sections from homozygous mutant and control mice were examined by transmission electron microscopy, which demonstrated that the JG cells of the *Ren-1*$^{d-/-}$ mice were completely devoid of the storage/secretory

granules typically present in wild-type controls (Fig. 4E and 4F). Nevertheless, *Ren-1$^{d-/-}$* JG cells contain an abundant rough endoplasmic reticulum (Fig. 4F).

DISCUSSION

Disruption of the *Ren-1^d* gene, described here, provides the first demonstration that inactivation of a gene encoding renin affects blood pressure homeostasis, exemplified by the sexually dimorphic hypotension seen in *Ren-1$^{d-/-}$* females. In addition, the *Ren-1^d* phenotype displays a decrease in the plasma concentration of active renin and an increase in plasma prorenin. Further, a discrete and reproducible change was observed in the morphology of the macula densa cells of the kidney distal tubular epithelium. This small group of cells which act as sensors in the tubular glomerular feedback loop, but do not express renin, show a 30% increase in basolateral to apical height. The physiological sequelae of this cellular change are presently unknown. Most striking is the complete absence of secretory/storage granules in the JG (modified smooth muscle) cells of the renal afferent arteriole. Thus, expression of the renin-1^d protein is a prerequisite for secretory granule formation and maturation, and the *Ren-2* gene product is unable to act as substitute in this role.

A novel feature of the current study is the successful inactivation of the *Ren-1^d* gene by homologous recombination using a targeting construct in which the regions of DNA providing *Ren-1^d* gene homology were generated by long-range PCR. Together with the use of a similar strategy to target the *Ren-2* gene (10), these data demonstrate the feasability of using homology regions generated entirely by PCR to target genes of interest. Optimised conditions for the efficient amplification of DNA fragments in the size range of 3-4kb, using a cloned genomic DNA template and the thermostable proof-reading *ULTma* DNA polymerase, included long (6.5 minute) extension times, an increased number of cycles (40) and primers of 28-32 nucleotides in length. To facilitate molecular manipulations and/or screening for homologous recombinants, restriction enzyme recognition sequences can be usefully built into primers, but they should be situated at least five nucleotides from the end of the PCR product to permit efficient digestion. Although this experiment utilised a 130kb bacteriophage P1 *Ren-1^d* genomic clone as the template for PCR, we have also demonstrated the efficient amplification of up to 10kb fragments from genomic DNA, using a mixture of proofreading and Taq DNA polymerases[4]. Consequently, the regions of homology required to target any gene for which a minimum amount of information is available can be generated, with the advantages of using isogenic genomic DNA from the ES cell strain to be targeted as template and the facile introduction of additional restriction sites to simplify molecular manipulations and screening strategies.

Juxtaglomerular cells, the principal site of renin synthesis, represent a specalised population of the smooth muscle cells of the renal afferent arteriole. In particular they contain abundant modified lysosomal granules, where prorenin is activated and stored (1). It is thought that the release of active renin from these secretory/storage granules is by regulated exocytosis in response to specific physiological stimuli, whereas an additional distinct secretory pathway mediates the constitutive secretion of inactive prorenin, via clear secretory vesicles. This concept is based on previous work on cultured tumoural JG cells and human kidney slices (17) and AtT-20 cells, a mouse pituitary cell line which expresses both regulated and constitutive secretory pathways,

and can process prorenin into active renin (18,19) (24,25). It has also been suggested that the mouse enzymes renin-1^d and renin-2 can each be sorted through distinct secretory pathways in AtT-20 cells (20, 21), but whether renin-1^d and renin-2 are secreted separately via distinct pathways or coordinately through both pathways *in vivo* has yet to be determined. In the current study, the most striking consequences of ablating expression of the renin-1^d protein are a change in renin immunostaining from a punctate, abundant granular pattern in JG cells of wild-type mice, to diffuse, weak cytoplasmic staining (Fig. 4C, D) and a complete lack of dense granule formation in *Ren-1^d*-null mice (Fig. 4E, F). Thus signals required for sorting renin to the regulated secretory pathway of mouse JG cells *in vivo*, reside exclusively in the renin-1^d protein and not in renin-2. This finding suggests that the trafficking and maturation of renin-2 protein within secretory granules in transfected AtT-20 cells (21,22) may not mirror the situation in the intact mouse, especially given that this cell line displays a different range of prorenin processing activities compared to the JG cells of the kidney [see (23)].

The reduced plasma concentrations of active renin and elevated prorenin seen in *Ren-1$^{d-/-}$* mice (Fig. 3) are the converse of the situation in *Ren-2$^{-/-}$* mice (10), The *Ren-1$^{d-/-}$* phenotype might be explained by a compensatory stimulation of the constitutive secretory pathway, in the absence of regulated secretion of renin-1^d, leading to enhanced secretion of renin-2 in the inactive form. This supports the idea that renin-1^d secretion is predominantly via the regulated (granular) pathway, and that renin-2 secretion is predominantly through the constitutive pathway. Higher rates of prorenin-2 secretion may be signalled by the deficit of active renin in the plasma, by the lack of JG secretory/storage granules, or directly by the absence of renin-1^d protein. The exact means by which one or other of these mechanisms stimulates *Ren-2* gene expression (2.8-3.9-fold) is not yet clear. However, this resembles a case of human familial elevated plasma prorenin (23), where a mutation in exon 10 of one allele of the human renin gene introduces a premature termination codon. The elevated levels of plasma prorenin in this phenotype are postulated to result from a compensatory mechanism which enhances expression from the normal renin allele (23). The data in Fig. 3 clearly show that homozygous *Ren-1^d*-null mice display reduced, but nonetheless detectable, levels of active renin in the plasma. This active renin must derive exclusively from the product of the *Ren-2* gene, although the means by which prorenin-2 is converted to activate renin-2 is presently not clear. The complete absence of storage/secretory granules in *Ren-1$^{d-/-}$* JG cells, the normal site of renin maturation activity, raises the possibility that prorenin-2 is activated in an extrarenal site in these mice.

The hypotension observed in female *Ren-1$^{d-/-}$* mice demonstrates that the renin-2 protein cannot accomplish all the functions of renin-1^d in maintaining basal blood pressure. The fact that reduced blood pressure is seen only in female mice might well be a consequence of the sexually dimorphic expression of the *Ren-2* gene (8). For example, male mice express much higher levels of renin-2 in the SMG than females, and this may compensate for a reduction in active renin concentration (Fig. 3) and the hypotension otherwise conferred by the *Ren-1^d* mutation. The altered macula densa cell morphology (Fig. 4) might reflect perturbations in the renin-angiotensin system in *Ren-1$^{d-/-}$* mice, leading in turn to changes in chloride and fluid balance and altered signalling via the tubuloglomerular feedback loop. Studies of ion and fluid balance in *Ren-1$^{d-/-}$* mice are presently underway to address these questions.

Recent gene targeting experiments have shown that an intact renin-angiotensin system is fundamental to maintaining basal blood pressure, since mice lacking genes for angiotensinogen [*Agt* (26,27)], angiotensin converting enzyme [*ACE* (28)], and angiotensin type 1A receptor [*AGTR1A* (29,30)] all share a reduction in blood pressure as common phenotypic feature. Ablation of angiotensinogen, or of angiotensin converting enzyme, also results in renal vascular damage and defects in kidney morphology (26-28,31). It is notable that while *Ren-1*$^{d-/-}$ mice also have altered renal morphology, these changes are much less severe, and more specific, than those seen in *Agt* and *ACE* knockout mice.

A critical feature of the present study is that all mouse stocks were maintained on the 129/Ola inbred genetic background onto which the original *Ren-1*$^{d-}$ gene mutation was introduced. This eliminates the risk of introducing modifier loci, inherent in cross-breeding to other genetic strains, which may mask any phenotypic change caused solely by the introduced mutation (32). Strategies to account for modifier gene effects in gene targeting experiments exist (33), but these involve large breeding populations to ensure random segregation of loci, coupled with genotype assessment. Importantly, the maintenance of a pure genetic background also permits direct comparison with different knock-out animals on the same 129/Ola background, for example, *Ren-2*$^{-/-}$ (10) and *Ren-1*^{d}/*Ren-2*$^{-/-}$ animals[5].

In conclusion, these studies demonstrate that the mouse *Ren-1*^{d} and *Ren-2* gene products fulfill distinct roles in renin secretory granule formation and blood pressure homeostasis and that the renin gene duplication in some strains of mice is not functionally redundant. The availability of both *Ren-1*$^{d-/-}$ (this study) and *Ren-2*$^{d-/-}$ (10) mice presents additional opportunities to dissect renin gene function and the mechanisms underlying the trafficking, storage, maturation and release of renin *in vivo*. Furthermore, *Ren-1*$^{d-/-}$ mice are likely to be especially useful in addressing the contribution of macula densa signalling in the tubuloglomerular feedback loop, especially in response to perturbations in the renin-angiotensin system.

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[1] The abbreviations used are as follows: PCR, polymerase chain reaction; Ang, angiotensin; JG, juxtaglomerular; SMG, submandibular gland; GMEM, Glasgow modification of Eagle's medium; DIA/LIF, differentiation inhibiting activity/leukaemia inhibitory factor; PRC, plasma renin concentration; PPC, plasma prorenin concentration.

[2] L. J. Mullins and J. J. Mullins, personal communication.

[3] M. G. F. Sharp and A. F. Clark, unpublished work.

[4] Expand kit (Boehringer Mannheim, Lewes, UK); data not shown.

[5] M. G. F. Sharp, A. F. Clark, D. F. Fettes and J. J. Mullins, unpublished work.

Fig. 1. **Gene targeting.** *A*, The mouse 129/Ola renin locus: The arrows represent the direction of transcription. Only relevant restriction sites are shown: *Hind* III (H), *Pvu* II (P), *Kpn* I (K), *Xba* I (X). *B*, PCR Amplification of homology arms: Enlarged view of the *Ren-1^{d}* gene, showing 5' and 3' gene homology arms generated by long-range PCR amplification. Primer pairs used (JJM 203/135 and JJM 224/204) are indicated by open triangles. The numbered black boxes represent exons. *C*, Targeting construct pR1*neo*KO: The construct deletes 92 bp of exon 3, the third intron and 35 bp of exon 4 and replaces them with a phosphoglycerokinase-1 neomycin phosphotransferase cassette (neo, not drawn to scale), flanked at the 3' end by an artificial *Pvu* II restriction site, specifically introduced during cloning to facilitate identification of a correctly targeted *Ren-1^{d}* allele. *D*, Targeted gene: The disrupted *Ren-1^{d}* gene after homologous recombination between the targeting construct and the endogenous gene. *E*, Confirmation of targeting: DNA (Southern) blots of tail DNA samples from offspring of a *Ren-1$^{d+/-}$* heterozygote intercross digested with *Pvu* II and hybridised with the 3' probe (shown in *A* and *D*) giving either a 10.6 kb fragment expected from the endogenous *Ren-1^{d}* gene or an 8.6 kb fragment diagnostic of the *Ren-1^{d}* targeted allele, confirmed correct targeting of the 3'arm. The 7.9 kb fragment, common to all, originates from the endogenous cross-hybridising *Ren-2* gene. Southern analysis, following *Sac* I digestion, also confirmed correct targeting of the 5' arm (not shown).

Fig. 2. **Renin mRNA analysis in targeted mice.** RT-PCR of total mouse kidney RNA, using primers that recognise both *Ren-1^{d}* and *Ren-2* cDNAs results in a 999bp product ('–' lanes). Following digestion with the restriction enzyme *Ear* I, *Ren-1^{d}* and *Ren-2* cDNAs can be distinguished by the presence of restriction fragments of 484bp, 347bp and 168bp specific to *Ren-1^{d}*, or 652bp and 347bp specific to *Ren-2*. Analysis of total kidney RNA from *Ren-1$^{d-/-}$* homozygotes demonstrates the presence of *Ren-2* mRNA, while products derived from *Ren-1^{d}* mRNA expression are completely absent. Parallel analysis of *Ren-1$^{d+/-}$* heterozygotes reveals the presence of both *Ren-1^{d}* and *Ren-2* gene products, while only *Ren-1^{d}* mRNA is detectable in *Ren-2$^{-/-}$* homozygous knockout mice (10).

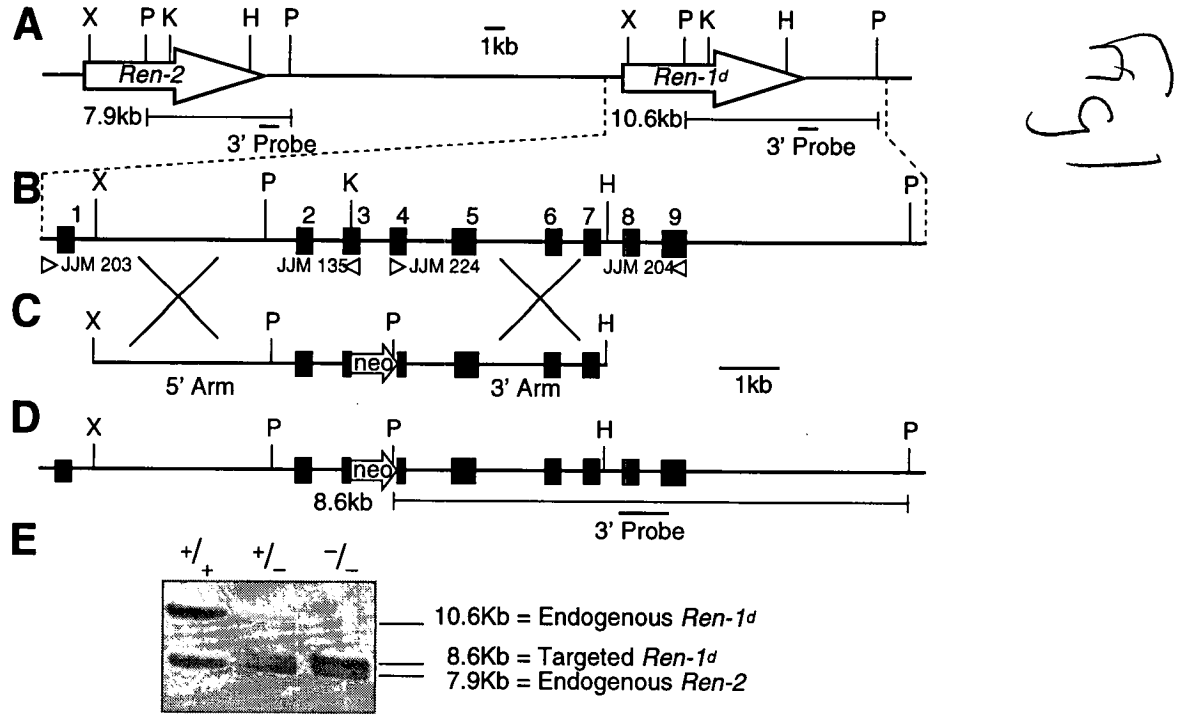
Fig. 3. **Circulating renin levels.** *A*, Plasma active renin concentration (PRC; solid bars) and plasma prorenin concentration (PPC; open bars) in male *Ren-1^{d}*-targeted mice. PPC in *Ren-1$^{d-/-}$* male mice (n=7) is significantly higher than *Ren-1$^{d+/-}$* (n=14; *, $P<0.027$) and wild-type (n=17; **, $P<0.0003$) mice. *B*, Female *Ren-1$^{d-/-}$* mice have significantly lower PRC than wild-type mice (*, $P<0.027$). Prorenin concentrations in the female *Ren-1$^{d-/-}$* mice (n=8) are significantly increased compared to wild-type (n=10; **, $P<0.0003$) and *Ren-1$^{d+/-}$* animals (n=14; *, $P<0.027$). Data represent mean±SEM.

Fig. 4. **Altered renal morphology.** *A-B*, in light microscopy of wild-type mice (*A*), differences were noted in the morphology of the cells of the macula densa compared with (*B*), *Ren-1$^{d-/-}$* mice, in which the epithelial cells of the macula densa (arrow) have a columnar morphology, the nucleus:cytoplasm ratio is increased and there is cell crowding. By contrast in the wild-type mice the same cells (arrow) have a cuboidal morphology with a lower nucleus:cytoplasm ratio and lower cell density. Sections were stained with haematoxylin and eosin; G=glomerulus. *C-D*, control mouse kidneys (*C*) show strong immunostaining for renin in the medial layer of the afferent arteriole (arrow). In contrast, sections from a *Ren-1$^{d-/-}$* mouse kidney (*D*) show only faint cytoplasmic immunoperoxidase staining for renin in a few cells near the vascular pole (arrow). *E-F*, electron microscopy of afferent arteriolar smooth muscle cells (SM) from a control mouse (*E*) show prominant dense cytoplasmic granules (arrows); A=afferent arteriole lumen, E=endothelial cell. The cells in the wall of this afferent arteriole from a *Ren-1$^{d-/-}$* mouse kidney (*F*), defined by tracing its origin from the interlobular artery in semi-thin sections, show no evidence of cytoplasmic granules. A=afferent arteriole lumen; E=endothelial cells; SM=modified smooth muscle cells (× 3,800).

TABLE 1: *Resting blood pressure in* Ren-1^d *gene knockout mice.*

Blood pressure was determined in conscious, restrained wild-type (+/+), *Ren-1^d* heterozygous (+/-) and *Ren-1^d* homozygous (-/-) mice by direct cannulation of the aorta as described (10). Values are mean±SEM (n). * $P<0.01$ by Student's *t* test.

	Mean arterial blood pressure (mmHg)		
	+/+	+/-	-/-
Males	93.6±5.2 (8)	93.0±4.9 (11)	92.3±3.8 (8)
Females	93.6±2.5 (8)	85.6±2.8 (7)	*80.9±3.4 (8)


A
X
P K
H
P
1kb
Ren-2
Ren-1d
7.9kb
10.6kb
3' Probe
3' Probe
B
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H
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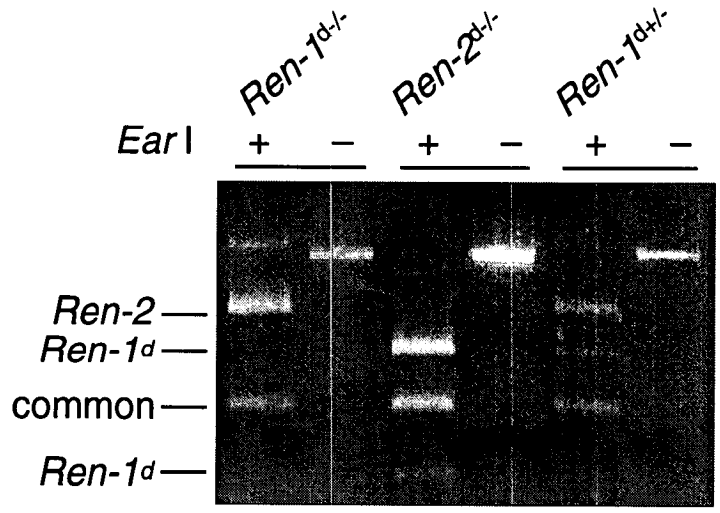
1
2
3
4
5
6
7
8
9
JJM 203
JJM 135
JJM 224
JJM 204
C
X
P
P
H
neo
5' Arm
3' Arm
1kb
D
X
P
P
H
P
neo
8.6kb
3' Probe
E
+/+
+/-
-/-
10.6Kb = Endogenous Ren-1d
8.6Kb = Targeted Ren-1d
7.9Kb = Endogenous Ren-2

Fig 1


Ren-1d-/-
Ren-2d-/-
Ren-1d+/-
Ear I
+
−
+
−
+
−
Ren-2
Ren-1d
common
Ren-1d

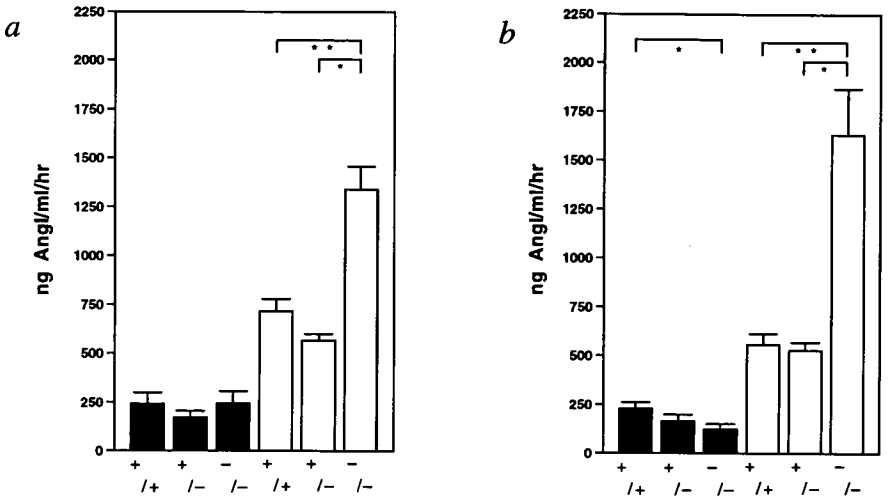


Fig 3

Fig 4