Investigation of the function of the SA gene by gene targeting

Vanessa Walsh B.Sc.

Department of Medicine, Division of Cardiology,
Glenfield General Hospital, Groby Road, Leicester.

Submitted for the degree of Ph.D. to the Faculty of Medicine, University of Leicester.
June 2000

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

Acknowledgements

I would like to thank my supervisor Professor Nilesh Samani for his guidance and encouragement throughout the duration of this project. His patience, constructive criticism and advice during the writing of this thesis have been invaluable.

Many thanks also to Catrin Pritchard of the Biochemistry Department whose guidance and expertise in embryonic stem cell technology were freely available throughout the duration of this work.

And to Brenda Leckie of the Department of Vascular Medicine for instruction in the technique of indirect tail-cuff measurement of mice and for allowing us the use of your blood pressure monitoring equipment.

Thanks to Kevin West of the Department of Pathology for preparing tissues for light and electron microscopy and to David Duckett of the Genetics Institute, LRI, for the karyotypic observation of our embryonic stem cell cultures.

Thanks to all the staff of the University of Leicester Biomedical Services Units, especially to Jane Brown, Annette, Ferrond and Bill in the Transgenic Unit who have been so helpful over the years.

Thanks to all the members of the Divisions of Cardiology and Medicine and to staff and students of the clinical Sciences Wing at Glenfield Hospital for much useful instruction, advice, discussion, and friendship over the years.

Finally a massive thanks to my husband Tom for being so patient in waiting for me to finish my studies, and for always being so enthusiastic, encouraging and supportive.

Abstract

The SA gene encodes a 578 amino acid protein of unknown function. Its main sites of expression are the kidney and liver with lower levels of expression in the brain and testes. SA was first identified as a candidate gene for hypertension and blood pressure regulation due to its increased expression in the kidneys of genetically hypertensive compared with normotensive rats. In F2 crosses between these strains the SA allele from the hypertensive strain co-segregated with increased blood pressure.

The aim of the work undertaken in this thesis was to investigate the function of the SA gene by gene targeting in the mouse and to assess any possible involvement of the SA protein in BP homeostasis. We utilised ES cell technology to generate a mouse model carrying a null mutation of the SA gene. Mice lacking the protein product of the SA gene are viable, reproductively normal and have no overt phenotype. Body weight and kidney, liver and heart weights are not affected by the absence of the SA protein.

Comparison of basal blood pressures (BP) revealed no differences between SA-null and wildtype littermate controls in either male or female mice. Exposure of male mice to a high salt diet caused an increase in BP in wildtype mice. However in SA-null mice no effect of salt intake on BP was observed. It therefore appears that absence of the SA protein may offer some protection against a sodium induced rise in BP. The mechanism for this remains to be elucidated but may include an involvement of the SA protein in sodium retention.

Administration of dihydrotestosterone (DHT) to female wildtype mice caused no increase in BP. However in SA-null mice BP was increased in response to DHT administration implying a protective effect of SA against a DHT induced rise in BP.

These finding provide for the first time direct evidence of the involvement of the SA protein in BP regulations under certain conditions.

Abreviations

ACE	Angiotensin converting enzyme
AMPS	Ammonium persulphate
AR	Androgen receptor
ARE	Androgen response element
AS	Albino Surgery (rat strain)
bp	Base Pair
BP	Blood pressure
BSA	Bovine serum albumin
BRL medium	Buffalo rat liver medium
cDNA	Complementary DNA
C	Degrees centigrade
CgA	Chromogranin A
cm	Centimetre
CO_2	Carbon dioxide
DA cells	Dopamine neurons
dATP	2'-Deoxyadenosine-5'-Triphosphate
dCTP	2'-Deoxycytidine-5'-Triphosphate
dd	Deionised
DEPC	Diethyl pyrocarbonate
DHT	Dihydrotestosterone
DIA	Differentiation inhibitory activity
DMEM	Dulbecco's Modification of Eagles medium
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxynucleotide-5'-Triphosphate
DTT	Di-thiothrietol
E. coli	Escherichia coli
ECL	Enhanced Chemiluminesence
EDTA	Ethylene diamine tetra acetic acid
ER	Oestrogen receptor
ES cells	Embryonic stem cells

EST	Expressed sequence tag
FCS	Foetal calf serum
h	Hour
HPRT	Hypoxanthine phosphoribosyl transferase
HRP	Horseradish peroxidase
I/D	Insertion/Deletion (alleles of the ACE gene)
IPTG	Isopropyl-1-thio-b-D-galactopyranoside
kb	Kilobases
Kda	Kilo Daltons
L	Litre
LB	Luria Bertani
$LiCl_2$	Lithium chloride
LINE	Linear insertion element
LIF	Leucocyte inhibitory factor
LMP	Low melting point
LOD	Logarithm of the odds
M	Molar
mA	Milliamps
MBq	Mega Bequerel
MEF cells	Mouse embryonic fibroblast cells
mg	Milligrams
µg	Micrograms
$MgSO_4$	Magnesium sulphate
min	Minute
ml	Millilitre
µl	Microlitre
mm	Millimetre
µm	Micrometre
mM	Millimolar
mmHg	Millimetres of mercury
NaCl	Sodium chloride
ng	Nanogram

nM	Nanomolar
nm	Nanometre
NZGH	Rat Strain
NZY medium	NZ-amine, yeast extract medium
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFU	Plaque forming units
pmol	Picomoles
PRA	Plasma renin activity
psi	Pounds per square inch
QTL	Quantitative trait locus
RAS	Renin angiotensin system
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcription PCR
SBH	Sabra Hypertensive Rat strain
SBN	Sabra Normotensive Rat strain
SDS	Sodium Dodecyl sulphate
Sec	Second
SEM	Standard error of the means
SHR	Spontaneously hypertensive rat
SHR-SP	Spontaneously hypertensive rat-Stroke prone
SM	Salt magnesium buffer (lambda diluent)
SNS	Sympathetic nervous system
SSC	Sodium, salt, citrate buffer
STO fibroblasts	Stretched-out fibroblasts

TAE	Tris, Acetate, EDTA buffer
TBE	Tris, Borate, EDTA buffer
TEMED	N, N, N', N'-tetramethylethylenediamine
TNE	Tris, Sodium, EDTA buffer
TBS	Tris buffered saline
TBS-T	Tris buffered saline-Tween
TE	Tris EDTA solution
temp	Temperature
6-TG	6-thioguanine
UV	Ultra violet
V	Volts
Vols	Volumes
v/v	Volume to volume ratio
WKY	Wistar Kyoto (rat strain)
w/v	Weight to volume ratio

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Chapter 1

INTRODUCTION

HYPERTENSION

What is blood pressure?

Blood pressure (BP) is a complex quantitative physiologic trait. In an individual, BP at any moment is the product of cardiac output x peripheral resistance (CO x PR) and is usually represented as systolic and diastolic readings. Systolic blood pressure is peak blood pressure as blood is ejected from the heart during contraction (systole). Diastolic blood pressure is the lowest blood pressure during each cardiac cycle, just before systole begins.

In the population BP follows a quasi-normal distribution. It rises progressively with age although this varies depending on gender, race, environmental factors and initial blood pressure (Whelton *et al.*, 1994). Generally BP increases with increasing age and is higher in men than women. Blood pressure levels are remarkably similar in developing and westernised countries with the exception of a few tribes leading primitive hunter-gatherer type life-styles. Such tribes (including Kalahari bushmen, Kenyan nomads and Congo pygmies) often have very low blood pressures, which do not show a characteristic increase with age (Poulter and Sever, 1994).

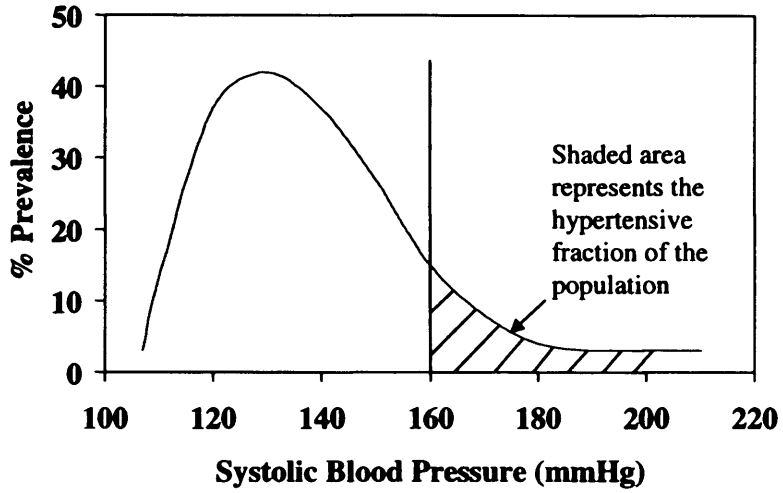
What is hypertension?

Hypertension represents one extreme of the normal distribution of blood pressure (Fig. 1.1). The cut-off point above which a person is considered to be hypertensive is dependant upon an arbitrary division of this normal distribution. The World Health Organisation (Chalmers *et al.*, 1999) provide the following guidelines for the definition of hypertension: systolic >160mmHg and/or a diastolic >99mmHg. Borderline hypertension is defined as systolic <160mmHg and diastolic 90-94mmHg or systolic 141-159mmHg and diastolic <90mmHg. Normotension is defined as systolic <140mmHg and diastolic <90mmHg.

Hypertension is a common disorder, effecting approximately 15% of the population in the U.S. (Hypertension Detection and Follow-Up Group, 1977).

FIG.1.1 Quasi-normal distribution of blood pressure within the population

Graph showing the distribution of blood presure in the population and the arbitrary division above which a person is considered hypertensive (adapted from Bannan, 1987).



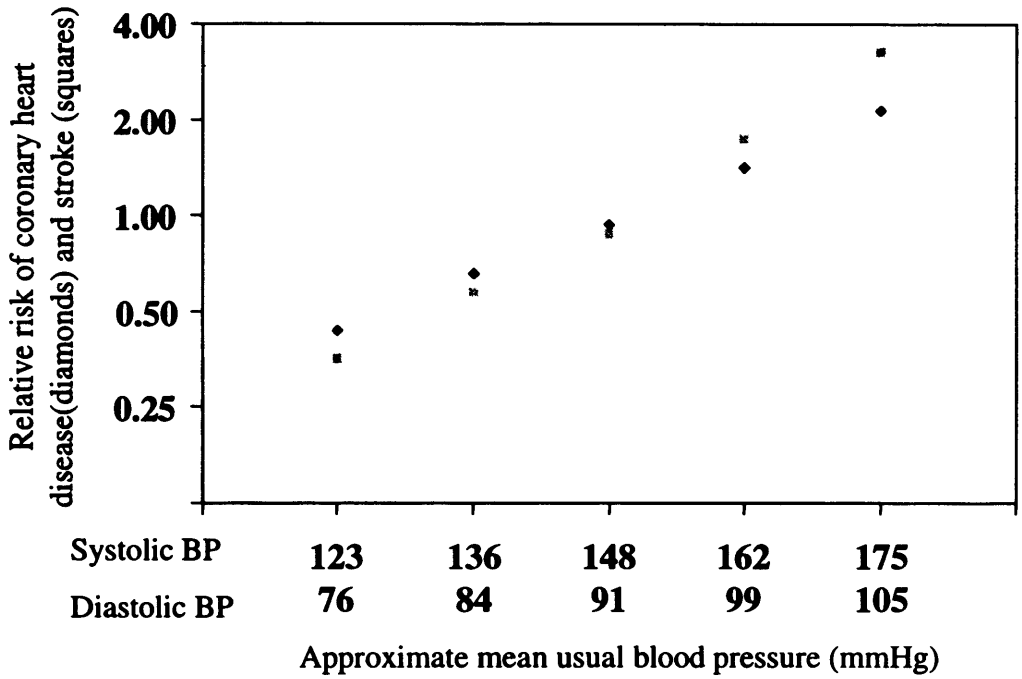
Significance of hypertension

Hypertension has been shown as a significant risk factor for stroke, coronary heart disease, heart failure, peripheral vascular disease and renal failure. Of these the most extensively studied have been stroke and heart disease. A stroke occurs when blood flow to the brain is disturbed due to a blockage in blood vessels leading to the brain. As a result the brain is starved of oxygen resulting in brain damage. Coronary heart disease is caused by blockage of coronary arteries and can result in deprivation of oxygen to heart tissues. This can result in a heart attack and subsequent heart tissue death leading to reduced cardiac function.

MacMahon *et al.* (1990) collated data from 9 previous studies in which initial blood pressure measurements were taken and patients subsequently monitored for a period of up to 10 years for coronary events or stroke. It was noted that the incidence of both stroke and coronary heart disease were related to blood pressure in a log-linear manner. Interestingly this relationship was maintained throughout the usual range of blood pressures showing that increased blood pressure increases risk of these conditions in normotensive as well as hypertensive individuals (Fig. 1.2). These results were found to be independent of age, blood cholesterol and smoking.

FIG. 1.2 Relationship between blood pressure and coronary events and stroke

Graph showing the log-linear increasing risk of a coronary event with increased blood pressure (adapted from MacMahon *et al.*, 1990)



In westernised countries coronary heart disease is a major cause of mortality, morbidity and loss of income. Loss of coronary function due to coronary heart disease can result in fatigue and shortness-of-breath which in turn can result in major changes in quality of life including the inability to continue in employment. Chest pains may occur on exertion (angina). In severe cases heart attack and death may occur. If an individual survives a heart attack, quality of life is often severely impaired as the damaged heart may no-longer be able to supply the required circulation throughout the body.

Studies into the causes of hypertension, which is a major risk factor for this condition, are therefore very important.

Causes of hypertension

In the majority of individuals a single cause of hypertension cannot be identified. This is referred to as *essential hypertension*. However in a small proportion of subjects (<5%) a cause of hypertension is apparent. This is referred to as secondary hypertension.

Secondary hypertension

Secondary hypertension is elevated blood pressure for which a cause can be identified. It is most common in young hypertensives and when hypertension is severe. Many types of secondary hypertension are now well-defined. A list of causes of secondary hypertension can be seen in Table 1.1. Treatment for these types of disorder is often surgical.

Table 1.1 Causes of secondary hypertension

Renal and renovasuclar disease
Renin secreting tumours
Primary aldosteronism
Cushing's Syndrome
Phaeochromocytoma
Thyroid disease and acromegaly
Coarctation of the aorta
Central nervous system disease
Drugs or oral contraceptives
Thyroid disease and acromegaly

More recently several monogenic forms of hypertension have been defined and added to the list of causes of secondary hypertension. A list of monogenic forms of hypertension can be seen in Table 1.2 and include Liddle's Syndrome, Apparent mineralocorticoid excess, and glucocorticiod-remediable hyperaldosteronism. These forms of hypertension are caused by a mutation in a single gene and therefore follow a simple Mendelian pattern of inheritance. Liddle's syndrome is an autosomal dominant form of hypertension caused by a mutation in either the β or γ subunit of the epithelial

sodium channel. A truncation in either of these proteins causes the constitutive function of the sodium channel, leading to an increased reabsorption of Na^+ ions and

Table 1.2 Mendelian forms of hypertension

Syndrome	Mode of inheritance	Gene responsible	Chromosomal location	Type of defect
Apparent mineralocorticoid excess	Autosomal recessive	HSD11B2 11 β -hydroxysteroid dehydrogenase	16q22	Steroid metabolism
Glucocorticoid-remediable hyperaldosteronism	Autosomal dominant	CYP11B1/CYP11B2 cytochrome 450; 11 β 1 hydroxylase/ 11 β 2 hydroxylase (aldosterone synthase)	8q22	Steroid metabolism
Gordon's syndrome	Autosomal dominant	? ?	1q31-q42 17p11-q21	Ion transport
Hypertension and brachydactyly	Autosomal dominant	?	12p	
Liddle's syndrome	Autosomal dominant	SCNN1B SCNN1G Amiloride sensitive sodium channel, β and γ subunits	16p12-p13	Ion transport

consequently an increase in blood pressure. Apparent mineralocorticoid excess is an autosomal recessive form of hypertension caused by the inactivation of the 11 β-hydroxysteroid dehydrogenase gene. In this condition active cortisol fails to be converted to inactive cortisone in the kidney. High levels of cortisol therefore activate the mineralocorticoid receptor. This has consequent effects on Na^+ ion and water reabsorption causing severe hypertension. Glucocorticiod-remediable hyperaldosteronism is caused by an unequal cross-over between 2 closely linked genes. This results in the control of the aldosterone synthase gene by the regulatory elements of the 11β-1 hydroxylase gene. As the promoter of the later is responsive to glucocorticoids, this form of hypertension can be controlled by the administration of dexamethasone.

Essential Hypertension

Essential hypertension is a complex disorder due to a combination of environmental and genetic factors (Ward, 1990). Several methods have been used for the assessment of relative contributions of genetic and environmental factors in the determination of blood pressure. Assessment of the distribution of blood pressure within a family, followed by the use of complex statistical methods to separate genetic and culturally determined factors has formed the basis of many studies (Krieger *et al.*, 1980; Moll *et al.*, 1983; Longini *et al.*, 1984). Twin and adoptive studies have also been used to separate genetic and environmental factors. Biron *et al.* (1976) compared blood pressures between parents, parents and children (adoptive and natural), natural siblings and adoptive siblings. From this study it was estimated that genetic factors account for 34% of variability within the population. Influence of a common household accounts for 11% of BP variability and random non-familial factors account for the remaining 55% of population variability. These figures correlate well with estimates made by statistical methods mentioned above. Twin studies have been used extensively in the study of BP and other heritable characteristics (Havlik *et al.*, 1979; Feinleib *et al.*, 1977). A higher degree of correlation between blood pressures is observable in monozygotic twins than in dizygotic twins. Use of this method tends to result in a higher estimate of the genetic component of BP compared to the other methods mentioned above. This is most likely due to greater extent of shared environment in early childhood. For this reason Ward (1990) cautions that twin studies are useful pointers towards characteristics with genetic heritability, however care should be taken in interpretation of data obtained in this manner.

Environmental factors

Environmental factors predisposing to essential hypertension include obesity, diet (including salt intake and alcohol consumption), a sedentary lifestyle and stress (Beilin, 1988). Obese people tend to have higher blood pressure than non-obese people. Reasons for this are unclear but may involve high calorie intake, high salt intake and the increasing incidence of insulin resistance with obesity (Stamler *et al.*, 1997). Effects of salt intake on BP were studied extensively by the INTERSALT Cooperative

research group (1988). Results showed that the increase in BP present in urban but not rural populations was due to the amount of salt in the diet. Migration studies have shown that blood pressures of Japanese people now living in America tend to be lower than BPs of Japanese people in Japan. Conversely BP of rural Africans migrating to urban areas tend to suffer an increase in BP. In both cases these changes are likely to be the result of alterations in diet, particularly salt intake which is known to be high in Japan and low in rural Africa. The INTERSALT study (1988) also showed an increase in BP with alcohol intake. Other studies have shown this to be a reversible effect with blood pressure levels reducing with a reduction in alcohol consumption.

Environmental factors do not always cause hypertension; not everybody who consumes a high salt diet suffers increased blood pressure. However in combination with certain genetic backgrounds environmental factors can significantly increase the risk of hypertension occurring.

Genetic factors influencing essential hypertension

Over the last 10 years a large effort has been made towards identifying genes involved in the pathogenesis of hypertension. Two main approaches have been utilised to this effect, association studies and linkage studies.

In an association study the distribution of a polymorphism is compared between cases and well-matched controls. If the frequency of a polymorphism (or the consequent genotype) is found to differ between cases and controls then it would suggest that either the polymorphism itself, or another variant close by with which it is in linkage disequilibrium, is causally related to hypertension.

Linkage studies in humans involve the study of families and sharing of alleles between affected members. The use of classical linkage studies is difficult in the study of hypertension as it is a late onset disorder and multi-generational pedigrees are generally not available. However linkage analysis of affected sib-pairs has been a frequently used technique. These studies allow loci increasing susceptibility to

hypertension to be identified by demonstrating excess sharing of alleles between affected hypertensive siblings than would be expected by chance.

Using these approaches many genes and loci have been identified as being associated and/or linked with essential hypertension. Some of these are listed in Table 1.3.

Table 1.3 Genes implicated in the pathogenesis of essential hypertension

α -adducin	low density lipoprotein receptor
α, β and γ sodium channels	leptin
angiotensin converting enzyme	lipoprotein lipase
angiotensin II receptor type 1a	methylenetetrahydrofolate reductase
angiotensinogen	natriuretic peptide receptor (type B)
apolipoprotein B	Na^+/K^+ transporter (α-1, α -3 β -2)
aldosterone synthase	Na^+/H^+ exchanger
bradykinin B2 receptor	$Na^+/K^+/Cl^{2-}$ cotransporter
cytochromes P450	Na^+/K^+ ATPase
endothelin-1	Na^+/Li^+ transporter
endothelin-2	endothelial nitric oxide synthase
G protein α subunit	constitutive nitric oxide synthase
G protein β subunit	neuronal nitric oxide synthase
growth hormone receptor	platelet-activating factor
glugagon receptor	proANP (atrial natriuretic peptide)
glucocorticoid receptor	prothrombin
11-β hydroxysteroid dehydrogenase type 2	plasminogen activator inhibitor
heat shock protein 27	renin
heat shock protein 70	SA
kallikrein	transforming growth factor β-1

However, association and linkage studies do have limitations. Association studies rely on case and control populations being well-matched as frequencies of non-functional mutations can vary between populations. Sometimes small differences in racial mixture between cases and controls may pass un-recognised leading to spurious results. A negative result in an association study shows that the specific polymorphism under observation is not responsible for hypertension and that it is not in linkage disequilibrium with a causative polymorphism. However a negative result for one polymorphism does not eliminate the possibility that other polymorphisms in the same gene may contribute to the observed phenotypic effect. Conversely a positive result for an association study only shows that a gene in the region of the candidate polymorphism is involved in BP determination. It does not mean that the

polymorphism is directly responsible for any effect on BP or is within the causative gene. Association studies are usually used for the investigation of dimorphic polymorphisms such as single nucleotide polymorphisms (SNPs) or restriction fragment length polymorphisms.

Linkage studies rely on the use of polymorphic microsatellite markers. The major advantage of this type of study is that a whole locus can be screened by the observation of a series of polymorphic markers close together. A linkage map for the quantitative trait locus (QTL) can then be generated. Similarly markers throughout the genome can be analysed in a genome-wide scan to identify chromosomal regions linked to blood pressure control. However the disadvantage of linkage studies is that large numbers of sib-pairs are required. If a sufficiently large sample is observed, this is a powerful approach capable of excluding a locus as harbouring a causative gene for hypertension. However false negatives or positives may still be obtained if too small a sample size is used.

Conflicting data is frequently generated by these approaches highlighting the above limitations and the difficulty of studying a complex genetic trait in an outbred human population. The angiotensinogen gene provides an example of a locus for which conflicting results have been obtained by linkage and association studies. Angiotensinogen (AGT) is the substrate of the Renin-angiotensin-system (RAS) and circulating levels of AGT have been found to be associated with hypertension in families (Watt *et al.*, 1992). Several polymorphisms have been identified within the *AGT* gene and analysed using association studies. The Methionine to Threonine polymorphism at amino acid 253 (M235T) in particular has been extensively studied. At least 6 different reports have shown the 235T variant to be positively associated with hypertension (including Jeunemaitre *et al.*, 1992 and Schmidt *et al.*, 1995). However a similar number of reports also exist for negative association studies (including Caulfield *et al.*, 1994 and Bennett *et al.*, 1993). Kunz *et al.* (1997) carried out a meta-analysis for the M235T polymorphism amalgamating the above data. A weak association was observed between this mutation and hypertension. It was pointed out that a meta-analysis is only as strong as the studies it utilises. Several

weaknesses and inconsistencies were reported in the M235T association studies contributing to this meta-analysis. Inconsistencies were observed in the definition of hypertension, choice of study and control groups (particularly failure to note family history in some studies) and other factors affecting choice of control groups (such as body mass index, age, sex, smoking, lipid status and salt consumption). All these factors will have affected the outcomes of the original studies, and thus also the outcome of the meta-analysis.

Two early linkage studies showed the *AGT* locus to be linked to hypertension (Jeunemaitre *et al.*, 1992; Caulfield *et al.*, 1994). However more recently negative results have been obtained in linkage studies of this locus. A large study amalgamating data from various Caucasian populations failed to show linkage between the *AGT* locus and hypertension (Brand *et al.*, 1998). Similarly Wang *et al.* (1999) failed to observe linkage between the *AGT* locus and hypertension in a Caucasian population. A negative result was also recently obtained for a linkage study in a Chinese population (Niu *et al.*, 1998).

The case of the *AGT* gene highlights the difficulties of studying complex multigenic traits in outbred genetically heterogeneous human populations. For this reason inbred genetic animal models of hypertension provide an attractive and useful alternative to, at least initially, study the genetics of this disorder.

Use of animal models for the study of hypertension

Many genetic models of hypertension have been generated, mainly in the rat (Kurtz *et al.*, 1994) and one in the mouse (Schlager, 1968). Each model has some similarities to sub-groups of essential hypertensives.

The spontaneously hypertensive rat (SHR) and the normotensive control (the Wistar Kyoto or WKY rat) have been widely used in studies of genetic hypertension. The SHR strain was selectively bred from the Wistar rat without dietary or environmental stimuli to further induce high blood pressure (Okamoto and Aoki, 1963). The WKY strain is the control strain derived from the Kyoto Wistar colony. During breeding of

the SHR strain, a sub-strain was found to be susceptible to an increased incidence of stroke. The SHRSP (SHR stroke prone) was maintained as a separate line. In addition, sub-strains of WKY and SHR also exist as these strains were initially exported from Japan before they were fully inbred.

Other rat strains frequently used in the study of the genetics of hypertension include the Milan hypertensive (MHS) and the Milan normotensive (MNS) (Bianchi *et al.*, 1975) and the Lyon hypertensive (LH) and Lyon normotensive (LN). These hypertensive strains were also selected for high blood pressure in the absence of additional stimuli to induce increased blood pressure. The Milan strains were derived from Wistar rats and the Lyon strains were derived from Sprague-Dawley rats.

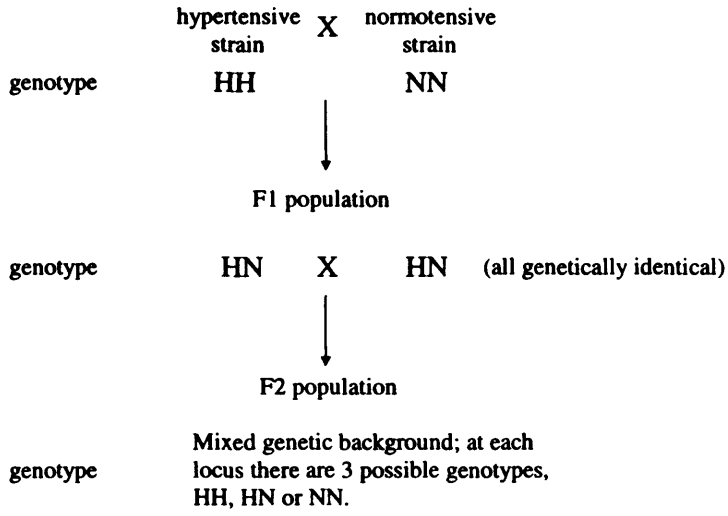
Dahl salt-sensitive (DS) and Dahl salt-resistant (DR) strains were selected for high and normal blood pressure after the administration of a high salt diet (Rapp and Dene, 1985).

In each of these models a fixed number of genes is consistently responsible for blood pressure phenotype. Specific experiments can therefore be devised to identify these genes and to assess the effect of genotype on phenotype.

Cosegregation analysis in animal models

To identify genetic loci predisposing to hypertension in these strains, experiments can be designed whereby animals of a hypertensive phenotype are crossed with those of a normotensive phenotype. Offspring are then inter-crossed to generate a large mixed F2 population from which DNA is extracted and blood pressure data generated (Fig. 1.3).

FIG. 1.3 Generation of an F2 population for cosegregation analysis

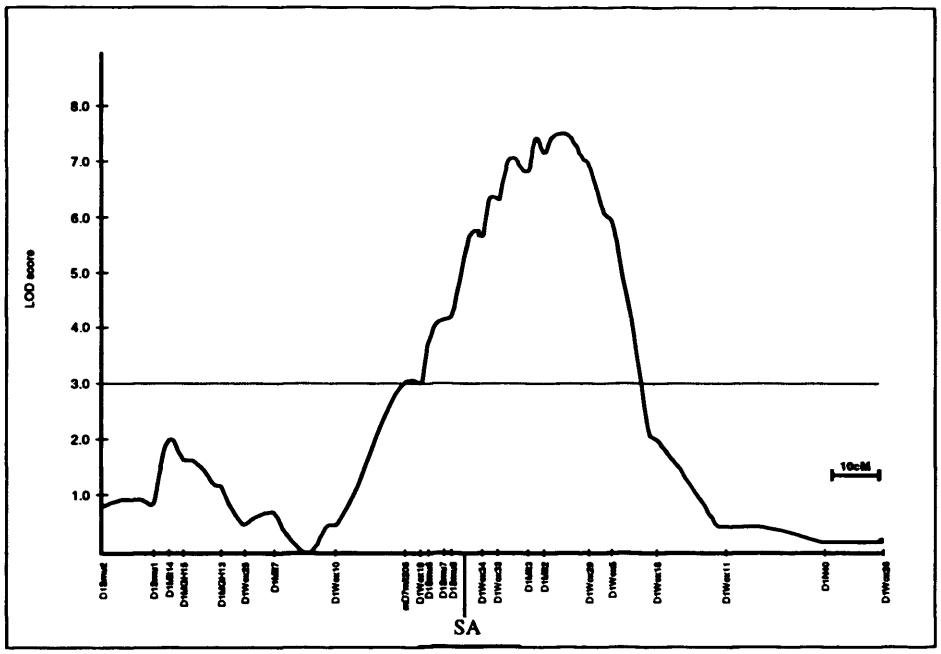


Genetic markers are identified which are polymorphic between the 2 progenitor strains. The genotype of these markers is determined in the F2 population. Statistical analysis is then performed to assess whether a marker co-segregates with increased blood pressure.

Co-segregation studies can be used for the investigation of individual polymorphisms (for example within a candidate gene). However a positive result in such a study only implicates the chromosomal region rather than the polymorphism under investigation. More recently it has become popular to carry out genome-wide scans using microsatellite markers which are polymorphic between the parental strains. For each marker a LOD score (logarithm of the ratio of the likelihood of there being a QTL present vs. the likelihood of no QTL being present at a particular position) can be calculated and a plot constructed for LOD scores of markers on the same chromosome. If a QTL is present then a significant peak is observed and the region of the peak defines the 95% confidence interval for the location of the QTL. An example of such a plot is presented in Fig 1.4 (adapted from Frantz *et al.*, 1998).

FIG. 1.4 Diagram showing LOD scores for chromosomal markers

Various micro-satellite markers are represented on the X-axis, LOD scores on the y-axis. The dotted line indicates the region of 95% confidence for the location of the BP QTL at this locus.



Using this type of approach, 22 individual QTL affecting blood pressure have been identified on 17 of the 20 autosomes in the rat, plus the X and Y chromosomes. These are reviewed by Rapp (2000a) and Dominiczak *et al.* (1998).

Once an area containing a blood pressure QTL has been identified, it can be further investigated by several means. An obvious focus would be any relevant candidate genes present within the QTL, for example the SA gene is present within the chromosome 1 QTL shown above. Mapping of additional microsatellite markers closer together gives a more detailed picture of the location of the QTL thus facilitating the identification of possible candidate genes within the region. The construction of congenic strains is another frequently used technique for isolating and narrowing a chromosomal region containing a blood pressure QTL (Rapp 2000b). This technique allows the exclusion of candidate genes and when the interval has been narrowed to 1-

2 centimorgans, physical mapping of genes harboured in the transferred region may be possible. Such studies are currently ongoing for several QTLs in the rat.

THE PRIMARY ROLE OF THE KIDNEY IN HYTPERTENSION

Identification of candidate genes has largely relied upon knowledge of the physiology of hypertension. Certain hormonal systems such as the renin-angiotensin system and the kallikrein-kinin system are known to be involved in blood pressure homeostasis. Therefore genes encoding substrates and enzymes within these cascades are obvious candidates for the pathogenesis of hypertension. Similarly genes known to be involved in sodium transport, such as the β subunit of the epithelial sodium channel and Na^+K^+ATPase, are also candidate genes.

Another method for the identification of candidate genes is to focus on gene expression in organs that may be involved in the determination of blood pressure. The secondary involvement of the kidney in hypertension is well recognised (as outlined above). However a primary role in the pathogenesis of hypertension has also been suspected for many years. Evidence for this comes from various sources, in particular transplantation studies.

Kidney transplantation studies in humans

Hypertension is common in renal transplant recipients and can be attributed to many factors including graft rejection, graft artery stenosis and treatment with immuno suppressant drugs. In addition genetic background of the donor kidney has been shown to affect blood pressure in the recipient after transplantation. In a frequently cited study Curtis *et al.* (1983) reported six cases of essential hypertension with nephrosclerosis leading to renal failure. In each case hypertension was eliminated by the transplantation of a kidney from a normotensive donor and remained normotensive at follow-up 4.5 years after transplantation. Elimination of hypertension by replacement of a failing kidney with a fully functioning kidney is not surprising. A follow-up time of 4.5 years may not be sufficient for the transplanted kidney to be affected by the background of the recipient. Further follow-up of these patients would prove interesting. Strandgaard

and Hansen (1986) compared transplant patients receiving kidneys from donors with subarachnoid haemorrhage and those from other donors (either having suffered head injury or cerebral tumours). They found BP to be significantly higher in the former than the latter group. The most comprehensive study to date was reported by Guidi *et al.* (1996). Eighty-five patients receiving kidney transplants were divided into 4 groups depending upon whether the donor did or did not have a hypertensive family background and whether the recipient did or did not have a hypertensive family background. BP was monitored over an 8 year follow-up period. Recipients with a normotensive family background receiving a kidney from donors with a hypertensive family background were found to have significantly higher BP than recipients in the 3 other groups. It was concluded that hypertension could be transferred with the kidney.

BP assessment of the kidney donor is an inherent difficultly in this type of study. The methods used in the later 2 studies above group donors on the likelihood rather than the certainty of being hypertensive. Even so, a correlation can still be observed between donor status and BP in the recipient, substantiating the view that the kidney has a primary involvement in BP control.

Kidney transplantation studies in rat models

In contrast to the necessarily observational nature of human studies, extensive studies have been undertaken in rat models to establish a primary involvement of the kidney in the pathogenesis of hypertension. Transplantation of a kidney from a hypertensive strain to a normotensive recipient has been shown to cause an increase in BP in several rat strains. Conversely transplantation of a kidney from a normotensive into a hypertensive strain results in a fall in BP. These results have been obtained using several different genetic models of hypertension in the rat: Bianchi *et al.*, 1974, using Milan Hypertensive and Milan normotensive rats; Kawabe *et al.*, 1979, using SHRSP, WKY and F1 hybrid rats; Morgan *et al.*, 1990, using DS and DR rats; Heller *et al.*, 1993, using the Prague hypertensive rat and Rettig *et al.*, 1990a, using SHR, WKY and F1 hybrid rats. Early studies of renal transplantation in rats transferred kidneys from hypertensive strains to normotensive strains using as donors mature rats already displaying the hypertensive phenotype (Bianchi *et al.*, 1974; Rettig *et al.*, 1989).

Problems arise in interpretation of data in these experiments as pre-existing hypertension may have altered the kidney prior to its use for transplantation. This likelihood is substantiated by experiments in which the transfer of kidneys from rats with surgically induced hypertension confer hypertension to the recipient indicating a secondary effect of transferring a kidney damaged by hypertension (Rettig *et al.*, 1990b). Later studies have attempted to limit this secondary effect by transferring kidneys from genetically hypertensive rats prior to their development of hypertension. When rats (SHR) were treated with ramipril to prevent the onset of hypertension prior to the removal of their kidneys for transplantation (Rettig *et al.*, 1990a) the hypertensive phenotype was still transferred with the kidney. Similarly when kidneys were explanted from young SHRSP rats prior to the development of hypertension, recipient rats showed an increase in BP (Kopf *et al.*, 1993). These studies were extended by the treatment of SHRSP female rats with enalapril or hydralazine whilst pregnant in an attempt to reduce early kidney damage occurring in utero as a result of the mother's high BP (Rettig *et al.*, 1991). Offspring were subsequently used for kidney explantation. The hypertensive phenotype was still conferred by the kidneys. Together these studies substantially support the hypothesis that the kidney has a primary involvement in the development of hypertension.

This primary involvement of the kidney in BP control formed the basis of the hypothesis on which SA was identified.

THE SA GENE

Discovery of the SA gene

Iwai and Inagami (1991) postulated that if the kidney plays a role in the determination of blood pressure, there must be genes differentially expressed between the kidneys of hypertensive and normotensive rats. To identify such genes the technique of differential plaque filter hybridisation was used. cDNAs from the kidneys of SHR or Sprague Dawley (SD) rats were immobilised onto duplicate filters. Filters were hybridised with total cDNA from SHR and WKY rats to compare expression levels between these 2 strains. Three genes were identified which gave a stronger hybridisation signal with the SHR cDNA probe than with the WKY probe indicating a

higher level of expression in the SHR than the WKY strain. Genes designated S2 and S3 were identified from filters with immobilised SD cDNA. SA was identified from filters with immobilised SHR cDNA. S3 was found to correspond to the cytochrome P450 IV A2 gene. SA and S2 did not show high levels of homology to any previously reported genes at the time. Of the 3 genes, SA showed the greatest difference in expression between SHR and WKY rats, expression being approximately 10-fold higher in the SHR kidney than the WKY kidney. For SA this difference in expression was maintained into adulthood where as differences in expression of S2 and S3 were apparent in young rats but were no longer significant by 16 weeks of age.

Co-segregation of the SA gene with genetic hypertension

Increased interest in the SA gene arose when a polymorphism within SA was subsequently found to co-segregate with blood pressure in several F2 crosses between hypertensive and normotensive rat strains. These crosses include SHR and WKY rats (Iwai and Inagami 1992; Samani *et al.*, 1993), DS and Lewis rats (Harris *et al.*, 1993) and SHRSP and WKY rats (Lindpaintner *et al.*, 1993). F2 crosses between SHR and Lewis rats showed co-segregation in female rats only (Iwai *et al.*, 1992). Between SHR and WKY rats the SA locus is responsible for 28% of variation in systolic BP (Samani *et al.*, 1993). A summary of F2 crosses can be seen in Table 1.4.

Structure and function of the SA gene

The SA gene encodes for an unknown protein of 546 amino acids in the rat (Iwai and Inagami, 1991), 578 amino acids in the human (Iwai *et al.*, 1994) and 579 amino acids in the mouse (Takenaka *et al.*, 1998). Sequence analysis shows very little homology with other known proteins. The carboxy-terminal region of the SA gene exhibits approximately 30% homology to acetyl co-A synthetase (Iwai and Inagami, 1991; Kaiser *et al.* 1994) indicating the possibility that its protein product may be an enzyme. An AMP binding domain consensus sequence, typical of these enzymes, has been identified within exon 6 of the SA gene (Kaiser *et al.* 1994). Alignment of the SA gene with acetyl-coA synthetase and position of the AMP binding domain are shown in Fig. 1.5. Absence of transmembrane domain sequences indicates this is not a cell surface receptor protein.

Table 1.4 Co-segregation analysis studies of the SA gene and genetic hypertension

Author	year	cross	result	number	comments
Iwai	1992	WKY x SHR	positive	n=61	Male F2 rats were studied at 5 weeks.
Samani	1993	WKY x SHR	positive	n=233	Male F2 rats were studied. Positive correlation was observed from 16 weeks onwards.
Iwai	1992	Lewis x SHR	positive	n=169	A positive result was seen only in female F2 rats. The overall effect was not significant.
Harris	1993	Lewis x DSS	positive	n=140	Male F2 rats studied.
		WKY x DSS	negative	n=156	Male F2 rats studied.
Lindpaintner	1993	WKY x SHRSP	positive	n=115	A positive correlation with systolic blood pressure was observed only after salt loading. No correlation was found with diastolic blood pressure.
Lodwick	1997	MNS x MHS	negative	n=251	Although no correlation was found between SA genotype and blood pressure, a correlation was found between genotype and body weight in the F2 males.
Kreutz	1997	WKY-1 x SHRSP	positive	n=139	WKY-1 contains a sub-fragment of SHRSP chromosome 10.

FIG. 1.5 Comparison of protein sequences of the mouse SA gene and *Bacillus subtilis* acetyl-coA synthetase

The length of the mouse SA gene is 578 amino acids. The length of acetyl-CoA synthetase (Bacillus subtilis) is 572 amino acids. Standard amino acid single letter code is used. The top line of sequence corresponds to the SA gene, the bottom line to the acetyl Co-A synthetase gene. The middle line highlights identical amino acids by letter and similar amino acids with a +. Comparison was generated by the BLAST program of the National Centre of Biotechnology Information (www.ncbi.nlm.nih.gov). The AMP binding domain is highlighted in grey.

Identities 194/562 (34%), Similarities 306/562 (53%), Gaps = 34/562 (6%)

```
31  NFSNYESMKQDFK-IEIPEYF--------NFAKDVLDQWTNMEKAGKRLSNPAFWWIDGN  81
    N  NYE   + F   E  ++F        N A + +D+     +  K     A ++ D
14  NLKNYEETYRHFDWAEAEKHFSWHETGKLNAAYEAIDRHAESFRKNK----VALYYKDAK  69

82  GEELRWSFEELGLLSRKFANILTEACSLQRGDRVMVILPKIPEWWLANVACLRPGTVLIP 141
     +E +++F+E+   S +  N+L    ++++GDRV + +P+ PE +   +  ++ G +  P
70  RDE-KYTFKEMKEESNRAGNVLRRYGNVEKGDRVFIFMPRSPELYFIMLGAIKIGAIAGP 128

142 GTTQLTQKDILYRLQSSKAKCIITDDTLAPAVDAVAAKCENLHSKLIVSQHSREGWG--N 199
          +  +  RL++S+AK ++T   L   +     K  +L     +V   +  G     N
129 LFEAFMEGAVKDRLENSEAKVVVTTPELLERIPV--DKLPHLQHVFVVGGEAESGTNIIN 186

200 LKEMMKYASDSHTCVDTKHDEMMAIYFTSGTTGPPKMIGHTHSSFGLGLSVNGRFWLDLI 259
      E  K  S            +   +++TSG+TG  PK + H H +  +     G++ LDL
187 YDEAAKQESTRLDIEWMDKKDGFLLHYTSGSTGTPKGVLHVHEAM-IQQYQTGKWVLDLK 245

260 ASDVMWNTSDTGWAKSAWSSVFSPWTQGACVFAHYLPRFESTSILQTLSKFPITVFCSAP 319
      D+ W T+D GW       +F+PW  GA        RF   S   T+ +  + V+ SAP
246 EEDIYWCTADPGWVTGTVYGIFAPWLNGATNVI-VGGRFSPESWYGTIEQLGVNVWYSAP 304

320 TAYRMLVQ--NDMSS-YKFNSLKHCVSAGEPINPEVMEQWRKKTGLDIYEGYGQTET--V 374
    TA+RML+   ++M++ Y   SL+H +S GEP+NPEV+    K     I++ +  TET
305 TAFRMLMGAGDEMAAKYDLTSLRHVLSVGEPLNPEVIRWGHKVFNKRIHDTWWMTETGSQ 364

375 LICGNFKGMKIKPGSMGKPSPAFDVKILDENGATLPPGQEGDIALQVLPERPFGLFTHYV 434
    LIC N+  M IKPGSMGKP P  +  I+D  G  LPP + G++A++      + +    H +
365 LIC-NYPCMDIKPGSMGKPIPGVEAAIVDNQGNELPPYRMGNLAIK----KGWPSMMHTI 419

435 -DNPSKTAST-LRGSFYITGDRGYMDEDGYFWFVARSDDIILSSGYRIGPFEVESALIEH 492
     +NP K  S  + G +Y++GD  YMDE+GYFWF  R DD+I++SG R+GPFEVES L+EH
420 WNNPEKYESYFMPGGWYVSGDSAYMDEEGYFWFQGRVDDVIMTSGERVGPFEVESKLVEH 479

493 PSIAESAVVSSPDPIRGEVVKAFIVLNPDYKSHDQEQLKKEIQEHVKKTTAPYKYPRKVE 552
    P+IAE+ V+  PDP+RGE++KAFI L   ++  D  +LK+EI+  VK+  A +  PR++E
480 PAIAEAGVIGKPDPVRGEIIKAFIALREGFEPSD--KLKEEIRLFVKQGLAAHAAPREIE 537

553 FIEELPKTVSGKVKRNELRKKE 574
    F ++LPKT SGK+ R  L+  E
538 FKDKLPKTRSGKIMRRVLKAWE 559
```

The SA gene in the rat is approximately 26kb in length and contains 15 exons (Frantz *et al.*, 1999). It is localised on chromosome 1 in the rat and chromosome 16 in humans (Lindpaintner *et al.*, 1993; Szpirer *et al.*, 1993; Samani *et al.*, 1994) in areas syntenic with chromosome 7 in the mouse (confirmed by microsatellite analysis) (Gu *et al.*, 1996).

Tissue distribution of the SA gene

Northern analysis of mRNA from various male rat tissues has shown that SA is expressed primarily in the kidney, with some expression in the liver, brain and testes (Kaiser *et al.*, 1994). Expression in the kidney is approximately twice that in the liver and several fold that in the brain and testes. The major SA transcript is 2.5kb in length, although other transcripts are present in a tissue and strain specific manner. In the kidney SA expression is localised to the proximal tubule (Patel *et al.*, 1994), where its distribution is strain specific (Yang *et al.*, 1996). This may indicate an involvement in tubular function or sodium uptake.

Regulation of SA gene expression

Genomic regulation of the SA gene

Several common rat models of hypertension have been used to study expression of the SA gene. Expression levels vary in a strain and tissue specific manner. At the time this work began high expression levels of SA had been reported in the kidneys of SHR (hypertensive) and DS (salt-sensitive hypertensive) rats compared to low levels in the kidneys of WKY and DR rats (both of which are normotensive) (Iwai and Inagami, 1991). In addition F2 crosses between SHR and WKY rats demonstrated SA expression to be largely dependent upon SA genotype (Samani *et al.*, 1993). This data supported the hypothesis that SA may be responsible for a major blood pressure QTL at this locus. However subsequent analysis of SA expression in other strains has lead to the realisation of a more complex scenario than originally anticipated. A study of Milan hypertensive versus Milan normotensive rats showed no co-segregation between genotype at the SA locus and hypertension despite a big difference in expression levels between these two strains (Lodwick *et al.*, 1998). Interestingly, in this model, expression levels of the SA gene are greater in the normotensive than the hypertensive

strain. Similarly, a study of SHR and WKY rats from the Izumo colony in Japan (Ishinaga *et al.*, 1997) revealed expression levels of SA in normotensive WKY rats to be as high as those in hypertensive SHR. (In the Izumo strains analysis of the F2 population for a BP QTL at the SA locus was not performed). Obviously a simple explanation of increased renal SA expression leading to increased BP is not the case. One possible explanation is that a closely linked gene, rather than the SA gene itself, is responsible for the major BP QTL at this locus. However the importance of genetic background in studies of this nature cannot be overlooked. It is possible that on certain genetic backgrounds SA plays a role in blood pressure homeostasis whilst on other backgrounds it is irrelevant. Only the generation and careful analysis of congenic and transgenic strains can provide direct evidence either for or against the involvement of the SA gene in blood pressure regulation

LINE elements within the SA gene

In certain rat strains including SHR a linear insertion element (LINE element) has been identified within intron one of the SA gene (Frantz *et al.*, 1996). This LINE element is responsible for many of the RFLPs between the SHR and WKY rat strains. It is currently not known whether these sequences are involved in the differential regulation of the SA gene between strains or if they have any other functional significance. However, a correlation has been observed between expression levels of the SA gene and the presence or absence of the LINE element. Strains in which the LINE element is present have high levels of expression of SA, whereas strains in which the LINE element is absent have relatively low levels of SA expression (Frantz *et al.*, 1996).

Alternative splicing within the SA gene

In the kidney of WKY rats several different sized transcripts of the SA gene have been reported. This has lead to the hypothesis of a novel method of transcriptional regulation for the SA gene. The size of the main SA transcript is approximately 2.5kb (Kaiser *et al.*, 1994), however at least three different size transcripts have been found in the kidney of the WKY rat (Iwai and Inagami, 1991; Samani *et al.*, 1993). This transcript was not observed in the kidney of SHR rats or the liver of either strain (Kaiser *et al.*, 1994). RT-PCR analysis of WKY mRNA shows there to be two additional transcripts

present (Shiota and Inagami, 1996; Frantz *et al.*, 1999). Shiota and Inagami, (1996) hypothesised that there is a transcriptional deficiency in WKY which may be responsible for the overall reduction in expression in this strain. Sequence analysis shows the extra transcripts to arise from the duplication of exon 2 and exons 2 and 4 respectively (Frantz *et al.*, 1999). As these duplications are not present at the DNA level it is hypothesised that they may have arisen due to a novel form of RNA splicing (Frantz *et al.*, 1999). Trans-splicing of this nature has previously been reported only once in higher eukaryotes (Caudevilla *et al.*, 1998). Whether the presence of alternative splicing mechanisms for the SA gene has any effect on blood pressure control remains to be elucidated.

Other factors regulating expression of the SA gene

Administration of a low salt diet for 4 weeks caused a 2.5-fold increase in renal SA expression in the DR rat compared to the administration of a high salt diet. However in DS and SHR no alterations in expression levels were noted (Iwai and Inagami, 1991). This again highlights the importance of genetic background in such studies. SA expression levels were investigated in the SHR after the administration of captopril to prevent the development of hypertension (Iwai and Inagami, 1991). SA expression was increased slightly in the kidney (by 1.5 fold) and by 2.5-fold in the liver by this treatment.

Alterations in expression of SA as a response to these treatments indicate a relationship between SA and factors known to be effected by these treatments, thus pointing toward an involvement in blood pressure homeostasis.

Regulation of the SA gene by androgens

More recently the SA gene was found to be androgen regulated by Melia *et al.* (1998). In these experiments, differential display was performed to compare the kidneys of C57BL/6 females with those of androgen treated females of the same strain. This approach identified the SA gene as being up-regulated in the kidney by the administration of androgens. Castration of male C57BL/6 mice and the subsequent

administration of androgens or androgens and flutamide (an androgen receptor antagonist) proved this irrefutably.

Human studies

A homologue of the SA gene was shown to be expressed in human kidneys and was assigned to the short arm of chromosome 16 (Samani *et al.*, 1994). However association studies for the SA gene in humans have yielded conflicting results. Iwai *et al.* (1994) identified a *Pst*I polymorphism within the human SA gene for which the rare allele was significantly more frequent in hypertensives than in normotensive controls (p=0.0001). This study was performed using a Japanese population. However 2 studies of this polymorphism in Caucasians have failed to show either association or linkage of the SA gene and hypertension. Zee *et al.* (1997) found the rare *Pst*I polymorphism allele to be present at a similar frequency in both hypertensive and normotensive study groups. Harrap *et al.* (1995) used a four corners approach to identify young adults likely to suffer from hypertension in the future. No difference in frequency of the *Pst*I polymorphism was observed between subjects most and least likely to develop hypertension. Nabika *et al.* (1995) studied a polymorphic microsatellite marker within intron b of the human SA gene. No linkage was found between this marker and hypertension in a French population. Reasons for these different results are unclear, however genetic background of the populations studied is likely to be a significant factor. Environmental differences, specifically a high salt diet in the Japanese population, may also be of significance. Experimental design, including sizes of study populations, choice of observed mutation, methods of statistical analysis and criteria for selection of study subjects, all contribute to the final results obtained in studies of this nature (as previously described).

Recent data from congenic models in the study of the chromosome 1 QTL

Congenic strains have been very useful for the confirmation and narrowing down of the BP QTL in the region of the SA locus on rat chromosome 1. Several congenic strains have been generated for this locus.

Reciprocal congenic strains between SHR and WKY have been constructed (Frantz *et al.*, 1998). Between 26.4cM and 54.5cM of SHR DNA around the SA locus was transferred into the WKY background and between 15.1cM and 34.9cM of WKY DNA was transferred into the SHR background. In both of these strains blood pressure was positively associated with genotype at the SA locus. Body mass was also positively associated with this area of the chromosome, with the SHR genotype conferring greater body mass. A similar strain was constructed by Iwai *et al.* (1998), also using SHR and WKY rats. This study also showed salt sensitivity to be conferred by an allele in the region of the SA gene.

A congenic strain was constructed by transferring a region of Brown Norway DNA in the region of the SA gene into an SHR background. Between 22cM and 33cM of DNA was transferred. Blood pressure was significantly lower than in the control SHR strain (St Lezin *et al.*, 1997).

Research has continued to narrow down the congenic regions at this locus. Recently two interesting strains have been reported. Hubner *et al.* (1999) have generated a congenic strain containing a portion of the SHRSP chromosome 1 on a WKY-1 background (WKY-1 is a congenic strain containing a region of SHRSP chromosome 10 on a WKY background). WKY-1 was used as the background strain in this case because co-segregation studies between it and SHRSP are positive for the SA locus (whereas similar crosses for SHRSP and WKY were negative). Obviously a certain genetic background is necessary in the chromosome 10 region for the hypertensive locus of chromosome 1 to be effective. Blood pressure data indicate that in this congenic strain the major blood pressure QTL in the region of SA has been separated from the SA gene. Similar results have been obtained by St Lezin *et al.* (2000) who generated congenic strains between SHR and BN rats. A major blood pressure QTL at this locus has been shown to exist in a portion of transferred DNA not including the SA gene. No congenic strain was generated however containing a reduced region around the SA gene. It therefore remains a possibility that the SA gene and another closely linked gene both affect blood pressure but in a non-additive manner.

The primary aim of this work was to define a function of the SA gene product. Many of the studies performed to date, including some during the course of the current work, provide only circumstantial evidence either for or against the involvement of the SA gene in the regulation of blood pressure and the pathogenesis of hypertension. The use of transgenic techniques can provide a direct link between a gene and its function. We therefore chose the approach of gene ablation using embryonic stem cell technology to address the function of the SA gene.

GENE TARGETING

The development of embryonic stem cell technology

Embryonic stem cells are undifferentiated cells derived from the inner mass of early blastocysts. They can be cultured *in vitro* under conditions allowing them to maintain their undifferentiated state and can later be re-introduced into a blastocyst to be incorporated in some or all tissues of the resulting animal as it develops.

In vitro culture of ES cells

Early attempts to culture cells *in vitro* directly from the inner cell mass of blastocysts were unsuccessful (Solter *et al.*, 1975; Atienza-Samois *et al.*, 1978). However in 1981 this was achieved by two separate groups using slightly different methods. Evans and Kaufman (1981) employed a technique whereby super-ovulated female mice were suspended in a pre-implantation stage allowing the inner cell mass of each blastocyst to increase substantially. Blastocysts were then cultured *in vitro*. After 4 days the inner cell mass had grown sufficiently to allow ES colonies to be picked off. ES cells were then trypsinised and plated out on an inactivated STO fibroblast feeder layer (Martin *et al.*, 1975). This method succeeded because a sufficient number of cells at the correct stage of development were generated. Also culture on a feeder layer was important as growth factors from the feeder cells contribute to maintaining an undifferentiated state.

Martin (1981) employed a method whereby the inner cell mass of blastocysts were removed by immuno-surgery and plated out on medium previously conditioned with teratocarcinoma cells. Thus factors produced by the growing teratocarcinoma cells were still present in the medium preventing differentiation. After several passages

when cell density had increased substantially, the use of pre-conditioned medium was no longer necessary. A feeder layer of STO fibroblasts was also used in these experiments.

Cultured ES cells were injected into blastocysts and were found to give rise to chimaeras in a high proportion of cases (30-40% by Robertson *et al.* in 1983 and over 50% by Bradley *et al.* in 1984). Bradley *et al.* (1984) found the germline transmission rate of these chimaeras was approximately 20%.

The discovery of (leukaemia inhibitory factor) LIF

Since ES cells were first grown in culture it has been know that a growth factor is necessary to maintain their undifferentiated state. Initially this factor was provided in the media by growth on a feeder layer of STO fibroblasts (Martin *et al.*, 1981; Evans and Kaufman, 1981). The process by which feeders inhibit differentiation is probably multifactorial involving both contact dependent mechanisms and secreted factors (Isacke and Deller, 1983). Differentiation is greatly reduced by growth on a feeder layer, however over time significant differentiation does still occur (Koopman and Cotton, 1984).

DIA (differentiation inhibitory activity) was isolated from medium conditioned by Buffalo rat liver cells (BRL-medium) (Smith and Hooper, 1987) and was found to support the undifferentiated growth of ES cells in the absence of a feeder layer. Maintenance of an undifferentiated state was supported to a much greater degree by media conditioned in this manner than by STO fibroblasts.

BRL-medium was subsequently used to maintain pleuripotency in cells prior to their passage through the germline of chimaeric mice (Hooper *et al.*, 1987).

In separate lines of study, LIF was identified as a haemopoietic molecule (Gearing *et al.*, 1987), as was Human interleukin for DA cells (Moreau *et al.*, 1987). Certain similarities were observed between these molecules with each other and with DIA. It was deduced that all three molecules were in fact the same. As recombinant LIF was

already available, it became widely used as a growth supplement for ES cells in culture (Williams *et al.*, 1988; Smith *et al.*, 1988; Moreau *et al.*, 1988).

Following its discovery, the use of LIF in the culture of ES cells has become routine, greatly facilitating the use of ES cell technology for experimental purposes.

Transgenesis utilsing ES cells with non-specific alterations

Initially ES cells were manipulated in vitro in a non-specific manner and found to still maintain their ability to colonise the germline. This was achieved by transfection with the neomycin resistance gene resulting in its random integration into the chromosome (Gossler *et al.*, 1986) and by transfection with genes in retroviral vectors (Stewart *et al.*, 1985; Robertson *et al.*, 1986). Both methods allowed transgenesis due to random integration of the transgene.

Hooper *et al.* (1987) selected spontaneous HPRT negative ES cells in vitro and utilised these in the generation of an HPRT null mouse, demonstrating the ablation of a gene with a selectable phenotype and the *in vitro* manipulation of ES cells prior to transgenesis.

Homologous recombination

At the same time as this work was taking place, homologous recombination was achieved in cell lines other than ES cells (Smith and Berg, 1984; Thomas and Cappechi, 1986). A specific mutation in a native gene was achieved by Smithies *et al.* in 1985.

These 2 technologies were brought together in 1987 with the first report of targeted mutations of ES cells by homologous recombination. Thomas and Capecchi (1987) generated ES cell lines carrying targeted mutations of the HPRT gene. These experiments took advantage of the selectable phenotype of the HPRT gene (wildtype cells are sensitive to the base analogue 6-thioguanine, 6-TG) and its location on the X chromosome allowing a mutation in the single allele of XY ES cells to generate a phenotype. Both replacement and insertion type vectors carrying the neomycin

resistance gene were used to target the HPRT gene. Selection was by neomycin and 6-TG.

Also in 1987, Doetschman *et al.* used homologous recombination to restore function to a spontaneous deletion mutant of the HPRT gene. This was achieved using an insertion vector and 6-TG selection. In these early experiments the targeted ES cells were not used to generate mutant mice.

Targeted disruption of non-selectable genes in ES cells; the use of thymidine kinase

The first use of a negative selection marker (the herpes simplex thymidine kinase gene, TK) was reported by Mansour *et al.* in 1988. A targeted disruption of the proto-oncogene *int-2* was described in an ES cell line. Unlike the HPRT gene, *int-2* has a non-selectable phenotype. The use of the TK gene to enrich for homologous over non-homologous recombination events allowed a strategy for the potential disruption of any gene. Targeted mutation of the *En-2* gene (Joyner *et al.*, 1989) was reported without the use of negative selection. In this case screening of a greater number of neomycin resistant clones was necessary after transfection in order to identify a correctly targeted clone. The first reported targeting of genes not expressed in ES cells was for the *adipsin* gene and the aP2 gene (Johnson *et al.*, 1989). It was noted that the lack of expression of these genes in ES cells did not affect the frequency of their targeting by homologous recombination.

Germline transmission of genes targeted by homologous recombination

Germline transmission of genes targeted by homologous recombination was achieved in 1989. Thompson *et al.* (1989) used an insertion style vector to correct a mutation in the HPRT gene. At a similar time, Schwartsberg *et al.* (1989) generated a targeted deletion of the c-*abl* gene using a replacement style vector containing a promoterless neomycin resistance gene. The absence of a neomycin promoter enriched for correctly targeted events over random events. The neomycin gene could only be expressed when inserted behind the promoter of a gene expressed in ES cells, for example the gene being targeted. This strategy can only be applied to the targeting of genes expressed in ES cells.

Other genes to be ablated by homologous recombination in these early days of knockout technology were β2M, (Koller *et al.*, 1990), *int*-1 (Thomas and Capecchi, 1990) and IGF-II (DeChiara *et al.*, 1990).

Since the early experiments outlined above manipulation of ES cells and the generation of targeted mouse lines has become a commonly used tool for the study of gene function. Many techniques for genetic manipulation of ES cells *in vitro* have been employed to address complex questions in many fields of biological research. The volume of publications utilising ES cell technology has increased substantially each year since the availability of this technology and is likely to continue increasing in the near future (Simpson *et al.*, 1997).

Transgenics and gene ablation in the study of hypertension

Classical transgenics and gene ablation have been used extensively in the study of the renin-angiotensin system (RAS) and other genes implicated in the cause of hypertension. These two approaches complement each other as overexpression will often yield different information about a gene to that generated by its ablation. One of the earliest uses of gene targeting in this field was for the study of the angiotensinogen gene (Tanimoto *et al.*, 1994; Smithies and Kim, 1994; Kim *et al.*, 1995). Rearrangement at the DNA level during ES cell manipulation in a targeting experiment gave rise to a cell line with two contiguous full copies of the *Agt* gene (Smithies and Kim, 1994). Use of this ES cell line, together with the wild type and null lines, allowed the production of mice with 0, 1, 2, 3 or 4 copies of the angiotensinogen gene. These mice were found to have 0-145% of the normal plasma angiotensinogen level, increasing in a gene specific non-linear manner (Kim *et al.* 1995). A corresponding result was obtained for blood pressure. Viability of the null phenotype was considerably reduced and kidney morphology showed pathological changes. In an independent study (Tanimoto *et al.*, 1994) systolic BP was found to be considerably reduced in the knockout as compared to the wildtype mice. Blood pressures of heterozygotes were however unaffected. The hypotensive effect of reduced *Agt* copy number confirmed the expected result of reduced BP due to a reduction in the activity

of the renin-angiotensin system. In addition the histological changes observed in the kidneys of these mice highlighted the importance of the RAS in organ development. Such renal abnormalities must however be taken into consideration when interpreting BP phenotypes in these mice.

At about the same time Kurihara *et al.* (1994) reported the generation of endothelin-1 (*ET-1*) null mice. Endothelin is a known vasoconstrictor, therefore null mice may have been expected to be hypotensive. However mice carrying only one copy of the endothelin gene are hypertensive (mice lacking both copies of this gene do not survive to adulthood). This clearly demonstrates how wider functions of a gene can affect many systems. The authors hypothesised that effects of ET-1 on determining BP levels via the CNS were altered in these heterozygous mice leading to hypertension, despite endothelin being a vasoconstrictor.

Since these early experiments many hypertension-related genes have been studied by gene targeting. These are summarised in Table1.5 below.

Table 1.5 Hypertension related genes studied by gene targeting

Gene	Blood pressure phenotype	Other phenotypes	References
Agt (Angiotensinogen)	Hypotensive	Reduced viability. Severe renal abnormalities	Kim *et al.*, 1995; Tanimoto *et al.*, 1994
Ace (Angiotensin converting enzyme)	Hypotensive	Male fertility reduced.	Krege *et al.*, 1995
Agtr1a (ANGII type 1a receptor)	Hypotensive	No response to ANGII infusion Mild renal abnormalities	Ito *et al.* 1995: Sugaya *et al.*, et al 1995.
Agtr1b (ANGII type 1b receptor)	Normotensive	Normal renal function	Chen *et al.*, 1997
Agtr1a/Agrt1b double knockout	Hypotensive	More severe renal abnormalities than *Agtr1* null mice	Tsuchida *et al.*, 1998 Oliverio *et al.*, 1998
Agtr2 (ANGII type 2 receptor)	Normotensive Hypertensive	Normal response to ANGII Altered response to ANGII and captopril	Hein *et al.* 1995 Ichiki *et al.*, 1995
Ren-1d (Renin)	Normotensive	Absence of renin storage granules in the juxtaglomerular cells. Altered plasma renin:prorenin concentrations	Clark *et al.*, 1997
Ren-2d (Renin)	Normotensive	Altered plasma renin:prorenin concentrations	Sharp *et al.*, 1996
Bradykinin B_2 receptor	Salt-sensitive hypertension	Increased hypertension in a 2-kidney/one-clip model	Alfie *et al.*, 1996 Alfie *et al.*, 1997
eNOS (endothelium nitric oxide synthase)	Hypertension	PRA doubled, kidney renin mRNA reduced. Decrease in BP with NOS inhibitors.	Huang *et al.*, 1995
iNOS (inducible nitric oxide synthase)	Normotensive	Altered immune responses	Laubach *et al.* 1995 Wei *et al.*, 1995
nNOS (neuronal nitric oxide synthase)	Normotensive	Normal renal histology Dilated stomachs. Aggressive behaviour. Altered sexual behaviour	Huang *et al.* 1993

ET-1 (Endothelin-1)	Lethal null. Heterozygotes hypertensive	Surprise phenotype as ET-1 is a vasoconstrictor (may be due to altered CNS responses)	Kurihara *et al.*, 1994
NHE3 (Na+/H+ exchanger, isoform 3)	Hypotensive	Increased plasma aldosterone, renin and other ion exchanger expression	Schultheis *et al.*, 1998
D_{1A} receptor (dopamine 1A receptor)	Hypertensive	Normal sodium and electrolyte balance	Albrecht *et al.*, 1996
D_3 receptor (dopamine 3 receptor)	Hypertensive	Increased renin (due to absence of dopamine's inhibitory effect on renin).	Asico *et al.*, 1998
proANP (atrial naturetic peptide)	Borderline hypertensive and salt sensitive hypertensive		John *et al.*, 1995
GC-A (atrial naturetic peptide receptor)	hypertensive	Cardiac hypertrophy and sudden death.	Lopez *et al.*, 1995 Oliver *et al.*, 1997

Limitations of gene targeting

Interpretation of phenotypes generated by gene targeting can often be difficult, especially if the targeted gene has numerous functions, as is the case with genes of the RAS. For example, both the AGT and ACE null mice show severe organ developmental abnormalities which make the functional relevance of the mutation difficult to establish.

Ablation of a gene from the beginning of embryo development can result in the up-regulation of other genes to compensate for the deleted protein. Thus the effect of a mutation may underestimate the function of the gene in the wildtype animal.

Gene targeting can sometimes give rise to completely unexpected phenotypes the explanation for which may not be obvious, for example the ET-1 null mice. Other possible outcomes of a gene targeting experiment are the generation of a lethal mutation which reveals little useful information about the target gene, or the generation of a mouse in which the effect of the mutation is not apparent. The outcomes of

targeting experiments are usually difficult to predict at the design stage of an experiment.

Despite these limitations, gene targeting is an extremely useful tool and has yielded a wealth of information in many fields of biological research including hypertension. At the design stage of our targeting experiment we were aware of the restrictions of this technique, however as so little is known about the function of the SA protein, this was a very appealing approach to follow.

AIMS OF THIS WORK

The aim of the current work was to generate a mouse model carrying a null mutation of the SA gene and to study the phenotypic effects of this mutation on blood pressure and cardiovascular phenotypes.

Chapter 2

MATERIALS AND METHODS

Chapter 2: Materials and Methods

Presented initially are general methods, followed by specific methods pertaining to manipulation of bacteriophage libraries, embryonic stem cell manipulation, genotyping of SA-null mice and blood pressure measurement. Other specific methods are detailed in individual chapters where relevant.

GENERAL METHODS

DNA MANIPULATION

Isolation of chromosomal DNA from tissue

Pieces of tissue not exceeding 2g in weight were chopped on sterile petri-dishes with clean razor blades. Chopped tissue was added to 700µl of TNE buffer. Proteinase K (to 0.5µg/µl) and SDS (to 0.5%) were added. Digestion was performed at 55°C with shaking for approximately 16 hours. DNA was extracted with phenol, phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). For each extraction 700µl of extractant was added, the sample shaken vigourously (but without vortexing) by hand for 10 minutes and spun at 13,000rpm. for 10-15 minutes at room temperature. At each stage the aqueous phase was removed to a fresh tube. Finally DNA was precipitated by the addition of 1ml of 100% ethanol to 500µl of aqueous phase and inverting the sample several times. If a sufficiently large amount of tissue was used initially, a clump of DNA was then hooked out using a sterile Gilson tip. This was placed in a fresh eppendorf tube and allowed to air dry before dissolving in an appropriate amount of sterile distilled water or TE. If DNA was extracted from a very small amount of tissue, too little was generated to hook out with a pipette tip. In this case, DNA was precipitated overnight at -20°C, pelleted by centrifugation at 13,000rpm and washed with 70% ethanol before air drying and dissolving in an appropriate amount of water or TE.

Preparation of plasmid DNA

For most applications plasmid DNA was prepared using the Qiagen mini-prep system following the manufacturers instructions. This yielded up to 20µg of plasmid DNA from 1.5ml to 5ml of culture, depending on the copy number of the plasmid. For large-scale plasmid preparation (to generate plasmid for gene targeting experiments) Qiagen maxi-prep kits were used, again following the manufacturers instructions. Using this method up to 500µg of plasmid DNA was generated.

Spectrophotometric determination of nucleic acid concentration

DNA and RNA concentrations were determined by spectrophotometry. Samples were diluted appropriately and OD_{260} and OD_{280} readings were measured in quartz cuvettes using a Pharmacia LKB Ultraspec III spectrophotometer. DNA concentrations were calculated as follows: for double stranded DNA, 1 OD_{260} unit is equivalent to a DNA concentration of 50µg/ml. Similarly for single stranded DNA and RNA, 1 OD_{260} unit is equivalent to a concentration of 40µg/ml. For oligonucleotides, 1 OD_{260} unit is equivalent to a concentration of 33µg/ml (Sambrook *et al.*, 1994).

Agarose gel electrophoresis

Agarose (0.6%-3%w/v) was dissolved in 1X TAE buffer. The w/v percentage gel used depended on the size of fragments to be resolved. Ethidium bromide was added to a final concentration of 100ng/ml and gels allowed to solidify. Sample volumes of up to 30µl were loaded with 10% gel loading buffer containing bromophenol blue. Gels were run at a constant voltage of between 12V and 100V, depending on the percentage of the gel and total running time required. DNA was visualised using a UV light box.

Restriction digestion of plasmid DNA

Restriction digestion was performed following the restriction enzyme manufacturers instructions. In brief, the following reagents were combined: up to 1µg of DNA, appropriate restriction buffer to 1/10 final volume, water and restriction enzyme (not exceeding 1/10 final volume). Final reaction volumes varied from 10 to 300µl. Reactions were incubated at 37°C for 2-16 hours.

Restriction digestion of bacteriophage DNA

Restriction digestion was performed following the manufacturers instructions (see above). Large reaction volumes were used in order to dilute any impurities in the DNA. Spermidine was added to a final concentration of 1.25mM to reduce secondary structure of the DNA and facilitate access of the enzyme to its recognition site.

Restriction digestion of ES cell DNA

ES cell DNA was prepared from approximately 400 clones per experiment, therefore phenol extraction of each DNA was not possible. Restriction digestion was consequently sometimes difficult. A final volume of 300µl per restriction digestion was used and spermidine was added to a final concentration of 1.25mM. RNase A was added to a final concentration of 0.2µg/µl and digestion was performed at 37°C for a minimum of 12 hours. Depending on the enzyme used up to 50U/µl of enzyme were used per reaction.

Restriction digestion of DNA with multiple enzymes

Multiple digestions were performed in a similar manner to single digestions. However care was taken to provide a digestion buffer suitable for all enzymes in a given reaction. Tables provided by the manufacturer, showing the efficiency of enzymes in the different buffers available, allowed a suitable buffer to be selected. In cases where no one buffer was appropriate for every enzyme in a reaction, the enzyme requiring the lower salt buffer was used first. Salt concentration was then increased to the level recommended by the manufacturer before adding the second enzyme.

Southern Blotting

DNA fragments were separated by agarose gel electrophoresis. For Southern blotting of chromosomal DNA approximately 10µg of restriction digested DNA was loaded per track. After electrophoresis, agarose gels were depurinated for 7 minutes in HCl (1:50 v/v concentrated HCl), denatured for 30 minutes (in 1.5M NaCl, 0.5M NaOH) and neutralised for 30 minutes (in 1.5M NaCl, 0.5M Tris-HCl, pH7.2). Gels were blotted in 20X SSC onto nylon membranes (Hybond) for approximately 15 hours. Membranes

were then rinsed briefly in 3X SSC, air dried and baked at 65°C for 10 minutes. DNA was cross linked for 1 minute on a UV light-box.

Hybridisation of filters with chromosomal or cDNA probes

20-30ng of probe DNA was labeled with 1.85 MBq ^{32}P dCTP using the Gibco Radprime labeling kit following the manufacturer's instructions. Unincorporated nucleotides were removed using the Qiagen Nucleotide removal kit. Hybridisation was carried out in a Hybaid oven using Hybaid bottles and meshes. Filters were pre-hybridised for approximately 2 hours prior to hybridisation. Probe DNA was denatured for 10 minutes at 100°C before adding to the hybridisation buffer. For Southern blotting both hybridisation buffer and pre-hybridisation buffer were composed of 3X SSC, 5X Denhardts, 0.6g/ml PEG, 0.5% SDS and 200µg/ml sonicated salmon sperm DNA. Salmon sperm DNA was denatured for 10 minutes at 95°C prior to addition to the buffer. Hybridisation and pre-hybridisation were performed at 65°C. (For Northern blotting Amersham Rapid-Hyb Buffer was used as per the manufacturer's instructions. Hybridisation and pre-hybridisation were performed at 42°C.)

Oligonucleotide labeling

Oligonucleotides were labeled using the Pharmacia TdT labeling kit following the manufacturers instructions. Essentially, the following reagents were combined to give a reaction volume of 20µl: 100ng oligonucliotide DNA, an appropriate volume of 5X one-for-all buffer, 1mM final concentration $MgCl_2$, TdT enzyme (not exceeding 1/10 of the final volume) and 1.85 MBq ^{32}P dCTP.

Hybridisation of filters with oligonucleotide probes

Hybridisation and pre-hybridisation buffers and apparatus were as for Southern blotting (see above, page 34). Filters were pre-hybridised at 42°C for 2 hours and hybridised at 42°C for 16 hours. Filters were washed as above.

Washing of filters

After hybridisation with a chromosomal or cDNA fragment, Southern blot filters were washed at 65°C in 3X SSC, 1X SSC and 0.5X SSC (all with 0.1% SDS). Each was carried out for 5-10 minutes. After hybridisation with an oligonucleotide probe, filters were washed at 42°C in conditions otherwise as above.

Stripping of nylon filters

Southern blot filters were stripped by placing in boiling 0.1% SDS and incubating for 2 hours at 70°C with gentle agitation.

Polymerase Chain Reaction (PCR)

As PCR is an extremely sensitive method of amplifying DNA it is prone to contamination. For this reason PCR-dedicated reagents, tips and tubes were used to prepare PCR reactions. PCR reactions were prepared in an area free from PCR products, usually a dedicated laminar flow hood.

In general, PCR reactions were performed in a 25µl volume. An appropriate volume of PCR Buffer was added to DNA (50-200ng) and water, followed by 10pmol of each primer and 1U of Biotaq (Bioline). Frequently during this work, PCR was used to genotype many samples. In this case a master-mix of water, PCR buffer, primers and Biotaq was prepared. An aliquot of master-mix was then added to each DNA sample. Samples were covered with 20µl of mineral oil to prevent evaporation during amplification. Amplification was performed in either a Perkin Elmer Cetus or a M.J. Research PTC-100 thermo-cycler. Amplification conditions for individual primer sets varied, but were based upon the following: an initial denaturing step of 94°C of 5 minutes followed by 1 minute denaturing at 94°C, 1 minute annealing at a temperature appropriate to the primers used, and 1 minute extension at 72°C. These last 3 steps were then repeated for a total of 30 cycles. A final step of 72°C for 7 minutes was performed to allow all products to be extended to completion. Annealing temperature for individual primer pairs were calculated as follows: 4(G+C) + 2(A+T).

Preparation of DNA probes specific to exons 2, 3 and 4

Primers designed from the rat cDNA sequence (supplied by Simon Frantz, Dept. Cardiology) were used to generate a small exon specific fragment for each of exons 2, 3 and 4 (for primer sequences, see page 62). The PCR reactions were performed using standard conditions in a Perkin Elmer Thermocycler. Rat chromosomal DNA was used as a template. The PCR reactions were run on a 2% LMP agarose gel, the specific bands cut from the gel, purified using a QIAquick gel extraction kit (Qiagen) following the manufacturer's instructions.

exon	Sense primer	Anti-sense primer	product size	total exon size
2	SA12	SA17	97 bp	148 bp
3	SA14	SA41R	133 bp	274 bp
4	SA15	SA6	170 bp	211 bp

Retrieval of DNA fragments from agarose gels

DNA was retrieved from agarose gels using either a QIAquick Gel Extraction kit (Qiagen) or a Sephaglas BandPrep kit (Pharmacia), following the manufacturers instructions. Using the Qiagen kit, DNA is retrieved in a final volume of 30µl. For some applications this was considered insufficiently concentrated. Using the Pharmacia kit, DNA can be retrieved in a final volume of 10µl if necessary, thus giving a higher concentration. This was useful for some cloning techniques.

End-filling of restriction digested DNA

Sticky ends generated by restriction digestion were end-filled using Klenow (DNA polymerase *Pol*I) (Gibco). DNA was dissolved in an appropriate volume of water (for example 300ng in 6µl). The following were added: dNTPs (to a final concentration of 20µM each), an appropriate amount of 10X Klenow buffer and Klenow (not exceeding 1/10 of the final reaction volume). The reaction was incubated at 37°C for 10 minutes. A 5 minute incubation at 65°C was then performed to inactivate the enzyme.

Cloning of DNA fragments

For each cloning experiment a cloning strategy was first carefully planned. DNA of the chosen plasmid vector was cut with the appropriate restriction enzymes (the amount of plasmid DNA restricted varied but was in the region of several hundred ng). If two different enzymes were used, the entire restriction digestion was run on an agarose gel to separate the cut plasmid from the remaining polylinker fragment (which would have compatible restriction ends to the linearised plasmid and due to its small size would be cloned preferentially, rather than band of interest). Cut plasmid vector was extracted from the agarose gel (see page 37). Insert DNA was generated by restricting the appropriate plasmid or bacteriophage and separating from unwanted sequences, again by agarose gel electrophoresis followed by band extraction. Relative concentrations of vector and insert were determined by running a small aliquot of each in parallel on an agarose gel. Absolute concentrations of vector were determined by running in parallel with a known amount of Gibco 0.24-9.5kb size markers. Ligations were then performed as follows: vector and insert DNA were mixed in a ratio of 1:8 (vector:insert) to give a total of 50-200ng of DNA per reaction. An appropriate volume of 5X ligation buffer (Gibco) and T4 DNA ligase (Gibco) were added. The reaction volume was kept to a minimum. Ligation was performed at 15°C in a waterbath in a 4°C room overnight. Transformation of an appropriate strain of *E. coli* was then performed (see below).

Transformation of *E. coli* with plasmid DNA

E.coli competent cells were obtained either commercially (Gibco sub-cloning efficiency competent cells, strain DH5α) or by the $CaCl_2$ method (see below). The *E.coli* strains used for cloning in this work all contained the *lac*Y gene which is necessary for the blue/white selection process in the presence of a plasmid borne *lac*Z gene.

After preparation or thawing, competent cells were kept on ice at all times. $CaCl_2$ competent cells were aliquotted into 200µl aliquots. Transforming DNA was added to an aliquot of cells and incubated on ice for 15 minutes. Heat shock was then performed

at 42°C for 2 minutes, followed by 30 minutes incubation on ice. 1ml of LB broth was added and the cells incubated at 37°C for 1 hour. Cells were then plated on selective medium and incubated at 37°C overnight. A similar procedure was followed for the Gibco competent cells, following the supplier's instructions. When plating transformed cells a volume of between 10 and 200µl was spread over a 10cm plate. A volume of ligation was transformed such that approximately 1ng of vector was later plated on a 10cm plate. Dilutions either side of this amount were also plated, to allow for error in estimation of the amount of vector DNA present in the ligation reaction. Transformation controls were performed in parallel to an experimental transformation.

Preparation of calcium chloride competent cells

10ml of LB broth was inoculated with *E. coli* from either an agar plate or a -80°C stock culture. After incubation overnight, 500µl of this culture was used to inoculate a 50ml culture in a 500ml conical flask. This was incubated with shaking at 37°C until it reached an OD_{550} of between 0.45 and 0.55 (approximately 4 hours). The culture was then incubated on ice for 10 minutes. From this point onwards, cells and all solutions were maintained chilled on ice. Cells were harvested by brief centrifugation (2 minutes at 2,500rpm) at 4°C, washed in 1/2 volume $MgCl_2$, washed in 1/10 volume $CaCl_2$ and finally resuspended in 1/25 volume of $CaCl_2$. Thus an initial culture volume of 50ml yielded 2ml of $CaCl_2$ competent cells.

Screening of bacterial clones

In most cloning experiments during this work, blue and white selection was used as a basis for identifying bacterial clones containing a plasmid of interest. Ampicillin was included in the agar plates (at a final concentration of 100µg/ml) to select for bacterial clones which had received a copy of the transforming plasmid, and thus the ampicillin resistance gene. X-gal (50µg/ml) and IPTG (1mM) were included in the agar plates to allow blue/white selection of those clones in which the plasmid-borne *lacZ* gene was interrupted by cloned sequences.

For cloning experiments where neither blue/white or antibiotic selection were available, it was necessary to screen a large number of bacterial colonies to identify clones of interest. This was achieved by growing bacterial clones on nitro-cellulose over agar and hybridising with potential insert DNA. Large LB agar plates were prepared and nitrocellulose filters cut to fit the surface of the agar. 10cm agar plates were also prepared as masterplates. Nitrocellulose was placed on the surface of the large plates and bacterial colonies were picked onto the nitrocellulose and onto a corresponding masterplate. Plates were incubated inverted overnight at 37°C after which masterplates were retained at 4°C. Nitrocelluose filters were processed as follows: 3MM Watmann paper was used to line the base of 3 trays. The papers were pre-wetted with either 10% SDS, denaturing solution or neutralising solution. Nitrocellulose filters were placed sequentially in each solution for 3-5 minutes. Filters were then rinsed briefly in 6X SSC. Bacterial DNA was fixed to the filter by exposure to UV light (using a light-box) for 1 minute. Hybridisation with DNA probes and washing of filters were performed as for Southern blotting (see page 34). When exposed to X-ray film, clones carrying insert sequences which were hybridised by the insert probe blackened the film. These clones were then identified on the masterplates.

RNA MANIPULATIONS

Precautions when handling RNA

Prior to commencing RNA experiments, fresh solutions were made using water previously treated with DEPC to remove RNases. All glassware was baked at 160°C to destroy RNases prior to use. All chemicals used were specifically kept free of RNases: chemicals were poured from their containers into baked glassware or handled using a baked spatula. Gloves were worn at all times during experiments involving RNA, and changed frequently. Bench surfaces were wiped frequently with presept solution or with RNase-away (Gibco). A separate set of Gilson pipettes were reserved entirely for use with RNA solutions.

DEPC treatment of solutions

DEPC was used to destroy RNase molecules in distilled water. DEPC was added to water at a concentration of 0.1% in a fume hood. The mixture was shaken vigorously in a sealed bottle and incubated at 37°C for approximately 16 hours. It was then autoclaved at 15psi for 15 minutes. All solutions used for RNA work were made with water thus treated.

Preparation of total RNA from mouse tissues

Preparation of total RNA was essentially by a modification of the method of Auffray and Rougeon (1980). A small amount of tissue (<0.4 g) was homogenised in 6ml of 6M LiCl with 3M urea, 10mM sodium acetate (pH5.2) and 0.1% SDS using a polytron homogeniser. The resulting suspension was incubated on ice, at 4°C for approximately 16 hours before centrifugation for 20 minutes at 12,000 rpm at 4°C. The supernatant was discarded and after airdrying for 2 minutes at room temperature, the pellet was resuspended in 750µl of 10mM Tris-HCl (pH7.4) with 0.5% SDS. Two extractions with phenol/choloform/isoamyl alcohol (25:24:1) and one with chloroform/isoamyl alcohol (24:1) were performed. For each wash the mixture was first vortexed for 10 seconds and the shaken vigorously for 10 minutes prior to centrifugation at 12,000rpm at 4°C for 20 minutes. Supernatants were removed to fresh tubes at each stage. Finally RNA was precipitated by the addition of sodium acetate (pH 5.2) to 0.3M and 2.5 volumes of absolute ethanol and incubation at -80 °C for >12 hours. RNA was pelleted by centrifugation at 4°C for 30 minutes at 12,000rpm. After the removal of the supernatant a wash with 70% ethanol was performed, centrifugation carried out again for 30 minutes at 4°C, 12,000rpm, and residual ethanol removed. The pellet was allowed to air dry for several minutes until no further traces of ethanol were visible. It was then resuspended in 50µl of RNase free water and stored at -80°C.

Northern blotting

Northern blotting was carried out essentially according to standard protocols (Sambrook *et al.*, 1994). 1.5% denaturing formaldehyde gels were prepared as follows:

1.5% (final w/v) agarose was dissolved in water prior to the addition of formaldehyde solution (37%) to a final volume of 0.174% v/v and MOPS buffer to 1X concentration. Electrophoresis was performed in 1X MOPS buffer at 70mA. Gels were pre-run prior to loading of samples, at 70mA for 10-20 minutes.

Prior to loading, samples were prepared as follows: to 60µg of RNA (in a volume not exceeding 6.75µl) the following were added: 50% v/v formamide, 10% v/v formaldehyde (37%) and 1X MOPS buffer (all percentages are of final volume). Samples were incubated at 65°C for 10 minutes to denature the RNA and then maintained on ice until loading on the gel. Formamide dye loading buffer was added to each sample to a final concentration of 10% v/v. Samples were loaded along with RNA size standards (Gibco) and gels run for approximately 4 hours or until the bromophenol blue dye front had traveled about 10-12cm. Gels were then soaked in 20X SSC for 1 hour to remove the formaldehyde prior to blotting. Blotting was performed in 20X SSC for approximately 16 hours onto Hybond-N nylon membranes (Amersham). After blotting, filters were rinsed briefly in 3X SSC to remove excess salt, allowed to air dry and baked for 10 minutes at 65°C. RNA was then fixed onto the filter by exposure to UV light for 1 minute.

Staining of RNA ladder

Sizing of transcripts on Northern blots was performed by comparison with a standard RNA ladder (Gibco). The ladder was run and blotted in parallel with experimental samples. After fixing of RNA to the hybond-N membrane, the ladder was cut from the remaining blot and markers were visualised as follows: the hybond-N strip was soaked in 10% glacial acetic acid for 10-15 minutes before soaking for a further 10-15 minutes in 0.5M sodium acetate (pH5.2) with 0.04% methylene blue. Destaining was performed with water for several short rinses until the background colour was reduced and the markers clearly visible. The ladder strip was then air-dried and reserved for later alignment with the Northern blot.

Densitometric analysis of Northern blot data

Densitometric analysis of Northern blot data was performed using the Flowgen AlphaImager 2000 Documentation and Analysis System. Density of RNA bands were standardised by linking to background, then expressed as a fraction of GAPDH. Density values were compared by oneway ANOVA.

Reverse-transcription PCR

Reverse transcription reactions were performed in a final volume of 20µl as follows: 1-5µg of total RNA and 500ng of Oligo $(dT)_{12-18}$ (Gibco) were diluted to 12µl with distilled water. The mixture was incubated at 70°C for 10 minutes, then placed on ice for 2 minutes. 4µl of 5X first strand buffer, 2µl of 0.1M DTT (final concentration 10mM) and 1µl of 10mM dNTPs (final concentration 0.5mM each) were added and the mixture incubated at 37°C for 2 minutes prior to the addition of 1µl (200U) of M-MLV reverse transcriptase. The reaction was then incubated for 1 hour at 37°C and heat inactivated by incubation at 70°C for 15 minutes. This reaction containing first strand cDNA was reserved at -20°C until it was needed.

PCR reactions were performed to generate the second strand of the cDNA molecule. Reactions using 1, 2 and 5µl of first strand cDNA were used as template for PCR reactions. PCRs were performed using conditions appropriate for the primers used.

PROTEIN MANIPULATIONS

Protein extraction

Tissue was homogenised in 10 volumes of 1% SDS with a polytron homogeniser. The suspension was centrifuged at 13,000rpm for 1 minute at 4°C to remove debris. The supernatent was stored at -80°C until required.

Quantification of protein

The concentration of protein samples was determined using the BioRad protein assay. A standard curve of absorbance (595nm) against protein concentration was generated using a series of BSA dilutions (0.1µg/ml to 200µg/ml). Protein samples (50µl) were mixed with 1 volume of PBS followed by 18 volumes of 1X protein assay reagent

(BioRad) (giving a total volume of 1ml). Absorbance at 595nm was measured and protein concentration read from the standard curve.

Running of SDS polyacrylamide gels

Proteins were separated by gel electrophoresis using standard protocols (Sambrook *et al.*, 1994). Gels were molded in 15 cm square gel plates. 8% separating gels were prepared as follows: 0.3625M Tris-HCl pH8.8, 26.7% v/v protogel acrylamide (National Diagnostics) (8% acrylamide, 0.213% bis-acrylamide), 0.1% w/v SDS, 0.02% w/v ammonium persulphate (AMPS) and 1.3 µl/ml TEMED. After polymerisation was complete the following stacking gel was applied over the separating gel: 0.125M Tris-HCl pH6.8, 13.5%v/v protogel acrylamide (National Diagnostics) (4% acrylamide, 0.106% bisacrylamide), 0.1% w/v SDS, 0.06% w/v AMPS and 1.5µl/ml TEMED. 50µg of protein was loaded in each track, in a total volume not exceeding 100µl. Prior to loading, buffer was to added to each protein sample to give the following final concentrations: 50mM Tris-HCl pH 6.8, 2% w/v SDS, 0.1% w/v bromophenol blue, 10% v/v glycerol and 100mM DTT.

Size standards, 10 KDa to 200KDa (Gibco), were included on every gel. 50µg were loaded per track. Electrophoresis was performed at a constant voltage (either at 60V for approximately 4 hours or at 5V for approximately 16 hours) in Laemelli buffer.

SA antibody 106

The SA106 antibody was kindly provided by Dr. R. I. Norman and Dr. D. Lodwick. It is a polyclonal antibody raised against a peptide with the following sequence:
C-G-K-V-K-R-N-E-L-R-R-K-E-W-T-T

This peptide corresponds to the C-terminal 15 amino acids of the rat SA protein. In addition the peptide also has an additional C residue at the amino terminus residual from its production process.

Western blotting

SDS gels were run as outlined above. The bromophenol blue dye-front was run from the end of the gel, electrophoresis was stopped and the gel dismantled. After equilibration in transfer solution (39 mM glycine, 48mM Tris-HCl, 0.0375 % w/v SDS, 20% methanol) for 30 minutes, a Western blot was assembled as follows. A semi-dry-transfer system (LKB Bromma, 2117 Multiphor II Electrophoresis unit) was assembled according to the manufacturers instructions; 6 pieces of 3MM paper and 1 piece of Hybond-C nitrocellulose were cut to the same size as the gel. These were pre-wet with transfer solution. Both the top and bottom plate of the transfer system were moistened with transfer solution. 3 pieces of the 3MM paper were placed on the anode (the bottom plate) followed by the nitro-cellulose. The gel was placed on top of this, followed by another 3 pieces of 3MM paper. Saran wrap was then placed surrounding this stack, in order to prevent short-circuiting of current during transfer. The cathode plate was placed on top and a voltage of 60V applied for 1 hour.

After dismantling the blot system, the transferred protein was visualised on the nitro-cellulose membrane using Ponceau S red stain. Membranes were incubated at room temperature in 1X Ponceau S red stain for 5-10 minutes. The position of the protein size standard markers were noted in pencil prior to destaining by rinsing briefly 2 or 3 times with distilled water. Filters were blocked overnight in 1X PBS with 2% Marvel and rinsed briefly in water before washing 3 times for 20 minutes each in PBS with 0.1% Tween-20. The area of the filters corresponding to the protein size standards were removed and retained and the remaining tracks containing proteins exposed to primary antibody. SA antibody 106 was used at a dilution of 1/50. If a peptide block control was performed, peptide was added with the primary antibody at a concentration of 5µg/ml. Filters were incubated with primary antibody by rocking gently at room temperature for approximately 90 minutes. Three 5 minute washes were performed in PBS with 0.1% Tween-20 before exposure to the secondary antibody. Secondary antibody (DAKO, peroxidase-conjugated, goat anti-rabbit immunoglobulins, 0.25g/L) was used at a dilution of 1/5,000. Incubation was for 60-90 minutes at room temperature with gentle rocking. Three further washes were performed for 20 minutes

each in PBS with 0.1% Tween-20 followed by one wash of 10 minutes in 20mM Tris-HCl pH8.0. As the secondary antibody is light-sensitive, it was protected from the light at all times.

Results were visualised using an ECL kit (Amersham) according to the manufacturers instructions. Equal quantities of the two reagents were mixed, poured over the filters and incubated at room temperature for 5 minutes. Filters were immediately wrapped in Saran wrap and exposed to X-OmatAR film for periods of 5 seconds to one minute. After blotting, protein remaining on the gel was visualised by staining with Coomassie blue (0.25g Coomassie Brilliant blue R250 dissolved in 45% methanol, 10% glacial acetic acid in water). The gel was incubated with gentle rocking in the Coomassie blue solution for 4 hours. It was then destained by incubation overnight in the above solution minus the Coomassie blue stain. The gel was dried onto 3MM Whatman paper on a gel drier (BioRad) with a water vacuum pump at 55°C for several hours.

SCREENING OF A BACTERIOPHAGE cDNA LIBRARY

A mouse liver chromosomal DNA library constructed in the λFIX II vector (Stratagene) was screened to obtain clones containing SA gene sequences. Clones in this library contain inserts of between 9 and 22kb of chromosomal DNA of mouse strain 129/sv.

Titration of the library

As the library had been kept in storage (at -70°C) for some time, it was necessary to verify the number of viable plaque forming units (PFU) present. Dilutions of bacteriophage were plated and PFU counts performed. The manufacturer stated library concentration of $1X10^{10}$ PFU/μl was assumed in deciding dilution factors. The number of viable PFU/μl was calculated for the library after storage.

Plating of bacteriophage

10ml of NZY medium (supplemented with $MgSO_4$ and maltose to final concentrations of 10mM and 0.2%v/v respectively) was inoculated with *E.coli* P2392 and incubated overnight at 30°C shaking at 200rpm (the temperature of 30°C ensures the culture does not become overgrown). Cells were pelleted by centrifugation at 2000rpm for 10 minutes and resuspended in 5ml (half the original volume) of 10mM $MgSO_4$. The OD of the cell suspension was measured at a wave length of 600nm and adjusted to 0.5 with 10mM $MgSO_4$. An appropriate volume of diluted bacteriophage DNA was added to 200μl of cells and allowed to adsorb for 15 to 30 minutes at 37°C. The cells were then added to 3ml of 0.7% agarose (melted and cooled to 48°C) mixed and poured onto TB plates (10cm) supplemented with $MgSO_4$ and maltose as above. Plates were incubated overnight at 37°C.

Identification of bacteriophage from a library

For the initial screening of the library, bacteriophage were plated out onto 20cm square agar plates at a density of approximately $40/cm^2$ (approximately 15,000 PFU per $20cm^2$ plate). At this density lysis was not confluent but plaques were just beginning to make contact with each other after overnight incubation. As the library has an average insert

size of 15kb and the total mouse genome size is 2.25X 10^3cM, it was calculated that a total of 90,000 library clones should be screened to guarantee coverage of the entire genome. Clones were therefore plated onto 6 agar plates.

Immobilisation of bacteriophage clones on nitrocellulose membranes

The method used was essentially that given in Sambrook *et al.* (1994) and is outlined as follows. After incubation, plates were chilled for one hour to allow the top agarose to harden. Hybond-N membranes were cut to fit the plate size. For each plate 2 membranes were used; duplicate plaque lifts were performed. The first membrane was placed onto the bacteriophage and left for 45 seconds. During this time it was marked with 2 asymmetric marks, as was the plate, to allow subsequent orientation of the filter. After removal from the agarose, DNA on the filter was then treated as follows; three trays were prepared containing 3MM Whatman filter paper pre-wetted with 20X SSC, denaturing solution and neutralising solution respectively. The nitro-cellulose was placed DNA side up, on each tray sequentially for 2 minutes before rinsing with 2X SSC and allowing to air dry. The repeat filter was treated in the same manner except that it was allowed to remain in contact with the bacteriophage for 2 minutes.
After air drying, filters were further dried for 10 minutes at 65°C and then fixed by exposure to UV light for 1 minute. Agar master plates were then stored inverted at 4°C until needed (less than one week).

Hybridisation of immobilised bacteriophage DNA

DNA hybridisation was performed with a ^{32}P labeled rat cDNA probe (a 1.6 kb *Pst*I fragment from plasmid pSA1). Hybridisation was performed as for Southern blotting (see above).

Retrieval of bacteriophage from agar plates

Positive clones were identified by exposure of filters to X-ray film (X-OmatAR). Clones giving a signal on both duplicate filters were considered positive. Filters were re-aligned with the agar master plates and the area of the positive clone marked. The area of each positive clone was removed from the agar plate in the large end of a sterile

1ml Gilson tip. The agar/top agarose plug generated was placed into 500µl of SM, shaken and incubated at room temperature for 30 minutes with occasional agitation. 1µl of chloroform was added to kill the host bacteria and any contaminants.
Thus a solution of bacteriophage DNA enriched for clones containing inserts of the gene of interest was created. This solution was then titrated by plating dilutions onto 10 cm plates as above. A plate with approximately 200 well-separated plaques was chosen and a further round of screening was performed as above, this time to isolate a single well-separated plaque containing sequences of the gene of interest. Once identified, the single bacteriophage plaque of interest was removed on an agar plug into lamda diluent, as above. To ensure no contamination with other bacteriophage, one final round of plating and hybridisation was performed. A plate with approximately fifty plaques was hybridised and every clone was positive for hybridisation to the sequence of interest.

Large scale preparation of bacteriophage DNA

Bacteriophage were plated as above. A single plaque was used to inoculate 500ml of LB supplemented with 10mM $MgSO_4$. The culture was incubated overnight in a 2L flask with good aeration at 37°C. When lysis of the culture had occurred and cell debris was visible in the culture, 1ml of chloroform was added and the culture shaken for 20 minutes. The culture was then transferred to centrifuge tubes, avoiding as much of the chloroform as possible, and centrifuged at 4,000rpm for 10 minutes. To the supernatant in a clean flask was added RNAse A to 10µg/ml and DNase to 10 units/ml. This mixture was incubated at room temperature for between 30 minutes and an hour before the addition of $NaCl_2$ to 2g/100ml and PEG-8000 to 8g/100ml. Gentle stirring for between 4-16 hours allowed the precipitation of the bacteriophage. Bacteriophage were then harvested by centrifugation at 8,000rpm, 4°C, for 20 minutes. The pellet thus generated was resuspended gently by the addition of 5ml of cold lambda diluent and incubation for a minimum of 3 hours at 4°C without agitation. After brief gentle swirling a clearing centrifugation step at 2,000 rpm was performed to remove debris. Bacteriophage were then proteinase K treated as follows; EDTA was added to 20mM, followed by SDS to 0.5% and proteinase K to 50µg/ml. This mixture was then

incubated at 55°C for 1 to 2 hours. Proteins were removed by extracting twice with an equal volume of phenol and once with an equal volume of chloroform. Bacteriophage DNA was then precipitated by the addition of sodium acetate (pH5.2) to 0.3M followed by the addition of 1 volume of isopropanol and harvested by centrifugation for 10 minutes at 7,000rpm. The resulting pellet was then washed in 70% ethanol, dried and resuspended in 1ml of TE. Yield from this method is expected to be approximately 1mg of phage DNA.

ES CELL MANIPULATION

Maintenance of ES cells in culture

ES cells are notoriously difficult to grow in cell culture. They are undifferentiated cells and their undifferentiated state must be maintained if they are to subsequently produce germline chimaeras. Whilst growing ES cells in culture care must be taken at all times to supply fresh growth medium (medium should be replaced at least once a day) and to monitor growth and morphology of cell clumps. ES cells should be divided when approaching confluence. Cells should not be allowed to become fully confluent or they will differentiate. If ES cells are maintained in culture over time, they can lose their ability to contribute to the germline of chimaeras. It is therefore important to manipulate ES cells as infrequently as possible, thus maintaining a low passage number, if they are subsequently to be used for blastocyst injection.

To prevent differentiation, ES cells were grown in ES medium containing LIF, on a monolayer of MEF cells. MEF cells were previously treated with mitomycin C (see below). Mitomycin C treated MEFs were prepared one day before the passaging of ES cells giving them time to adhere to the plate before adding the ES cells. ES cell medium was pre-warmed before placing on ES cells. ES cells were plated at a ratio of approximately 1:1, ES cells to MEFs. Growth was monitored frequently and cells passaged when cell clumps became large. Both ES and MEF cells were incubated in a humidified atmosphere of 10% CO_2/90% air, at 37°C.

Preparation and culture of mouse embryonic fibroblast (MEF) cells

A 14 day pregnant female mouse was sacrificed and the foetuses removed from the womb. All membrane material was removed from the foetuses as well as the livers and eyes. Embryos were placed in a sterile cell sieve and mashed. For every 3 or 4 embryos, 10ml of MEF media was added and the resulting cell suspension incubated (37°C, 5% CO_2) in a gelatin treated 10cm plate. The following day cells were rinsed several times with PBS to remove debris. Cells were rinsed with PBS and medium changed daily until cells were confluent. This took several days. Each plate was then divided 1 in 3,

re-plated on gelatin-treated plates and allowed to grow to confluence again. Cells were then frozen in aliquots (approximately 2 aliquots per embryo).

Freezing of MEF cells

2 confluent 10cm plates of MEFs (approximately 10^6 cells each) were trypsinised, combined and resuspended to a total volume of 10ml with MEF medium. Cells were centrifuged at 2,000 rpm and resuspended in 4ml MEF freezing medium. 1ml aliquots were frozen at -80°C over night and then transferred to liquid nitrogen storage.

Gelatin treatment of cell culture plates

Prior to plating of MEF cells, plates were gelatin treated as follows. This provides an anchor allowing the cells to adhere to the surface of the plate. Sufficient 0.1% gelatin solution (Sigma) was placed in each plate to cover the bottom surface. This was allowed to stand at room temperature for a minimum of 15 minutes. Excess gelatin was removed by aspiration. The suspension of MEF cells in MEF medium was then placed in the plate and incubated at 37°C with 10% CO_2. As the MEF cells settle they adhere to the gelatin and grow attached to the surface of the culture dish.

Recovery and culture of frozen MEF cells

Cells were taken from liquid nitrogen or -80°C storage and thawed quickly in a 37°C waterbath. Freezing medium was removed by washing; cells were placed in 10ml of pre-warmed ES cell medium and centifuged for 3 minutes at 1,500rpm.. The pellet was resuspended in an appropriate volume of MEF medium, plated on gelatin treated plates and incubated at 37°C.

Trypsinisation of MEF and ES cells

Growth medium was removed from the cells which were then rinsed once in PBS. 1ml of 1X trypsin (in PBS) (Gibco) per 10cm plate was added to the cells which were then incubated at 37°C for approximately 5 minutes. Cells were resuspended by pipetting to generate a single cell suspension. When trypsinising culture plates of other sizes, the amount of trypsin added was varied accordingly. When working with ES cells, trypsin was removed by resuspending the trypsin suspension in 10ml of media, centrifuging

and resuspending in fresh medium. When working with MEF cells, cells were plated without removing the trypsin solution.

Mitomycin C treatment of MEF cells

80% confluent 10cm plates of MEFs (approximately 10^6 cells) were treated with mitomycin C (Sigma) at a concentration of 2µg/ml for between 2 and 3 hours. Cells were rinsed 3 times with 25ml of pre-warmed PBS, trypsinised, re-suspended in 10ml of MEF medium and counted using a haemocytometer. Cells were then centrifuged at 1,500rpm, re-suspended in fresh MEF medium and plated at a density of 10^6 cells per 10cm plate or equivalent (see table). Mitomycin C treated MEFs were plated on plates pre-treated with 0.1% gelatin (Sigma) for at least 15 minutes.

Size of plate	Number of MEFs
96-well	$1.4X\ 10^4$
48-well	$4X\ 10^4$
24-well	10^5
12-well	$2.5X\ 10^5$
6-well	$4X\ 10^5$
60 mm	$5\text{-}6X\ 10^5$
10 cm	10^6

Preparation of plasmid DNA for electroporation

DNA for transfection experiments was prepared by Qiagen maxi-prep kit following the manufacturer's instructions. This method yielded up to 500µg of plasmid DNA from a 500ml bacterial culture containing a low copy number plasmid. Concentration of plasmid DNA was determined by spectrophotometry. 120µg of plasmid DNA was linearised by restriction digestion with *Not*I. DNA was cleaned by extraction twice which phenol and once with chloroform/isoamyl alcohol (24:1), precipitated by the addition of 1/10 volume of 3M sodium acetate (pH5.2) and 2 volumes of ethanol, harvested by centrifugation at 13,000rpm for 15 minutes and washed several times with

70% ethanol to remove any remaining salt. DNA was resuspended in 50µl and concentration determined again by spectrophotometry.

ES cell transfection by electroporation

Four confluent 10cm plates of ES cells (approximately 4-5 x 10^6 cells) were trypsinised, washed in 1X PBS and resuspended to a final volume of 1.6ml in PBS. 60µg of plasmid pVaX6 DNA (linearised with *Not*I) was added and the mixture incubated at room temperature for 5 minutes. Cells were electroporated in 1ml cuvettes as follows: 200V, 500µFD capacitance (a BioRad electroporation system was used). After electroporation the time constant was monitored to be in the region of 6.0. Cuvettes were incubated for a further 5 minutes at room temperature prior to resuspending in 41ml of ES medium and plating on a mono-layer of mitomycin C treated MEFs. Cells were plated onto 4 x 10cm plates (10ml each) and 1 x 6cm plate (1ml). Cells were allowed a recovery period of 24 hours before the application of G418 at a concentration of 330µg/ml. Two days after the application of G418, gancyclovir was applied to the 4 x 10 cm experimental plates at a concentration of 2µM (the 1cm plate was left without gancyclovir as a control for the negative selection).

Picking of potentially targeted ES clones

Discrete clones with good morphology were chosen for picking. Approximately 400 clones were picked for each transfection experiment. Each clone was taken from the feeder layer in approximately 3µl of ES medium, added to 30µl of 1X trypsin and incubated at 37°C for 5 minutes. Cells were pipetted to give a single cell suspension and each clone transferred to a 48-well plate (onto a monolayer of treated MEFs). Growth of clones was monitored over several days. When clones became confluent they were frozen as follows; ES medium was removed, the well washed with 1X PBS and 75µl of 1X trypsin added. After incubation for 5 minutes cells were pipetted to create a single cell suspension. 50µl of Hyclone serum was added, giving a total volume of 125µl. 100µl of the mix was transferred to a 96 well plate on ice, 100µl of 2X freezing mix was added. After mixing, 50µl of paraffin oil was added to seal the well. Up to 48 clones were frozen per 96 well plate at -80°C. The remaining 25µl of

cell suspension was added to ES medium in a gelatin treated 12 well plate. Cells were incubated at 37°C until confluent and used for DNA extraction.

Isolation of chromosomal DNA from ES cells

ES cells for DNA preparation were not grown on a feeder layer as morphology was not important at this stage. A confluent 12-well plate was washed with 1X PBS and 500µl of ES cell lysis buffer added. Digestion was at 37°C overnight. 1ml of 100% ethanol was added and DNA harvested by spooling with a Gilson tip. DNA was rinsed briefly in 70% ethanol, allowed to air dry and resuspended in 500µl of TE (pH 8.0).

Recovery of ES clones from frozen storage

Cells were taken from liquid nitrogen or -80°C storage and thawed quickly in a 37°C water bath. Freezing medium was removed by washing; cells were placed in 10ml of pre-warmed ES cell medium and centrifuged for 3 minutes at 1,500rpm. The pellet was resuspended in an appropriate volume of ES medium and plated on pre-prepared mitomycin C treated MEF cells.

Preparation of ES cells for blastocyst injection

ES cells were grown on mitomycin C treated MEF cells on a 6-well plate. When 70% confluence was reached (i.e. the cells were actively growing and dividing) they were trypsinised, resuspended in 10 ml of ES medium, pelleted (at 1,500rpm. for 3 minutes) and resuspended in 500µl of CMRL 1066 (supplemented with 20% Hyclone FCS, 2mM L-glutamine, 1mM sodium pyruvate, 100U/µl penicillin and 100µg/µl streptomycin). From this point cells were kept on ice at all times.

Blastocyst injection

Injection of blastocysts with ES cells and their subsequent implantation into pseudo-pregnant females was performed as part of a service provided by the Biomedical Services Unit at the University of Leicester.

GENOTYPING OF SA-NULL MICE

A piece of tail approximately 0.5cm in length was removed from the end of the mouse's tail. This was immediately chopped finely using a razor blade and placed in TNE buffer. DNA was extracted (see above). For each sample, 2 separate PCRs were performed, one amplifying a fragment of exon 2 of the SA gene (only present in mice carrying the wildtype SA allele), the other amplifying a fragment of the neomycin resistance gene (only present in mice carrying a copy of the targeted SA gene). By considering both these results together it was possible to distinguish wildtype mice from both SA null mutants and mice heterozygous for this mutation. Details of these PCR reactions are given below.

Primer pair	Denaturing	Annealing	Extension	Cycles	Product size
OCP49/OCP50	95°C, 1 min	58°C, 1 min	72°C, 30s	30	504bp
MSA12/MSA17	95°C, 1 min	58°C, 1 min	72°C, 12s	30	95bp

BLOOD PRESSURE MEASUREMENT AND PHYSIOLOGICAL STUDIES

Indirect blood pressure measurement

Johns *et al.* (1996) validated the method we have used in this study for the indirect measurement of mouse blood pressure. He compared indirect blood pressure readings with direct pressures obtained by intra-arterial measurement of the iliac artery. Readings by the two methods were found to correlate well. This method of indirect blood pressure measurement uses a pulse amplifier and computerised blood pressure monitor (available from IITC) to measure blood pressure photoelectrically by recording the cuff pressure when the occluded blood flow returns to the tail.

For each reading the tail cuff was inflated to a pressure of 150mmHg using the automated inflation setting on the pulse amplifier. Pressure is maintained automatically at 150mmHg for several seconds before gradually releasing. Two traces, one of cuff pressure, the other of pulse, are produced on the computer monitor. If the return-of-pulse point is obvious on the second trace it can be recorded. In certain cases 150mmHg was insufficient cuff pressure to occlude blood flow in the tail. In these instances the cuff was inflated manually to approximately 180mmHg and gradually deflated manually. Pressure was held at 180mmHg just long enough to occlude blood flow as such a high pressure can damage a mouse's tail if maintained for longer periods.

Prior to blood pressure measurement each mouse was acclimatised to the blood pressure monitoring equipment on two occasions. On day one, mice were placed in the restrainers for approximately 3 minutes before returning to their cages. The following day they were restrained for 6 minutes. Blood pressure measurement commenced the following day. Restrained mice were pre-warmed to 32°C for approximately 5 minutes prior to blood pressure measurement and were maintained at this temperature throughout the procedure. For each mouse, blood pressure measurements were taken on three separate consecutive days. On each day approximately ten successful blood pressure readings were obtained. All blood pressure readings were taken between the times of 9am and 6pm. An average blood pressure for each mouse was then calculated.

12 week readings were taken within 3 days of the 12 week age point; likewise for 16 week readings.

Administration of dihydrotestosterone (DHT)

DHT was administered to 12 week old female mice as a subcutaneously implanted pellet. Pellets and placebo pellets were obtained from American Research International and were designed to release 0.02mg of DHT day per day for 21 days. Surgery was performed under halothane anaesthetic. The back of the mouse's neck was shaved and a small incision (3mm in diameter) was made approximately 8mm behind the right ear. A pocket was made between the skin and the peritoneal lining and the pellet was positioned with forceps at the side of the neck behind the left ear, approximately 1cm from the incision site. The incision site was closed with a metal suture clip. This clip was removed after 7-10 days when the wound was fully healed.

Administration of high salt intake

1.5% NaCl was dissolved in tap water and offered to the mice *ad libitum*, in place of tap water. Mice were maintained on a diet of standard 0.7% rat chow (RM1 from Special Diets Services).

Measurement of fluid intake

Water bottles were weighed prior to placing in the mice's cages. They were re-weighed prior to refilling. Water consumption was measured over several days and the average daily consumption per mouse calculated.

Tissue collection

Mice were culled by cervical dislocation. Organs were removed immediately and flash frozen in liquid nitrogen. Organs were weighed whilst still frozen prior to use for RNA extraction.

COMMONLY USED SLOUTIONS

ddH_2O	Distilled and sterile water
TE	10mM Tris-HCl, 1mM EDTA, pH7.6
SSC (20X)	3M NaCl, XM sodium citrate, pH 7
PBS (20X)	2.6M NaCl, 60mM Na_2HPO_4, 140mM NaH_2PO_4, pH 7.4
TBS (20X)	0.05M Tris-HCl, 0.15M NaCl, 0.1% (w/v) BSA, pH 7.65
TAE Buffer (50X)	2M Tris-HCl, 1M Glacial Acetic Acid, 0.005M EDTA, pH 8.0
TBE Buffer (10X)	108g Tris-HCl, 55g Boric Acid, 40ml 0.5M EDTA
TNE	150mM NaCl, 100mM EDTA, 50mM Tris-HCl, pH8
MOPS buffer (10X)	200mM MOPS, 50mM sodium acetate, 10mM EDTA, pH 7
Neutralising solution	1.5M NaCl, 0.5M Tris-HCl, 0.001M EDTA, pH7.2
Denaturing solution	0.5M Sodium hydroxide, 1.5M NaCl
Agarose loading buffer	0.025% Bromophenol blue, 0.025% Xylene cyanol, 2.5% Ficoll in water
PCR buffer	45mM Tris, 11mM ammonium sulphate, 4.5mM magnesium chloride, 6.7mM β-mercaptoethanol, 4.4mM EDTA, 113µg/ml bovine serum albumin, 1mM each dNTP, pH8.8
Denhardts solution (50X)/500ml	5g Ficoll, 5g polyvinylpyrrolidine, 5g BSA (fraction V)
PAGE loading buffer	95% formamide, 20mM EDTA, 0.05% Bromophenol blue 0.05% Xylene cyanol
SDS-PAGE sample buffer (2X)	0.05M Tris-HCl, 4% SDS, 20% glycerol, 0.2% bromophenol blue, 1.4M β-mercaptoethanol, pH 6.7.
Laemelli buffer (10X)	0.25M Tris-HCl, 0.192M glycine, pH8.3
Ponceau Red solution (10X)/100ml	2g Ponceau S, 30g trichloroacetic acid, 30g sulphosalicylic acid
TBS-T	As TBS, without BSA, and with 0.1% Tween-20.

Lambda diluent per litre	0.1M NaCl, 10mM MgSO4, 50mM Tris-HCl, 0.01% gelatin, pH 7.5
Blocking solution	TBS-T and 5% dried skimmed milk powder
NZY Agar/litre	5g NaCl, 2g $MgSO_4.7H_2O$, 5g yeast extract, 10g NZ amine, pH7.5
Formamide dye loading buffer	50% gycerol, 1mM EDTA (pH8), 0.25% bromophenol blue, 0.25% xylene cyanol.
TB medium/litre	5g NaCl, 10g bactotryptone, pH7.4
ES cell medium	DMEM (with 4,500mg/ml D-glucose) supplemented with the following:15% foetal calf serum (Hyclone), 20mM HEPES buffer, 2mM L-glutamine, 100U/µl penicillin, 100µg/µl streptomycin, 1mM sodium pyruvate, 0.1mM non-essential amino acids, 0.11mM β-mercaptoethanol, 2500U/ml recombinant LIF (ESGRO)
MEF medium	DMEM (with 1,500mg/ml D-glucose, Gibco) supplemented with the following:10% foetal calf serum, 2mM L-glutamine, 100U/µl penicillin, 100µg/µl streptomycin, 0.11mM β-mercaptoethanol
ES cell freezing mix (2X)	20% Dimethyl sulphoxide (DMSO), 40% foetal calf serum, 40% ES cell medium
MEF freezing mix	20% Dimethyl sulphoxide (DMSO), 40% foetal calf serum, 40% MEF cell medium
ES cell lysis buffer	50 mM Tris-HCl, 1mM EDTA, 100mM NaCl, 0.2% SDS, 100µg/ml proteinase K
LB/litre	10g bactotryptone, 5g yeast extract, 10g NaCl, 1g glucose, (15g agar/litre for plates).

OTHER MATERIALS

Rat SA 1.6kb cDNA clone, pSAF1

The region of rat cDNA included in this clone is bounded by a 5' *SstI* site (located in exon 2) and a 3' *StuI* site (located in exon 14). This fragment is cloned into pBluescript vector and was kindly supplied by Simon Frantz, Department of Cardology. The size of pSAF1 is approximately 4.6kb, containing approximately 1.6kb of cloned SA cDNA.

Primers

Rat SA primer sequences were supplied by Simon Frantz (Dept. Cardiology). OCP49 and OCP50 primer sequences were supplied by Catrin Pritchard (Dept. Biochemistry). KS1, KS2, MSA12 and MSA17 primer sequences were designed using the Primer 3 package (http://www -genome.wi.mit.edu). Default settings were used on both occasions. All primers were supplied by Leicester University Proteins and Nucleic Acids Chemistry Laboratory (PNACL). Primers were supplied in solution and required de-salting by ethanol precipitation prior to use. To 1 volume of primer solution, 1/10volume of 3M sodium acetate (pH5.2) was added followed by 2 volumes of ethanol. Precipitation was performed for 2 hours at -20°C. Precipitated primers were pelleted by centrifugation at 13,000rpm for 15 minutes at 4°C, washed in 70% ethanol and resuspended in 1 volume of distilled water. Quantification of primers was by spectrophotometry.

Miscellaneous primer sequences

Primer	Sense	Sequence (5' to 3')
MSA12	Sense	AGATCACCGACTCGTGAGGT
MSA17	Anti-sense	GGTGGCTAGGCATCATGCTG
OCP49	Sense	GGTTCTCCGGCCGCTTGGGTGGAG
OCP50	Anti-sense	GCGCGCCTTGAGCCTGGCGAACAG
KS1	Sense	CGGAGACTCTGGAGAGTTGG
KS2	Anti-sense	GTCTGTCCGTCCCATGAAGT

Rat SA primer sequences

Primer	Sense	Sequence (5' to 3')	exon
SA1	Sense	TGGCTTTCTCTCCATTAAG	1
SA4	Anti-sense	AAAAGTTCTGAGGAATCGTGTTCTGTAAT	3
SA5	Sense	GGTGATTCTGCCCAAGATCCCAGAGTGGTGG	4
SA6	Anti-sense	CACCACTCTGGGATCTTGGGCAGAATCACC	4
SA10	Anti-sense	CTGGAGTGCTGAGACACAAT	5
SA12	Sense	AGATCACTGACTTGTGAGCT	2
SA14	Sense	GTAGTCTGCATGGAAATCTC	3
SA15	Sense	CTTTCCAATCCAGCCTTCTG	4
SA17	Anti-sense	GGTGGTTAGGCATCGTGTTG	2
SA41R	Anti-sense	AAGATTCTCCTGGG	3
SAEX2	Anti-sense	GGGCCAAGTTTAAGTTGTTG	15

***E. coli* strains**

Strain	Genotype
E. coli LE392	hsdR514, supE44, supF58, lacY1or Δ(lacIZY)6, galK2, galT22, metB1 trpR55, mcrA
E. coli P2392	P2 lysogen of LE392
E. coli NM522	F′ endA+ lacI^q Δ (lacZ)M15 proA$^+$B$^+$/supE thi Δ (lac-proAB) Δ (hsdMS-(mcrB)5 ($r_k^-m_k^-$ McrBC$^-$)
E. coli TG1	F′ traD36 lacI^q Δ (lacZ)M15 proA$^+$B$^+$/supE Δ (hsdM-mcrB)5 (r_k^- m_k^+McrB$^-$) thi Δ (lac-proAB)
E. coli DH5 α (Gibco)	F′ X80dlacZΔM15Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(r_K^-,m_K^+), phoA, supE44, λ$^-$ thi-1, gryA96, relA1

Chemical reagents and enzymes

All chemicals used in this work were 'Analar' grade, from Sigma, Fisher Scientific, or ICN. Most restriction and DNA modification enzymes were supplied by Gibco with the following exceptions; Taq polymerase was supplied by Bioline and TdT by Pharmacia. ^{32}P dCTP was from ICN. 2'-Deoxynucleoside 5'-Triphosphates (dNTPs) were from Pharmacia.

Tissue culture reagents

All reagents were supplied by Gibco with the exception of the following; Hyclone FCS (supplied by Hyclone), DMSO and β-mercaptoethanol supplied by Sigma and gancylovir kindly provided by Catrin Pritchard (Dept. Biochemistry).

Cell lines

ES cell line E14TG2a was kindly provided by Dr. Catrin Pritchard (Dept. Biochemistry). MEF cells were prepared as primary cultures from A-raf:b-geo transgenic mouse lines (Luckett *et al.*, 2000).

Bacteriological reagents

Tryptone, yeast extract and BactoAgar were purchased from Difco.

Mouse diets

Diets were supplied by Special Diets Services. Mice were routinely maintained on Rat and Mouse No. 1 Maintenance (RM1) which contains 0.25% sodium, corresponding to approximately 0.7% sodium chloride. Breeding pairs were maintained on Rat and Mouse No. 3 Breeding Diet (RM3) which contains 0.32% sodium. Pups were weaned using RM1 moistened in tap water.

Chapter 3
Targeting of
the mouse SA gene

CHAPTER 3: Targeting of the mouse SA gene

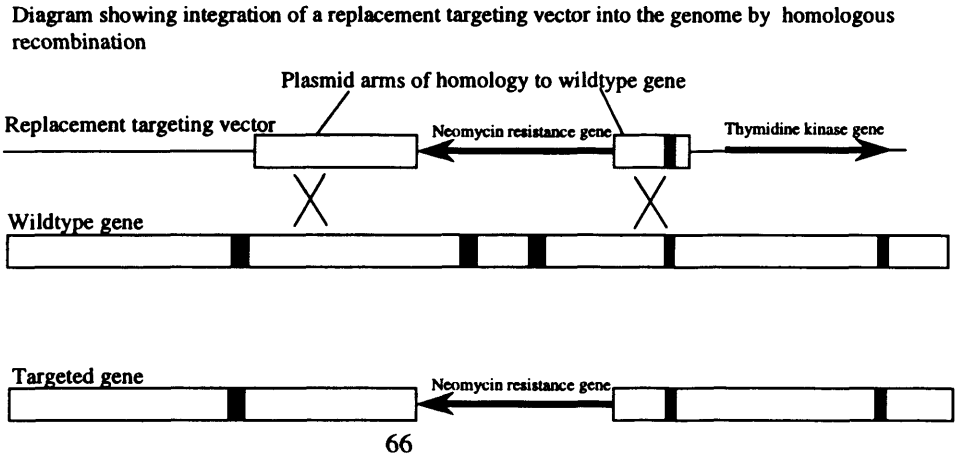
Mapping of the mouse SA gene and construction of targeting vectors

At the time this work was undertaken, sequence data for the human and the rat SA cDNAs had been published (Iwai *et al.*, 1991; Iwai *et al.*, 1994), however the mouse SA cDNA sequence was not available. It has since been submitted to Genbank with the accession numbers AF068246 (Melia *et al.*, 1998) and AB022340 (Takenaka *et al.*, 1998).

In order to construct a targeting vector for the SA gene, more information was needed about its chromosomal structure, specifically in the mouse. A restriction map of the full-length gene was constructed and this was utilised in designing targeting vectors. Prior to this work, no restriction map for the chromosomal SA gene was available in any organism although intron sizes within the rat gene were known (Frantz, personal communication).

In the design of our gene targeting experiment we utilised a replacement vector to introduce a specific mutation into the SA gene. A targeting vector was constructed including 2 homologous arms of DNA from the mouse SA gene (strain 129/sv). These were separated by a selectable marker, the neomycin resistance gene. As a means of negative selection, the *Herpes simplex* thymidine kinase gene was also present in the plasmid (Fig. 3.1).

FIG. 3.1 Integration of a classic replacement vector into the chromosome by homologous recombination



Several factors were taken into consideration in the design of replacement targeting vectors:

The SA gene is approximately 26kb in length. There have been reports of the use of homologous recombination to remove large sections of chromosomal DNA for certain loci, however a much higher recombination frequency is obtained when shorter sections of DNA are removed. Therefore in our experimental strategy we aimed to remove only a portion of the SA gene. A frequently employed strategy in gene ablation experiments is the elimination of the active site of the protein. However as very little is known of the structure or function of the SA gene product this was not possible here.

A vector was therefore designed to result in the elimination of the amino terminus of the protein. Removal of exons 2 and 3 eliminated the ATG translation initiation codon (located in exon 3). It was hypothesised therefore that if, despite the elimination of these 2 exons, a message was still produced, initiation of protein synthesis would be unlikely to occur. Synthesis of a truncated protein would require the presence of an alternative initiation codon (with a Kozak consensus sequence) inframe with the remainder of the protein. In addition this sequence would need to occur upstream of any possible out-of-frame translation initiation sites. It was hypothesised that if an SA mRNA were to be generated in an experiment of this design, coding sequences would begin at the start of exon 4. It was noted that from this point, no potential translation initiation codons were present with high levels of homology to the Kozak consensus sequence (Kozak, 1991). Generation of a truncated SA message was possible in this model due to the splicing of exons 1 to exon 4.

Screening of clones for targeted versus random integration events

Southern blotting and PCR are commonly used methods for screening ES cell clones for correctly targeted mutations. PCR analysis offers the advantage of speed; this is especially useful as screening of a large number (200-400) of clones is usually necessary. However when PCR is to be used as the method of screening, it is usually necessary to limit the size of one of the arms of homology of the targeting vector to a

maximum of 1kb. Screening by Southern blotting, although more laborious, allows the use of a targeting vector with arms of homology of equal length. This gives the advantage of increasing the frequency of homologous recombination compared to vectors with arms of unequal length. In addition the likelihood of aberrant chromosomal rearrangements is reduced. Southern blotting was therefore our chosen method of screening ES cell clones.

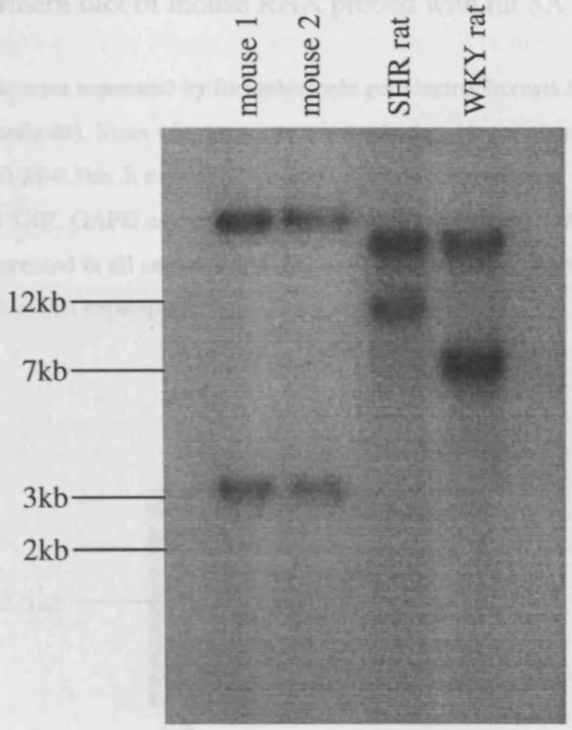
RESULTS

Hybridisation between a rat SA cDNA probe and mouse DNA sequences

At the time this project was undertaken, no work had been published on the mouse SA gene. This experiment was performed to confirm the presence of the SA gene in the mouse and to show that hybridisation can occur between the rat and mouse homologues of this gene. Mouse chromosomal DNA from strain BalbC was restriction digested, Southern blotted and hybridised with a 1.6 kb *Pst*I fragment of rat cDNA from plasmid pSA1 (see materials). Filters were washed at 65°C, in 3X SSC for 15 minutes, 1X SSC for 15 minutes and 0.5X SSC for 15 minutes. It can be seen in Figure 3.2 that even at this high stringency of washing, hybridisation occurs between the mouse chromosomal DNA and the rat SA cDNA probe. This indicated the presence of a mouse homologue of the SA gene with sequence homology to the rat SA gene.

FIG. 3.2 Southern blot of mouse and rat genomic DNA restriction digested with *Bgl*II and hybridised with the rat SA cDNA probe

In this experiment restriction fragments were separated by agarose gel electrophoresis and immobilised on a nylon membrane by Southern blotting (see above and methods). 1kb ladder (Gibco) was used a size standard here and in all further experiments utilising the technique of agarose gel electrophoresis. In all 4 samples 2 fragments are hybridised by the 1.6kb rat partial cDNA fragment indicating that *Bgl*II recognises sequences within the chromosomal copy of the SA gene. It can be noted that the sum of the fragment sizes varies between samples and does not correspond to the total size of the SA gene. This is because positions of the *Bgl*II sites peripheral to the SA gene vary between samples and because a partial cDNA was used as a probe in this experiment.

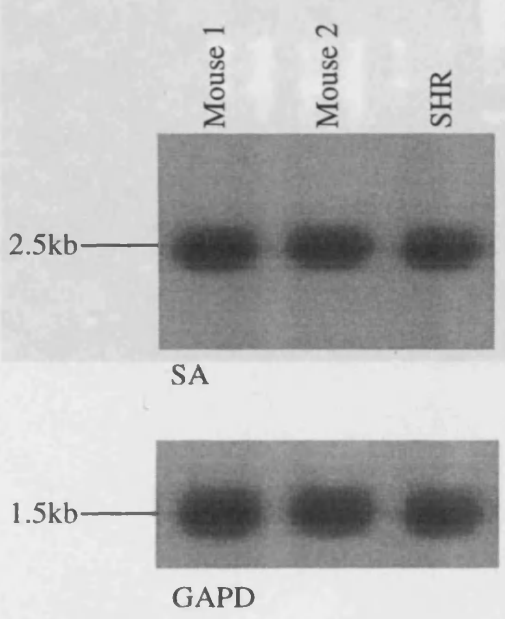


Hybridisation between a rat SA cDNA probe and mouse SA RNA

This experiment was performed to confirm the expression of the SA gene in mouse tissues. A Northern blot of total kidney RNA from male mice (strain MF1) was hybridised with a 1.6 kb *Pst*I fragment of rat cDNA from plasmid pSA1. SHR kidney total RNA was also hybridised as a positive control. Washes were performed at 42°C in 3X SSC for 15 minutes, 1X SSC for 15 minutes and 1X SSC for 15 minutes. It can be seen that there is strong hybridisation between mouse kidney RNA and the rat cDNA probe (Fig. 3.3). A band of 2.5kb can be seen, indicating a single transcript of the SA gene in the kidney of this species of mouse. The level of expression is comparable to that of SHR.

FIG. 3.3 Northern blot of mouse RNA probed with rat SA cDNA

RNA fragments were separated by formaldehyde gel electrophoresis following the protocol for Northern blotting (see methods). Sizes of transcripts were calculated by comparison with Gibco RNA standards in the size range 0.24-9.5kb. It can be noted that only one SA transcript is present in the kidneys of both MF1 mice and SHR. GAPD expression levels were also observed. GAPD is a housekeeping gene which is similarly expressed in all samples and therefore demonstrates that the same quantity of each sample RNA was used in this experiment.



Isolation of mouse SA gene clones from a lambda FIX II bacteriophage library

A commercial library (Stratagene) was used for the identification of SA gene clones. This library contained genomic inserts of mouse strain 129/sv in a λFIXII vector. The library was screened in *E. coli* strain P2392 using a 1.6kb *Pst*I fragment of rat cDNA from pSA1. Six clones hybridised with the rat SA gene probe; these were designated PH1, PH2, PH4, PH5, PH6 and PH7.

Restriction mapping of the Mouse SA gene

Insert sizes within the bacteriophage clones were determined by *Not*I digestion to release the mouse chromosomal DNA insert. As expected each clone had an insert in the 9-22kb range indicated by the library manufacturer (Figs. 3.4 and 3.5).

FIG. 3.4 Agarose gel of phage clones restriction digested with *Not*I

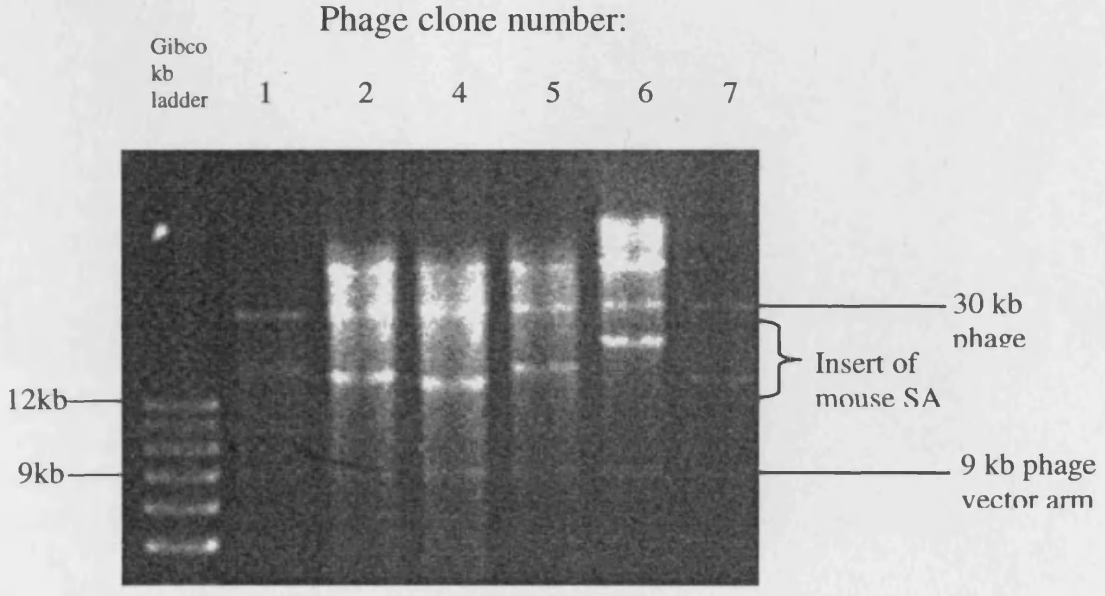
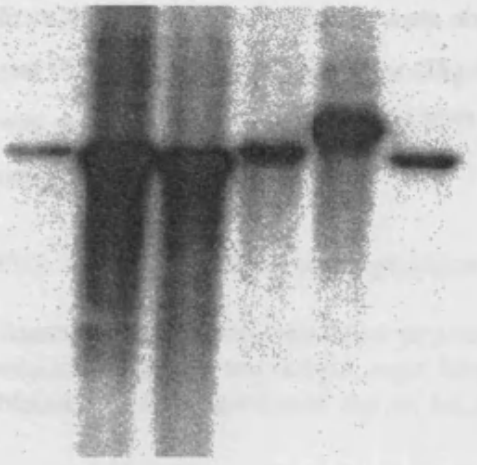


FIG. 3.5 Southern blot of the above gel hybridised with the 1.6kb rat cDNA probe

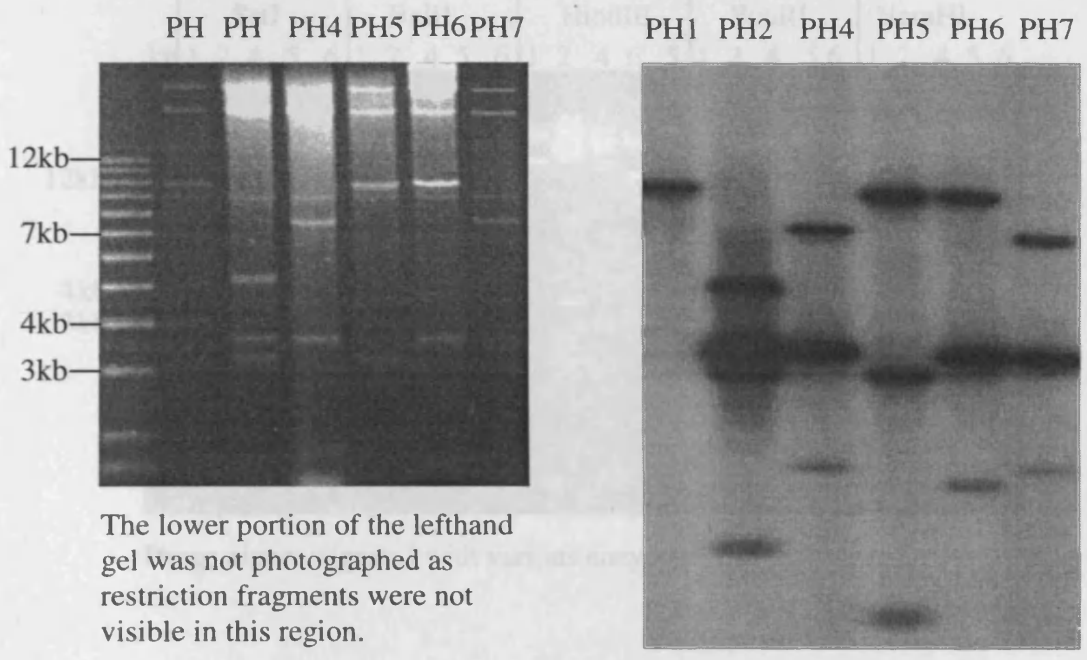


12-18kb inserts of mouse SA gene DNA excised with *Not*I and hybridised with the rat SA cDNA probe

As several of the insert sizes were quite similar further analysis was carried out by *Xba*I digestion. This enzyme restricts the insert DNA as well as excising the insert from the vector. Similar bands were observed in each clone corresponding to the 30kb and 9kb arms of the phage vector (Fig.3.6). In addition, each insert was also cleaved. It was apparent that clones PH4 and PH7 were identical; no further analysis was carried out on PH7.

FIG. 3.6 *Xba*I digestion of phage clones

Bacteriophage clones restriction digested with *Xba*I. Left hand figure shows ethidium bromide stained gel; right hand figure shows the same gel Southern blotted and hybridised with the rat SA cDNA probe.



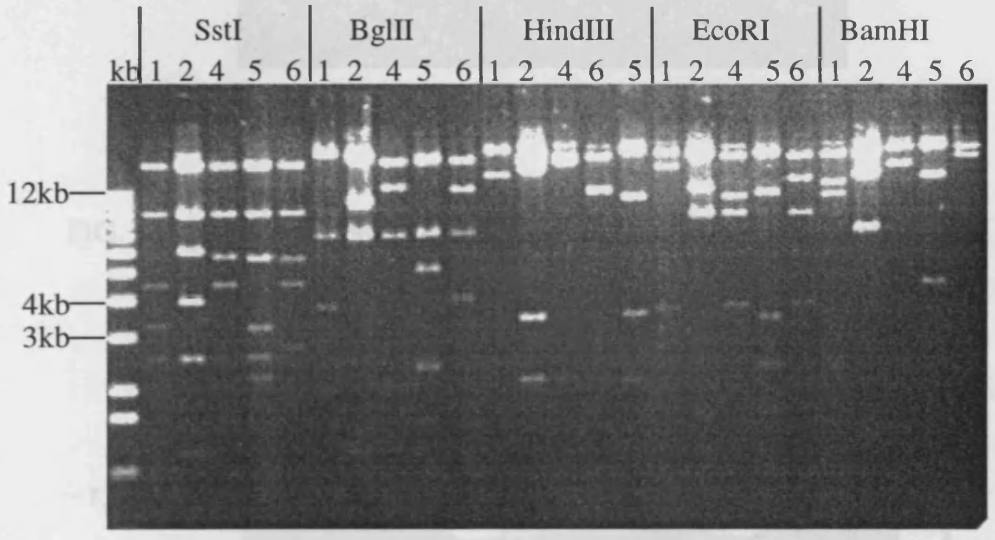
The lower portion of the lefthand gel was not photographed as restriction fragments were not visible in this region.

Orientation and restriction analysis of 5 clones representing the entire SA gene

The 5 phage clones were further analysed by restriction digestion with the following enzymes: *Eco*RI, *Hin*dIII, *Bam*HI, *Sst*I, *Xba*I and *Bgl*II. Single digestions were compared to digestions of each enzyme used in conjunction with *Not*I (which excises the insert sequences from the vector) (Figs. 3.7 to 3.10). Fragments that were apparent in the double digests but absent in the single digests were placed on the restriction map

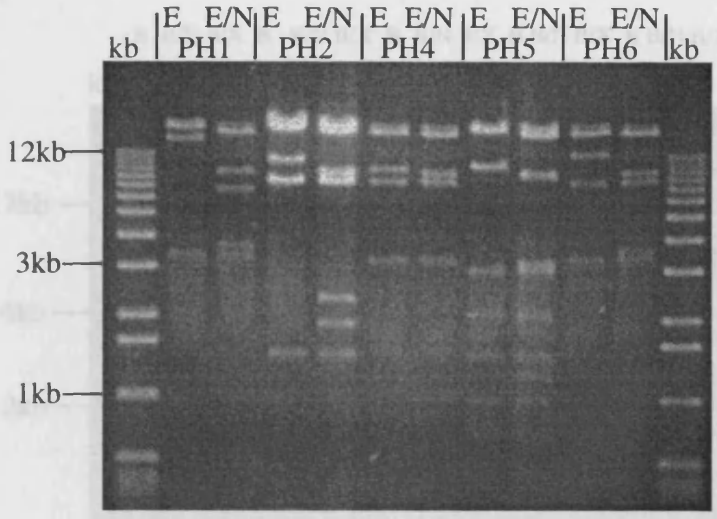
at the extremities of each insert. Comparison of fragment sizes between each clone pointed to regions of overlap between clones. Sizes of bands were calculated and a restriction map generated to include the relationship between the clones. All agarose gels were Southern blotted and probed with the 1.6kb rat cDNA probe as this often resolved much smaller bands than were visible on the gel. Difficulty in visualising small bands is due to the large size of the phage vector (30kb). Small bands constitute a very low percentage of the total DNA loaded per track. In addition, hybridisation of each gel with the rat cDNA probe gave us some indication which fragments were of entirely intronic origin, as these were not hybridised.

FIG. 3.7 Gel showing phage clones digested with various enzymes



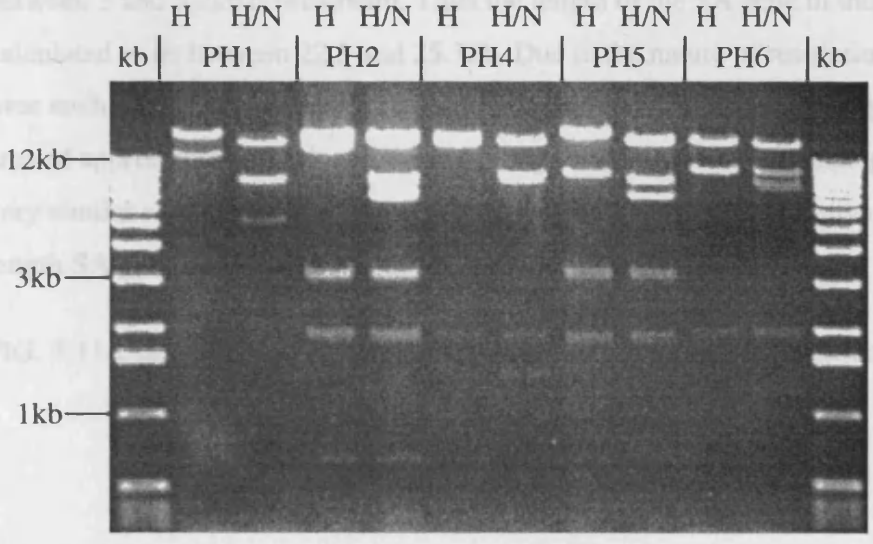
Phage clones digested with various enzymes.

FIG. 3.8 Gel showing phage clones digested with *Eco*RI and *Not*I



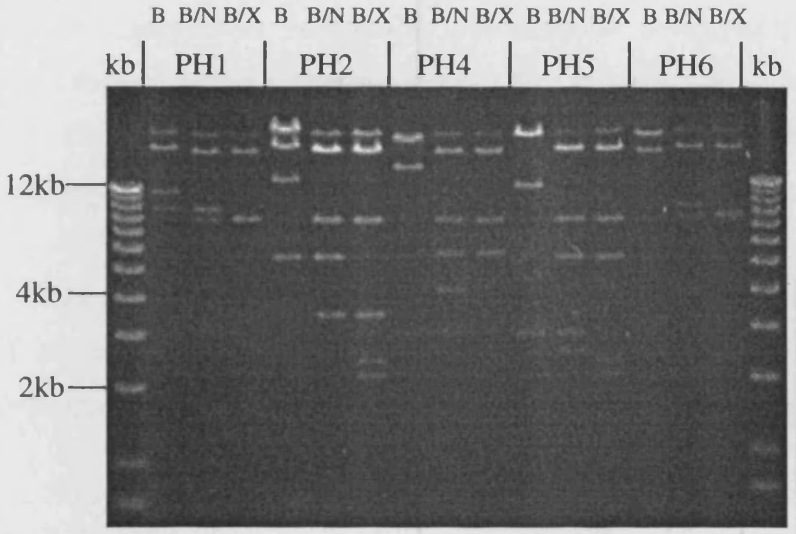
Phage clones digested with *Eco*RI and *Eco*RI/*Not*I (as double digests)

FIG. 3.9 Gel showing phage clones digested with *Hin*dIII and *Not*I



Phage clones digested with *Hind*III and *Hind*III/*Not*I (as double digests)

FIG. 3.10 Gel showing phage clones digested with *Bam*H1, *Xba*I and *Not*I

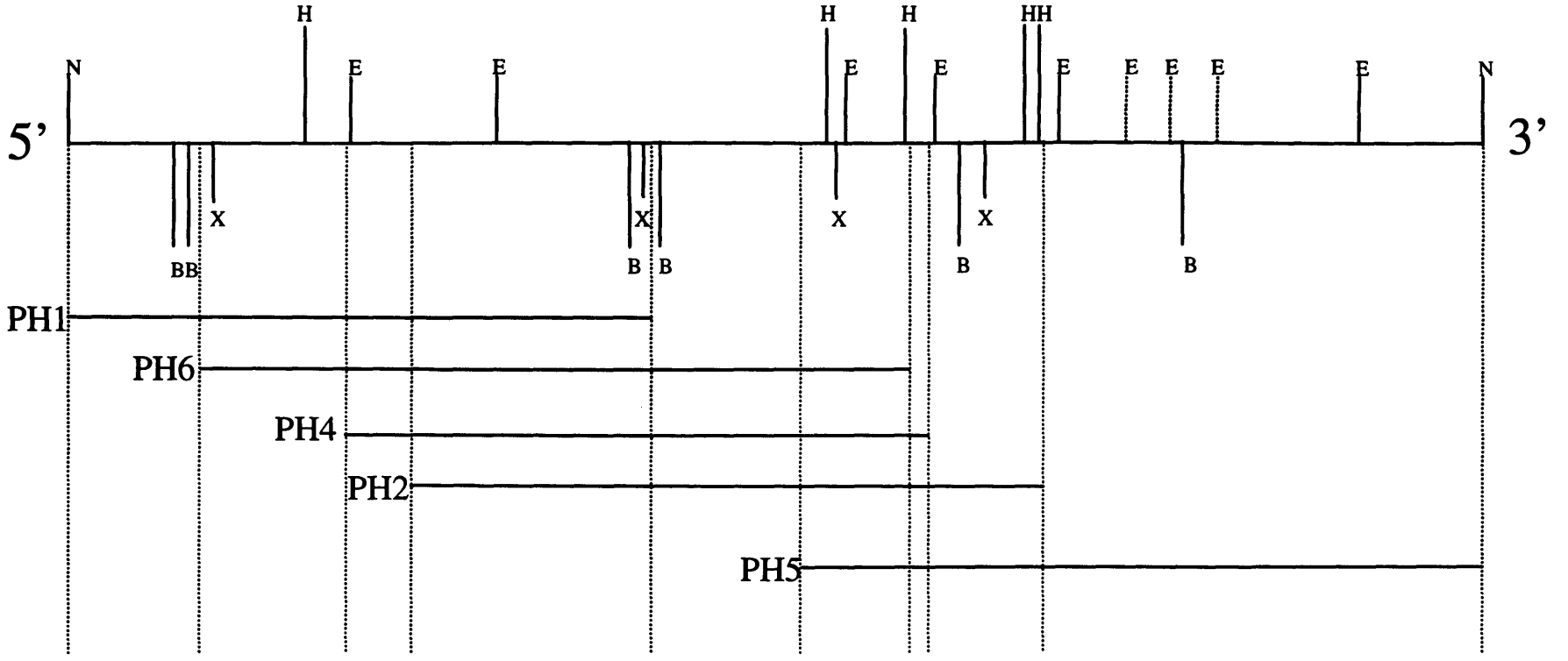


Phage clones digested with combinations of *Bam*HI, *Not*I and *Xba*I

The 5 clones cover an area of approximately 31kb of mouse chromosomal DNA. This includes the entire SA gene as well as an area of between 2.7 and 3.3kb upstream and between 3 and 5.2kb downstream. Thus the length of the SA gene in the mouse was calculated to be between 22.5 and 25.3kb. Due to the nature of restriction mapping over such a large area, a degree of inaccuracy is inevitable. The rat SA gene spans an area of approximately 26kb and these findings indicate the mouse analogue to be of a very similar size. A diagram of the position of each phage clone relative to the full-length SA gene can be seen in figure 3.11.

FIG. 3.11 Diagram showing relationship between the 5 bacteriophage clones

Restriction map of the region covered by the 5 bacteriophage clones



Localisation of exons one to four on the mouse SA restriction map and confirmation of the presence of the 3' region

A specific probe was generated for each of exons 2, 3 and 4 by PCR amplification of rat DNA with rat primers (see Methods). These probes were hybridised against Southern blots of the 5 bacteriophage clones digested with various enzymes (Figs. 3.12 to 3.14). Similarly the oligonucleotide SA1 was used to localise exon one (Fig. 3.15) and oligonucleotide SAEX2 was used to confirm the presence of the 3' end of the gene (Fig. 3.16). It can be noted that only bacteriophage clone PH1 contains sequences hybridising to the exon 1 specific probe corresponding to the 5' end of the gene (Fig. 3.15). Similarly only PH5 contains sequences hybridising to the exon 15 specific probe corresponding to the 3' end of the gene (Fig. 3.16).

FIG. 3.12 Southern blots for the localisation of exons 2; phage clones cut with various enzymes and hybridised with an exon 2 specific probe.

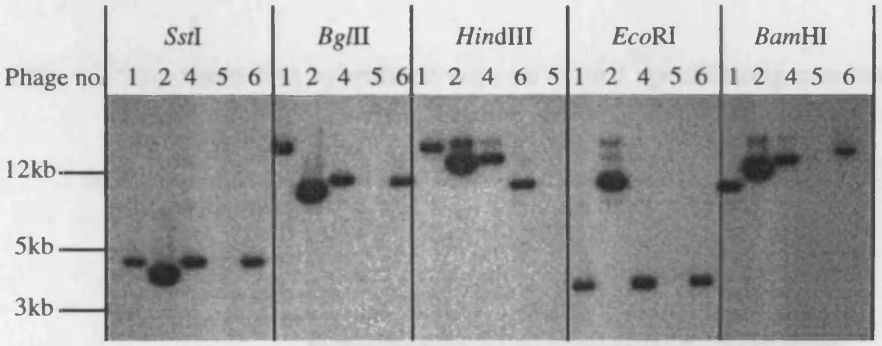


FIG. 3.13 Southern blot for the localisation of exons 3; phage clones cut with various enzymes and hybridised with an exon 3 specific probe

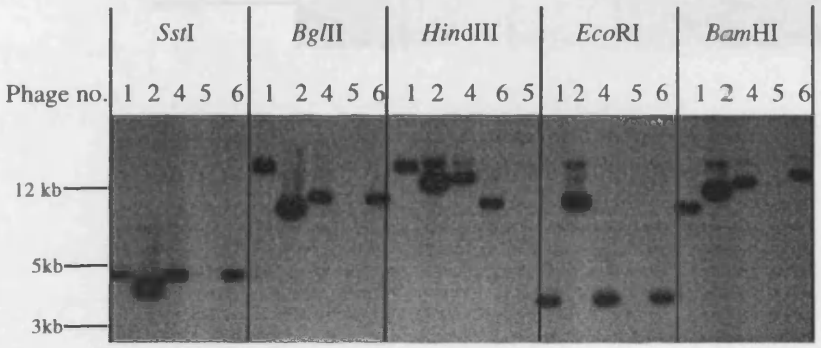


FIG. 3.14 Southern blot for the localisation of exons 4; phage clones cut with various enzymes and hybridised with an exon 4 specific probe

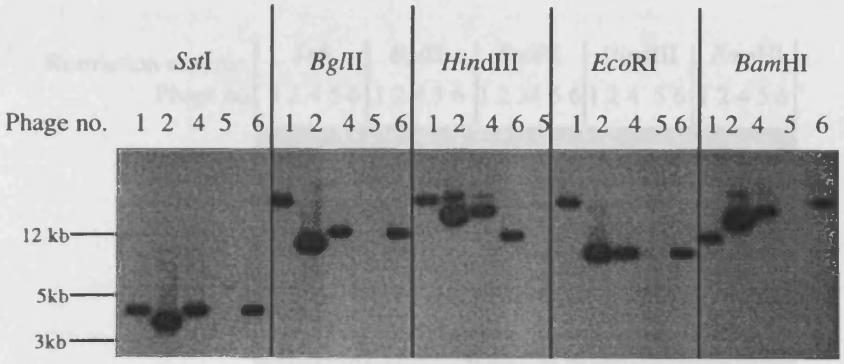


FIG. 3.15 Southern blot hybridised with an exon 1 specific oligonucleotide

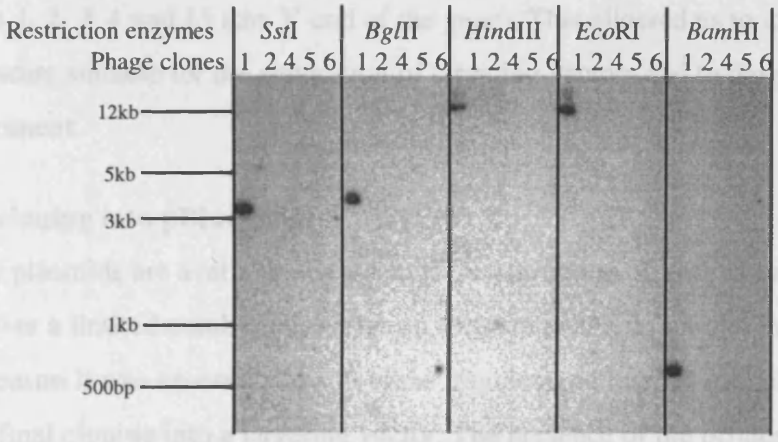
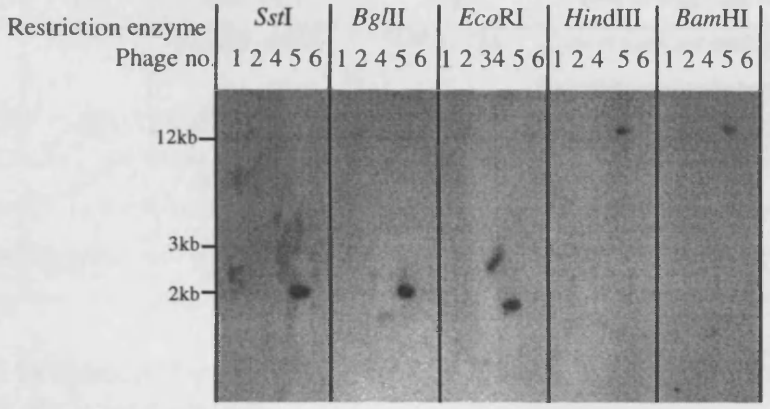


FIG. 3.16 Southern blot hybridised with an exon 15 specific oligonucleotide confirming the presence of the 3' end of the SA gene



Summary

Together the above experiments allowed us to generate a detailed restriction map of the mouse SA gene including the identification of restriction fragments incorporating exons 1, 2, 3, 4 and 15 (the 3' end of the gene). This allowed us to identify restriction fragments suitable for the generation of targeting vectors and to design our targeting experiment.

Sub-cloning into pBluescript

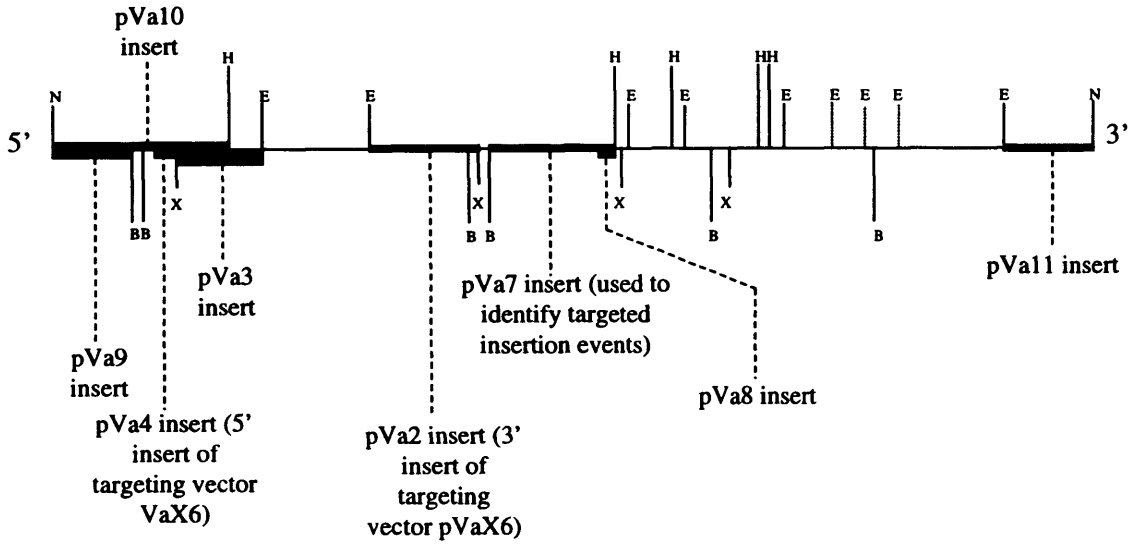
Many plasmids are available for use in the construction of gene targeting vectors. Often however a limited number of restriction sites are available for cloning purposes. For this reason it was necessary to sub-clone fragments of interest into pBluescript prior to their final cloning into a targeting vector. The presence of the pBluescript polylinker then increased subsequent cloning options substantially.

The plasmids shown in Table 3.1 were constructed by cloning fragments from bacteriophages 1-6 into pBluescipt. A diagram showing their placement relative to the full-length SA gene can be seen in Fig. 3.17.

Table 3.1 pBluescript clones containing SA gene sequences

Plasmid	Insert size (kb)	Restriction sites used for cloning	Bacteriophage of origin	Comments
pVa2	3.2	*Xba*I/*Eco*RI	PH1	3' arm of targeting vector (pVaX6)
pVa3	3	*Xba*I/*Eco*RI	PH1	Vector and insert are of similar size
pVa4	3.8	*Eco*RI/*Sal*I	PH6	5' arm of targeting vector (pVaX6)
pVa7	1.6	*Bam*HI/*Hind*III	PH4 or PH6	Insert used for probing Southern blots to identify correctly targeted ES clones
pVa8	0.6	*Hind*III/*Not*I	PH5	Possible alternative to pVa7
pVa9	2.7	*Bam*HI/*Not*I	PH1	
pVa10	5.5	*Hind*III/*Not*I	PH1	
pVa11	3.2	*Eco*RI/*Sal*I	PH5	

FIG. 3.17 Restriction map of the region covered by the 5 phage clones showing alignment of cloned fragments



Construction of targeting vectors

Plasmid pVaX6 was designed as our targeting vector (targeting exons 2 and 3 of the SA gene) (Fig. 3.20). The plasmid pX53 was chosen as a cloning vector as it contains the neomycin resistance gene as positive selection and the thymidine kinase gene as negative selection (Fig. 3.19). Two inserts of SA sequences were cloned into this vector from pBluescript as detailed below.

FIG. 3.18 Restriction map showing relative positions of homologous arms of targeting vector pVaX6 and position of pVa7 insert (which was used to identify ES cell clones correctly targeted by pVaX6)

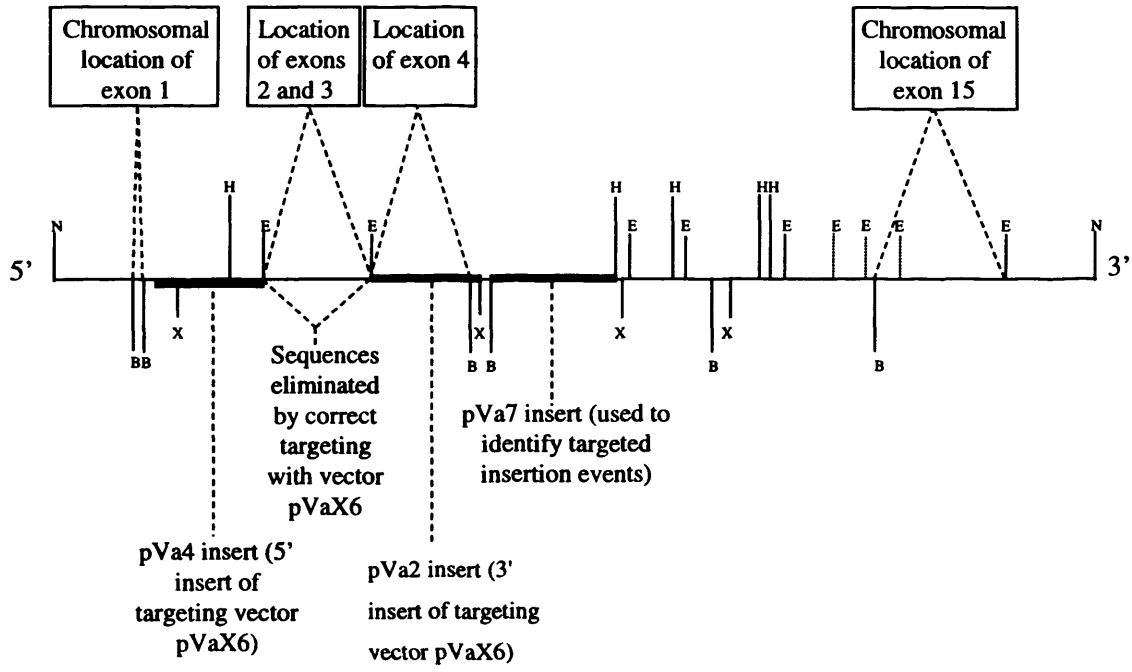


FIG. 3.19 Plasmid pX53

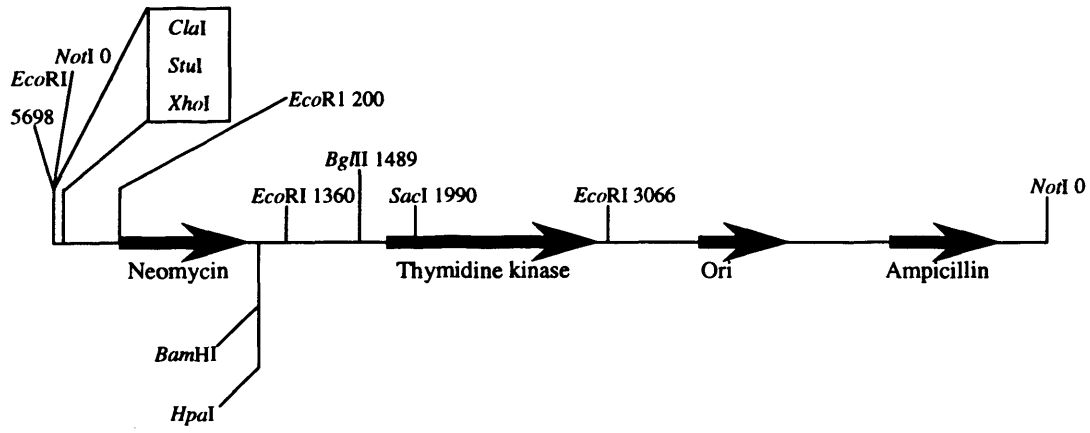
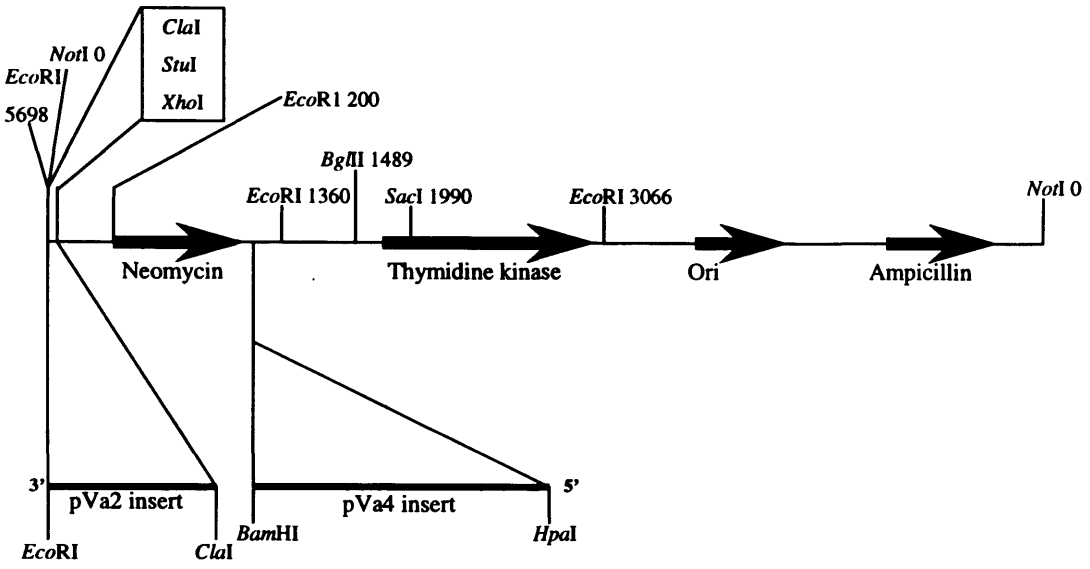


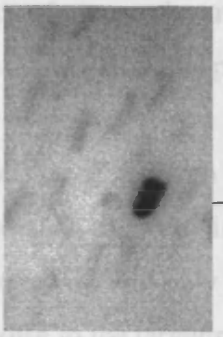
FIG. 3.20 Plasmid pVaX6



Cloning of the first arm of the targeting vector

The 5' arm of homology of plasmid pVaX6 was cloned as follows. A fragment of mouse DNA was first cloned from phage clone PH6 into pBluescript to create the plasmid pVa4; for localisation of plasmid insert see Figs. 3.17 and 3.18. This step was necessary due to the limited number of restriction sites in pX53. Plasmid pVa4 was then linearised with *SalI*, end filled to create a blunt end, and cut with *Bam*HI. After gel purification the resulting 3.8kb fragment of mouse chromosomal DNA was cloned into pX53 restriction digested with *Bam*HI and *Hpa*I. As pX53 contains no colour or antibiotic selection, cloning into this vector can prove difficult. Screening of a large number of putative clones was carried out by streaking colonies onto a nylon membrane, and hybridising with the expected insert (Fig 3.21). The resulting plasmid was designated pVaX5.

FIG. 3.21 Hybond-N colony hybridisation for the identification of pVaX5



Cloning of the second arm of homology of pVaX6

A fragment of mouse chromosomal DNA from bacteriophage PH6 was cloned into pBluescript to create pVa2. The pVa2 insert was then removed on a *Not*I/*Cla*I fragment and cloned into pVaX5 restricted with *Not*I and *Cla*I. Again screening of a relatively large number of putative clones was carried out as above.

Manipulation of embryonic stem cells and production of chimaeras

Transfection of ES cells with pVaX6

Approximately 5X 10^6 ES cells were transfected with 60µg of pVaX6 which had been linearised with *Not*I. Cells were resuspended in 41ml of growth medium. Four 10cm dishes were plated with 10ml each of cell suspension. The remaining 1ml was plated on a 6cm plate as a control for gancyclovir selection. After the application of positive and negative selection 294 ES clones were picked onto individual 48-well plates. DNA from each clone was screened by Southern blotting for the presence of a targeted insertional event.

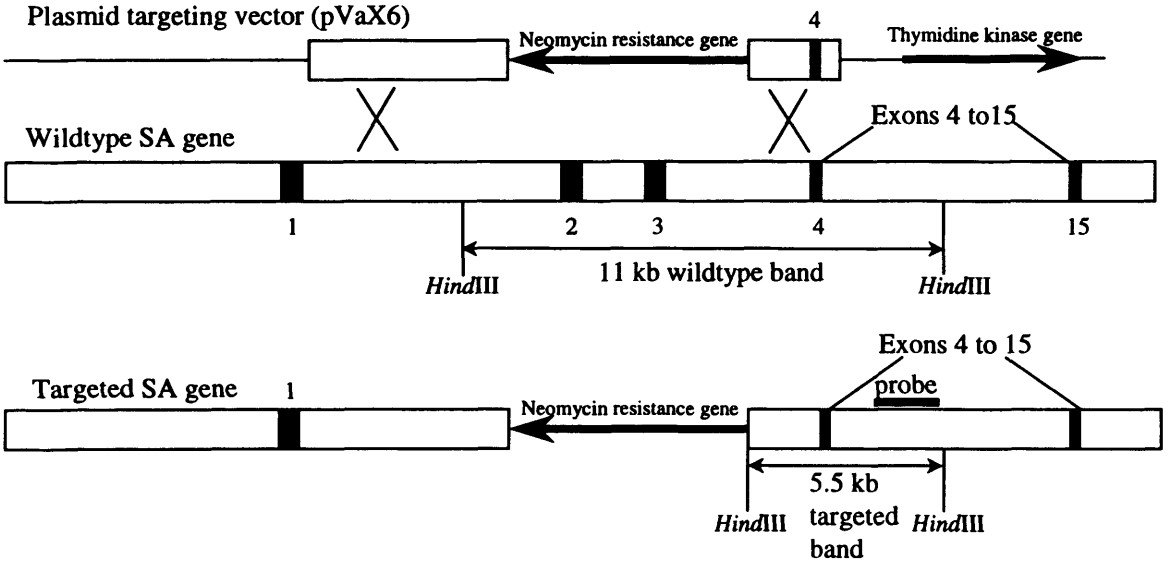
On the four experimental plates a total of 1394 clones survived G418 and gancyclovir selection representing a frequency of 23.8 per µg of DNA transfected.
On the control plate 118 clones survived representing a frequency of 80.63 per µg of transfected DNA. Thus the gancyclovir enriched in the selection of targeted over non-targeted insertion events by 3.39 fold.

Identification of ES clones targeted by pVaX6 using Southern blotting

A 1.7kb *Bam*HI/*Hin*dIII fragment of mouse chromosomal DNA was cloned into pBluescript from bacteriophage clone PH6. This plasmid was designated pVa7 (Fig. 3.18).

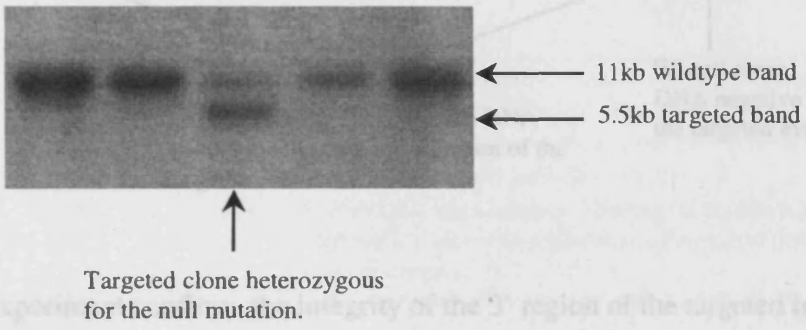
DNA from each ES cell clone was restricted with *Hin*dIII, Southern blotted and probed with the pVa7 chromosomal insert. As the pVa7 insert is outside the region of mouse DNA included in the targeting vector, it will only hybridise to the chromosomal copy of the SA gene. It will not hybridise to the targeting vector when it has inserted by random integration. When targeted homologous recombination takes place, one copy of the wildtype 11kb *Hin*dIII band is replaced by a 5.5kb band due to the introduction of an additional *Hin*dIII site from the targeting vector. The pVa7 insert hybridises to this smaller fragment in a correctly targeted event. In clones where targeted recombination has not taken place, only the 11kb wildtype band is hybridized (Fig. 3.22).

FIG. 3.22 Targeted homologous recombination of the SA gene by plasmid pVaX6



Chromosomal DNA from 294 clones was screened by Southern blotting after restriction digestion with *Hin*dIII. Of these 294 clones, 11 positives were identified containing the targeted insertion into the SA gene (Fig.3.23). This represents a targeting frequency of 1 in 26.7. The following clones contained targeted insertion events: 51, 67, 74, 139, 140, 141, 151, 182, 237, 266, and 285.

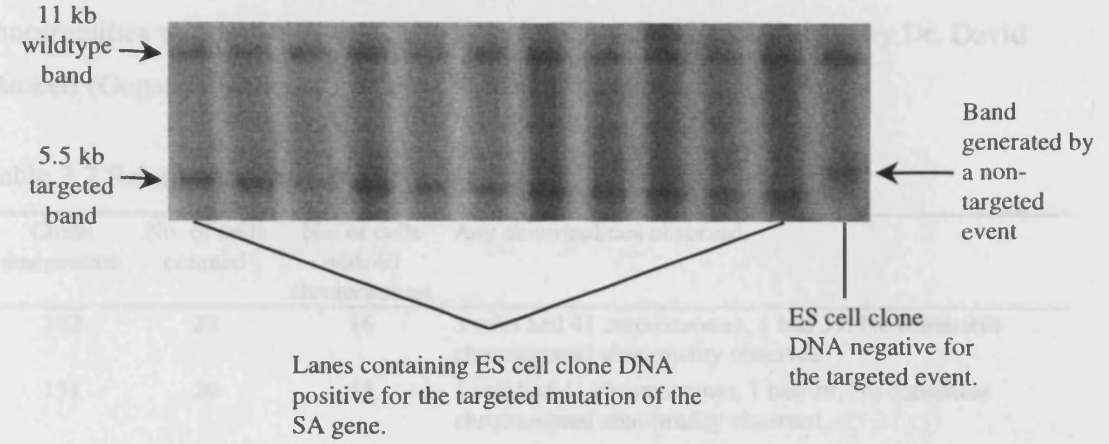
FIG. 3.23 Example of an ES cell clone positive for a targeted insertion event



Further analysis of positive ES clones

In order to ensure the targeting vector had not inserted into the chromosome at multiple sites, further analysis of the ES cell clone DNA was performed. ES cell DNA was digested with *Hin*dIII and probed with the pVa2 insert. Thus fragments generated by non-targeted as well as targeted integration of pVaX6 into the chromosome were hybridised (Fig. 3.24). No additional bands to those created by a targeted event were observed in any of the positive clones.

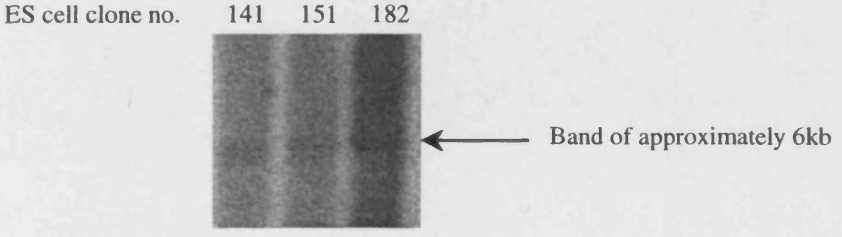
FIG. 3.24 Autoradiogram of targeted ES cell clone DNA hybridised with pVa2 insert DNA (the 3' arm of targeting vector pVaX6)



The above experiment confirms the integrity of the 3' region of the targeted integration within the ES cell clones; it was also necessary to eliminate the possibility of rearrangements of the 5' region. This was achieved by hybridising *Hind*III restricted ES cell clone DNA with an up-stream fragment of mouse chromosomal DNA. Plasmids pVa9 and pVa10 were generated (Fig. 3.17). Hybridisation was carried out using the 600bp *Bam*HI fragment from pVa10. For each clone only one band (of approximately 6kb) was observed indicating the likelihood of no 5' rearrangement in any clone (Fig. 3.24).

FIG. 3.25 ES clone DNA hybridised with pVa10 600bp *Bam*HI fragment

Autoradiograph showing *Hind*III restricted ES cell clone DNA hybridised with a fragment from the insert of pVa10 (a 600bp *Bam*HI fragment). A single 6kb fragment appeared alone in each lane. This was expected as the probe hybridises a band just peripheral to the region of the targeted insertion event. If however a rearrangement had occurred in the five prime region during homologous recombination, an additional band would have been observed.



Chromosomal configuration of targeted ES clones

Prior to the use of a clone for blastocyst injection a chromosomal spread was observed. Chromosomal counts were performed and the absence of gross karyotypic abnormalities eliminated (Table 3.2). This work was kindly performed by Dr. David Duckett (Genetics Institute, Leicester Royal Infirmary).

Table 3.2 Results of chromosomal observation

Clone designation	No. of cells counted	No. of cells with 40 chromosomes	Any abnormalities observed.
182	20	16	3 cells had 41 chromosomes, 1 had 39. No consistent chromosomal abnormality observed.
151	20	18	1 cell had 41 chromosomes, 1 had 39. No consistent chromosomal abnormality observed.
67	20	<1%	Most cells were tetraploid having 82 chromosomes. Some variability was seen with cells having slightly fewer chromosomes.
141	20	17	No consistent chromosomal abnormality observed.

Injection of blastocysts with ES cells and generation of chimaeric mice

Clones 151, 182 and 141 were chosen for blastocyst injection. 460 blastocysts were injected and implanted into 49 females over a period of approximately 1 year. From these injections a total of 14 chimaeras were generated; these were designated 1/97 to 3/97 and 4/98 to 14/98. Photographs of chimaeras 1/97 and 3/97 can be seen in Figs 3.26 and 3.27. Blastocyst injection and implantation into pseudo-pregnant females was performed by staff of the Transgenic Unit at Leicester University.

FIG 3.26 Chimaera 3/97; the founder of our colony.

FIG 3.27 Chimaera 1/97 showing high levels of chimaerism. Unfortunately however this mouse was infertile.

Breeding of Chimaeras and generation of mouse strains

An initial breeding strategy for each chimaera was determined by the genetic background of the blastocyst from which it was derived in order to allow the detection of germline transmission by coat colour observation. Within the transgenic unit at Leicester University, blastocysts of two different origins are variously available; C57BL/6 blastocysts and F1 blastocysts generated by crosses between C57BL/6 and CBA mice. Chimaeras derived from 129/Ola ES cells injected into C57BL/6 blastocysts were mated with C57BL/6 mice; offspring derived from the blastocyst (C57/BL6) component would therefore be black and easily distinguishable from offspring derived from the ES cell (129/Ola) component which would be brown (having a genetic make-up of 50% 129/Ola and 50% C57BL/6). Similarly, chimaeras derived from F1 blastocysts from strains C57BL/6 and CBA were mated such that coat colour observation could be utilsed to detect germline transmission. Such chimaeras were mated with MF1 mice; offspring derived from the host blastocyst were therefore either black or brown in colour whilst offspring derived from the ES cell component were champagne/grey in colour (having a genetic make-up of 50%129/Ola and 50% MF1). MF1 mice are outbred in their genetic make-up and are in some ways not the best strain to use for genetic studies of this nature. However the necessity of detection of germline transmission by coat colour observation rather than by laborious DNA screening and the robust nature of the strain makes this a good first choice for chimaera breeding. Presence or absence of gross phenotypic alterations arising from our introduced mutation could therefore be assessed relatively rapidly on this background.

All chimaeras were mated to assess their ability to germline transmit their ES cell component. Resulting offspring are summarised in table 3.3. Only chimaera 3/97 achieved germline transmission and was used to generate mice homozygous for the SA gene mutation.

Table 3.3 Offspring of chimaeras generated from ES cells targeted with pVaX6

	DOB	Blasto.	ES clone	sex	mated with	no. of litters	no. of off-spring	no. of germ-line off-spring	percentage gemline offspring
1/97	18/06/97	F1	182	male	MF1	0	0	0	-
2/97	18/06/97	F1	182	male	MF1	8	79	0	0
3/97	31/07/97	F1	151	male	MF1	14	138	3+2	3.62%
					C57BL/6	5	34	1	2.94%
					129/sv	7	57	1	3.33%
					Total	26	229	5+2	
4/98	29/08/97	C57	141	male	C57BL/6	2	18	0	0
5/98	19/09/97	F1	151	female	MF1	10	84	0	0
6/98	26/09/97	C57	151	female	C57BL/6	3	8	0	0
7/98	28/02/97	F1	141	male	MF1	7	79	0	0
8/98	28/02/98	F1	141	male	MF1	5	59	0	0
9/98	26/02/98	F1	141	male	MF1	0	0	0	-
10/98	26/02/98	F1	141	female	MF1	4	45	0	0
11/98	11/03/98	F1	182	male	-	-	-	-	-
12/98	11/03/98	F1	182	female	MF1	3	31	0	0
13/98	11/04/98	F1	141	male	-	-	-	-	-
14/98	10/04/98	F1	141	female	MF1	3	26	0	0

Generation of an out-bred KO line by heterozygous crosses

Germline transmitting chimaera 3/97 was used as the founder of an out-bred line of 50% MF1 origin and 50% 129/Ola origin. This line was used for initial characterisation of the SA null mutation.

Two F1 heterozygote offspring of chimaera 3/97 (SA1 male and SA2 female) were mated to give several litters i.e. a mixed F2 population. Heterozygous mating pairs were arranged from these F2 mice, resulting in large numbers of F3 mice. As these F3

mice consisted of a mixed population of knockout, wild type and heterozygous mice, they were used for experimental purposes.

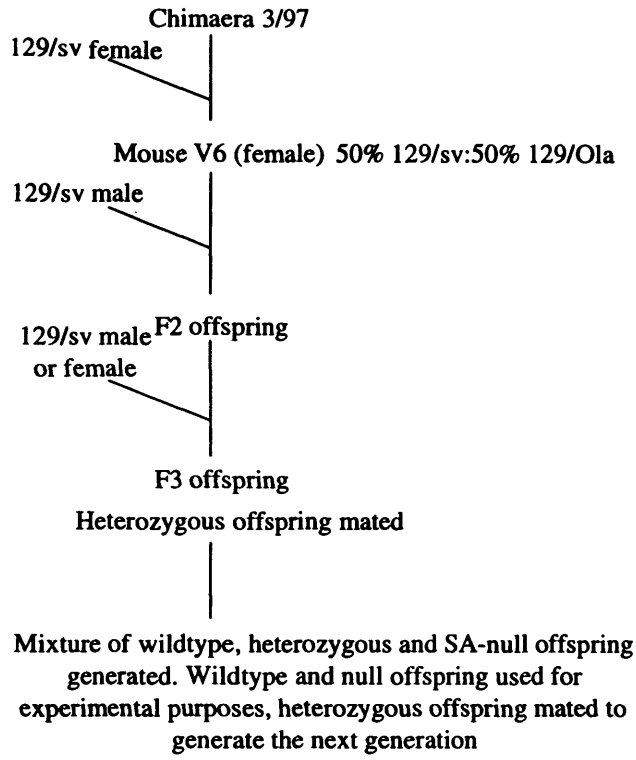
An F4 generation was generated by non-brother-sister mating of F3 heterozygotes. Three heterozygote pairs were mated to give a mixed F4 population which were used to maintain this line.

Generation of a partially-inbred mouse strain 129/sv:129/OlaSA-null

Chimaera 3/97 was mated with 129/sv females. Offspring generated were first screened by coat colour; black offspring were discarded. Screening of the remaining brown offspring was performed by PCR analysis for the presence of the neomycin resistance gene. This method of screening showed germline offspring carrying a targeted copy of the SA gene; germline offspring carrying a wildtype SA gene were not detected.

Six litters were produced from 129/sv females; one female was identified carrying the SA gene knockout (mouse V6). Mouse V6 has a genetic make-up of entirely 129 origin; 50% 129/Ola and 50% 129/sv. Heterozygous mutant offspring of mouse V6 and a 129/sv male were mated with 129/sv mice to generate a large number of offspring heterozygous for the mutation in the SA gene. From these mice breeding pairs were established and the offspring thus produced were a mixture of wildtype, heterozygotes and homozygotes for the SA mutation. These mice were utilised for the study of blood pressure, body mass and organ weight with respect to genotype at the SA locus. The genetic background of these mice is of entirely strain 129 in origin; approximately 87.5%129/sv and 12.5% 129/Ola (Fig 3.28).

FIG 3.28 Generation of strain 129/sv:129/OlaSA-null



DISCUSSION

Choice of gene targeting strategy

When designing our gene targeting experiment we decided upon the use of a classic replacement vector to introduce the neomycin resistance gene into the SA gene in place of a section of chromosomal DNA. As very little is known about the structure and function of the SA gene we decided that a simple experiment to generate a mouse strain completely lacking the SA protein would potentially be very revealing. Various gene targeting approaches are available allowing the introduction of mutations into a gene of interest. Some of the options available to us when designing our experiment are outlined below, together with reasons behind our choice of strategy.

There are 2 broad classes of gene targeting vector, the insertion vector and the replacement vector. An insertion vector contains one region of homology to the target gene, together with one or more selectable markers. Before transfection, the vector is linearised within the region of homology. Homologous recombination then results in the integration of the selectable markers within the target gene. No sequences are lost from the target gene using this method. The insertion of exogenous sequences, especially into an important exonic region of the target gene, is frequently sufficient to prevent the production of the target gene product. In addition the insertion of the neomycin resistance gene can be arranged in the same orientation as the target gene so that its termination sequences prevent the generation of any downstream target gene mRNA. An advantage of this type of vector is the need to clone only one insert of target gene DNA into the vector. A major disadvantage however is that no target gene sequences are actually deleted from the target gene, therefore the potential will remain for the production of a wildtype protein product. For this reason insertion vectors are no longer frequently used for the ablation of a gene by gene targeting. Insertion vectors are useful in the first stage of a knock-in/knock-out type of experiment for the generation of subtle mutations.

Replacement vectors generally include two sections of target gene DNA separated by a selectable marker and are designed such that the selectable marker replaces target gene

sequences when integration occurs by homologous recombination. Thus if a gene is well characterised it is possible to design a vector to replace crucial sequences with a selectable marker gene. In such a case there is no possible chance of a wildtype protein product being produced by a mutant mouse strain. Even though the location of the functional regions of the SA gene are not known, this was our preferred strategy. We generated a replacement vector to cause the deletion of genomic sequences corresponding to the first 65 amino acids of the SA protein. As the translation initiation codon was also deleted it was hypothesised that no SA protein would be produced. This was infact the case, as is discussed in Chapter 4.

One popular modification of the replacement vector strategy uses the *Lac*Z gene as a marker gene, introducing this along with the neomycin resistance gene in place of chromosomal sequences. This can be useful in the identification of sites of expression of the targeted gene. The *Lac*Z gene is placed to utilise the promoter of the targeted gene and so is expressed in tissues of expression of the target gene. With appropriate staining a blue dye can be generated in sites of expression of the *Lac*Z gene. This strategy is useful if little is known about the expression pattern of a gene or for studying developmental changes in expression patterns of a gene. As the major expression sites of the SA gene have been identified and we had no plans to further study expression patterns, we decided not to pursue this approach. One argument for including the *Lac*Z gene is that during experimental design it is not always possible to predict the usefulness of this gene downstream. However, the *Lac*Z is a relatively large gene. Cloning targeting vectors can be technically challenging as large plasmids are generated, routinely including both the neomycin resistance and the thymidine kinase genes as well as sequences homologous to the target gene. If the *Lac*Z gene is also to be included in a targeting vector, in order to facilitate cloning, it is often necessary to keep target gene sequences to a minimum. As this can reduce the efficiency of gene targeting we decided to increase the size of SA gene insert DNA, rather than include the *Lac*Z gene in our targeting vector.

The use of the Cre-recombinase system is another commonly used strategy in gene targeting. *Lox*P sites can be introduced into the genome such that when a cell (either an

ES cell in vitro or cells within an organ in vivo) is exposed to Cre-recombinase, recombination occurs between the *lox*P sites, deleting any sequences between them. One major use of this strategy is the generation of conditional mutants. A mouse strain can be generated in which critical regions of a gene are surrounded by *lox*P sites. The mouse still generates the target protein. However when crossed with another mouse strain expressing Cre-recombinase in a temporal or tissue specific manner, the gene will be ablated conditionally. This is useful if ablation of a gene is lethal at the embryonic stage. It is possible to generate one replacement targeting vector (and thus one targeted ES cell line) which can give rise to both standard and conditional gene ablation models after an additional in vitro step exposing the targeted ES cells to Cre-recombinase. This is a strategy which could have been a used in our experiments as it was not known whether ablation of the SA gene would generate an embryonic lethal mutation, although due to its highly localised expression pattern this seemed unlikely. Had ablation of the SA gene proved lethal, it would have been useful to have the Cre-lox system in place. However at the time these experiments were undertaken embryonic stem cell technology was not used extensively at Leicester University and no gene-targeted mouse had been produced by the Leicester Transgenic Unit. It was therefore decided to keep our experiment as simple as possible. The use the Cre-recombinase system for gene ablation as outlined above requires an additional step in the manipulation of the targeted ES cell line. The less passages ES cells are subjected to prior to re-injection into a blastocyst, the more likely that germline transmission will be achieved. It was therefore decided to use a classic replacement vector to generate a null mutation of the SA gene.

Various factors were taken into consideration when deciding on our design of replacement vector for the SA gene. Sequences of up to 15kb have been deleted by the use of a single replacement vector, however it is generally accepted that targeting efficiency is higher for smaller deletions. Our replacement vector targeted sequences of 3.3kb of the chromosomal SA gene for deletion and contained SA gene sequences of 3.2kb and 3.8kb for homologous recombination. The greater the length of homologous sequences within a targeting vector, the higher the frequency of homologous

recombination between that vector and genomic DNA (up to 10kb). A total of 7kb was cloned into our targeting vector.

Germline transmission rates

Early work using ES cells for gene targeting reported germline transmission rates of 20% (Bradley *et al.*, 1984). In this work we have experienced much lower transmission rates. Only one of 14 chimaeras transmitted its ES cell component through the germline and then at a very low frequency. Only 3.3% of offspring from chimaera 3/97 contained cells of ES cell origin. Reasons for this are unclear. Quality of ES cells used for blastocyst injection is vitally important if germline transmitting chimaeras are to be obtained. Passage number of ES cells should be kept to a minimum; batches of ES cells should be frozen at a low passage number. Ours was the first ES cell line to be used for blastocyst injection within the Transgenic unit at Leicester University. Generation of quality blastocysts, blastocyst injection and reintroduction of blastocysts into pseudo pregnant females are skilled tasks. When our ES cells were first used for blastocyst injection these techniques had not been practised in Leicester. One possible reason for our lack of success in generating a high percentage of germline transmitting chimaeras may have been due to this lack of experience. By the time the staff of the transgenic unit were well-practised in the necessary techniques, our ES cells had undergone many passages jeopardising their chances of contributing to the generation of germline transmitting chimaeras.

Choice of genetic background for breeding of mutant mouse strains

The choice of genetic background onto which a targeted mutation is bred is vitally important, especially in the case of quantitative traits determined by multiple loci. In the case of any null mutation there can be differences in phenotype dependent upon the genetic background, for example on a predominantly C57BL/6 background *raf*A null mice displayed neurological and intestinal abnormalities, dying soon after birth. However when maintained on a 129/Ola background, mice survived to adulthood displayed no obvious intestinal abnormalities, but did have some neurological defects (Pritchard *et al.*, 1996). Both of these phenotypes are however quite striking and although different on different backgrounds, there can be no doubt that the null

mutation is responsible for the effects observed. In such a case it is useful to present a mutation on different backgrounds to observe differences in penetrance. On one genetic background a phenotype may be masked whereas on another it may be much more apparent. Often maintaining a mutation on an outbred or partially outbred background can allow the observation of a phenotype that would otherwise have been unapparent. The genetic variety available within such a model allows that in some mice a phenotype may be observed. One other advantage of maintaining a mutation on such a background is that this more closely resembles the human situation.

When studying quantitative multigenic traits genetic background considerations become paramount. If a phenotype follows a normal distribution in an outbred population (such as blood pressure) then small phenotypic differences due to a specific mutation can be much more difficult to detect. Blood pressure is subject to variability between different mouse strains, however this is not a field which has been extensively studied. Classic transgenic approaches usually involve the use of 129/Ola ES cells injected into a C57BL/6 blastocyst, with resulting chimaeras mated with C57BL/6 mice to generate heterozygous mutant mice which are F1 between strains 129/Ola and C57BL/6. Breeding of an F2 population then generates a mixture of homozygous and heterozygous mutant mice as well as wildtype mice. As this background is partially outbred the range of blood pressure within such a population could be expected to be quite wide. This in itself presents a problem as it necessitates the blood pressure measurement of many animals for small differences in blood pressure to become significant (especially by indirect BP measurement). More important however is the fact that using this strategy, null mice always receive a 129/Ola copy of the chromosome containing the null mutation, wildtype mice always receive a C57BL/6 copy of this chromosome. Thus if other genes variable between the 2 strains and affecting blood pressure are harboured on the same chromosome as the null mutation, problems of data interpretation will arise.

Several strategies are available to alleviate this problem. The construction of inbred lines by backcrossing for several generations results in a line which is entirely inbred except for the region immediately surrounding the mutation. For the study of single

gene traits and resulting differences of penetrance between strains, this is an ideal and frequently used strategy. The major disadvantage with this approach is the time required for the generation of such a line. It is widely accepted that backcrossing for 10 generations is required for a strain to become completely inbred. Thus a minimum period of 90 weeks is required to generate such a line. Additional limitations occur when backcrossing is applied to multigenic traits. To use our work as an example, we are currently breeding a C57BL/6 backcross line for the SA-null mutation. The chromosomal region immediately surrounding the mutation was derived from a 129/Ola ES cells and although successive backcrossing reduces this region within the C57BL/6 background, the amount of 129/Ola DNA remaining linked to the SA-null mutation will depend upon the position of crossover at each generation. In effect a congenic strain is generated with a portion of 129/Ola sequences on a C57BL/6 background. The SA-null mutation acts as a marker for sequences of 129/Ola origin. Thus any problems inherent with the use of congenic strains for the study of multigenic traits can potentially apply to a model of this type. If a phenotypic difference is observed between such a backcross strain and wildtype C57BL/6 mice, it could potentially be a gene closely linked to the SA gene rather than the SA-null mutation itself that is causing the observed effect. For the construction of congenic strains, 2 strains are selected which have distinct phenotypes, for example SHR and WKY rats strains. Problems arising due to closely linked genes are less likely to occur in mice strains which have not been deliberately selected for distinct phenotypes, however it is necessary to take these considerations into account when interpreting data.

The complete elimination of genetic background interference can be achieved by breeding a germline transmitting chimaera directly onto a 129/Ola background. Thus a mutation can be maintained on an entirely 129/Ola background. In this case any observed phenotype can be attributed unequivocally to the introduced mutation. The only problem with this approach is that the mouse strain 129/Ola is a very poor breeding strain. Litters are frequently lost or very small. A problem can then arise generating sufficient mice for experimental purposes.

One elegant method of eliminating background interference was utilised by Kim *et al.* (1995) in the breeding of angiotensinogen null mice. Chimaeras were bred with C57BL/6 mice to generate an F1 generation of germline offspring receiving either a wildtype or mutant chromosome of ES cell origin, thus being either wildtype or heterozygote in genotype. These F1 offspring were identical except for the presence or absence of the targeted mutation and were used for the comparison of heterozygous and wild type phenotype on identical backgrounds. An F2 generation was bred by intercrossing these F1 mice. As the F2 wildtype and heterozygous offspring showed a similar phenotype to F1 wildtype and heterozygous offspring, it was surmised that effects observed were due to the mutation in the *AGT* gene rather than a genetic background effect. Results were extrapolated statistically to include homozygous mutant mice. One limitation of this strategy is that it requires the presence of an intermediate heterozygote phenotype. In addition a germline transmitting chimaera must transmit its ES cell component with high efficiency to generate enough offspring for experimental purposes.

The use of mouse strain 129/sv to generate a partially inbred strain can provide a degree of compromise between the use of 129/Ola (poor breeders) and a backcross line. 129/sv mice are closely related to 129/Ola mice but are a much stronger breeding stock. Thus we designed our breeding strategy to utilise this strain. A study of microsatellite variability between 129 sub-strains (Simpson *et al.*, 1997) showed variability in 26% of markers between 129/sv and 129/Ola sub-strains. As our experimental mice are derived from an F3 backcross between 129/sv and 129/Ola they are 87.5% 129/sv and 12.5% 129/Ola in origin. Thus the microsatellite variability within our 129 sub-strain can be calculated to be 26% of 12.5%, i.e. 3.25%. Although this is a small degree of variability we chose to maintain our stocks by heterozygous matings so that wildtype littermates were available as controls for our experiments. In addition the generation of a backcross line carrying our mutation on a second genetic background will substantiate our results.

Chapter 4

Confirmation of SA-null status and initial phenotypic observations of SA-null mice

Chapter 4: Confirmation of SA-null status and initial phenotypic observations of SA-null mice

INTRODUCTION

The design of our gene targeting experiments generated mice lacking part of intron 1, exon 2, intron 2, exon 3, and part of intron 3 of the SA gene. It was necessary to determine the effect of this mutation on the transcription of the SA mRNA and translation of the SA protein. Total RNA and protein were extracted from kidney and liver. Analysis was performed by Northern blotting, RT-PCR and Western blotting.

RESULTS

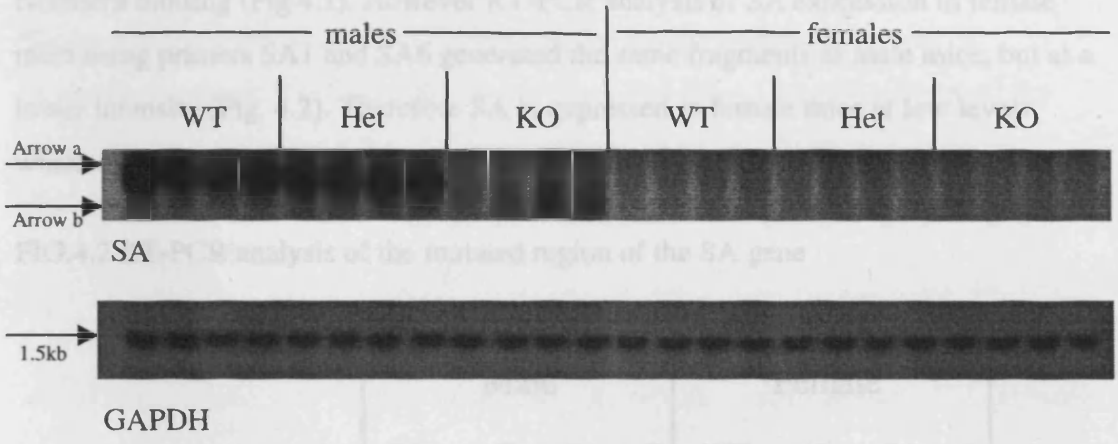
A. Confirmation of SA-null status

Northern blot analysis of SA expression in the kidneys of wildtype, heterozygous and SA-null mice

RNA from 22 week old wildtype, heterozygous and SA-null male and female mice (strain MFI:129/Ola) was Northern blotted and hybridised with the rat 1.6kb cDNA probe from plasmid pSAF1. A transcript of 2.5kb was observed in the wildtype and heterozygous males, corresponding to the expected size of the SA message (Fig. 4.1, arrow a). In the SA-null male mice a smaller transcript of approximately 2.1kb was observed (Fig. 4.1, arrow b). Expression levels of this aberrantly spliced transcript vary but are approximately 10% of that of the SA transcript in wildtype males on this genetic background. Expression levels of the SA transcript in heterozygous males are similar to those in wildtype males. From this Northern blot experiment it appears that the mutant transcript is not produced by mice heterozygous for the SA-null mutation. However analysis by reverse transcription PCR (see below) indicate that a truncated transcript is produced by these mice (Fig. 4.2). As the larger transcript is much more abundant than the truncated transcript and the 2 are similar in size, it is likely that the presence of the truncated transcript is obscured by the wildtype transcript in this Northern blot experiment.

Expression levels of the SA transcript are much higher in males than in females (Fig. 4.1). Expression levels of SA in the kidneys of female mice are below the sensitivity of Northern blotting.

FIG.4.1 Expression of SA in the kidney of wildtype and SA-null mice



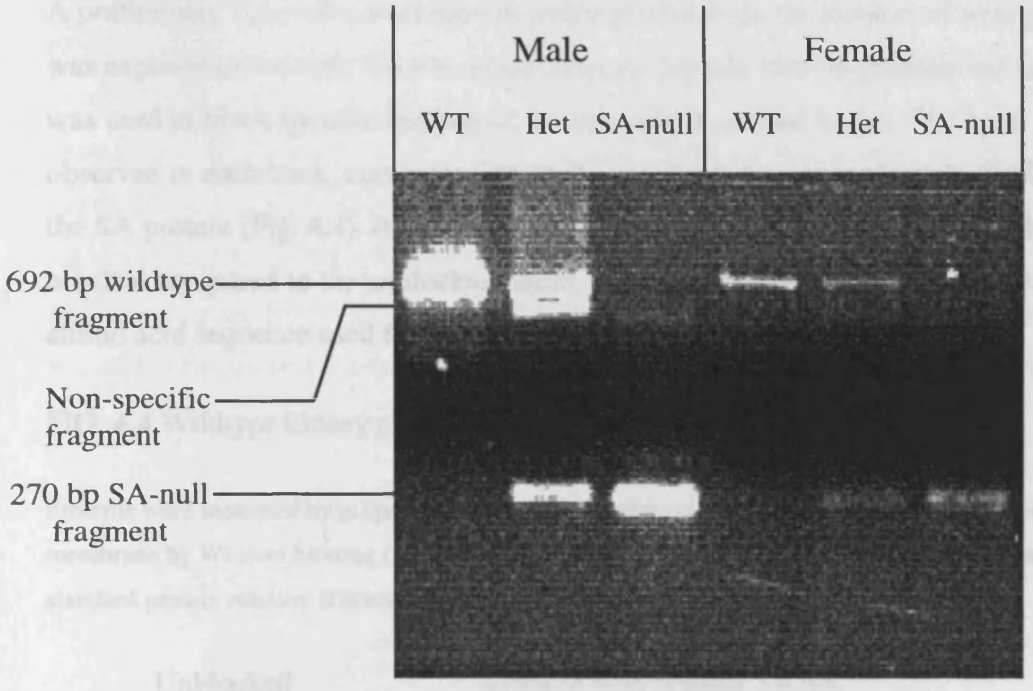
RT-PCR analysis of SA mRNA

Northern blot analysis has shown that SA-null mice transcribe the SA gene. It was therefore hypothesised that the truncated transcript consists of exon 1 spliced directly to exon 4, as exons 2 and 3 are absent in the chromosomal DNA of SA-null mice. To confirm this hypothesis mRNA from the kidneys of wildtype, heterozygote and SA-null mice was reverse transcribed and PCR was performed. Sense primer SA1 (within exon 1) and antisense primer SA6 (within exon 4) were used to generate a PCR product spanning the potential splice junction. RNA from wildtype mice generated a PCR product of 692bp corresponding to the expected size of a wildtype cDNA fragment. However RNA from SA-null mice generated a fragment of 270bp, corresponding to the size of the wildtype fragment minus exons 2 and 3 (422bp) (Fig. 4.2). RT-PCR analysis of RNA from heterozygote kidneys generated both bands. Further confirmation of the direct splicing of exon 1 to exon 4 was obtained by sequence analysis of these RT-PCR products. Products were cloned into pBluescript and sequence data generated (PNACL, University of Leicester). Sequence data from the 692bp wildtype band corresponded to the wildtype cDNA sequence. However sequence data for the 270bp SA-null band

showed exon 1 spliced directly to exon 4. Both forward and reverse sequence data were generated. Sequence data showing the splicing of exon 1 to exon 4 can be seen in appendix 1 to this chapter.

Expression of the SA transcript in the kidney of female mice was not detected by Northern blotting (Fig 4.1). However RT-PCR analysis of SA expression in female mice using primers SA1 and SA6 generated the same fragments as male mice, but at a lower intensity (Fig. 4.2). Therefore SA is expressed in female mice at low levels which are detectable by RT-PCR but not by Northern blotting.

FIG.4.2 RT-PCR analysis of the mutated region of the SA gene



From these experiments it can be concluded that we have generated a mouse strain producing a truncated SA transcript lacking exons 2 and 3. This transcript is apparent in lower levels than the wildtype transcript, showing it to be either produced in lower amounts or to be more unstable than the wildtype transcript.

Due to the presence of this truncated SA message in SA-null mice the possibility remained that a truncated SA protein was produced in our model (see discussion). It was therefore necessary for us to investigate the presence of SA protein in SA-null mice.

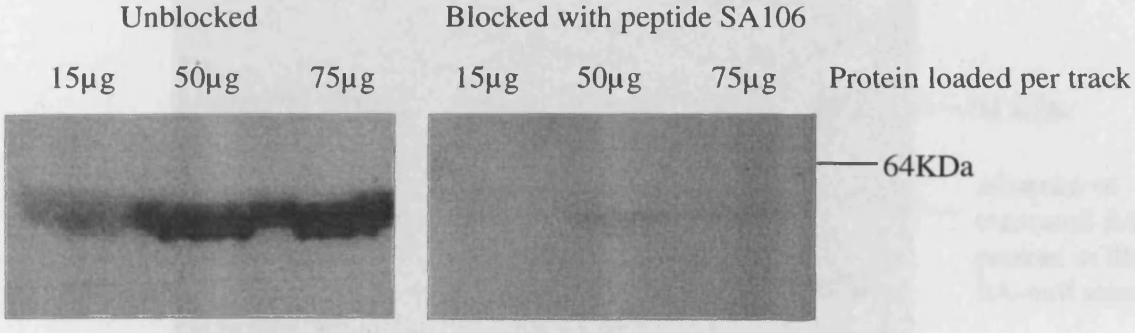
Western blot confirming the specificity of the SA106 antibody

To determine whether truncated mRNA produced by the SA-null mice allowed the production of a truncated SA protein, it was necessary to study protein levels by Western blotting. Antibody SA106 (see Methods, page 44) was kindly provided by Bob Norman (Division of Vascular Medicine).

A preliminary blot was undertaken in which protein from the kidneys of wildtype mice was exposed to antibody SA106. In addition the peptide used to generate the antibody was used to block specific binding of the antibody in control tracks. One band can be observed in each track, corresponding to the expected size approximately (64KDa) of the SA protein (Fig. 4.4). It can be noted that binding levels are vastly reduced in the blocked compared to the unblocked tracks thus showing that binding is specific to the amino acid sequence used for the generation of the antibody.

FIG. 4.4 Wildtype kidney protein hybridised with antibody SA106

Proteins were separated by polyacrylamide gel electrophoresis and immobilised onto a nitrocellulose membrane by Western blotting (see methods). Protein sizes were calculated by comparison with standard protein markers (Gibco) stained with Ponceau Red solution.



Western blot of kidney extracts of wildtype and SA-null mice

Kidney protein extracts from 3 wildtype and 3 SA-null mice were observed by Western blotting with the SA106 antibody. A single band of approximately 64kDa was observed at a similar intensity in each lane (Fig. 4.5).

This was initially a perplexing finding for several reasons. Firstly we did not expect translation to occur in this model due to the absence of the translation start site. Also analysis of SA transcription showed lower expression levels in SA-null than wildtype mice (Fig 4.1), a finding that did not appear to be paralleled at the protein level. Finally, as we had eliminated almost 10% of the coding sequences for the SA protein, we expected any protein generated to be noticeably smaller than the wildtype protein. Literature review revealed the recent identification of a protein, KS (kidney specific), of exactly the same size as SA (Hilgers *et al.*, 1998). KS is 55% identical and 71% similar to SA at the amino acid level. Therefore its presence in the kidney and binding to antibody SA106 could have obscured the absence of SA protein in SA-null mice. Further observation of the reported KS amino acid sequence showed that over the epitope of antibody SA106, 9 out of 15 amino acids are common between the SA gene and the KS gene (Fig. 4.6). This level of homology between the SA and KS proteins would undoubtedly allow the SA106 antibody to bind to the KS protein. Importantly however, no truncated SA protein is present in the SA-null mice.

FIG. 4.5 SA antibody binding to proteins isolated from kidney

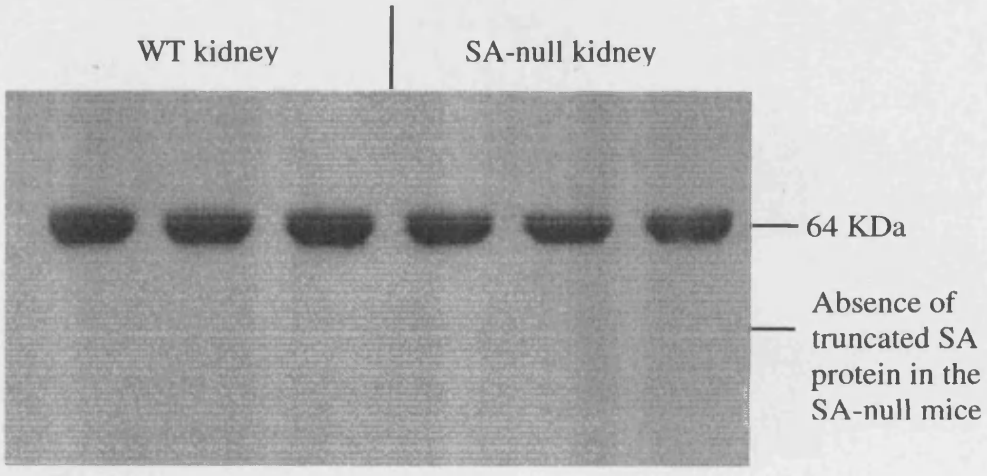
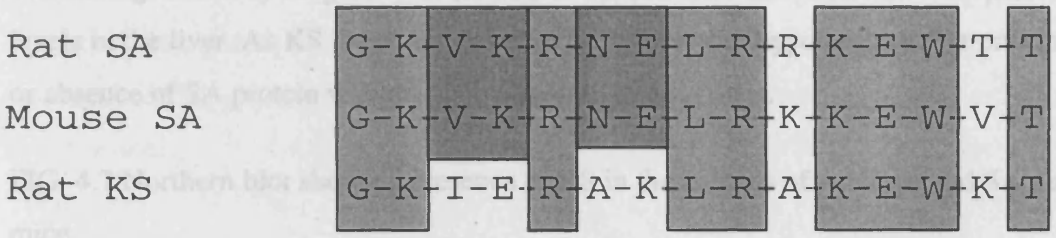


FIG. 4.6 Comparison of amino acid sequences in the region to which antibody SA106 was raised



We confirmed high and equal levels of KS expression in the kidneys of wildtype and SA-null mice (Fig. 4.7). Expression was however absent in the liver (Fig. 4.8), confirming results by Hilgers *et al.* (1998). It was therefore decided to observe protein levels in the liver. As KS is not expressed in the liver it was hypothesised that presence or absence of SA protein would not be obscured in this tissue.

FIG. 4.7 Northern blot showing presence of KS in the kidneys of wildtype and SA null mice

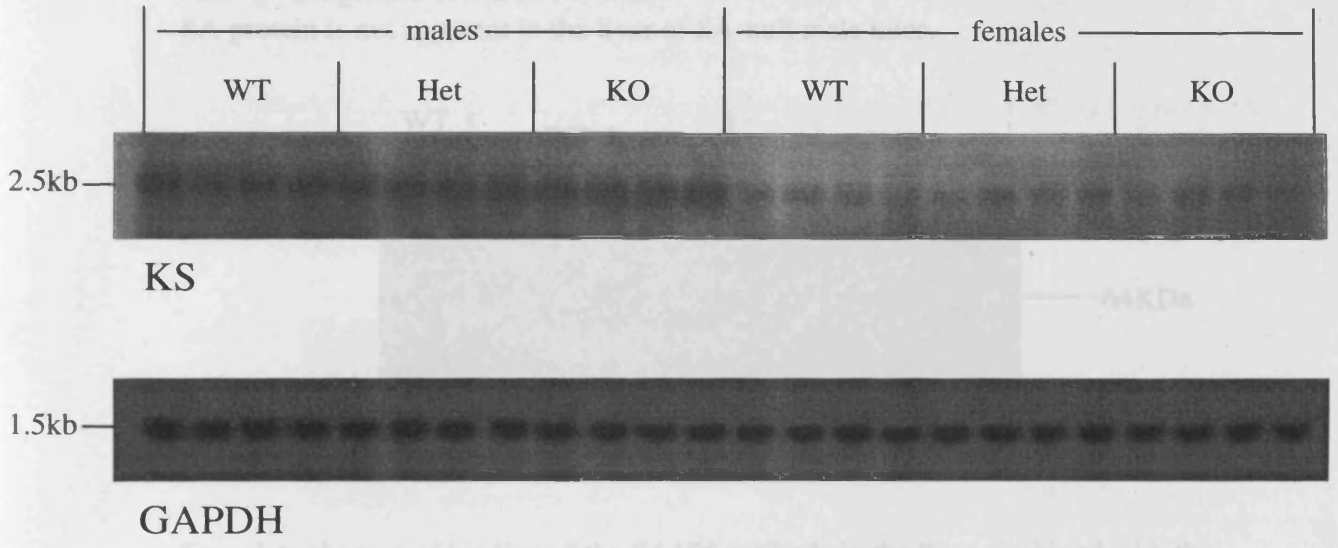
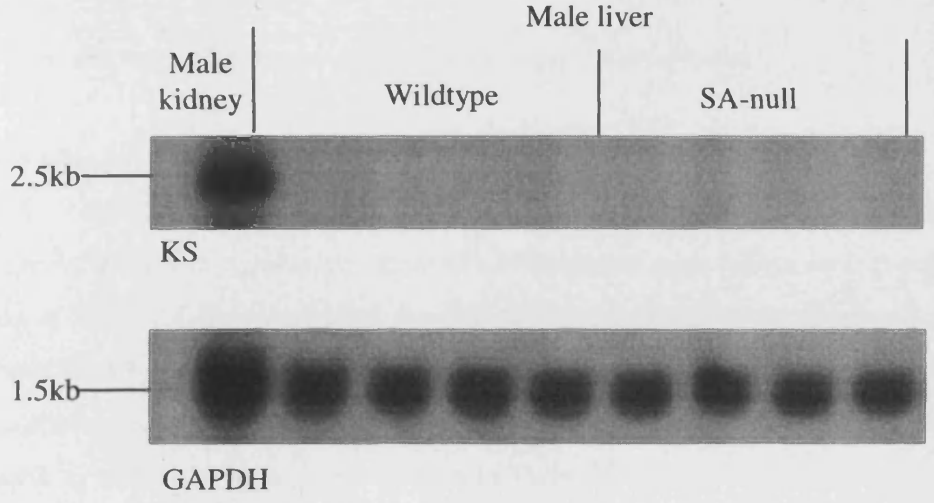


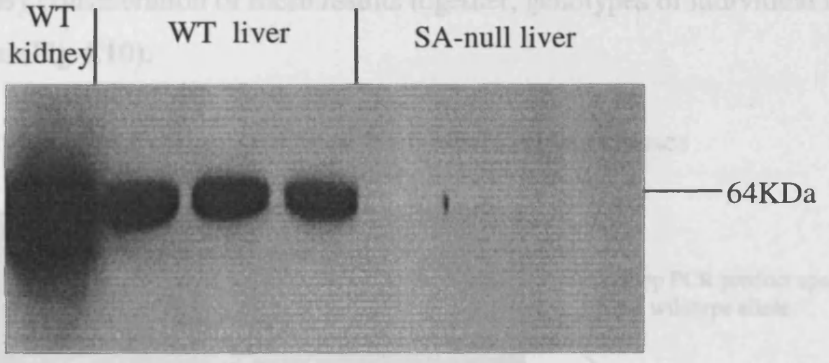
FIG. 4.8 Northern blot showing absence of KS in the livers of wildtype and SA null mice



Western blot of liver extracts of wildtype and SA-null male mice

As KS is produced only in the kidney, we confirmed the absence of SA protein in the liver. Liver protein extracts from 3 wildtype and 3 SA-null male mice were analysed by Western blotting with the SA106 antibody. A single band of approximately 64kDa (corresponding to the expected size of the SA protein) can be seen only in the wildtype protein samples. No band is present in the null protein samples showing an absence of SA protein in the liver.

FIG. 4.9 SA protein levels in the liver
SA protein is not apparent in the liver of SA-null male mice.



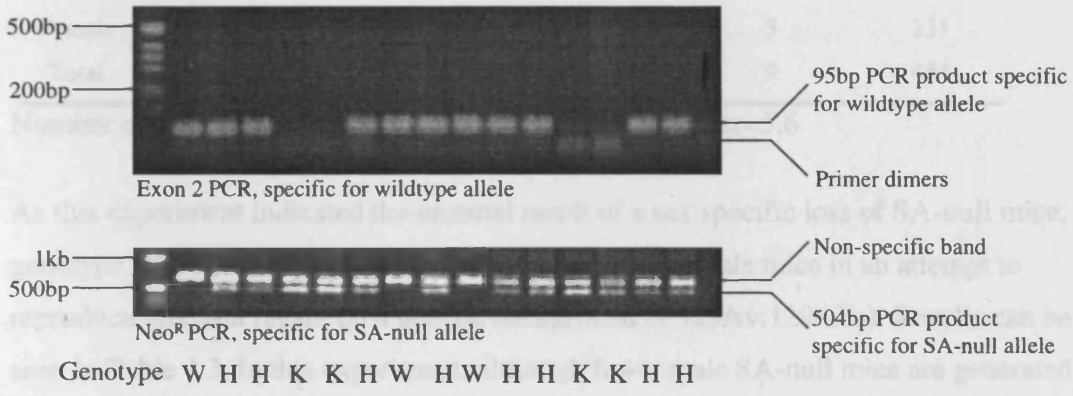
Complete absence of binding of the SA106 antibody in the liver combined with the lack of a truncated SA protein in the kidney can be considered together as definitive evidence that the SA-null mouse strain does not produce SA protein.

B. Initial phenotypic observations of SA-null mice

Initial observation showed the SA-null mice to have no overt phenotype and to be fertile. For the majority of breeding purposes within this work mouse lines were maintained by heterozygous matings to allow the use of wildtype litter mates as controls for experimental animals. Using this strategy we would expect a ratio of 1:2:1 for WT:HET:KO(SA-null) mice to be generated.

Identification of wildtype, heterozygote and SA-null offspring was performed by PCR (see Methods, page 56). For each mouse, 2 separate PCR reactions were performed, one to show the presence of the wildtype allele, the other to show the presence of the SA-null allele. By consideration of these results together, genotypes of individual mice were determined (Fig 4.10).

FIG.4.10 Genotyping of offspring generated from heterozygous crosses



W refers to wildtype, H to heterozygote and K to SA-null (knockout) mice.

When the SA-null mutation was maintained on an outbred background (in MF1:129/OlaSA-null mice) a ratio of 1:2:1 was obtained, with figures corresponding to Hardy Weinberg equilibrium (p=0.82). Numbers of mice within each group can be seen in Table 4.1. However when the SA-null mutation was maintained on a partially inbred background (129/sv:129/Ola) fewer wildtype males were produced than were expected (p=0.011). Genotype ratios of female mice were as expected (p=0.89). Numbers of mice generated can be seen in Table 4.2.

Table 4.1 Offspring generated by heterozygous crosses of mice carrying the SA-null mutation on an outbred background (in MF1:129/OlaSA-null mice)

	Wildtype	Heterozygote	SA-null	Sub-total	Genotype not determined	Total
Male	45	86	44	175	4	179
Female	55	94	53	202	4	206
Total	100	180	97	377	8	385

Number of litters=49 Average number of mice per litter=7.9

Table 4.2 Offspring generated by heterozygous crosses of mice carrying the SA-null mutation on a partially inbred background (129/sv:129/Ola). Genotype was determined for both male and female mice.

	Wildtype	Heterozygote	SA-null	Sub-total	Genotype not determined	Total
Male	69	120	30	219	4	223
Female	55	110	61	226	5	231
Total	124	230	91	445	9	454

Number of litters=81 Average number of mice per litter=5.6

As this experiment indicated the unusual result of a sex specific loss of SA-null mice, genotype ratios were determined for a further cohort of male mice in an attempt to reproduce this data (again on a genetic background of 129/sv:129/Ola). Results can be seen in Table 4.3. In this experiment, although fewer male SA-null mice are generated than wildtype mice, this effect is not significant (p=0.14). Reasons for this are unclear. Amalgamation of data from both these cohorts (Tables 4.2 and 4.3) are presented in Table 4.4. Statistical analysis of these combined results reveal the loss of SA-null males still to be significant (p=0.009).

Table 4.3 Offspring generated by heterozygous crosses of mice carrying the SA-null mutation on a partially inbred background (129/sv:129/Ola). Genotype was not determined for female mice from these litters.

	Wildtype	Heterozygote	SA-null	Sub-total	Genotype not determined	Total
Male	59	72	38	169	20	189
Female	-	-	-	-	153	153
Total	-	-	-	-	-	342

Number of litters=62 Average number of mice per litter=5.5

Table 4.4 Data from tables 4.2 and 4.3 above combined

	Wildtype	Heterozygote	SA-null	Sub-total	Genotype not determined	Total
Male	128	192	68	388	24	412
Female	-	-	-	-	-	384
Total	-	-	-	-	-	796

Number of litters=143 Average number of mice per litter=5.6

Breeding of SA-null homozygous mice

Three breeding pairs of SA-null homozygous mice were established (on an outbred background in MF1:129/OlaSA-null mice). These mice were allowed to produce several litters each, which they appeared to rear in a normal manner. The average litter size for these breeding pairs was 6.8. This is not significantly different from the average litter size of 7.9 obtained by heterozygous crosses on the same genetic background (p=0.33). It can therefore be concluded that on this genetic background the absence of the SA gene product has no effect on fertility.

Table 4.5 Offspring produced by SA-null homozygous breeding pairs

	Pair 1		Pair 2		Pair 3	
Sex	male	female	male	female	male	female
L1	4	3	4	3	5	2
L2	3	5	2	4	1	2
L3	3	3	5	1	5	1
L4	2	4	7	4	2	1
L5	4	6	5	4		
Total	16	21	23	16	13	6
Total	37		39		19	
Average No. of mice/Litter	7.4		7.8		4.8	

Total number of litters=14 Average number of mice per litter=6.8

The initial experiments presented above were carried out on the more robust outbred MF1:129/Ola genetic background. Subsequent observation of more subtle phenotypes were undertaken on the partially inbred background of strain 129/sv:129/Ola.

Body weight of SA-null mice

Body weights of male SA-null mice were recorded at 12 and 16 weeks of age (on a partially inbred background of 129/sv:129/Ola). Wildtype litter mates were used as controls. No significant difference in body weights was observed between the 2 groups at either age (p=0.11 and p=0.18). Similarly, body weights of female SA-null mice were recorded at 12 and 15 weeks of age (on the same partially inbred background). Again, no significant difference in body weight was observed between SA-null and wildtype litter mate controls (p=0.41 and p=0.11) (Table 4.6).

Table 4.6 Body weights of SA-null and wildtype mice (±SEM)

		Wildtype	SA-null	Significance
Male	12 week (g)	25.0±0.3 (n=24)	25.7±0.3 (n=23)	p=0.11
	16 week (g)	26.3±0.6 (n=12)	27.4±0.4 (n=12)	p=0.18
Female	12 week (g)	21.0±0.3 (n=25)	21.4±0.3 (n=25)	p=0. 41
	15 week (g)	21.6±0.7 (n=12)	22.9±0.4 (n=13)	p=0. 11

Organ weights of SA-null mice

As kidney and liver are the main sites of expression of the SA gene we observed these organs for weight differences between wildtype and SA-null mice. Organ weights of male SA-null mice were recorded at 16 weeks of age (on a partially inbred background of 129/sv:129/Ola). Wildtype litter mates were used as controls (Table 4.7). No significant differences in weights were observed between wildtype and SA-null mice for either organ. Similarly, kidney and liver weights of female SA-null mice were recorded at 15 weeks of age (on the same partially inbred background). Again, no significant difference in weight was observed between SA-null and wildtype litter mate controls. As increased ventricular mass is often associated with elevated blood pressure, ventricular heart weights were also recorded in this study. No significant differences were observed between wildtype and SA-null mice in either males or females (Table 4.7).

Table 4.7 Comparison of wildtype and SA-null mice (±SEM)

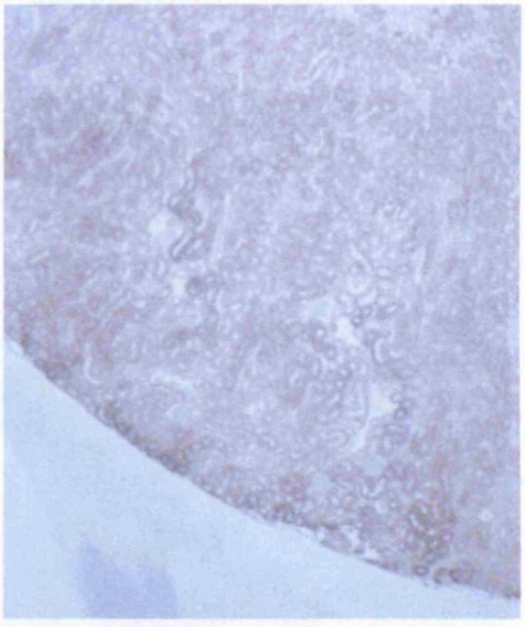
Organ weights (mg)	Wildtype	SA-null	Significance
Male 16 week kidney	374.7±11.0 (n=11)	379.1±12.3(n=11)	p=0.79
Female 15 week kidney	247.5±9.2 (n=12)	259.1±6.4 (n=13)	p=0.31
Male 16 week liver	1156±45 (n=9)	1087±40 (n=11)	p=0.27
Female 15 week liver	778.4±35.0 (n=12)	819.6 (n=13)	p=0.42
Male 16 week left ventricle	92.0±4.1 (n=12)	96.1±7.6 (n=10)	p=0.63
Female 15 week left ventricle	67.4±2.9 (n=12)	70.4±2.1 (n=13)	p=0.41
Male 16 week right ventricle	21.2±1.4 (n=12)	22.0±1.5 (n=10)	p=0.70
Female 15 week right ventricle	18.4±1.6 (n=12)	16.9±0.9 (n=13)	p=0.41

Microscopic observation of organs from SA-null mice

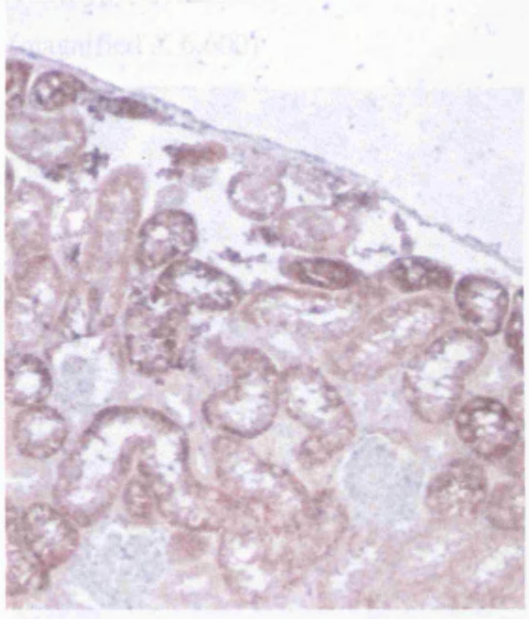
We investigated the possibility that absence of the SA protein may affect the structure of the kidney. Kidney from SA-null and wildtype mice were observed by light and electron microscopy for structural abnormalities. Preparation of tissues and generation of electron micrographs was kindly performed by Dr. Kevin West, Department of Pathology. No differences were apparent between SA-null and wildtype mice in the kidney by either light (Figs. 4.11) or electron microscopic examination (Fig. 4.12).

FIG 4.11 Light microscopic examination of wildtype and SA-null renal cortex

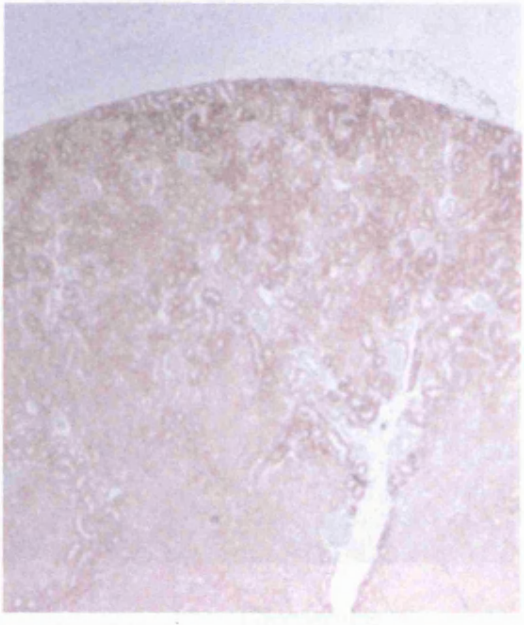
Low power magnification of wildtype renal cortex



High power magnification of wildtype renal cortex



Low power magnification of SA-null renal cortex



High power magnification of SA-null renal cortex

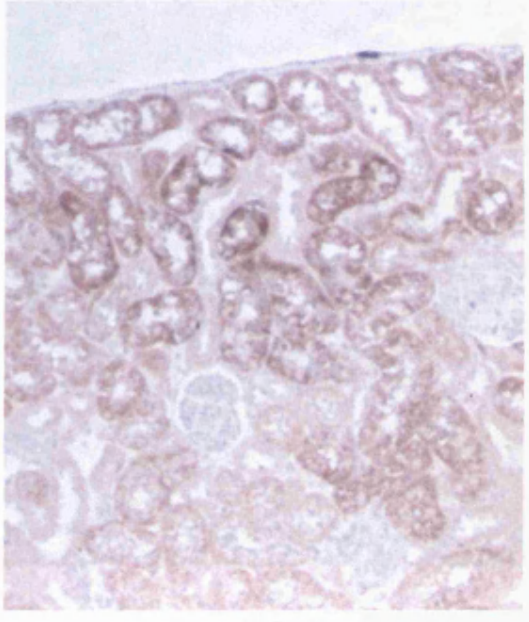
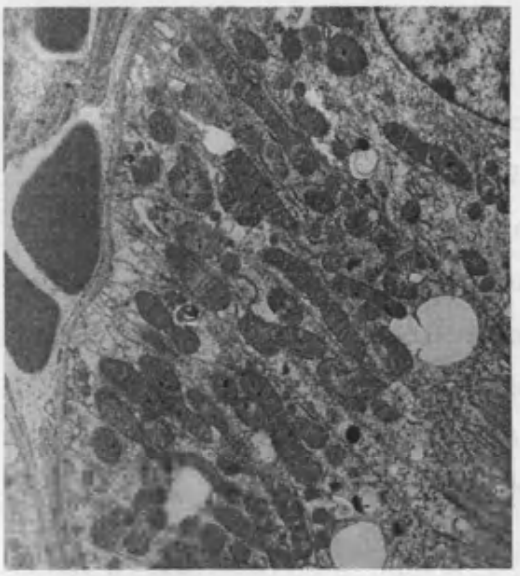
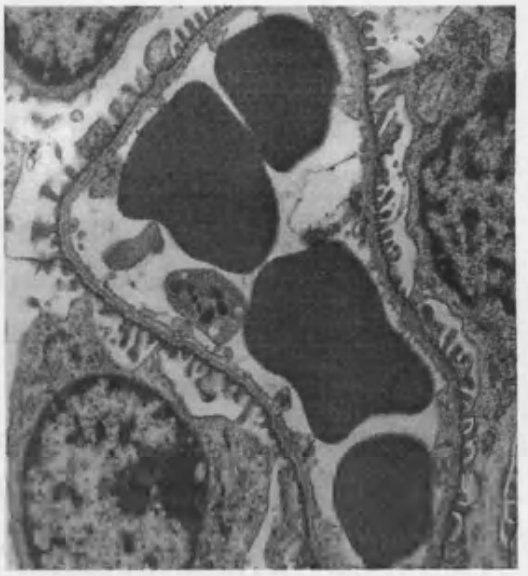


FIG 4.12 Electron microscopic examination of wildtype and SA-null renal cortex

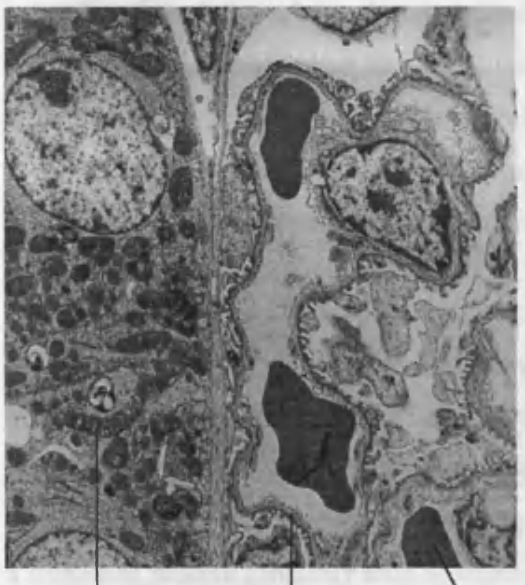
Wildtype proximal tubular cell (magnified X 4,400)



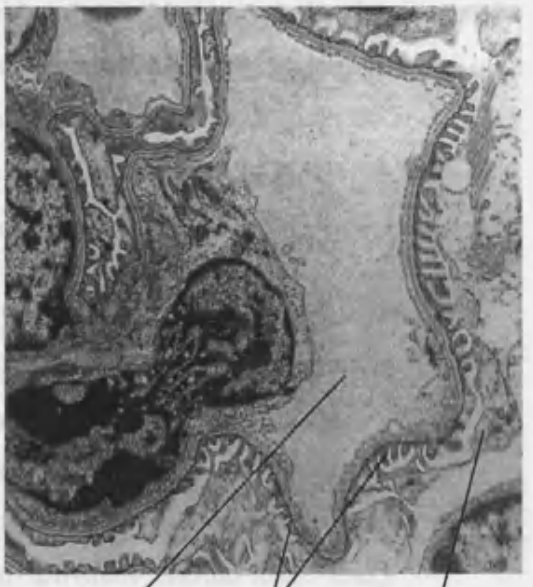
Wildtype glomerulus (magnified X 6,600)



SA-null proximal tubular cell and glomerulus (magnified X 3,7000)



SA-null glomerulus (magnified X 6,600)



DISCUSSION

Experiments within this chapter were performed to confirm the presence of the SA-null mutation within our transgenic mouse strain. Initial experiments were also performed to identify obvious morphological phenotypic differences between SA-null and wildtype mice.

Northern blot analysis showed that a truncated SA gene mRNA is produced by SA-null mice, corresponding to the loss of exons 2 and 3. Our targeting vector was designed to result in the elimination of exons 2 and 3 of the SA gene, however the terminal splice junction of exon 1 and the initial splice junction of exon 4 remained intact allowing the possibility of a truncated message being generated. Splicing of exons 1 to exon 4 is not a naturally occurring event. In SA-null mice direct splicing of these exons is forced to take place in the absence of exons 2 and 3. In the kidney of SA-null mice the truncated message is present at much lower levels than levels of the wildtype message in wildtype mice. There are several possible reasons for this. Inefficiency of splicing between exons 1 and 4 is the most likely explanation. Another possibility is reduced stability of the truncated transcript. Alternatively, sequences regulating transcription may have been deleted due to the absence of part of introns 1 and intron 2 of the SA gene. The SA gene has been shown to be androgen regulated (Melia *et al.*, 1998) however sequences affecting this regulation have not been studied. Other androgen regulated genes have been shown to contain control elements within intron 1 (Ho *et al.*, 1993; Rennie *et al.*, 1993). Therefore if such control elements exist within the SA gene, they may have been affected by our gene targeting strategy.

Review of the literature available on gene targeting in mice, reveals that most authors show absence of transcription or absence of protein production, but not necessarily both. For example, mice lacking the A-Raf protein kinase produced a truncated *A-Raf* transcript, however no protein was generated (Pritchard *et al.*, 1996 and personal communication). Cases where targeting results in the generation of reduced amounts of a truncated protein (possibly with reduced function) are often referred to as "leaky knockouts". This is not the case with our model as no protein is generated, as shown by Western blotting (Figs. 4.5 and 4.9).

SA protein was unlikely to be generated in our model as the translation start site of the SA gene had been deleted. In addition the remaining SA gene sequences in our model do not contain ATG codons with high levels of homology to the Kozak consensus sequence (Kozak, 1991). However it was not possible to completely eliminate the possibility of a truncated protein being generated without analysis of proteins by Western blotting. Kozak consensus sequences can give some guideline as to which Methionine codons will allow translation initiation. For example, comparison of the hypothesised start codon of the wildtype mouse SA protein with the Kozak consensus sequence reveals that only 6/10 bases are conserved. In some cases high levels of consensus will be required for initiation to occur. In other cases initiation will occur with less homology. Thus comparison of sequences surrounding potential ATG protein initiation codons within the SA gene can offer only an indication of whether translation initiation will occur. ATG codons down-stream of the beginning of exon 4 were compared with Kozak consensus sequences (Chapter 4, Appendix 2, page 120). In the portion of the protein remaining in SA-null mice, the highest level of homology of any ATG codon with the Kozak consensus sequence is 5/10 base pairs. Thus hypothetically translation of a truncated protein seemed unlikely.

However, it was necessary to confirm the absence of a truncated SA protein in our model. We therefore compared SA-null and wildtype proteins from both the kidney and the liver by Western blotting. The SA106 antibody used for these experiments was previously generated within our department and had been optimised for Western blotting (by Dr Bob Norman). At the time the strategy for generating this antibody was devised, there were no known sequences with high levels of homology to the SA protein. Therefore the typical strategy was employed of designing a peptide corresponding to the carboxy-terminal amino acids of the SA protein and using this to generate an antibody. During the course of this study, the KS gene was identified and noted to have high levels of homology to SA (Hilgers *et al.*, 1998). If sequence data had been available for KS at the time the SA106 antibody was generated, it would have been possible to design a peptide corresponding to a region of the SA gene with low

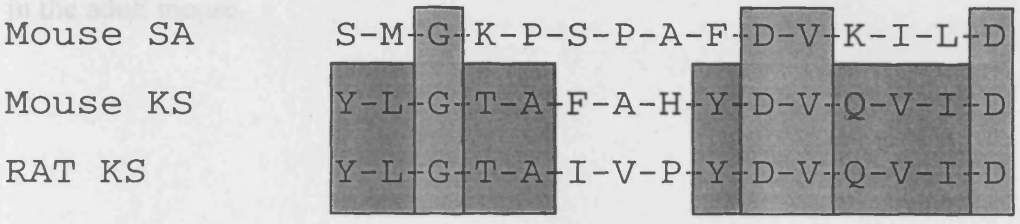
homology to KS. This was however not the case and as presented above (Fig. 4.5), cross-reaction occurs in the kidney between the SA106 antibody and the KS protein.

The similar amounts of protein binding to antibody SA106 in Western blots of kidney protein was also perplexing (Fig. 4.5). In the wildtype mice the antibody binds to both SA and KS, whereas in the SA-null mice it binds only to KS. Two factors are likely to contribute to this observation. Firstly Western blot analysis using heavily loaded protein gels is not a quantitative technique. Saturation could occur at many stages in the procedure, for example in the amount of antibody SA106 to which the blot was exposed. This is substantiated by the observation that when antibody was reserved at the end of an experiment and used in subsequent experiments, no binding was seen. Secondly, data presented in Chapters 5 and 6 of this work indicate that the expression level of KS in the kidney is substantially higher than that of SA (Chapter 5, Fig. 5.4 and Chapter 6, Fig. 6.4). Therefore SA protein levels in the kidney could easily be masked by the higher concentrations of KS protein.

In SA-null mice however DNA sequences are absent corresponding to 65 amino acids of the SA gene. Therefore if any SA protein were generated it would be substantially truncated and would migrate distinctly from the KS protein. Our results clearly show that no such truncated SA protein is generated. Western blotting of liver protein showed a complete absence of both SA and KS in SA-null mice as expected. Taken together these 2 results provide substantial evidence of the absence of SA protein in the SA-null mouse strain. We did consider the possibility of developing a more SA specific antibody. As the rat KS cDNA sequence and mouse SA cDNA sequence are both available, it is possible to identify potential regions of non-homology between these genes. A peptide could then be generated for an SA specific antibody. Comparison of protein sequences of rat KS and mouse SA genes shows very few potential regions for the design of such an antibody. I have identified a region spanning exons 10 and 11 of the mouse SA gene for which only 4 of 15 amino acids are identical between the 2 proteins (Fig. 4.15). A peptide produced to correspond to this region of the SA gene could be used to generate an antibody specific to the SA protein. Sequence data was generated for this region of the mouse KS gene as potentially 1 or 2 amino

acid differences between the rat and mouse KS genes could result in cross-reaction of an SA specific antibody with KS. However, as generation of a peptide and antibody is an expensive and time-consuming process, and our data were convincing, it was felt to be unnecessary for the purpose of this work.

FIG. 4.15 Comparison of amino acid sequences in the region to which an SA specific antibody could be generated



Another approach which may have been taken for the separation of SA and KS is isoelectric-focusing. This electrophoretic technique relies upon differences in charges of proteins for their separation, rather than differences in size. Iso-electric focussing gels are available commercially and can be blotted by modified Western blotting techniques.

Phenotypic observations

When heterozygous breeding pairs were used to generate a mixture of wildtype, heterozygote and SA-null mice, the ratio of offspring generated was dependent upon genetic background. On an outbred background frequencies of allele occurrence corresponded to Hardy-Weinberg equilibrium. However on a partially inbred background fewer male SA-null mice were generated than would be expected. As this was an interesting result, we attempted to repeat this observation but were unable to do so. Further mice are currently being generated from heterozygous crosses in an attempt to clarify this point. If preferential loss of male SA-null mice is a genuine occurrence, this represents an unusual finding which, to our knowledge, has not been previously reported for any targeted gene. An important point that may be relevant is that SA is expressed in the testes. This result could imply that male SA-null sperm are less viable than wildtype sperm or SA-null sperm carrying an X chromosome. Alternatively male SA-null embryos may be less successful at implantation than wildtype or female

embryos or be selectively lost during gestation. Clearly this effect is dependant on genetic background effects present in 129 verses MF1 mouse strains.

Despite the absence of the SA protein, it is clear that SA-null mice are fertile, grow normally and do not have any detectable morphological abnormalities in several relevant tissues including the kidney and liver. Thus these animals provide an appropriate model for studying the physiological effects of absence of the SA protein in the adult mouse.

Chapter4, Appendix 1

Sequence analysis of SA cDNA showing direct splicing of exon 1 to exon 4

Chapter 4, Appendix 2

Potential protein initiation sequences downstream of the beginning of exon 4 in the mouse. Surrounding sequences are given for comparison with the Kozak consensus sequence. The most important bases for Kozak consensus are highlighted in green. Other bases conforming to consensus sequence are highlighted in yellow

ATG number (down-stream of exon 3)	Distance (amino acids) from beginning of exon 4	Exon		In or out of frame	Most important surrounding bases in consensus	Other surrounding bases in consensus
			GCC GCC A/GCC AUG G Kozak consensus sequence			
	-65	3	TCT GTC ATC ATG G Wildtype SA protein initiation site	IF	2 of 2	4 of 8
1	13	4	GGT GGA TAG ATG G	OF	1 of 2	2 of 8
2	20	4	AGA GCT GAG ATG G Weak consensus	OF	2 of 2	2 of 8
3	50	4	GAC AGA GTA ATG G Weak consensus	IF	2 of 2	2 of 8
4	63	4	GGC TTG CAA ATG T	OF	0 of 2	2 of 8
5	100	5	TTA TTA CCG ATG A	OF	0 of 2	1 of 8
6	108	5	CAG CAG TAG ATG C	OF	0 of 2	0 of 8
7	113	5	GGC AGC TAA ATG T	OF	0 of 2	3 of 8
8	137	5	CTC AAG GAG ATG A	IF	1 of 2	1 of 8
9	138	5	AAG GAG ATG ATG A	IF	1 of 2	1 of 8
10	155	6	CAC GAC GAG ATG A	IF	1 of 2	3 of 8
11	156	6	GAC GAG ATG ATG G Weak consensus	IF	2 of 2	3 of 8
12	170	6	CCT CCT AAG ATG A	IF	1 of 2	2 of 8
13	185	6	TGT CTG TCA ATG G	OF	1 of 2	1 of 8
14	196	7	TAG CCT CCG ATG T	OF	0 of 2	2 of 8
15	198	7	TCC GAT GTG ATG T	IF	1 of 2	3 of 8
16	210	7	TCT GCA TGG AGT A	OF	0 of 2	3 of 8
17	217	7	TTT TTC TCC ATG G	OF	1 of 2	3 of 8
18	222	7	CCA AGG AGC ATG T	OF	1 of 2	2 of 8
19	258	8	GCC TAC CGG ATG C	IF	0 of 2	4 of 8
20	262	8	TTG TTC AGA ATG A	OF	1 of 2	1 of 8
21	264	8	CAG AAT GAC ATG A	IF	1 of 2	1 of 8
22	287	9	CCT GAA GTG ATG G Weak consensus	IF	2 of 2	2 of 8
23	289	9	GAT GGA ACA ATG G Moderate consensus	OF	2 of 2	3 of 8
24	299	10	TAG ACA TCT ATG A	OF	0 of 2	2 of 8
25	317	10	TTC AAG GGG ATG A	IF	1 of 2	1 of 8
26	324	10	CCC GGC TCA ATG G	IF	1 of 2	5 of 8
27	332	10	CTG CTT TTG ATG T	OF	0 of 2	0 of 8

28	337	11	GAT TTT	TAG ATG A	OF	0 of 2	1 of 8
29	339	11	TAG ATG	AAA ATG G	OF	2 of 2	0 of 8
30	367	11	TTA CTC	ATT ATG T	OF	1 of 2	1 of 8
31	392	12	GAT ATA	TGG ATG A	OF	0 of 2	1 of 8
32	394	12	TGG ATG	AAG ATG G	OF	2 of 2	0 of 8
33	404	12	CAA GAT	CAG ATG A	OF	0 of 2	1 of 8
34	459	14	ACA AGT	CCC ATG A	OF	0 of 2	3 of 8
35	502	15	TCA AAA	GAA ATG A	OF	1 of 2	1 of 8
36	508	15	GAA AAA	GGA ATG G	OF	2 of 2	1 of 8

Chapter 5

Observation of indirect blood pressure and response to increased salt intake in SA-null and wildtype mice

Chapter 5

Observation of indirect blood pressure and response to increased salt intake in SA-null and wildtype mice

Chapter 5: Observation of fluid intake, fluid deprivation, indirect blood pressure and response to increased salt intake in male SA-null and wildtype mice

INTRODUCTION

As it has been suggested that the SA gene may be implicated in the pathogenesis of hypertension, an important part of my studies was to examine the effect of absence of SA protein on blood pressure phenotype. Differences in SA expression levels between rats subject to high and low salt diets (Iwai and Inagami, 1991) and the proximal tubular location of SA expression (Patel *et al.*, 1994) lead to the hypothesis that SA may be involved in sodium and fluid homeostasis. We therefore compared basal blood pressures of wildtype and SA-null mice and examined the effect of high salt intake on blood pressure in wildtype and SA-null mice.

METHODS

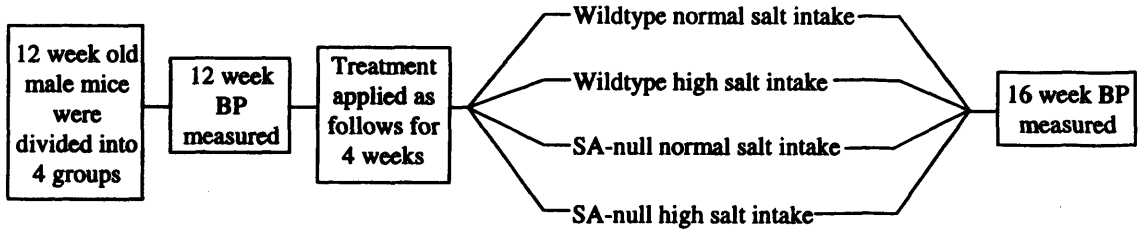
Experimental outline

Breeding pairs were established using mice heterozygous for the SA-null mutation on a 129/sv:129/Ola background. Genotypes of mice were identified by PCR analysis at 4 weeks of age (see Chapter 2, Methods). 24 wildtype and 23 SA-null male mice were randomised into 4 experimental groups:

Wildtype normal salt intake (n=12)	SA-null normal salt intake (n=12)
Wildtype high salt intake (n=12)	SA-null high salt intake (n=11)

Animals were numbered anonymously and genotypes were not known during subsequent experiments. Blood pressures and body weights of mice were determined at 12 weeks of age. Each mouse was then subject to either a normal or high salt intake for 4 weeks. Normal salt intake was provided in the form of a 0.7% salt diet (Diet RM1, Special Diet Services), high salt intake was provided by supplementing this diet with 1.5% saline in place of tap water for drinking. After 4 weeks of treatment, 16 week blood pressure and body weight were determined. A diagrammatic representation of this protocol is given in Fig. 5.1.

FIG. 5.1 High salt intake protocol



Mice were then sacrificed and kidney, liver, heart, brain and testes were flash frozen in liquid nitrogen. Organs were later weighed and RNA extracted.

Blood pressure measurement

Systolic blood pressure measurements were made by the indirect tail-cuff method using the IITC system as described in Chapter 2 (Methods). Measurements made using this system have previously been validated by Johns *et al.* (1996); tail-cuff measurements were shown to correlate well with direct blood pressure measurements by arterial cannulation.

For each mouse blood pressure was measured at 12 weeks of age on three separate days. Each day between 8 and 10 separate measurements were obtained and an average taken for each day. The average of the 3 days was then assumed as the 12 week blood pressure. 16 week blood pressure measurements were carried out in a similar manner.

Statistical analysis

Blood pressure and data was analysed by oneway ANOVA using the Minitab statistical software package. Averages are expressed as ±SEM.

RESULTS

12 week body weight and blood pressure analysis

Average basal systolic blood pressures and weights at 12 weeks of age for wildtype and SA-null mice are presented in Table 5.1.

Table 5.1 Basal systolic blood pressure and body weights of wildtype and SA-null male mice at 12 weeks of age (±SEM)

	Wildtype	SA-null
12 week basal systolic BP (mmHg)	111.0±2.0 (n=24)	116.7±1.7 (n=23)
12 week body weight (g)	25.0±0.3 (n=24)	25.7±0.3 (n=23)

Basal blood pressures of SA-null mice at 12 weeks of age were slightly elevated compared to wildtype mice (p=0.037).

No genotype dependant effect on weight was observed at 12 weeks of age (p=0.11).

16 week blood pressure and body weight analysis

Average systolic blood pressures for the 4 treatment groups are presented in Table 5.2

Table 5.2 Systolic blood pressure and body weights of the 4 treatment groups at 16 weeks of age (±SEM)

	Wildtype	SA-null
16 week BP: normal salt intake (mmHg)	114.0±2.0 (n=12)	113.6±2.5 (n=12)
16 week BP: high salt intake (mmHg)	127.1±2.7 (n=12)	116.5±4.3 (n=11)
16 week body weight: normal salt intake (g)	26.3±0.6 (n=12)	27.4±0.4 (n=12)
16 week body weight: high salt intake (g)	26.7±0.5 (n=12)	27.5±0.2 (n=11)

At 16 weeks of age there was no difference between blood pressures of wildtype and SA-null mice subjected to normal salt intake (p=0.82).

In wildtype mice administration of high salt intake caused an increase in BP of an average of 13mmHg (p=0.001). SA-null mice however showed no increase in BP due to high salt intake (p=0.79). Consequently a significant effect of genotype was observed on BP in high salt intake animals (p=0.008 for comparison between wildtype and SA-null mice after high salt intake). Graphical representation of 12 and 16 week average blood pressure data for each group can be seen in Fig. 5.2.

No significant effect on body weight was observed by either genotype or salt intake at 16 weeks of age.

FIG. 5.2 Graphical representation of 12 and 16 week blood pressures

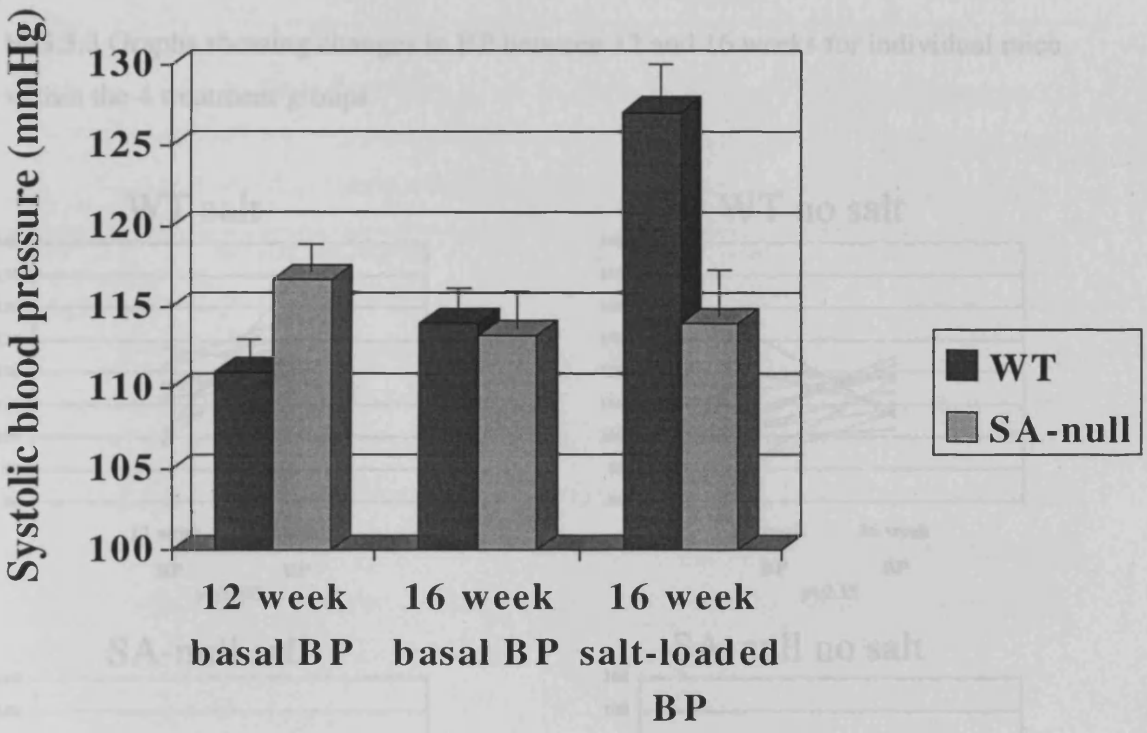
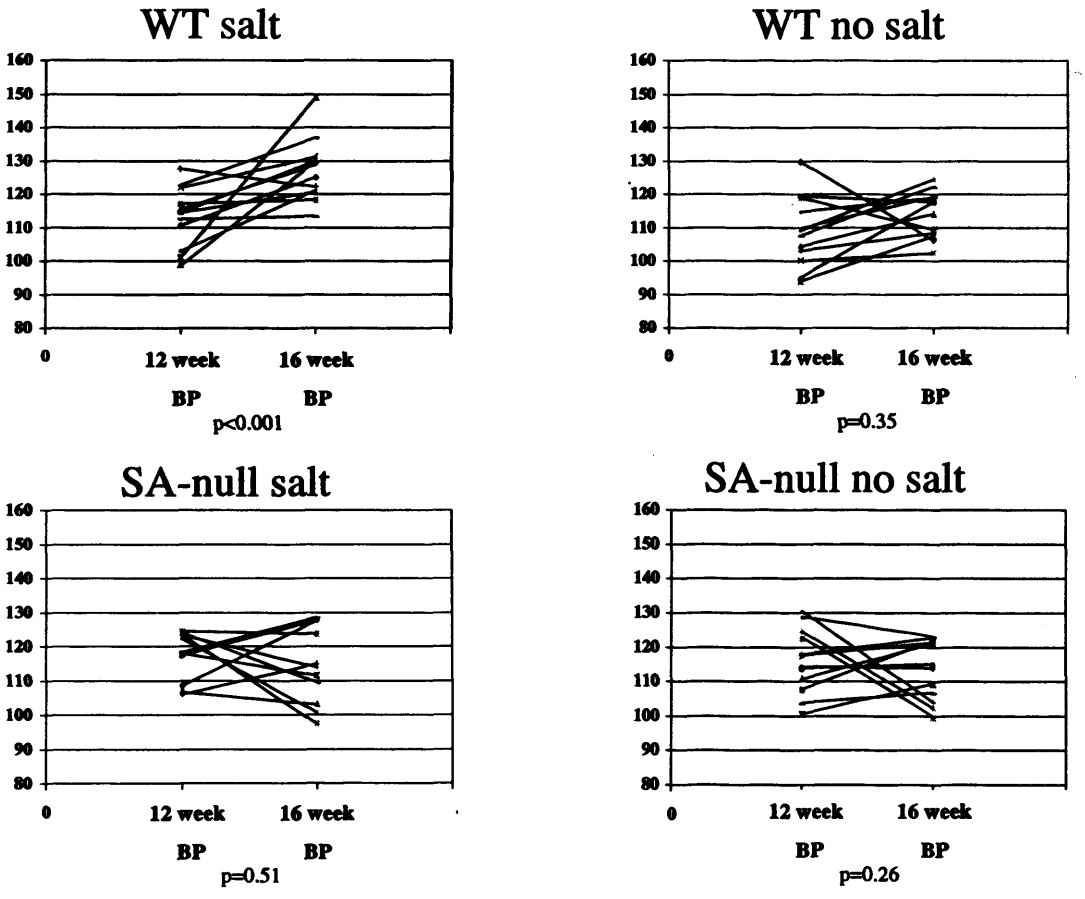


Figure 5.3 shows changes in blood pressure between 12 and 16 weeks of age for individual mice within the 4 treatment groups. For each group the average 12 and 16 week blood pressures were compared by oneway ANOVA. A significant change in BP was observed in the wildtype high salt intake mice ($p<0.001$). No other group showed a significant change in blood pressure between 12 and 16 weeks of age.

FIG.5.3 Graphs showing changes in BP between 12 and 16 weeks for individual mice within the 4 treatment groups



Kidney and liver weight analysis in SA-null and wildtype mice

The effect of high salt intake on organ weights in wildtype and SA-null mice are presented in Table 5.3.

Table 5.3 Comparison of organ weights between wildtype and SA-null mice

		Wildtype	SA-null
kidney	normal salt diet (mg)	374.7±11.0 (n=11)	379.1±12.3 (n=11)
	high salt diet (mg)	375.6±17.5 (n=7)	402.1±9.0 (n=7)
	normal salt diet (mg/g body weight)	14.4±0.3 (n=11)	13.9±0.3 (n=12)
	high salt (mg/g body weight)	14.3±0.6 (n=12)	14.6±0.3 (n=11)
liver	normal salt diet (g)	1.16±0.05 (n=9)	1.09±0.04 (n=11)
	high salt diet (g)	1.09±0.06 (n=6)	1.09±0.07 (n=8)
	normal salt diet (mg/g body weight)	44.2±1.9 (n=9)	39.9±1.3 (n=11)
	high salt (mg/g body weight)	41.2±2.2 (n=6)	39.9±2.3 (n=8)

As kidney and liver are the main sites of expression of the SA gene, it was possible that absence of SA protein may have affected the weights of these organs. Kidney and liver weights were therefore measured. However no significant effect of genotype or high salt intake was observed on either kidney or liver weights.

Expression data

Expression levels within the kidney

Kidney RNA was extracted from 5 animals from each of the 4 experimental groups and used for Northern blot analysis (60μg per sample). RNA was hybridised with probes specific for the following genes: SA, KS, renin and GAPDH.

Expression of the SA gene

Expression levels of SA in the 4 treatment groups are shown in Fig. 5.4.

In wildtype mice a single SA transcript was apparent in animals subject to both the high and normal salt intakes (Fig 5.4). The administration of a high salt intake had no effect on SA expression in wildtype mice.

In the SA-null mice low levels of the truncated SA transcript were observed (corresponding to the full-size transcript minus exons 2 and 3). Again administration of a high salt intake had no effect on SA expression levels.

Densitometric analysis of Northern blot data for the SA gene is presented in Fig. 5.5. In wildtype mice expression of the full-length transcript is approximately 9 times that of the truncated transcript in SA-null mice, irrespective of salt intake.

Expression of the KS gene

No effect of genotype or salt intake was observed on KS expression levels in wildtype or SA-null mice (Fig. 5.4).

Expression of the renin gene

No differences in expression levels of renin were observed between wildtype and SA-null mice in either the high or normal salt groups. However a small reduction in expression (of approximately 20%) could be observed in the mice subject to high intake compared to those subject to normal salt intake (Fig. 5.4). This effect was further characterised by densitometric analysis (Fig. 5.6) and ANOVA. High salt intake has slightly suppressed the expression of the renin gene in both wildtype and SA-null mice ($p=0.019$).

FIG. 5.4 Northern blot of kidney RNA hybridised with various cDNA probes

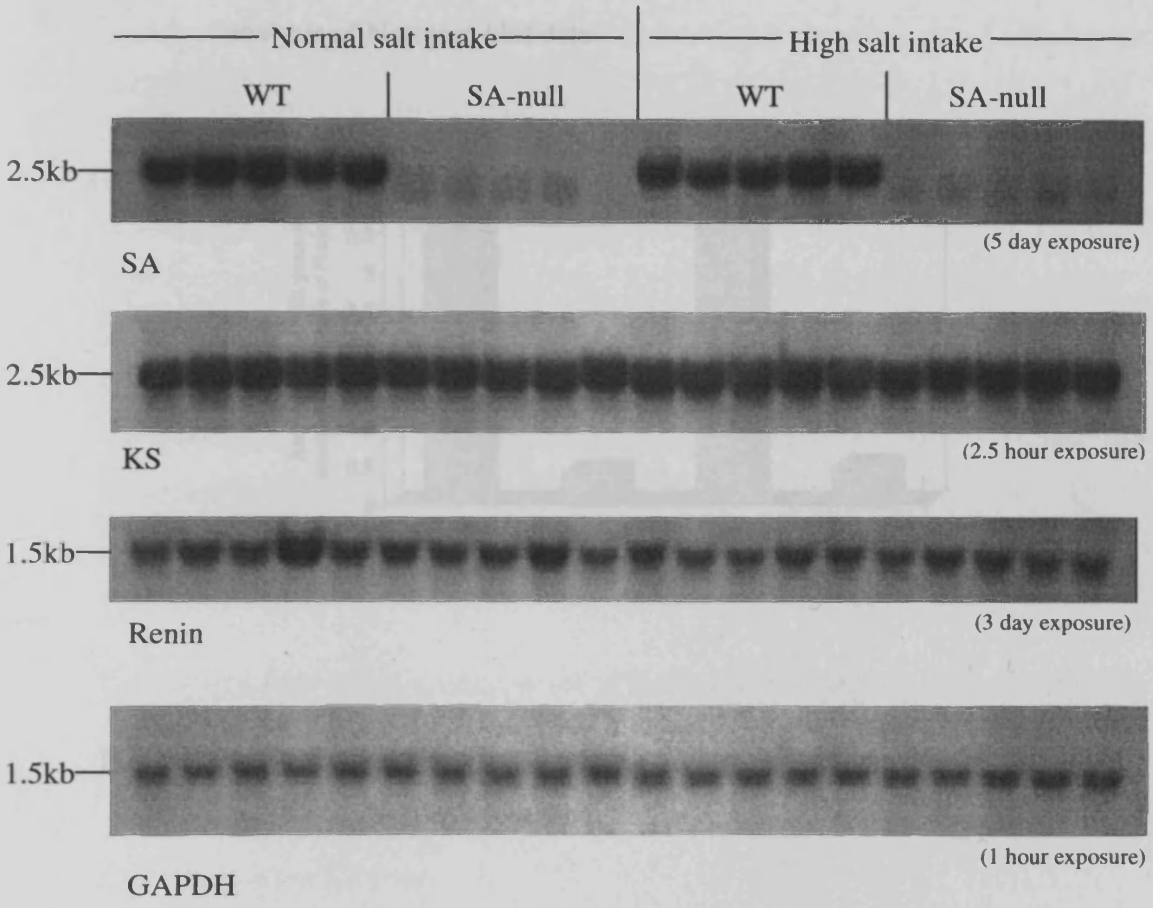


FIG. 5.5 Changes in SA expression in the kidney as determined by densitometric analysis of Northern blot data

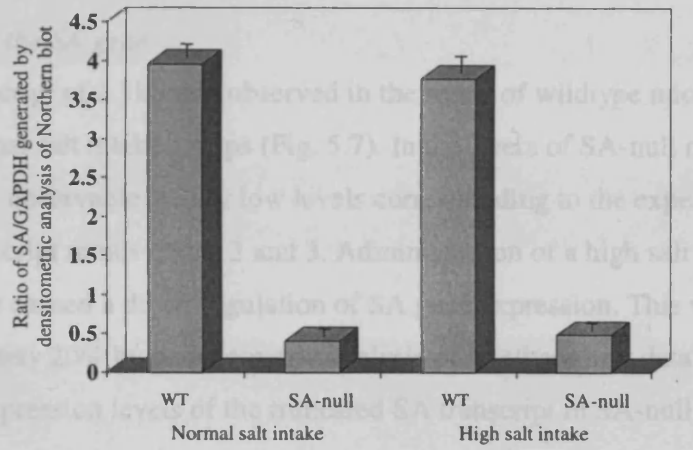
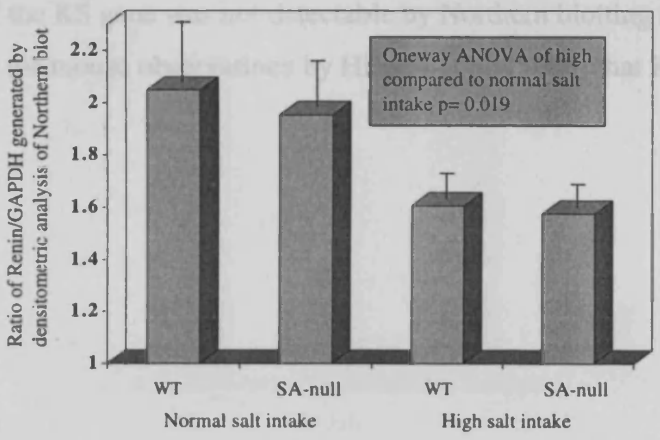


FIG. 5.6 Changes in Renin expression in the kidney as determined by densitometric analysis of Northern blot data



Expression levels within the liver

Liver RNA was extracted from 4 mice from each of the 4 experimental groups and used for Northern blot analysis. RNA was hybridised with probes specific for SA and GAPDH.

Expression of the SA gene

A single transcript of 2.5kb was observed in the livers of wildtype mice in both the high and normal salt intake groups (Fig. 5.7). In the livers of SA-null mice a single transcript was observable at very low levels corresponding to the expected size of the wildtype transcript minus exons 2 and 3. Administration of a high salt intake to wildtype mice caused a down-regulation of SA gene expression. This was confirmed to be approximately 20% by densitometric analysis of Northern blot data (Fig. 5.8) ($p=0.036$). Expression levels of the truncated SA transcript in SA-null mice were not affected by the administration of a high salt diet ($p=0.57$).

Comparison of levels of SA expression in the liver and kidney (positive control, Fig. 5.7) show that levels of expression of the SA gene are substantially lower in the liver than in the kidney in male mice.

Expression of the KS gene

Expression of the KS gene was not detectable by Northern blotting in this experiment confirming in the mouse observations by Hilgers *et al.* (1998) that KS is not expressed in the liver.

FIG. 5.7 Northern blot of liver RNA hybridised with various cDNA probes

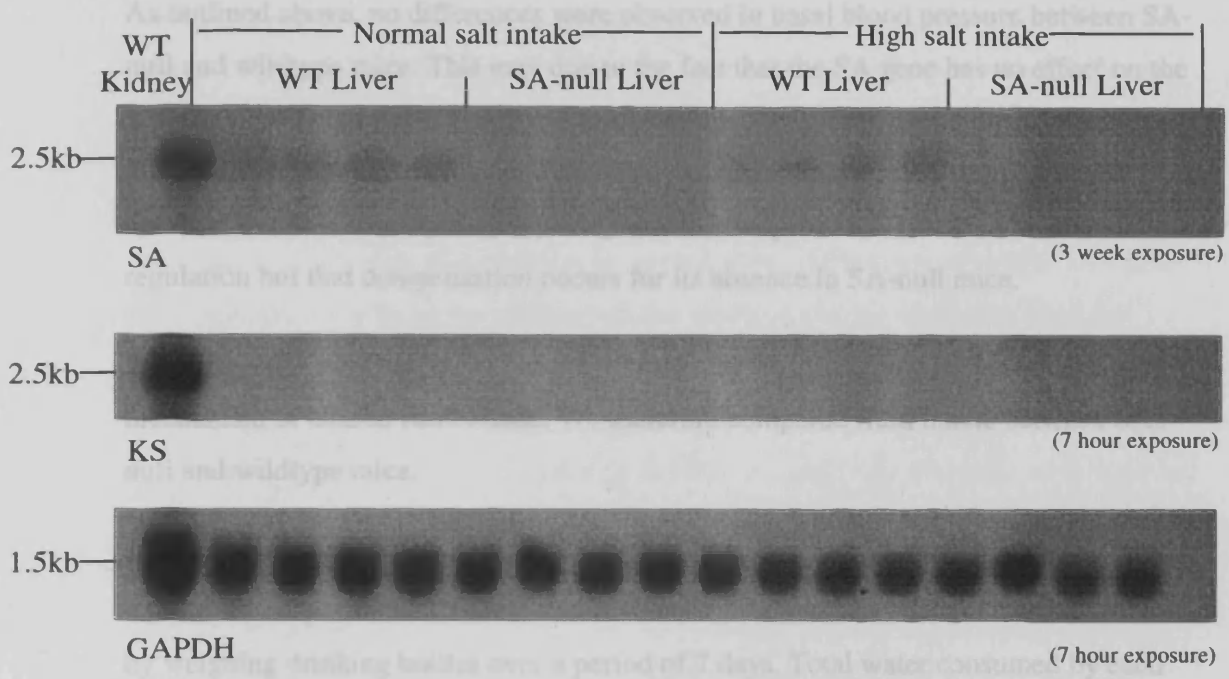
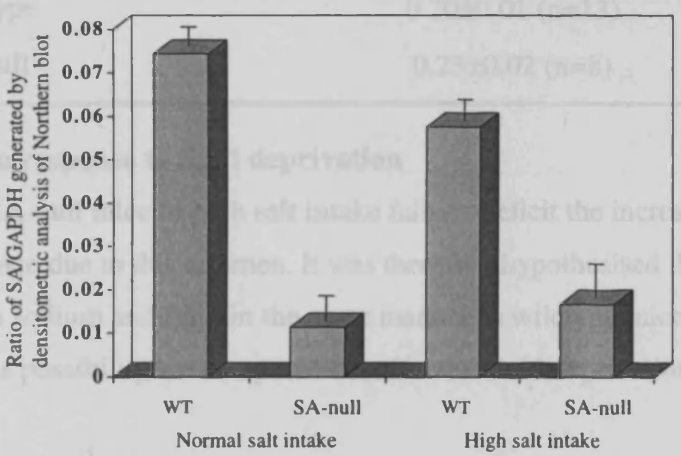


FIG. 5.8 Changes in SA expression in the liver as determined by densitometric analysis of Northern blot data



Fluid consumption by SA-null and wildtype mice

As outlined above, no differences were observed in basal blood pressure between SA-null and wildtype mice. This may due to the fact that the SA gene has no effect on the maintenance of basal BP. However as differences were observed between genotypes after high salt intake, the SA gene obviously has an intimate relationship with BP homeostasis. It is therefore a possibility that the SA protein is involved in basal BP regulation but that compensation occurs for its absence in SA-null mice.

It was hypothesised that basal blood pressure may be maintained in SA-null mice by a mechanism of altered fluid intake. We therefore compared fluid intake between SA-null and wildtype mice.

15-17 week old SA-null and wildtype litter mate controls (strain 129/sv:129/Ola) were used in this experiment. Crude measurement of fluid intake for each mouse was made by weighing drinking bottles over a period of 7 days. Total water consumed by each mouse per day was calculated. No difference in fluid intake was observed between wildtype and SA-null mice ($p=0.14$) (Table 5.4).

Table 5.4 Fluid intake by SA-null and wildtype mice (±SEM)

	Fluid intake per day/gram body weight (g)
Wildtype	0.20±0.01 (n=13)
SA-null	0.23±0.02 (n=8)

Weight loss in response to fluid deprivation

Exposure of SA-null mice to high salt intake failed to elicit the increase in BP observed by wildtype mice due to this regimen. It was therefore hypothesised that SA-null mice may not retain sodium and fluid in the same manner as wildtype mice. In an attempt to investigate this possibility we compared the effect of fluid deprivation on wildtype and SA-null mice.

13 week old male SA-null mice and wildtype littermate controls (strain 129/sv:129/Ola) were deprived of water for a period of 22 hours. Mice were weighed

before and after water deprivation and percentage loss in body weight calculated. Water was then restored and body weight measured again after 1 hour of exposure to water *ad libitum*. Weight gained in this hour was calculated as a percentage of weight lost.

All mice seemed lethargic after dehydration, irrespective of genotype. After 1 hour of rehydration no lethargy was apparent in any mice. No differences in weight loss after fluid deprivation were observed between the wildtype and SA-null mice (p=0.68) (Table 5.5). However levels of rehydration after 1 hour did vary between the wildtype and SA-null groups. SA-null mice regained an average of 74.4% of lost body weight compared to 55.4% for wildtype mice (p=0.019). As relatively few mice were used in this experiment it is difficult to draw conclusions from this apparent trend. It is however possible that whilst SA-null mice suffer the same level of dehydration as wildtype mice, this is tolerated less well. Mice therefore drink more to rehydrate more rapidly. The repetition of this experiment with a larger number of mice to confirm results would be interesting. It can be concluded that SA-null mice are capable of conserving fluid after water deprivation in the same manner as wildtype mice.

Table 5.5 Weight loss in response to fluid deprivation in SA-null and wildtype mice (±SEM)

	% body weight lost	% lost weight regained
Wildtype	9.8±0.6 (n=8)	55.4±3.9 (n=8)
SA-null	9.4±0.4 (n=5)	74.4±6.2 (n=5)

DISCUSSION

Early studies implicating the SA gene in the pathogenesis of hypertension showed higher levels of SA expression in hypertensive compared to normotensive rats (Iwai and Inagami, 1991; Samani *et al.*, 1993). However during course of the current studies, expression levels of the SA gene in other strains were reported which confound the simple hypothesis that increased renal expression of SA causes an increase in BP. In the Milan hypertensive rat expression levels of SA are low compared to the Milan normotensive rat (Lodwick *et al.*, 1998) and in WKY rats from the Izumo colony in Japan expression levels are similarly high to those of SHR/Izm (Ishinaga *et al.*, 1997). Obviously no simple relationship exists between SA gene expression levels and blood pressure. The presence of certain genetic backgrounds may be necessary for an effect of the SA protein to be observed on blood pressure. Investigation of blood pressure in our SA-null model provides a useful direct method for elucidating the relationship between the presence of the SA protein and blood pressure.

Our main observations resulting from these experiments were as follows:

- Absence of the SA-protein caused a small increase in basal blood pressure at 12 weeks of age. This effect may however be spurious as it was no longer apparent at 16 weeks of age.

- Administration of a high salt intake caused an increase in blood pressure in wildtype mice. This effect was completely ameliorated by the absence of the SA gene in SA-null mice.

- Administration of a high salt intake caused no change in SA expression levels in the kidney of wildtype or SA-null mice. However in the liver of wildtype mice expression of SA was decreased by approximately 20% due to the administration of a high salt diet.

- No effect on expression of renin or KS was observed due to the absence of the SA protein. Administration of a high salt intake caused a decrease in renin expression in mice of both genotypes.

Blood pressure analysis and expression data

Average basal blood pressures in wildtype and SA-null mice were 111mmHg and 116.7mmHg respectively at 12 weeks of age. Although this difference is significant (p=0.037), it is possible that this is a spurious result as a difference was no longer observed at 16 weeks of age (p=0.82) (however, see Chapter 6, Discussion, page 163). It can therefore be concluded that absence of the SA protein has no profound effect on basal BP in SA-null mice. If the small effect on basal BP apparent at 12 weeks of age is a genuine result, compensation for absence of SA protein has occurred by 16 weeks of age. If however the apparent effect on basal BP at 12 weeks of age is a spurious result, this does not necessarily mean that SA has no role in maintaining basal BP. Compensation for its absence may occur by other systems in SA-null mice. A possible explanation for the difference in basal BP at 12 weeks of age in male mice is discussed in Chapter 6 (page 163).

The most significant result from this work is the observation that SA-null mice fail to respond to an increase in dietary salt in the same manner as wildtype mice. High salt intake in wildtype mice caused an increase in BP of approximately 13mmHg (p=0.001). However in SA-null mice no increase in blood pressure was observed after high salt intake (p=0.79). Thus in this model the absence of SA protein offers a protective effect against salt-induced increase in blood pressure.

Compared to some salt-sensitive models of hypertension, for example the Dahl salt-sensitive rat in which the sodium induced rise in BP is approximately 70mmHg (Rapp and Dene 1985), the BP increase observed in our experiment was modest. This may more closely mimic the scenario in some humans. Interestingly this increase was completely ameliorated by the absence of the SA protein thus implying that the

predominant mechanism for the sodium induced BP increase in this strain is mediated via the SA protein.

Increase in blood pressure in response to high salt intake has been reported in several strains of rat, for example Dahl salt-sensitive and salt-sensitive SHR and has also been observed in some strains of mice. The effect of high salt intake in mice has been most studied when blood pressure in various transgenic and knockout mouse models have been compared with wildtype mice. Alfie *et al.* (1996) observed salt sensitivity in bradykinin B_2 Receptor knockout mice. Blood pressures of 129/J wildtype mice used as positive controls in this experiment were observed to increase by an average of 12mmHg after administration of a high salt diet for 6 weeks, a result consistent with our findings. John *et al.* (1995) administered a high salt intake to C57BL/6 X 129 F1 hybrid mice during investigations of salt sensitivity in *proANP* null mice. In these experiments however no change in blood pressure was observed between wildtype mice subject to normal and high (8%) salt diets for 2 weeks. Similarly in several other studies no effect was observed on blood pressure due to high salt intake (Kennedy *et al.*, 1999; Morita *et al.*, 1999; Melo *et al.*, 1998; Lopez *et al.*, 1995). These experiments also investigated mice on a mixed C57BL/6 and 129 genetic background, a combination which appears to be salt resistant. Interestingly however C57BL/6 mice have been reported to be salt sensitive (Paigen *et al.*, 1999; Carlson and Weiss, 2000). In addition, method of salt administration (diet, saline or both) and duration of high salt administration may affect experimental results. However these explanations may not fully explain the apparent differences in salt sensitivity between these studies; in 1997 Alfie *et al.* presented data contradicting their earlier work (Alfie *et al.*, 1996); wildtype 129/J mice were reported to show no increase in BP after administration of a high salt intake for 6-8 weeks. Reasons for discrepancies between results in these experiments are not obvious. Very few studies have been performed subjecting 129 mice to an increased salt intake. The strain on which we have studied the SA-null mutation (129/sv:129/Ola) appears to be sensitive to an increase in salt intake in our experiment. We have confidence in our observed increase in BP in wildtype 129 mice for several reasons. Our final BP measurements are based on a large number of readings. For each

mouse investigated, approximately 30 individual BP readings were taken over a period of 5 days to determine BP at a given time point. Therefore, as each experimental group consisted of 12 mice, a total of approximately 360 BP readings were used to generate an average group BP. Despite some variability between the BP readings for individual mice, the overall data were highly statistically significant (p=0.001).

Our observations raise the question of how salt increases BP in these animals, such that this effect is ameliorated by the absence of the SA protein. Sensitivity to salt is a complex phenotype and many factors have been implicated in its etiology. Sodium retention due to the kidney's impaired ability to excrete salt and promote natriuresis, an increase in peripheral vascular resistance and alterations of the sympathetic nervous system have been implicated (Campese, 1994). As SA ablation has ameliorated salt-sensitivity and the main site of SA gene expression is in the proximal tubule, it is likely that this effect is caused by altered sodium and water homeostasis in the proximal tubules, possibly by increased excretion of sodium. However, SA is also expressed in the brain, which is involved in BP control via the CNS and the testes, which are the main site of production of androgens. It is therefore also a possibility that other mechanisms besides those mediated via the kidney are responsible for the effect observed in SA-null mice.

As the mechanism for ameliorated salt sensitivity is most likely to be mediated via the kidney, we investigated expression of renin in the kidney in order to determine any alterations in the renin angiotensin system caused by the absence of the SA protein. No genotype dependent effect was observed on renin expression in either the control or salt treated mice in this experiment indicating that alterations in the RAS are unlikely to compensate for absence of the SA gene in SA-null mice. Further confirmation of this could be obtained by observation of angiotensinogen expression levels. Blockade of the RAS by an angiotensin II antagonist and subsequent observation of BP would also highlight any interaction between the absence of the SA protein and the RAS.

As the KS gene shows high levels of homology to SA we investigated the possibility that it may be up-regulated to compensate for the absence of SA in SA-null mice. However no difference in expression levels of KS were observed between wildtype and SA-null mice. As KS is highly expressed in the kidney, it is possible that compensation for lack of SA may occur without the necessity of up-regulation.

Possible kidney related mechanisms for the absence of salt sensitivity in SA-null mice were further investigated by observation of fluid intake and fluid deprivation. It was hypothesized that maintenance of BP in SA-null mice may be mediated via increased fluid intake. Comparison of fluid intake between wildtype and SA-null mice however revealed no gross differences between the 2 genotypes, within the constrains of the experimental protocol for measuring fluid intake.

The possibility that SA-null mice are incapable of retaining sodium in the same manner as wildtype mice was investigated by the observation of fluid deprivation. Sodium excretion is accompanied by water loss, therefore we investigated response of SA-null mice to water deprivation. No differences were observed between weight loss by wildtype and SA-null mice due to fluid deprivation for a period of 22 hours implying that SA-null mice are as capable as wildtype mice of conserving sodium and fluids under duress. Interestingly, after 1 hour of rehydration, SA-null mice had replaced an average of 74% of lost body weight compared to only 55% in wildtype mice ($p=0.019$). After fluid deprivation, increased osmotic pressure of cellular fluids is perceived by the hypothalamus, triggering the production of ADH by the pituitary gland, which allows the reabsorption of fluids by the distal tubules in the kidney. In addition the hypothalamus also triggers a feeling of thirst. It is unlikely that the mechanism of ADH production is affected by the absence of the SA-gene, otherwise a difference in weight loss due to excess fluid loss would have occurred in SA-null mice. However the perception of thirst may be altered somehow by the absence of this gene. After drinking, nerve impulses from the distended stomach usually quench the thirst response before fluid is actually absorbed. It is possible that transmission of such nerve impulses is altered in SA-null mice. Alternatively the mechanism by which the hypothalamus

triggers the thirst response may be altered. Another possibility is that the osmotic pressure in the cells of SA-null mice may be higher than in wildtype mice after fluid deprivation. This could be caused by an inability to concentrate sodium in the urine in the same manner as wildtype mice. Thus wildtype mice and SA-null mice may generate the same decreased volume of urine (causing the same weight loss), however less sodium may have been excreted by SA-null mice. If this is the case SA-null mice exposed to a high salt diet would be expected to drink substantially more than wildtype mice. In our BP experiment we administered salt as saline, providing no source of tap water. SA-null mice showed no increase in BP implying that they can excrete excess sodium successfully with no parallel increase in fluid intake. It therefore seems unlikely that decreased ability to concentrate sodium in the urine is responsible for the observed difference in fluid intake after dehydration in these mice. An interesting experiment may be to compare fluid intake between wildtype and SA-null mice subject to a high salt diet.

Alternatively alterations in the renin-angiotensin system between wildtype and SA-null mice may be responsible for the difference in drinking response after fluid deprivation. Angiotensin II is known to invoke thirst, therefore up-regulation of the RAS could cause SA-null mice to feel increased thirst after fluid deprivation.

Expression levels of SA in the kidney were found to be similar in wildtype mice subject to normal and high salt intake. This is in contrast to observations by Iwai and Inagami (1991) who noted SA expression to be slightly reduced in the kidneys (30%) by high salt intake. In the same study Iwai and Inagami observed SA expression in the liver to be 60% reduced on a high compared to low salt diet. We observed a 20% reduction of SA expression in the liver from a normal to high salt diet. The significance of this is unclear.

Other observations on the SA locus and salt-sensitivity

As well as being implicated as a locus for hypertension, the SA locus has also been implicated as a locus for salt sensitivity in rats. Iwai *et al.* (1998) generated a congenic

strain containing the chromosomal region around SA from an SHR background on a WKY background. This strain was observed to have increased BP compared to wildtype WKY rats. In addition, administration of a high salt diet further increased BP in congenic but not wildtype rats in these experiments. It is not possible to conclude whether SA or another closely linked gene is responsible for these observations. However it is interesting to note that increased salt sensitivity is conferred by SHR sequences which also produce increased levels of SA expression.

In humans the only positive study to date to find an association between the SA gene and hypertension studied a Japanese population (Iwai *et al.*, 1994). As the Japanese traditionally consume a high salt diet this result may also implicate the SA locus in the pathogenesis of salt-sensitive hypertension.

Harrap *et al.* (1995) studied urinary sodium excretion in relation to SA genotype in a Caucasian population. No differences in excretion levels were observed between groups carrying different genotypes, implying that the SA gene variation, as defined by this polymorphism, has no effect on sodium retention or excretion. However if the effect of SA genotype is only apparent on certain genetic backgrounds or under specific environmental conditions, genetic and environmental variability may have masked potential positive results in this study. Also, if the RFLP under observation did not represent a causative mutation, a positive result may have been masked. This study also showed absence of correlation between BP and genotype at the SA locus. An interesting result may have been obtained if subjects with a high urinary sodium excretion (presumably indicative of a high sodium intake) were observed independently to those with low sodium excretion with respect to blood pressure. This sub-set of the population would share the environmental factor of a high sodium intake with the Japanese population in which the SA gene was found to cosegregate with hypertension (Iwai *et al.*, 1994).

There is considerable variation in expression levels of the SA gene between different rat strains. However no obvious correlation exists between expression levels and either

basal BP or salt sensitive BP. Not all rat strains expressing high levels of the SA gene are salt sensitive. Therefore whilst elimination of the SA gene has conferred a protective effect against salt, presence of the SA gene does not in itself cause salt sensitivity. Expression levels of SA are high in Dahl salt sensitive rats and low in Dahl salt resistant rats, corresponding to the hypothesis that lower levels of SA may protect from salt sensitive hypertension, however this is obviously a huge over-simplification. No simple relationship exists between SA levels and salt sensitivity. As SA is androgen regulated, female mice have low levels of expression and it is likely that this is also the case in women. However, salt sensitive hypertension still exists in women. It therefore seems likely that the protective effect of lowering SA levels may only be relevant in certain cases of salt sensitive hypertension. Alternatively complete absence of the SA gene may be necessary for salt sensitivity to be inhibited. A combination of both of these scenarios is also a possibility.

Body weight analysis in SA-null and wildtype mice

Body weight was compared between SA-null and wildtype mice. This was important to determine if the above differences in blood pressure were independent of increased body weight. In addition, an association has been observed between the SA gene locus and body weight in both human and rat studies (Zee *et al.*, 1997; Lodwick *et al.*, 1998). Our SA-null mouse model presented an opportunity to study the SA-gene in relation to body mass. No significant effect of genotype or high salt intake was observed on body weight at either 12 or 16 weeks of age (Tables 5.1 and 5.2) indicating that on this genetic background the SA gene is unlikely to contribute to the determination of body weight.

Chapter 6

Administration of dihydrotestosterone (DHT) to SA-null and wildtype female mice

Chapter 6: Administration of dihydrotestosterone (DHT) to SA-null and wildtype female mice

Differences in blood pressure between males and females have been reported both in animal models and in humans. In pre-menopausal women the prevalence of hypertension is lower than for age-matched men (Hypertension Detection and Follow-up program Co-operative Group, 1977). In several mouse strains (for example CBA and DEA/2J) the male develops higher blood pressure than the female (Schlager, 1968). This is also the case for some rat models including the Dahl salt-sensitive rat and the SHR (Dahl *et al.*, 1962; Iams and Wexler, 1977; Iams and Wexler, 1979; Ganten *et al.*, 1989). The protective effects of oestrogens are believed to contribute to the sexual dimorphism of blood pressure. In women after the menopause, levels of hypertension and heart disease increase to those of men as their oestrogen levels fall. In DS rats the Na^+ induced rise in blood pressure is slower in females than in males. However, when females are ovariectomised, the blood pressure rise becomes the same as for males implying an oestrogen protecting effect. In addition to the protective effect of oestrogens, androgens have also been shown to contribute to the sexual dimorphism of blood pressure. The mechanism for sexual dimorphism in the SHR has been shown to be androgen dependent. Blood pressures of young SHR and SHRSP given testosterone receptor antagonists did not rise in the same manner as blood pressures of control animals; similarly castration was found to reduce blood pressure significantly (Ganten *et al.*, 1989; Chen and Meng, 1991).

In this work we investigated a possible involvement of the SA protein in the sexual dimorphism of blood pressure determination. Recently the SA gene was shown to be testosterone regulated in the kidney (Melia *et al.*, 1998). We therefore hypothesised that the SA protein may be involved in mediating the role of androgens in the sexual dimorphism of blood pressure determination. In the experiments of Melia *et al.* (1998), dihydrotestosterone (DHT) pellets were implanted subcutaneously into C57BL/6 female mice and an up-regulation of the SA gene noted. Blood pressure was however not studied. We devised an experimental strategy to study the effect of DHT administration on SA expression levels and blood pressure in female mice. It was our

intention to confirm data by Melia *et al.* (1998) showing SA to be up-regulated by DHT and to further investigate any possible relationship between expression levels of SA and blood pressure. This allowed us to directly confirm any effect of absence of the SA gene on blood pressure. Our hypothesis was that if the SA protein is involved in mediating the androgen derived sexual dimorphism of blood pressure, SA-null mice should not respond to DHT treatment in the same manner as wildtype mice.

METHODS

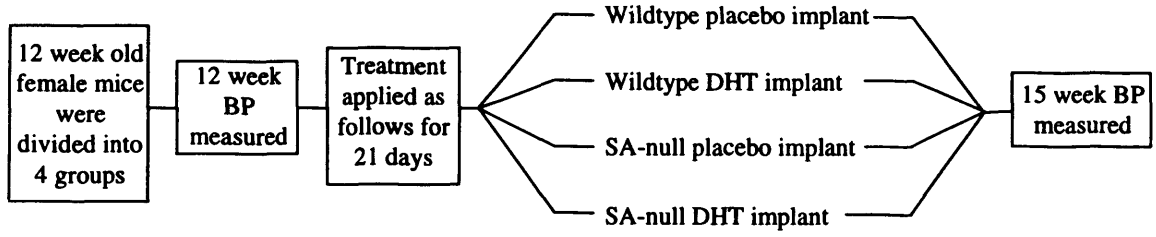
Experimental outline

Heterozygous breeding pairs (strain129/sv:129/OlaSA-null) were utilised for the generation of mice for these experiments. SA genotypes of mice were identified by PCR amplification at 4 weeks of age (see Chapter 2, Methods). 25 wildtype and 25 SA-null mice female mice were randomised into 4 experimental groups:

Wildtype placebo treated (n=12)	SA-null placebo treated (n=13)
Wildtype DHT treated (n=13)	SA-null DHT treated (n=12)

Animals were numbered anonymously and genotypes were not known during subsequent experiments. Blood pressures of mice were measured at 12 weeks and then each mouse was implanted with either a DHT or placebo implant (see Chapter 2, Methods). Implants were obtained from Innovative Research of America and were designed to deliver 0.02mg of DHT per day for 21 days. Placebo implants contained carrier only. Blood pressure was measured again at 15 weeks of age. A diagrammatic representation of this protocol is given in Fig. 6.1. After final BP measurement mice were sacrificed. Kidney, liver, heart, and brain were flash frozen in liquid nitrogen. Organs were later weighed and RNA was extracted (see Chapter 2, Methods).

FIG 6.1 Dihydrotestosterone treatment protocol



Blood pressure measurement

For each mouse blood pressure was measured at 12 weeks of age on three separate days as described in Chapter 2 (Methods). Each day between 8 and 10 separate measurements were obtained and an average taken for each day. The average of the 3 days was then assumed as the 12 week blood pressure. 15 week blood pressure measurements were carried out in a similar manner. Blood pressure data was analysed by oneway ANOVA using the Minitab statistical software package.

RESULTS

12 week body weight and blood pressure analysis

Average basal systolic blood pressures and weights at 12 weeks of age for wildtype and SA-null mice are given in Table 6.1.

Table 6.1 Basal systolic blood pressure measurements and body weights of SA-null and wildtype female mice at 12 weeks of age (±SEM)

	Wildtype	SA-null
12 week basal systolic BP (mmHg)	103.7±2.0 (n=25)	103.6±2.7 (n=25)
12 week body weight (g)	21.0±0.3 (n=25)	21.4±0.3 (n=25)

No differences were observed between wildtype and SA-null mice in either blood pressure ($p=0.98$) or weight ($p=0.41$) at 12 weeks of age.

15 week body weight and blood pressure analysis

Average systolic blood pressures and weights for the 4 treatment groups at 15 weeks of age are given in Table 6.2.

Table 6.2 Systolic blood pressure measurements and body weights of the 4 treatment groups at 15 weeks of age (±SEM)

	Wildtype	SA-null
15 week placebo treated BP (mmHg)	108.6±3.9 (n=12)	103.6±3.0 (n=13)
15 week DHT treated BP (mmHg)	104.5±2.7 (n=13)	116.5±4.3 (n=12)
15 week placebo treated body weight (g)	21.6±0.7 (n=12)	22.9±0.4 (n=13)
15 week DHT treated body weight (g)	22.8±0.4 (n=13)	22.9±0.5 (n=12)

At 15 weeks there was no difference between blood pressures of wildtype and SA-null placebo treated mice (p=0.32).

DHT treatment surprisingly had no effect on blood pressure of wildtype mice (p=0.4). Interestingly however DHT treatment did have an effect on blood pressure of SA-null mice (p=0.02 compared to placebo treated mice). Consequently a significant effect of genotype was also observed in DHT treated animals (p=0.026 for comparison of wildtype and SA-null DHT treated mice). Graphical representation of 12 and 15 week blood pressures can be seen in Fig 6.2.

No significant effect on body weight was observed by either genotype or DHT administration in this experiment (Table 6.2).

FIG.6.2 Graphical representation of 12 and 15 week systolic blood pressures

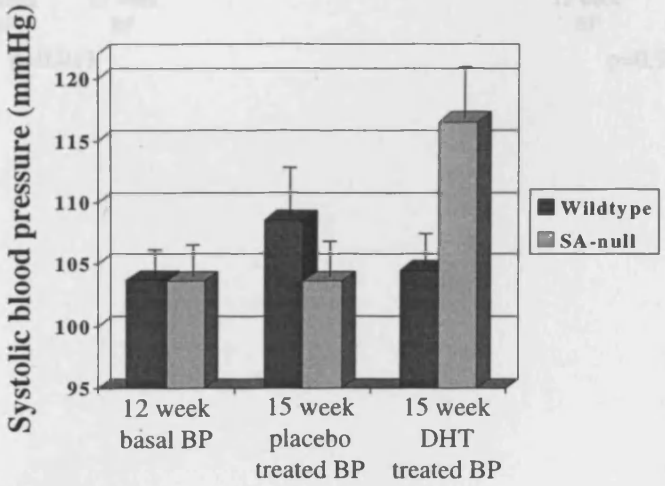
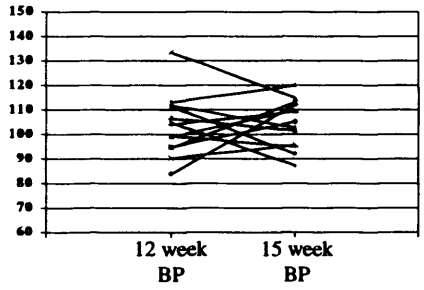


Figure 6.3 shows changes in blood pressure between 12 and 15 weeks for each individual mouse within the 4 treatment groups. For each treatment group average 12 week and average 15 week blood pressures were compared. Although there is some variability in blood pressures of individual mice, only in the SA-null DHT treated mice is the change in BP from 12 to 15 weeks significant (p=0.013).

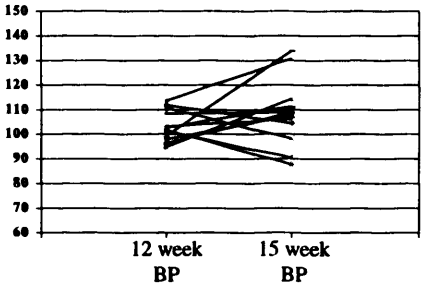
FIG. 6.3 Differences in blood pressure between 12 and 15 weeks of age in the 4 treatment groups

WT dihydrotestosterone



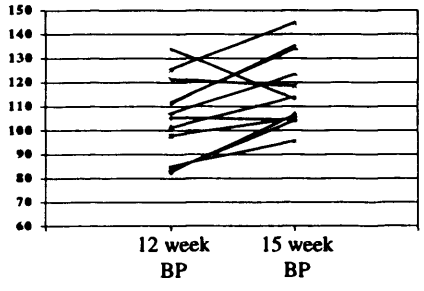
p=0.80

WT placebo



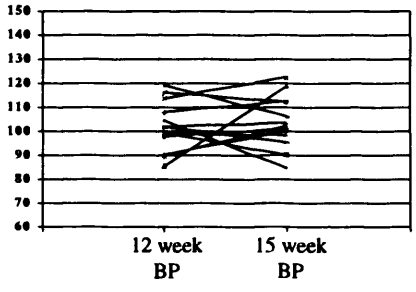
p=0.22

SA-null dihydrotestosterone



p=0.013

SA-null placebo



p=0.99

Organ weight analysis

The effect of DHT treatment on kidney and liver weights in wildtype and SA-null mice are presented in table 6.3.

Table 6.3 Organ weight comparisons of wildtype and SA-null mice (±SEM)

		Wildtype	SA-null
kidney	Placebo (mg)	247.5±9.2 (n=12)	259.1±6.4 (n=13)
	DHT (mg)	283.1±10.5 (n=13)	269.8±12.3 (n=12)
	Placebo (mg/g body weight)	11.45±0.16 (n=12)	11.32±0.16 (n=13)
	DHT (mg/g body weight)	12.38±0.29 (n=13)	11.72±0.32 (n=12)
liver	Placebo (mg)	778±35.0 (n=12)	819.6±35.1 (n=13)
	DHT (mg)	804±37.1 (n=13)	834.2±42.2 (n=12)
	Placebo (mg/g body weight)	20.6±0.5 (n=12)	19.9±0.4 (n=13)
	DHT (mg/g body weight)	20.1±0.3 (n=13)	19.9±0.3 (n=12)

A significant effect was noted on kidney weight due to the administration of DHT in wildtype mice (Table 6.3). Average kidney weight for wildtype placebo treated mice was 247.5mg compared to 283.1mg for DHT treated wildtype mice ($p=0.018$). Average kidney weight for treated SA-null mice also showed a trend towards being heavier in DHT treated animals ($p=0.062$). When kidney weights were expressed as a fraction of body weight for each animal the effect of DHT treatment on wildtype mice remained significant ($p=0.011$). For SA-null mice however, this trend was lost after adjustment for body weight ($p=0.27$).

Expression data

Expression levels within the kidney

Kidney RNA was extracted from 5 animals from each of the 4 experimental groups and used for Northern blot analysis (60μg per sample). RNA was hybridised with probes specific for the following genes; SA, KS, renin and GAPDH.

Expression of the SA gene

Expression levels of SA in the 4 treatment groups can be seen in Fig. 6.4. In wildtype female mice treated with placebo implants, SA expression was not detectable by Northern blotting, confirming observations presented in Chapters 4 of this work in which levels of SA expression in females were shown to be below the sensitivity of Northern blotting (Chapter 4, Fig. 4.1). After treatment with DHT for a period of 3 weeks, SA expression was detectable in the kidneys of female mice. However, as can be seen in Fig 6.4, levels of upregulation of SA due to DHT administration vary markedly between individual animals. For this reason we extracted RNA from all mice treated with DHT (both wildtype and SA-null) in order to further assess variability of SA expression between individual mice. Northern blot showing expression of SA in DHT treated mice is presented in Fig. 6.5.

In some individual mice expression is barely detectable by Northern blotting, in others expression levels are much higher. Possible reasons for this variability are discussed below (Chapter 6, Discussion). SA expression was not detectable by Northern blotting in SA-null mice irrespective of DHT treatment.

Expression of the KS gene

KS is obviously expressed in the kidneys of female mice (Fig 6.4). Administration of DHT caused an increase in expression levels of KS in both the wildtype and SA-null mice. As with expression of the SA gene in this experiment, there was some variation in the extent to which KS was upregulated in this experiment. Variability of KS expression in DHT treated mice was less marked than for the SA gene. Average KS expression due to DHT treatment was increased 2.3-fold in wildtype mice and 2.4-fold in SA-null mice. Absence of the SA protein had no effect on expression levels of KS.

Expression of the renin gene

Levels of renin expression in the 4 treatment groups can be seen in Fig. 6.4. No gross effect of DHT treatment could be observed. Densitometry was performed to elucidate small differences in expression levels between groups. Graphical representation of renin expression standardised by comparison with GAPDH is shown in Fig. 6.7. No significant genotype dependant effect on renin expression levels was observed in either the placebo or DHT treated groups (p=0.2 and p=0.96 respectively). However administration of DHT caused small but significant drops in renin expression in both genotypes (of 30% for wildtype, p=0.033 and 15% for SA-null, p=0.024). Oneway ANOVA of the effect of DHT on renin expression, considering all 4 treatment groups together, showed a highly significant effect of DHT treatment on expression levels (p=0.004).

FIG. 6.4 Northern blot of female kidney RNA hybridised with various cDNA probes

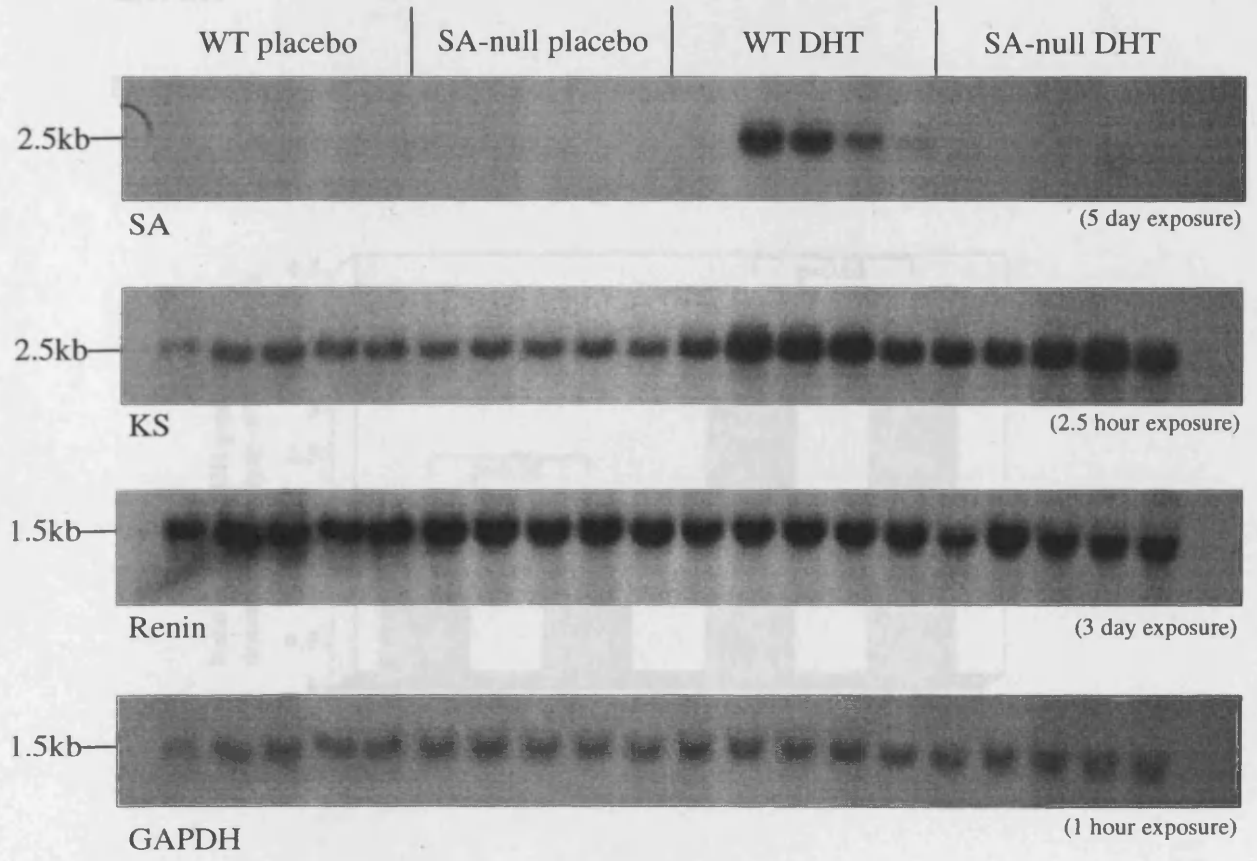


FIG. 6.5 Northern blot of kidney RNA from DHT treated female mice hybridised with various cDNA probes

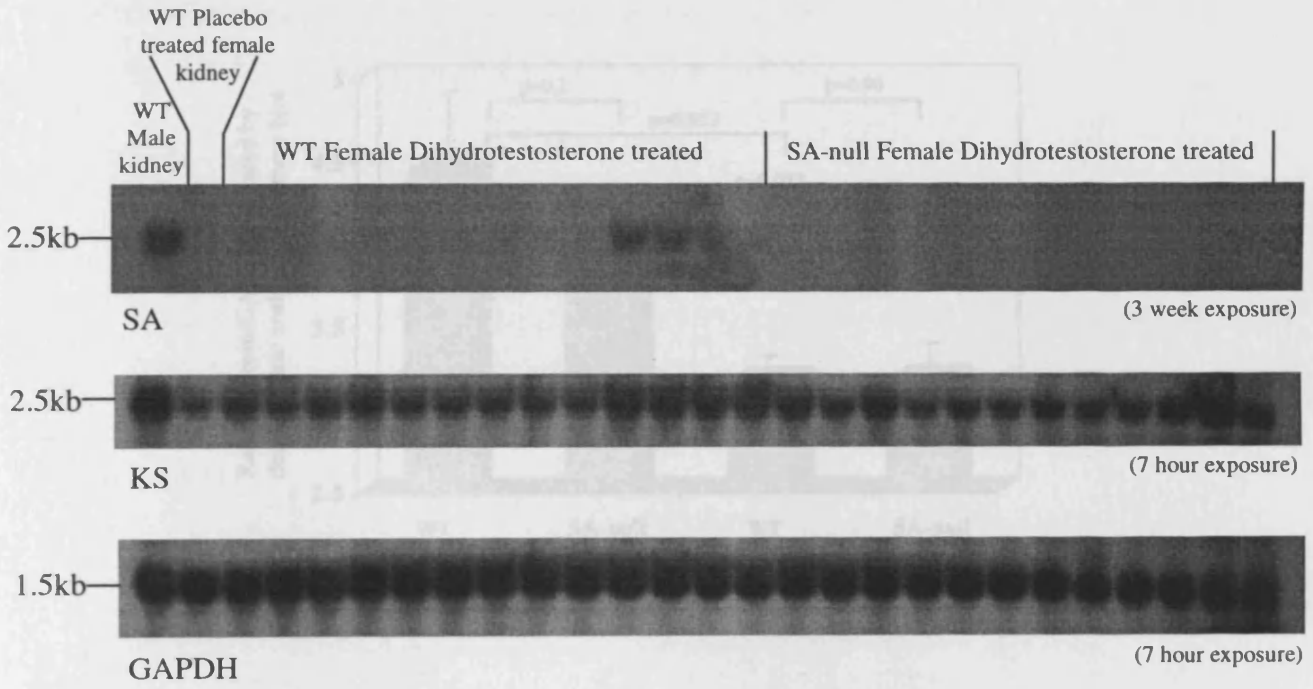


FIG. 6.6 Densitometric analysis of KS expression levels within the kidney of SA-null and wildtype mice

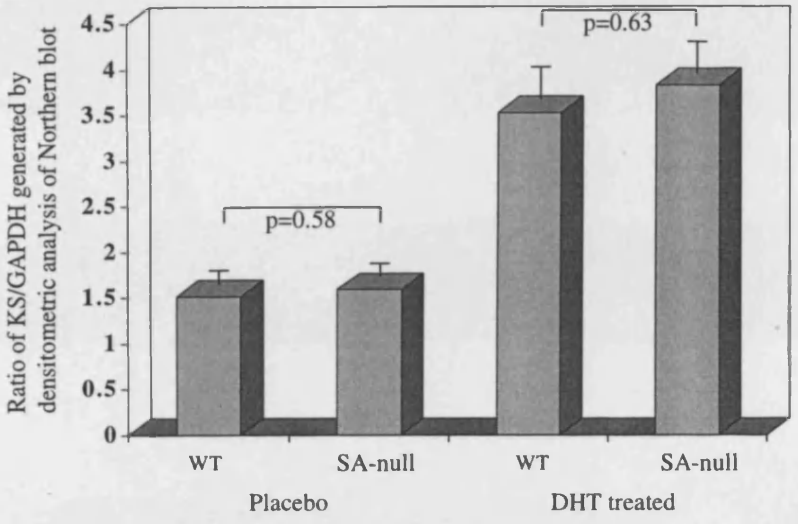
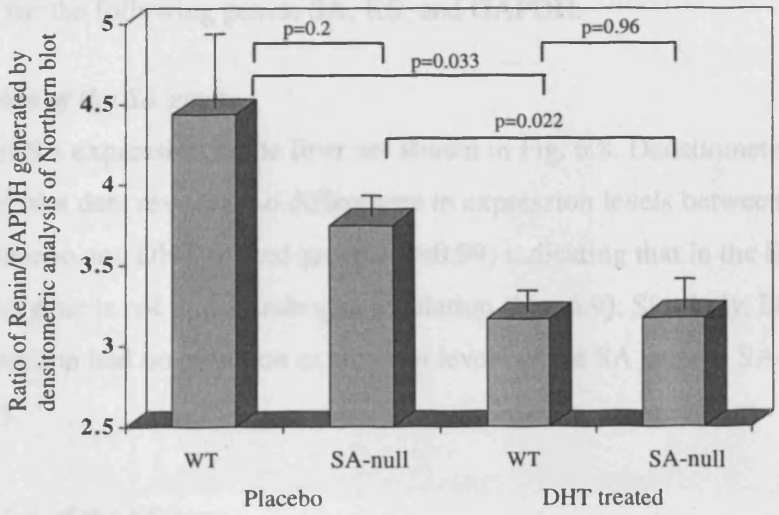


FIG. 6.7 Densitometric analysis of Renin expression levels within the kidney of SA-null and wildtype mice



Expression levels within the liver

Liver RNA was extracted from 4 animals from each of the 4 experimental groups and used for Northern blot analysis (60µg per sample). RNA was hybridised with probes specific for the following genes: SA, KS, and GAPDH.

Expression of the SA gene

Levels of SA expression in the liver are shown in Fig. 6.8. Densitometric analysis of Northern blot data revealed no differences in expression levels between wildtype mice in the placebo and DHT treated groups (p=0.99) indicating that in the liver expression of the SA gene is not under androgen regulation (Fig. 6.9). Similarly, DHT administration had no effect on expression levels of the SA gene in SA-null mice (p=0.45).

Expression of the KS gene

No expression of KS in the livers of female mice could be detected (Fig.6.8).

Fig. 6.8 Northern blot of liver RNA hybridised with the rat SA 1.6kb cDNA probe

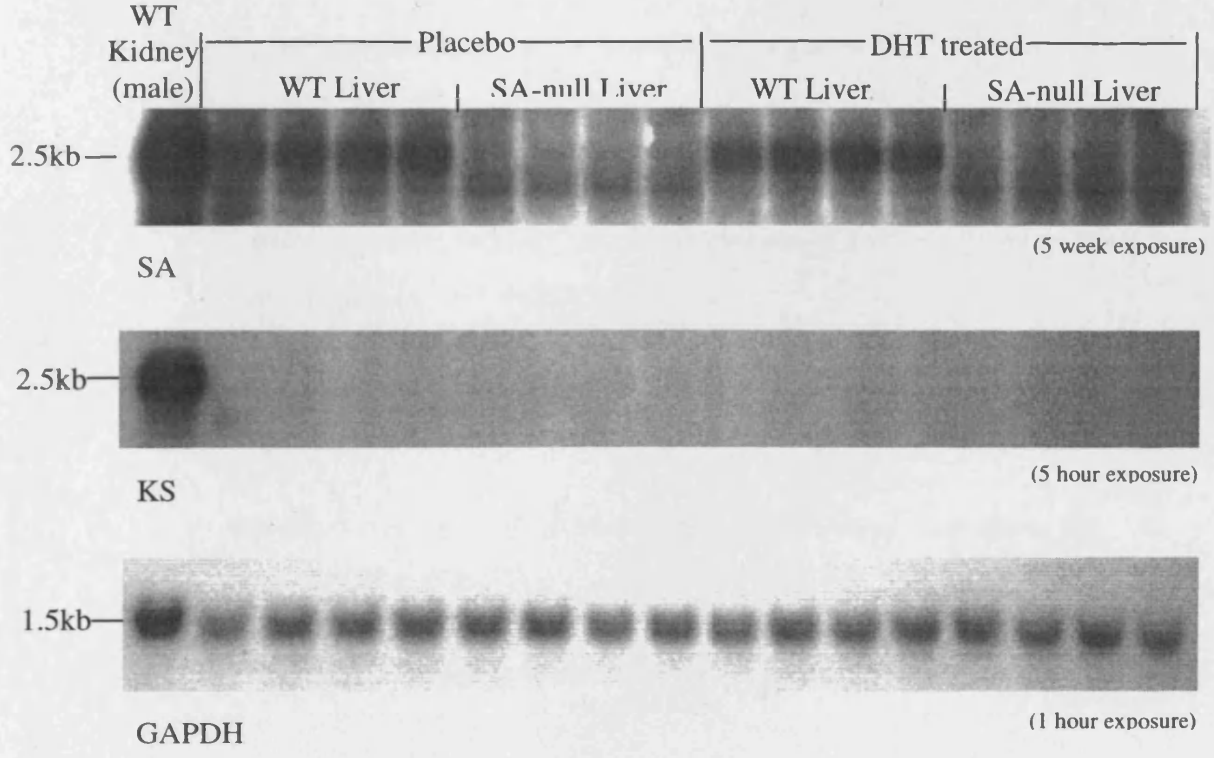
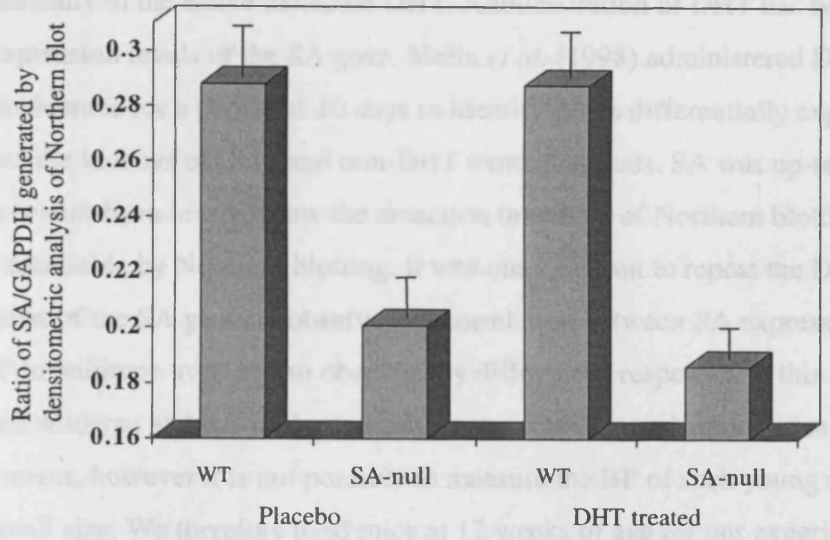


FIG. 6.9 Densitometric analysis of SA expression levels within the liver of SA-null and wildtype mice



DISCUSSION

In order to observe the effects of androgen administration on blood pressure and gene expression levels in our SA-null model we chose the method of subcutaneous administration of dihydrotestosterone (DHT). *In vivo* testosterone is converted enzymatically to the active molecule DHT. Administration of DHT has been shown to alter expression levels of the SA gene. Melia *et al.* (1998) administered DHT to 6 week old female mice for a period of 10 days to identify genes differentially expressed between the kidneys of DHT and non-DHT treated animals. SA was up-regulated by this treatment from levels below the detection threshold of Northern blotting to levels easily detectable by Northern blotting. It was our intention to repeat the DHT up-regulation of the SA gene, to observe any correlation between SA expression levels and BP in wildtype mice and to observe any differential responses to this treatment between wildtype and SA-null mice. Melia *et al.* (1998) used young mice in the above experiments, however it is not possible to measure the BP of such young mice due to their small size. We therefore used mice at 12 weeks of age for our experiments. We administered DHT for a period of 21 days in order to maximise any differences in SA expression or blood pressure caused by this regimen.

The main observations resulting from these experiments were as follows:

- Administration of DHT caused an increase in SA expression in the kidneys of wildtype (but not SA-null) mice. There was however marked variability in expression levels between individual animals. In the liver SA expression was not affected by DHT administration.

- A more consistent up-regulation of KS was observed in the kidneys of individual mice of both wildtype and SA-null genotypes.

- No effect was observed on blood pressure in wildtype mice due to the administration of DHT.

- Paradoxically, blood pressure was increased in SA-null mice treated with DHT.

Effects of DHT treatment on expression levels within the 4 experimental groups

Our study confirmed observations by Melia *et al.* (1998) that SA expression in the kidneys of female mice is androgen regulated. In our experiment, although levels of SA expression in the kidneys of all mice increased in response to androgens, upregulation varied substantially between individual mice. Possible factors contributing to this variation may be slight differences in positioning of the implant resulting in differential administration of DHT or the failure of implants to deliver hormone throughout the duration of the experiment. Some variation in levels of KS expression in the kidneys was also noted in our experiments, but to a lesser degree than those observed for SA. Comparison of expression levels between SA and KS in wildtype mice revealed that individual mice exhibiting the greatest increase in SA expression also show the greatest increase in KS expression. This may point towards some effect of differential administration of DHT in our experiment. However as SA expression levels vary more markedly than KS expression levels, differential administration of testosterone can only represent partial explanation for the wide variability observed in SA expression. Also an increase in kidney weight was observed due to administration of DHT, suggesting that hormone was delivered effectively throughout the course of the experiment. Another possibility is that differences in sensitivity to DHT between individual mice could similarly affect the control of both genes. Control of SA and KS may be intimately linked and vary in a similar fashion dependant upon genetic background. As we generally observed a more modest upregulation of SA than Melia *et al.* (1998), it is possible that the SA gene is less sensitive to androgens on a 129 genetic background than on the C57BL/6 background used by Melia *et al.* (1998). The inducibility of other androgen regulated genes has been reported to vary markedly between mouse strains (Melanitou *et al.*, 1987; Lund *et al.*, 1988). Observation of expression levels of another androgen regulated gene such as KAP to see if expression levels also parallel those of KS and SA would prove interesting.

Figs. 6.4 and 6.5 clearly show KS to be upregulated by DHT in the kidneys of female mice compared to non-DHT treated controls. This is the first report of androgen regulation of this gene. KS expression is not altered by the absence of the SA protein in

either wildtype or SA-null mice, therefore upregulation of KS is unlikely to compensate for absence of SA.

Transcription of the truncated SA message in SA-null mice does not respond to DHT administration in the same manner as wildtype mice. Several factors may be responsible for this observation. The absence of intron 2 and part of intron 1 in SA-null mice may have eliminated sequences containing androgen response element enhancers. Reports have been made of AREs within the first introns of various genes (Ho *et al.*, 1993; Rennie *et al.*, 1993; Claessens *et al.*, 1989) and it is possible that the SA gene may also contain regulatory sequences within these regions. Elimination of these would result in reduced expression of the SA gene in response to androgens. Other possible explanations for the low levels of expression of the SA-null transcript are inefficiency of splicing between exons 1 and 4, an event not occurring naturally, and reduced stability of the truncated SA transcript. In male mice expression of the SA-null transcript is also several fold less than in wildtype mice (Chapter 5, Fig. 5.4).

Expression of renin in the kidney was slightly but significantly decreased by the administration of DHT in both wildtype and SA-null mice (Fig. 6.4). Comparison of renin expression levels in wildtype females (Fig. 6.4) and wildtype males (Chapter 5, Fig. 5.4) reveals a higher level of expression in females than in males. This is in accordance with observations of NMRI mice (Wagner *et al.*, 1990) showing higher levels of renin expression in female than male mice and decreased renin expression in ovariectomised females after testosterone administration. Studies performed using Swiss mice (Catanzaro *et al.*, 1985) also revealed higher renin content and renin activity in the kidneys of female than male mice. Interestingly a different result was obtained in a study using rats. Chen *et al.* (1992) observed expression levels of renal renin between intact male and female rats to be similar. Ovariectomy reduced renin expression in female rats whilst testosterone administration to ovariectomised female rats restored expression levels to those found in males. In this study Chen *et al.*, (1992) also showed PRA (plasma renin activity) levels to be enhanced in SHR rats by the administration of exogenous testosterone, as were hepatic angiotensinogen mRNA levels. One possible explanation for these differences may be the high levels of

androgen dependent renin expression in the sub-mandibular gland (SMG) of mice. It has been hypothesised renin from the SMG causes an elevation in PRA which down-regulates renin expression in the kidneys upon androgen administration in female mice. Such high levels of renin expression in the SMG of male mice are thought to be linked to the presence of 2 copies of the renin gene in the strains studied.

We observed no genotype dependent effect of DHT administration on levels of renin expression. It therefore seems unlikely that any effect of absence of the SA gene is compensated for by up-regulation of the renin-angiotensin system. To further substantiate this result, it would be interesting to observe angiotensinogen expression levels within the 4 treatment groups.

Regulation of SA gene expression in the liver does not appear to be altered by the administration of DHT. In contrast to expression levels in the kidney of female mice, expression levels in the livers were detectable by Northern blotting indicating greater expression in the liver than the kidney in female mice (Fig. 6.8). This is in contrast to the accepted expression pattern in male rats (Kaiser *et al.*, 1994) where the kidney is the main organ of SA expression, with lower expression levels in the liver. No direct comparison was made between expression levels in the liver of male and female mice in this work. However comparison with positive controls indicates greater expression of SA in the livers of female mice than in the livers of male mice (Fig. 6.8 and Chapter 5, Fig. 5.7).

The response of many genes to androgen stimulation is species, strain and tissue specific and at any given time will also depend upon other transcription factors which may be activated. Any one gene may contain more than one androgen response element as well as recognition sites for additional transcription factors. Other transcription factor recognition sites may be close to an ARE such that activation of one will affect the other. For example, in the promoter of the mouse vas deferens protein gene, site-directed mutagenesis of the NF1 and Sp1 transcription factor binding sites abolishes or greatly reduces the control of this gene by androgens (Darne *et al.*,

1997). Thus a very complex picture of transcriptional regulation emerges. This is also likely to be the case for the SA gene.

In order for a gene to exhibit androgenic control, the androgen receptor protein must be expressed in the same cell type as that gene. DHT binds to the androgen receptor to form an active complex, which can then bind to the regulatory elements of an individual gene (androgen response elements, AREs). AREs are found in the promoter region of genes and conform to a consensus sequence (Roche *et al.*, 1992), although non-consensus AREs have been reported (Rennie *et al.*, 1993; Zhou *et al.*, 1997). The consensus sequence for the androgen response element is the same as that for the gluccocorticoid response element and similar to that of the oestrogen response element (Beato, 1989; Roche *et al.*, 1992). In the kidney the main site of androgen action is the epithelial cells of the proximal convoluted tubule. This is also the location of SA gene expression. Many classic kidney androgen regulated genes are localised in these cells, for example β-glucuronidase, kidney androgen regulated protein (KAP), alcohol dehydrogenase and ornithine decarboxylase. For all of these genes, sequences similar to the gluccocorticoid response element have been identified in the promoter regions. In the liver the exact location of SA gene expression has not been determined. Androgen regulation of genes within the liver has been reported, for example the angiotensinogen gene (Chen *et al.*, 1992), but it is possible that the location of SA expression in the liver is spatially separated from the expression of the androgen receptor explaining the absence of androgen control of SA expression in this organ. Alternatively other transcription factors present in the liver but absent in the kidney may compete with the binding site of the androgen/androgen receptor complex.

Blood pressure analysis

The effect of steroid hormones on blood pressure has been studied extensively in rat models. Cambotti *et al.*, (1984) showed that neonatal administration of testosterone to female SHR caused an increase in BP similar to that observed in males. In addition, exposure to testosterone during the neonatal period was found to be important in setting sensitivity of BP to testosterone later in life. Chen *et al.* (1992) administered testosterone to male and female orchidectomised rats (SHR) at 4 weeks of age. Blood

pressure was recorded weekly until the age of eighteen weeks and found to be elevated in both males and females by testosterone treatment. Orchidectomy lowered BP in males and testosterone treatment elevated BP to the levels of intact males. In females orchidectomy had no effect on BP but testosterone treatment increased BP to levels similar to those of intact males. Reckelhoff *et al.* (1998) made similar observations and also showed the pressure-natriuresis shift of SHR rats to be androgen dependent. The effect of testosterone on BP in SHR rats was shown to be mediated via the androgen receptor and to be independent of the conversion of testosterone to DHT (Reckelhoff *et al.*, 1999).

These studies and others point towards an important role of androgens in the pathogenesis of hypertension in the SHR. In addition testosterone induced alterations in expression levels of angiotensinogen and renin indicate this effect may be mediated via the renin-angiotenisin system. However the exact mechanisms of this effect are poorly understood.

Very little work has been undertaken on the effects of testosterone administration on BP in strains of rat other than SHR or in mice, although sexual dimorphisms have been reported for example in DOCA-salt hypertensive rats, DS rats, New Zealand hypertensive rats, CBA mice and DEA/2J mice. Differences in expression data for genes of the RAS between rats (SHR) and NMRI mice (discussed above) highlight the possibility of different regulatory effects in different species. Also, in the SHR, oestrogens have been shown to have little protective effect against hypertension (Chen *et al.*, 1992), whereas this is not the case in humans. Much variability exists in response to sex hormones across species and even between strains.

The effect of testosterone administration on blood pressure in mice has not been studied, although as mentioned above, males have been reported to have higher BP than females in some strains. This was also the case in our studies. 12 week basal blood pressures in males and females of both SA genotypes are directly compared in Table 6.4 below.

Table 6.4 Comparison of 12 week basal systolic BPs between male and female mice

12 week basal systolic BP (mmHg)	Wildtype	SA-null
Male	111.0±2.0 (n=24)	116.7± (n=23)
Female	103.7±2.0 (n=25)	103.6±2.7 (n=25)

In both wildtype and SA-null mice blood pressures are higher in males than females ($p=0.012$ and $p=0.002$ respectively). As males produce androgens naturally, this could be taken to mean the absence of the SA gene is not involved in mediating the androgen dependent dimorphism of BP; the androgens produced by male SA-null mice produce higher BPs than in female SA-null mice. However it is also a possibility that SA is involved in mediating the sexual dimorphism of BP under certain circumstances and in conjunction with many other systems. Compensation may occur in SA-null male mice by other systems mediating the effect of androgens.

As studies in rat models have shown an increase in testosterone to cause an increase in BP in female rats, we hypothesised that DHT treatment was likely to cause an increase in blood pressure of female wildtype mice. This was however not the case in our study. Several factors may contribute to this. It is possible that in the 129 mice used in our experiment, increased androgen levels must be present at a certain developmental stage to cause an increase in BP. Therefore blood pressure in male mice is increased by naturally occurring androgens but administration of androgens to mature females did not increase BP. Studies in rats showed neonatal exposure to testosterone to sensitise females to androgen administration later in life. However control females did responded with increased BP to a lesser degree when testosterone was administered to mature animals (Cambotti *et al.*, 1984). Another possibility is that whilst higher levels of androgens in males than females are likely to contribute to the sexual dimorphism of BP in our model, it is possible that other mechanisms also contribute to this effect. For example the protective effect of oestrogens may have inhibited a testosterone induced rise in BP in our experiment. In the rat studies outlined above female rats were ovariectomised prior to administration of testosterone. The consequent absence of oestrogens in these rats may have allowed BP to rise in these experiments. This was not the case in our study.

The above mentioned studies of BP in rats used testosterone for the administration of androgens. However as dihydrotestosterone was shown by Melia *et al.* (1998) to alter expression levels of SA, we chose this androgen for our experiment. In SHR the effects of androgens on BP have been shown to be mediated via the androgen receptor, however similar information is not available for our model. As testosterone has been shown to exert effects both via the androgen receptor and independently it is possible that an effect on BP may have been observed if testosterone had been used in place of DHT in our experiment.

As expression levels of KS were upregulated and kidney weights were increased in mice receiving DHT treatment in our experiment, it is unlikely that our administration of hormone was ineffective. It is however possible that fundamental differences in blood pressure regulation exists between rats and mice and that these are reflected in our results.

The most interesting observation from this experiment is that whilst blood pressure in wildtype female mice was not affected by administration of DHT, blood pressure of SA-null mice was increased by this treatment ($p=0.02$). This was an unexpected result. Rat studies lead us to expect an increase in BP in wildtype mice upon androgen administration and we were interested to assess whether this effect was ameliorated in SA-null mice. In reality the opposite occurred with an increase in BP occurring only in SA-null DHT treated animals. This implies a *protective* effect against a DHT induced increases in BP by the SA gene in female mice.

One hypothesis is that in female mice DHT causes an up-regulation in a system with the potential to increase BP. However the presence of the SA protein and its concomitant up-regulation prevents this BP rise from occurring. Thus absence of the SA protein allows other systems to elevate BP. A similar hypothesis is that other systems may become predominant in the absence of the SA protein without necessarily becoming elevated. Alternatively DHT may cause down-regulation of a system that lowers BP in the absence of the SA protein. These potential mechanisms are

speculative and further work is required to confirm the observed effect of DHT and to investigate the effects of upregulation of SA on other homeostatic systems.

It is possible that the effect on BP we observed due to the administration of DHT is spurious. However one observation that suggests this is not the case is the 12 week blood pressure data of male mice. The elevation in BP of SA-null female mice due to the administration of androgens appears to be paralleled in the 12 week BP data of male mice. As observed in Chapter 5 of this work, and presented in comparison with female BP data above (Table 6.4), BP is slightly higher in SA-null male mice than wildtype male mice at 12 weeks of age (116.7mmHg compared to 111mmHg). This difference is statistically significant ($p=0.037$). In chapter 5 we hypothesised that this was likely to be a spurious result (especially as it was not repeated at 16 weeks of age in male mice). However it is a possibility that the initial exposure of male mice to testosterone at puberty causes an increase in BP in SA-null male mice, in a similar fashion to the DHT induced BP rise in mature SA-null female mice. In male mice compensation could have occurred for this effect by the age of 16 weeks. Thus BP may initially rise in SA-null male mice at puberty, and decline thereafter. Further BP measurements would be necessary to confirm or dispute this hypothesis.

If further studies do confirm an increase in blood pressure response to androgens due to the absence of SA, this would be a major novel observation that may shed new light on the mechanisms leading to the sexual dimorphism of blood pressure and the effect of sex hormones on cardiovascular regulation.

Chapter 7

DISCUSSION

Chapter 7: FINAL DISCUSSION

Specific results generated during the course of this thesis have been discussed in individual chapters. Therefore in this general discussion I will focus on more general issues including future work involving the SA gene and my SA-null mouse model.

Elucidating gene function on the post-genomic era

The SA gene was first identified almost ten years ago, yet despite knowledge gained from sequence data and experiments in this thesis, the precise function of this gene still remains unknown.

Historically, prior to the advent of molecular genetics, proteins were identified on the basis of physiological observations. For example the renin gene was first identified over a century ago due to the ability of rabbit kidney extracts to raise blood pressure in healthy rabbits (Tigerstedt and Bergman, 1898). Careful research over many years revealed the details of the renin-angiotensin system, however it was not until 1982 that the gene for renin was cloned and sequenced (Panthier *et al.*, 1982). Similarly, although in a somewhat shorter timeframe, endothelin was first identified on the basis of its properties as a vasoconstrictor, thus leading to the cloning and sequencing of the endothelin gene (Itoh *et al.*, 1988).

More recently, this historical pattern has been reversed, with genetics preceding physiology, in what has been termed reverse genetics. Identification of new genes potentially involved in disease processes is becoming faster by direct study of gene expression. However, as the SA gene illustrates, assignment of a function to these genes can be difficult. This is likely to be an increasing issue for the scientific community as technologies such as the use of microarrays and expressed sequence tag (EST) database mining are more widely applied.

Future work

In the current study several interesting observations have emerged with regard to the SA-null mouse model and blood pressure:

- Ablation of the SA gene has no profound effect on basal blood pressure in either male or female mice.
- SA-null male mice fail to show the salt induced increase in BP present in wildtype controls.
- SA-null female mice show an increase in BP in response to androgen treatment which is absent in female wildtype mice.

The blood pressure data presented in this thesis were generated by the indirect tail-cuff method. Whilst this method has been validated (Johns *et al.* 1996), substantiation of data by direct methods would be beneficial. There are 2 alternatives available for the measurement of direct blood pressure in rodents. Firstly cannulation of a superficial artery followed by measurement of blood pressure after recovery, and secondly longitudinal measurement of blood pressure by radiotelemetry. The use of this latter technique has only recently been developed for use in mice (Carlson and Wyss, 2000). Although these techniques offer the advantages of measuring direct blood pressure, limitations must also be recognised. Direct artery cannulation involves general anaesthesia from which the animal may not be fully recovered by the time of blood pressure recording. Also post-operative discomfort and tethering of animals may effect blood pressure.

Radiotelemetry is a technically demanding technique in which a substantial sized probe is placed in a major blood vessel where it occupies significant volume. In addition, using current technology, mice must be over 27g before a probe can be inserted. This is not practical in female mice of strain 129 which do not achieve such a weight.

If data presented in this work are substantiated, several key questions arise.

Why is basal blood pressure unaffected by the absence of the SA protein?
As absence of SA protein affects response of blood pressure to increased sodium intake and DHT administration, it seems likely that SA is involved in the regulation of blood pressure. The lack of effect on basal blood pressure is therefore intriguing. One possible reason for this is that other mechanisms compensate for absence of SA in SA-null mice. Elucidation of such mechanisms will form an important part of future studies.

Blockade of the renin-angiotensin system (for example by angiotensin II receptor blockade) may generate interesting results. In the absence of the SA protein, blood pressure homeostasis may rely more heavily on the RAS. Blockade of this cascade may therefore result in a more profound drop in BP in SA-null than in wildtype mice. In this thesis compensation for lack of SA by the RAS was investigated by comparison of renin expression levels within the kidneys of wildtype and SA-null mice. No differences were observed between SA-null and wildtype mice, either after a normal or high salt diet. However circulating levels of renin are affected by extra-renal as well as renal production of renin. Therefore altered regulation of the RAS in this model may not necessarily be accompanied by an alteration in renin expression in the kidney. Changes in regulation of circulating renin or other local renin-angiotensin systems may occur in SA-null mice. Observation of RAS blockade *in vivo* would allow a more comprehensive study of the effect of absence of SA protein on the RAS. Investigation of plasma renin activity under normal and altered salt conditions would also be of interest.

In mice angiotensinogen is the rate limiting step of the RAS (in contrast to the rat where renin is the rate limiting step) (Weaver *et al.*, 1991). Therefore comparison of angiotensinogen expression levels between wildtype and SA-null mice would be of interest. This could be achieved by hybridisation of Northern blots generated in this work.

Other systems affected by absence of the SA gene can be potentially identified by comparison of expression profiles between the wildtype and SA-null mice under appropriate experimental conditions, for example, after salt-loading. In such experiments the kidney would be a key tissue in which genes may be differentially expressed between wildtype and SA-null mice. In the past a commonly used technique has been differential display (Liang and Pardee, 1998). More recently however microarray technology, which allows the simultaneous screening of several thousand genes, has become available. These 2 techniques are complimentary. Differential display, whilst being more labour intensive, allows the systematic screening of transcripts in the tissue of interest and thus has the capacity to identify completely novel genes. Microarray technology whilst having the advantage of being fast and efficient, screens only previously identified genes. In the field of hypertension, Aitman *et al.* (1998) have recently shown the feasibility of the microarray approach. Cd36 was thus identified as the gene responsible for defective catecholamine and insulin action on rat chromosome 4. In these experiments RNAs were compared between SHR and an SHR chromosome 4 congenic strain. Cd36 was seen to be 90% lower in SHR compared to the congenic strain carrying a Brown Norway segment of this chromosome. Thus further investigations could be carried out with respect to this gene.

By what mechanism does absence of the SA protein ameliorate salt sensitivity?

Salt sensitivity is a complex phenotype and can be mediated by both renal an extra-renal mechanisms (Campese, 1994). SA is highly expressed in the proximal tubules of the kidney, a key site of sodium and water reabsorption. It therefore seems likely that in the SA-null model, renal mechanisms are responsible for the observed differences between wildtype and SA-null mice after high sodium intake. A key issue is whether absence of the SA protein affects proximal tubular function. Sodium reabsorption in the kidney under conditions of different sodium loading may vary between wildtype and SA-null mice, and this could be a focus of future studies. Simple observations of urine volume, sodium content and plasma sodium content under various salt conditions may be revealing. One possible reason for the observed difference in BP between wildtype and SA-null mice after high salt intake may be the failure of SA-null mice to

conserve salt. If this is the case, administration of a low salt diet to SA-null mice may result in hypotension or possibly severe sodium depletion and death. Observation of SA-null mice subjected to a low salt diet would be interesting in this respect. Alterations in proximal tubular sodium and water reabsorption could be measured directly using kidney micropuncture techniques (Harris *et al.*, 1987). Briefly, this technique involves the puncture of a proximal tubule with a double-barrelled micro-pipette and injection of oil into the tubule followed by injection of artificial proximal tubular fluid, splitting the oil column. Reabsorption of the split-drop by the tubule is then captured digitally.

Another interesting approach would be to use our SA-null mouse model to study the combined effects of genes known to affect sodium sensitivity. Bradykinin 2 receptor knockout mice (B_2KO) develop salt sensitive hypertension (Alfie *et al.*, 1996). If double-null mice could be generated for this gene and SA, it would be interesting to observe whether the substantial salt induced increase in B_2KO mice is ameliorated by absence of SA, implicating an interaction between the 2 systems. However as many factors are involved in the aetiology of salt-sensitive increases in blood pressure, it is quite possible that the 2 mechanisms are unrelated and BP in double null mice would respond in the same manner as B_2KO mice to salt administration. Administration of pharmacological B_2 receptor blockade to SA-null mice may also prove interesting. *ProANP* is another gene for which the null mouse model develops salt sensitive hypertension (John *et al.*, 1995). Similar investigations of this gene in SA-null mice could be undertaken.

What is the reason for the increase in blood pressure observed in female SA-null mice due to the administration of androgens?

One possible extension of our experimental protocol could be to include groups of mice subjected to ovarectomy prior to the administration of testosterone. Thus the presence of oestrogens, which may have confounded blood pressure results in our experiment, would be eliminated.

If our previous findings are reconfirmed it would appear that the SA-protein provides a protective effect against androgen induced increases in blood pressure. Differential expression profiling, as outlined above, could be used to investigate genes differentially expressed between wildtype and SA-null mice after testosterone treatment. This approach would highlight systems interacting with the SA gene to cause this effect.

Other future approaches

One of the limitations of our SA-null model is that the absence of the SA gene is absent from the beginning of embryogenesis in all tissues of the mouse. Ablation of a gene from early in development may result in the up-regulation of other mechanisms to compensate for the absent protein. Generation of a conditional mutant, for example using a tetracycline-inducible system, would allow the ablation of the SA gene in the adult animal only (Kitamura, 1998). Thus the effects of compensatory effects of other systems would be minimised.

The use of an inducible system such as the Cre-recombinase system could be used to generate conditional mutants which fail to express the SA gene in a specific organ. Use of this technology would require the introduction of *lox-P* sites into the SA gene flanking the region to be conditionally deleted. A mouse line generated from such ES cells could then be bred with mice conditionally expressing Cre-recombinase. Offspring would fail to express the SA gene in the tissues of Cre-recombinase expression. A mouse could be generated lacking SA expression in the kidney only, comparison of BP data with data from our total-null strain would demonstrate whether our observed phenotype was mediated via kidney or extra-renal expression of SA (Stec and Sigmund, 1998; Stricklett *et al.*, 1999).

The generation of a transgenic model over-expressing the SA gene in the kidney would also be of interest. There are several well-characterised kidney specific promoters to facilitate this approach, as reviewed by Cvetkovic and Sigmund (2000).

Apart from differential expression studies which may identify genes that interact with the SA system, another possible way of identifying systems interacting with SA is the yeast 2 hybrid system (reviewed by Allen *et al.*, 1995), which can be used to identify protein-protein interactions. If the substrate of the SA gene is a protein, it would be identified by this approach, as would other proteins binding to the SA protein. It is however possible that SA does not interact with other proteins. As the yeast 2-hybrid system is a lengthy and time-consuming technique, some risk would therefore be involved with this approach. Elucidation of the substrate of the SA-protein would be a major breakthrough in this field.

Concluding remarks

At the outset of this work our aims were to generate a mouse model lacking the SA protein product and to study the phenotypic effects of this mutation, specifically with respect to blood pressure. These objectives have been achieved and we are now able to conclude that the SA protein is involved in the regulation of blood pressure under certain conditions.

In the future many genes are likely to emerge for which no function is apparent. The use of gene targeting strategies provide a direct method of studying gene function, and will continue to contribute significantly in this field. However integrated approaches combining physiological, biochemical, clinical and genetic techniques will be necessary to fully characterise the many genes which will be rapidly identified in the post-genomic era.

Publication pending

In the near future we are intending to submit a manuscript, as outlined below, to the journal Hypertension.

Title: Absence of the SA protein product completely ameliorates the increase in blood pressure induced by a high sodium intake in 129 mice.

Authors: Vanessa Walsh, Catrin Pritchard, Nilesh Samani

Abstract: The SA gene encodes a 578 amino acid protein of unknown function. Its main sites of expression are the kidney and liver with lower levels of expression in the brain and testes. SA was first identified as a candidate gene for hypertension and blood pressure regulation due to its increased expression in the kidneys of genetically hypertensive compared with normotensive rats. In F2 crosses between these strains the SA allele from the hypertensive strain co-segregated with increased blood pressure.

We investigated the function of the SA gene *in vivo* by generating a mouse model carrying a null mutation of the SA gene.

Mice lacking the protein product of the SA gene are viable, reproductively normal and have no overt phenotype. Body weight and kidney, liver and heart weights are not affected by the absence of the SA protein.

Comparison of basal blood pressures (BP) revealed no differences between SA-null and wildtype littermate controls in either male or female mice. Exposure of male mice to a high salt diet caused an increase in BP in wildtype mice. However in SA-null mice no effect of salt intake on BP was observed. It therefore appears that absence of the SA protein may offer some protection against a sodium induced rise in BP. The mechanism for this remains to be elucidated but may include an involvement of the SA protein in sodium retention.

These finding provide for the first time direct evidence of the involvement of the SA protein in BP regulations under certain conditions.

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