

**THE RENIN GENE AND ITS EXPRESSION IN HYPERTENSION**

**by**

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**Published works and critical review submitted for the Degree  
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I dedicate this Thesis to my wife and my parents for their  
unfailing support and encouragement

#### DECLARATION

All the work recorded in this thesis is original and the result of the applicant's personal observations unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other University.

Signed..........

Date... 15<sup>th</sup> February 1994

#### ACKNOWLEDGEMENTS

I would like first of all to thank Professor WJ Brammar for allowing me to work in his laboratory, for teaching me molecular jargon, and giving me continual support and encouragement throughout my three years with him when the majority of the work presented was carried out. I would also like express my sincere gratitude to Professor JD Swales for his guidance and unrelenting support throughout my career. This work would not have taken place without him. Several people helped with various aspects of the work. They are acknowledged in the individual papers. Finally, I would like to thank the Medical Research Council for providing financial support through a Training Fellowship.

## THE RENIN GENE AND ITS EXPRESSION IN HYPERTENSION

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## INTRODUCTION

The majority of the work presented in the enclosed papers (apart from that in Papers 7 and 8) was carried out between November 1985 and October 1988 while I worked in Professor Brammar's laboratory in the Department of Biochemistry, University of Leicester. The findings described in the papers should therefore be considered in the context of the state of knowledge that existed at the time. Significant advances have occurred in the field in the period since then. This review attempts to relate the findings to the current state of knowledge in the field.

## PAPERS

A brief synopsis of each of the papers included in the thesis is given below.

(1) Samani NJ, Swales JD, Brammar WJ. Expression of the renin gene in extra-renal tissues of the rat. *Biochem J* 1988; 253: 907-910. This paper reports the initial observations made on the widespread extent of extra-renal renin gene expression in the rat. In addition, it includes the results of a subsequent study that showed the contrasting effects of changes in dietary salt intake on renal and extra-renal renin mRNA levels.

(2) Miller C, Samani NJ, Carter A, Brooks C, Brammar WJ. Modulation of mouse renin gene expression by dietary sodium chloride intake in one-gene, two-gene and transgenic mice. *J Hypertens* 1989; 7: 861-863. This paper reports the results of a further investigation carried out on the effect of changes in salt intake on kidney renin mRNA levels. Using a novel transgenic mouse strain containing an additional renin gene developed locally we were able to conclude that any transcriptional effect on renin gene expression in response to change in salt intake must occur through a *cis*-acting element(s) located within 5 kb either side of the gene. The northern blots in this study were carried out by Dr C Miller.

(3) Samani NJ, Swales JD, Brammar WJ. A widespread abnormality of renin gene expression in the spontaneously hypertensive rat: modulation in some tissues with the development of hypertension. *Clin Sci* 1989; 77: 629-636. This paper reports the results of observations made on renin gene expression in the spontaneously hypertensive rat, a widely studied genetic model of human essential hypertension. Compared to its normotensive control, the Wistar-Kyoto (WKY) rat, the SHR was found to have increased renin mRNA levels in several tissues (brain, adrenal and liver) both at an early age (5 weeks) when blood pressure is just beginning to diverge between the strains and later (at 12 weeks) when hypertension is established in the SHR. In other tissues (kidney and heart), an early increase in renin gene expression in the SHR appeared to be modulated as

hypertension develops. The relevance of these findings to the hypertension in the SHR are discussed in the paper and later in this review (Pages 35-51). The findings directly prompted the studies on the renin genes of the SHR and WKY reported in paper 6.

(4) Samani NJ, Godfrey NP, Major JS, Brammar WJ, Swales JD. Kidney renin mRNA levels in the early and chronic phases of two-kidney, one-clip hypertension in rats. *J Hypertens* 1989; 7: 105-112. In this paper, the marked and contrasting effects of clipping the left kidney on renin mRNA levels in the two kidneys of this model of renovascular hypertension, which persist into the chronic phase of the hypertension despite its decreasing dependency on circulating renin, are reported. The persistent increase in renin mRNA in the clipped kidney into the chronic phase most likely reflects the continued importance of enhanced local angiotensin generation within the kidney in maintaining adequate renal perfusion. The surgery on the animals was performed by Mr N Godfrey and the plasma renin activity measurements were carried out by Dr J Major.

(5) Samani NJ, Brammar WJ, Swales JD. Renal and extra-renal levels of renin mRNA in experimental hypertension. *Clin Sci* 1991; 80: 339-344. This paper reports the observations made on extra-renal renin mRNA levels in the two-kidney, one-clip Goldblatt model and in DOC-salt hypertension and compares these with the changes seen in kidney renin mRNA levels in both models. The findings again indicate that different



factors regulate renal and extra-renal renin gene expression and particularly suggest that a change in the level of circulating angiotensin II, which is characteristic of both these models, does not significantly affect extra-renal renin gene expression. An intriguing finding was a tissue-specific increase in adrenal renin mRNA level in the chronic phase of the Goldblatt model. Given that adrenal renin mRNA is also increased in other models of hypertension, this raises the possibility that an adrenal renin-angiotensin system may play an important role in several forms of hypertension (see Pages 47-49).

(6) Samani NJ, Brammar WJ, Swales JD. A major structural abnormality in the renin gene of the spontaneously hypertensive rat. *J Hypertens* 1989; 7: 249-254. This paper reports the comparison of the renin genes of the SHR and WKY rat carried out by Southern blot analysis of restriction enzyme DNA digests. Polymorphisms were detected with several enzymes. By comparing the Southern blot patterns observed with those predicted from the published sequence of the rat renin gene, most of the polymorphisms between SHR and WKY could be attributed to a 650 base pair "deletion" in the SHR renin gene compared with that of the WKY. By further mapping, the difference was localised to a region of tandemly repeated sequence in the first intron (intron A) of the gene.

(7) Samani NJ, Vincent M, Sassard J, Henderson IW, Kaiser M, Brammar WJ, Swales JD. Analysis of the renin gene intron A

tandem repeat region of Milan and Lyon hypertensive rat strains. *J Hypertens* 1990; 8: 805-809. This paper reports an analysis of the renin gene intron A tandem repeat region of two other rat genetic models of hypertension, the Milan and Lyon strains. The findings indicated that variation in this region of the gene is unlikely to by itself be a major causal factor in the hypertension in these models.

(8) Dubay C, Vincent M, Samani NJ, Hilbert P, Kaiser MA, Beressi J-P, Kotelevtsev Y, Beckmann JS, Soubrier F, Sassard, J, Lathrop GM. Genetic determinants of diastolic and pulse pressure map to different loci in Lyon hypertensive rats. *Nature Genet* 1993; 3: 354-357. Notwithstanding the finding in paper 7, this paper reports the results of a formal co-segregation analysis carried out to investigate the involvement of the renin locus in the hypertension of the Lyon model of genetic hypertension. Significant linkage of the locus was found to both systolic and diastolic blood pressures in F2 rats derived from a cross of Lyon hypertensive and Lyon normotensive rats. The finding was part of a systematic linkage study of the hypertension in the Lyon strain using a panel of microsatellite markers, whose other (major) conclusion was that different blood pressure phenotypes may have different genetic determinants. The study was a multi-center exercise in which my role together with that of Mr M Kaiser (my research assistant) was in analysing the involvement of the renin locus.

(9) Samani NJ, Swales JD, Jeffreys AJ, Morton DB, Naftilan AJ, Lindpaintner K, Ganten D, Brammar WJ. DNA fingerprinting of spontaneously hypertensive and Wistar-Kyoto rats: implications for hypertension research. *J Hypertens* 1989; 7: 809-816. This study reports the results of a comparison of SHR and WKY rats from several sources using probes to hypervariable minisatellite regions of DNA which identify multiple loci scattered over the genome and produce a complex southern blot pattern of fragments termed a DNA "fingerprint". While the DNA fingerprints of SHR from different sources were the same, those of WKY showed considerable variability indicating that different stocks of WKY rats were not genetically identical. There were also several inter-strain differences. It was concluded that results of experiments involving WKY rats from different sources may vary because of the genetic heterogeneity. While the work is not directly on the renin-angiotensin system it is relevant in providing the most likely explanation for a variant result observed in co-segregation analysis of the renin locus with blood pressure (see page 57). In addition, in a wider context, it was suggested that the inter-strain differences may serve as useful markers in locating the chromosomal sites of genes involved in blood pressure regulation and this has also proved to be the case (see page 58). Some of the DNAs used in the analysis were extracted by Drs AJ Naftilan and K Lindpaintner.

(10) Samani NJ, Ruprai AK, Brammar WJ, Swales JD. The renin gene in patients with malignant hypertension and raised

plasma renin activity. Clin Sci 1989; 76: 151-155. This study tested the hypothesis that the raised plasma renin activity seen in patients with malignant hypertension, and which plays a major role in the pathogenesis of the condition, is the consequence of expression of a duplicate renin gene. The study was prompted by the finding that gene duplication may also play a role in other clinical conditions. However, no evidence of duplication of the gene was found in several patients with "primary" malignant hypertension.

For brevity individual papers are referred to from now on with the number given to them in the above list.

#### BACKGROUND INFORMATION

When I joined Professor Brammar's group the renin probe available for use was a mouse submandibular gland cDNA, pSMG5913, containing sequence from exon 2 to exon 9 of the mouse Ren-2<sup>d</sup> gene (see footnote Page 21) which had been cloned in his laboratory [1]. Given the strong homology between the coding regions of the renin genes of different species [2], the probe readily detected renin mRNA from rat kidney by Northern blotting (see papers 3 and 4). However it soon became apparent that Northern blotting was not a sensitive enough technique to detect the much lower levels of renin mRNA in extra-renal tissues despite attempts to maximise the yield by loading large amounts of RNA (up to

250 µg total RNA) and using RNA enriched for mRNA species (poly A+ RNA) [3].

Various alternative strategies were considered and it was thought that the best chance was with an RNAase-protection assay [4] (The polymerase chain reaction had not been described at this stage). The increased sensitivity of the RNAase-protection assay, assessed to be greater than ten-fold higher than Northern blotting [3,4], is mainly related to the lack of need for electrophoresis and transfer of the RNA to a membrane with limited nucleic acid binding capacity and because hybridization occurs in solution rather than to a species bound to a membrane.

Initial attempts to use pSMG5913 in an RNAase-protection assay proved futile because, unlike the situation in Northern blotting, RNAase-protection requires absolute homology between the probe and the mRNA species being assayed; otherwise the hybrid is cleaved by the ribonuclease digestion step which is an integral part of the assay [4, also see Paper 1]. Fortunately, in early 1987 we were kindly provided with a 4 kb *EcoRI* rat renin genomic fragment cloned into plasmid pBR322 by Dr Hiroaki Ohkubo, Kyoto University, in advance of the publication of the rat renin gene sequence by him and his collaborators [5]. He informed us that the 4 kb insert contained several exons including a 750 base pair (bp) *RsaI* fragment containing exon 9 of the gene (350 bp) and adjacent intronic sequence. This fragment was isolated and subcloned into a transcription vector in the correct

orientation to generate the antisense RNA required for the RNAase-protection assay (see Paper 1). This probe proved most useful for the detecting renin mRNA in extra-renal tissues (see papers 1, 3, 5) but considerable work was required to characterise the probe and to optimise the conditions of hybridization (temperature and formamide concentration) and ribonuclease digestion (temperature) [4] which is not reflected in the work presented in the papers. An important characteristic of the probe that needs to be emphasised was the presence of intronic sequence. This meant that in the presence of renin mRNA, the protected "hybrid" fragment (350 bp = exon 9 size) was a different size compared with the original probe (750 bp) (see Figure 1, Paper 1). Thus, one could exclude incomplete digestion by the ribonucleases as a cause of persistent probe, which would have been a concern if the probe had only contained exonic sequence. An approach that was considered for assaying the "protected" probe was scintillation counting of the residual radioactivity [6]. While having the virtue of providing better quantitative information, it was decided that autoradiography after running the products out on an acrylamide gel provided more reliable information. One could directly see that the protected probe was there due to hybridization to renin mRNA (band at 350 bp) and not present from hybridization to heterologous mRNA species or from incomplete digestion. pSMG5913 continued to be used for Northern blot hybridization and proved particularly useful in the comparative analysis of the renin genes of the SHR and WKY rat (see Paper 6).

#### RENIN GENE EXPRESSION IN EXTRA-RENAL TISSUES

The classical view of the renin-angiotensin system (RAS) is one of an endocrine system in which renin secreted by the kidney acts on circulating angiotensinogen produced by the liver to release angiotensin I; this is converted to the active angiotensin II, by angiotensin converting enzyme (ACE), mainly in the pulmonary circulation. Angiotensin II then mediates the two main roles of the RAS - blood pressure maintenance and body water and electrolyte balance - by actions on multiple target organs including the vascular wall, adrenal gland and kidney [7]. There is overwhelming evidence that such an endocrine RAS, responsive to perturbations in blood pressure and body sodium content, participates in acute cardiovascular homeostasis [7].

In 1964 Gould *et al.* [8] showed the presence of renin like enzymatic activity in blood vessel walls. This was soon followed by the demonstration of similar activity in a wide range of tissues [9-30]. Interest began to develop into the possibility that there existed, in addition to the circulating renal-derived RAS, local (extra-renal) RASs in several tissues, subserving functions complementary to as well as distinct from the endocrine RAS [31,32]. The possible involvement of tissue RASs in some sites in hypertension attracted particular interest [31,32]. This was spurred on by the demonstration in the late 1970s that ACE inhibitors lowered blood pressure not only in hypertensive subjects with raised plasma renin activity (PRA), the group

that would have been predicted to respond on the basis of the classical endocrine concept of the system, but also in patients with normal or indeed low PRA [33-34].

Apart from possible functions, two issues dominated the investigations of tissue renin in the 1970s and early 1980s. First, the nature of the renin-like material and second, its source. Since tissue renin was measured through its enzymatic activity and assay of generated angiotensins, initially bioassay of angiotensin II and later immunoassay of angiotensin I [9-30], concern was expressed fairly early on [35] that what was being measured was not renin activity but release of angiotensins from angiotensinogen by other tissue angiotensinogenases. Several studies documented the presence of such angiotensinogenases e.g. cathepsin D, which were particularly active at acidic pHs [18,20-22,27,29]. Many of the later studies took this on board and measured angiotensin I generation at the optimal pH for renin (6.5) and several obtained additional support by using a renin antibody to block angiotensin I generation [22-25,28,36]. As this issue to some extent was being resolved, the question of the source of the renin came to the forefront. Several studies had provided both indirect [37-39] and direct [40,41] evidence of uptake of circulating renin into the vascular wall and considerable data pointed to physiologically relevant generation of angiotensins from such renin [20,27,38-40]. Indeed it was proposed that the major site of angiotensin production from the endocrine RAS itself was actually outside the vascular compartment [42].



Thus these findings suggested that, at least in some tissues, the renin detected was renally-derived. On the other hand several types of other observations suggested a local source for at least part of the renin detected in some tissues (Table 1).

**Table 1. Types of evidence pointing to a local source of renin in extra-renal tissues**

- 
- [a] Large amounts of tissue renin e.g. mouse salivary gland [43], human chorion [9,11,44]
  - [b] Renin in locations outside the blood-tissue barrier e.g. brain [17,21,22,24,30,45], testes [17,23,46,47]
  - [c] Rise in tissue renin after nephrectomy e.g. adrenal gland [25,48-50], vascular wall [15,19,26,29,51]
  - [d] Veno-arterial increase in renin across tissue bed e.g. ovary [52], adrenal [52]
  - [e] Intracellular localisation of tissue renin e.g. adrenal [53], brain [54], pituitary [55]
  - [f] Renin synthesis by cultured cells e.g. vascular endothelial [56] and smooth muscle [57] cells, chorionic cells [58], adrenocortical cells [59]
- 

However, the most direct evidence for local synthesis of a protein is the demonstration of its precursor messenger RNA

(mRNA) in the tissue of interest. The main requirement for this is the availability of a nucleic acid sequence complementary to the mRNA being investigated that specifically hybridizes with it. Given the debate about both the nature and source of the renin-like activity in tissues, the first priority of the work undertaken in this thesis was therefore to determine the extent of extra-renal renin gene expression in the rat. The findings are presented in Paper 1. As this work was being carried out, some reports were published on extra-renal renin gene expression using Northern and dot blotting [60-63]. The data presented in Paper 1 were however the first comprehensive evaluation of such expression in the species more widely used for cardiovascular physiological studies, the rat, using the more sensitive RNAase-protection assay.

For some sites e.g. the adrenal gland and the brain, the findings essentially provided support for the conclusion from biochemical studies (see Table 1) that the renin detected was at least partly locally derived. However, somewhat surprisingly, renin gene expression was also demonstrated in other sites e.g. liver, where hitherto local renin synthesis had not been suspected, suggesting a role for renin extending beyond the circulation (see later). Several studies have since confirmed the widespread expression of the renin gene in extra-renal tissues in several species including humans [64-71].

While the detection of renin mRNA in various tissues provides strong evidence for extra-renal renin synthesis, the correct interpretation of this observation requires several issues to be discussed. The presence of an mRNA for a protein, although an absolute requirement for its synthesis, does not necessarily imply that the protein is actually produced. For this to occur, the mRNA has to be translated. There is evidence [72,73] that many, if not all genes, are transcribed at a low level in all cells (so called illegitimate transcription) without the protein actually being synthesized. However, such low levels of RNA transcripts are only detectable by optimal application of extremely sensitive techniques such as PCR amplification, and the levels of renin mRNA that have been found in most sites far exceed these. Additionally, in several sites *in situ* hybridization has localised renin gene expression to specific cell types [69,74,75] which also argues against illegitimate transcription being the explanation for renin mRNA in tissues. It is also particularly important to note that these studies have shown that the cells that stain positive for renin mRNA in different tissues are not the same indicating that the presence and levels of renin mRNA in various tissues do not simply reflect the frequency of a particular, for instance vascular, cell type. Another important point to note is that while most studies and reviews in this field refer to the presence of tissue renin mRNA, as indeed is done here, current evidence [61-63] indicates that, as in the case of the kidney, the transcribed RNA in most, if not all, tissues actually codes

for prorenin. Thus for renin to be produced further processing is necessary. The evidence that this actually occurs in tissues is controversial (see later).

#### REGULATION OF TISSUE RENIN GENE EXPRESSION

Because of their inability to distinguish locally derived renin from that derived from the circulation, another major limitation of biochemical techniques was in studying the regulation of tissue renin. Many stimuli whose effects on locally derived renin were worthy of investigation caused such a stimulation of the endocrine RAS that it was difficult to detect any independent effects on local renin. Studies had to be carried out under rather non-physiological conditions such as after bilateral nephrectomy or after isolation of vascular beds from the circulation and the relevance of findings from such studies were difficult to interpret [76].

A major advantage afforded by molecular biology techniques has been the ability to examine the regulation of locally derived renin, at least at the mRNA level, independently of the circulating system. Since the level of salt intake had been long known to influence the activity of the circulating RAS [1], I thought it would be interesting to first look at the effects of this stimulus on renal and extra-renal renin mRNA levels. The data are also presented in paper 1. While a low salt intake for two weeks dramatically increased, and a

high salt intake suppressed renin mRNA levels in the kidney, neither type of modification of salt intake had any effect on renin mRNA levels in the brain or liver.

Several studies have since reported the effects of a variety of stimuli on renin mRNA levels in mouse and rat tissues [77-93]. Some of the more interesting and relevant findings from these studies are summarised in Table 2.

**Table 2. Regulation of tissue renin gene expression. The table show the effects of various stimuli on renin mRNA levels in rat and mouse tissues. Data derived from several published studies [Paper 1, 77-93]**

Stimulus	Tissue									
	K	Ad	Br	Li	Ao	Ht	T	SMG	Ut	Ov
Low salt	↑	↑	↔	↔		↑	↔	↔		
Nephrectomy		↑	↔	↔	↔	↔	↔			
ACEI	↑	↔	↔↓	↔	↔	↔↑	↔			
DHT	↓	↑	↑			↑	↑	↑	↔	↓
Frusemide	↑	↑	↑			↑				

↑, increased; ↓, decreased; ↔, unchanged; K, kidney; Ad, adrenal; Br, brain; Li, liver, Ao, aorta; Ht, heart; T, testes; SMG, mouse submandibular gland (see footnote on page 21); Ut, uterus, Ov, ovary; ACEI, ACE inhibitor; DHT, dexamethasone.

As in the case of actual detection of renin mRNA some caution is again necessary in the interpretation of these findings. Some results, for instance, are based on single reports and require further confirmation. The studies also by and large do not distinguish a change in renin mRNA level due to alteration of gene transcription from that due to a change in mRNA stability and finally they do not exclude the possibility of regulation at the translational or post-translational levels for which considerable evidence now also exists [78,94.95].

Despite these limitations, the studies allow some important conclusions to be drawn about tissue renin gene expression. First, they clearly demonstrate that renin gene expression is regulated in a *tissue specific* manner, opening up the possibility of modifying individual tissue systems. At present, however, the precise nature of the *cis* and *trans* acting factors that underlie this tissue specificity are poorly understood (see below). Second, the findings indicate that renin expression in several (if not all) extra-renal sites is not affected by some of the main factors known to regulate its expression in the kidney. Specifically, the studies appear to rule out a negative feedback effect of *circulating* angiotensin II on extra-renal renin gene expression. This was suggested to some extent by the findings in the dietary salt study (Paper 1). However, in this study, plasma renin activity and angiotensin II level were not measured. In another study, an ACE inhibitor, perindopril, given for 5 days did cause a marked fall in

circulating angiotensin II levels. This was associated with a fourfold increase in kidney renin mRNA levels. However, there were again no changes in the level of renin mRNA in the brain, testes, liver, heart or adrenal gland [91]. A lack of effect of circulating angiotensin II on extra-renal renin gene expression is further supported by the findings in the Goldblatt two-kidney, one-clip and DOC-salt hypertensive rats presented in paper 5. Apart from anything else, this dissimilar regulation of renin gene expression in renal and extra-renal sites, raises interesting teleological questions about renin. Intuitively, it seems most likely that renin first had a role as a local hormone and that the more widespread cardiovascular role came later in association with the development of a specialised secretory apparatus in the kidney under precise circulatory regulation. One is tempted to draw an analogy with the local and systemic roles of the sympathetic nervous system.

While the nature of the *trans*-acting cellular factors through which the effects of physiological stimuli, even on renal renin gene expression, are mediated remain virtually unknown, the nature of or at least the location of some of the putative *cis*-acting elements regulating renin gene expression have been defined. By sequence alignment of the mouse and human genes, Pratt *et al.* [96] identified eleven conserved blocks upstream of the genes, 9-29 bases long, with greater than 80% homology which may represent important regulatory elements in basic renin gene function. In block 7 (at position - 144 in human) sequence homologous to a cAMP

responsive element is present and in block 3 (at position -396 in human) the sequence GTTAAT, homologous to a *cis*-acting element thought to be necessary for liver specific gene expression [97] is seen. This later observation is particularly interesting in view of my finding on hepatic renin gene expression (see above). Outside these blocks, several other sequences with potential regulatory roles have also been identified. Thus sequences resembling hormone responsive elements (HREs) for glucocorticoid, oestrogen and progesterone are present and may play a role, for example, in the tissue-specific effects of androgens and thyroxine on renin gene expression [77,82,88].

Detailed studies of the putative regulatory sequences discussed above have been hampered by the lack of renin synthesizing cell lines. To circumvent this problem, two approaches have been taken - transient transfection studies and transgenesis. Using the first approach, in which the sequences are connected to a heterologous promoter (e.g. thymidine kinase promoter) and reporter gene (e.g. chloramphenicol acetyl transferase) and the effect of the additional fragment on expression of the reporter gene studied during transient transfection of suitable cultured cells, Nakamura *et al.* [98] confirmed the presence of the putative CAMP responsive element in the upstream region of both *Ren-1<sup>d</sup>* and *Ren-2<sup>d</sup>* mouse genes (see footnote on Page 21). They also showed the presence of a negative element in the *Ren-1<sup>d</sup>* upstream region not present in *Ren-2<sup>d</sup>*, perhaps as a consequence of inactivation by a 143 base pair insert in



the latter, that may play a role in the differential expression of the two genes in the sub-mandibular gland of some mice strains. Using a similar approach, Burt *et al.* [99] mapped a negative control element in the human gene between -149 to +13 that modulated the effects of two positive control sequences (at -892 to -453 and -453 to -149). As in the case of the mouse genes a cAMP responsive element was also detected (at -149 to +13).

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**FOOTNOTE :** Rats and humans have a single renin locus and the gene is not expressed in the sub-mandibular gland (SMG). In the mouse all strains express the gene to some extent in SMG [1,95]. Certain strains that produce low amounts of renin in the SMG (for example, C57-BL10) have a single renin locus (*Ren-1<sup>C</sup>*) whereas other strains that contain high SMG renin activity (for example, DBA/2) have a duplication of the renin structural gene (*Ren-1<sup>d</sup>*, *Ren-2<sup>d</sup>*). *Ren-1<sup>d</sup>* is highly homologous to and largely (although not entirely [100]) resembles *Ren-1<sup>C</sup>* in terms of its tissue pattern of expression while it is the high level of expression of *Ren-2<sup>d</sup>* in the SMG that accounts for the increased amount of renin activity in the SMG of two-gene mice [1,95,100]. The physiological relevance of the duplication is still unclear. All the mouse renin genes are on chromosome 1 and in the two-gene strains, the duplicate genes are very tightly linked. The human gene is also located on chromosome 1 while the rat gene is located on chromosome 13 [1,95].

As discussed later, transgenesis has provided unique insights into the potential physiological effects of extra-renal renin gene expression. The technique has however also provided important information on the regulation of renin gene expression. Tronik and co-workers [101] were the first to show that a 16kb fragment containing the *Ren-2<sup>d</sup>* mouse gene, 2.5 kb of upstream sequence and 3 kb of downstream sequence had all the *cis*-specific elements required to direct the correct tissue expression of the gene in transgenic mice and also to respond to stimulation by androgens and thyroxine. In an analogous experiment Miller et al. [100] also found that a 19 kb fragment of *Ren-1<sup>d</sup>* introduced into a one gene mouse background displayed the same tissue specificities for expression as seen in the native two gene mouse. Interestingly, they were unable to demonstrate expression of a *Ren-1<sup>d</sup>*-CAT transgene construct containing the same *Ren-1<sup>d</sup>* upstream sequence up to the signal sequence as in the above experiment. This suggests that sequences downstream of the cap site may be important in renin gene expression as has been demonstrated for several other genes [102]. With this in mind, and particularly in relation to the co-segregation data discussed later, it is notable that the presence of a number of potential regulatory sequences have been reported in the introns of the renin gene [5] although as with most of the sequences flanking the gene any functions for such sequences remains to be established.

Nonetheless, as described in Paper 2, the renin transgenic mouse created locally by Miller et al. [100] allowed a question of interest to be pursued : namely the location of any *cis*-acting elements involved in mediating the effect of dietary salt intake on renal renin gene expression. In the study undertaken on these mice, we showed that, just as in the case of the native renin genes, mRNA level of the transgene in the kidney varied with salt intake indicating that any transcriptional regulation of *Ren-1d* in the kidney in response to changes in salt intake must occur through an element or elements located within the 19kb introduced fragment.

#### TISSUE EXPRESSION OF OTHER COMPONENTS OF THE RENIN-ANGIOTENSIN CASCADE

##### Angiotensinogen

For tissue renin-angiotensin systems to exist, other components of the renin-angiotensin cascade, apart from renin, need also to be present (although see later). The presence of renin substrate in tissues has been much less extensively investigated compared with renin and has largely been inferred from the presence of angiotensins for which it is the only known precursor. Nevertheless several studies have provided both biochemical and immunohistochemical evidence of its occurrence in a wide variety of tissues [30,69,103-107]. Particularly notable is its recent immunological demonstration in specific areas of the heart

[108], a tissue where, as in the case of the vascular wall, there is considerable controversy about the presence of an intrinsic RAS [108].

However, as in the case of renin, the most direct evidence that at least some of the angiotensinogen detected in such tissues is synthesized locally has been provided by studies looking at angiotensinogen gene expression. Like renin, angiotensinogen mRNA has been found in a wide variety of tissues (Table 3) and expression has been localised to specific cell types in several sites [62,63,69,107,109-115]. Levels of angiotensinogen mRNA in some sites such as the brain and adipose tissue are between 30-50% of those seen in the liver, when expressed as a proportion of total RNA [109,110,112,114]. In contrast, at best, the amount of renin mRNA in extra-renal sites is no more than 5-10% of the level seen in the kidney (e.g. compare levels of renin mRNA in kidney, liver and brain in Figure 1, paper 5). Interestingly, if one compares levels of angiotensinogen and renin mRNA levels reported in various sites there does not appear to be a simple relationship between the two (Table 3). Also, *in situ* hybridization studies suggest that at least in some sites e.g. the aorta [112,114], the adrenal gland [69,74,113] and the kidney [74,115] cells expressing renin and angiotensinogen may not be the same and be located in different areas. Further studies are required but these observations have important implications for the organisation of tissue renin-angiotensin systems which are discussed in the next section.

**Table 3. Distribution of renin and angiotensinogen mRNAs in rat tissues. Compiled from results in papers 1, 3, and 5 and Refs 62-65, 109, 110, 112 and 114.**

	Renin	Angiotensinogen
Kidney	+++++	++
Liver	++	+++++
Brain	++	+++
Adrenal	++	+
Aorta	+	++
Heart	+	+
Testis	++	-
Adipose tissue	-	+++

#### **Angiotensin converting enzyme**

ACE is present in virtually all mammalian organs and body fluids [116]. While some ACE circulates in blood, its presence in tissues undoubtedly reflects local synthesis, a fact confirmed by the demonstration of ACE mRNA in several sites [117]. Both the size of ACE mRNA [117] and protein [118] in testes is smaller than in other sites. Cloning of the respective cDNAs [119] has revealed that this is due to the use of different transcription start sites from a single ACE gene. The significance of the variation in ACE gene products is unclear. ACE is widely distributed on the endothelial surface of blood vessels including microvessels [120,121]. Thus access to it of any angiotensin I generated within tissues is likely to be rapid. There is in addition

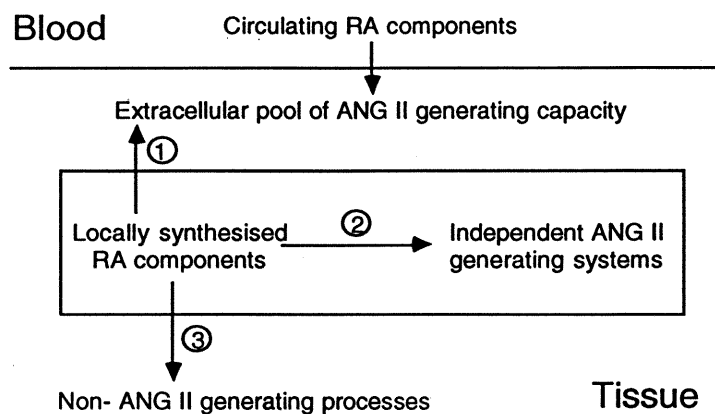
considerable evidence for its occurrence in non-endothelial sites including the epithelial brush borders of the kidney, placenta, intestine, choroid plexus, prostate and epididymis, certain neuroepithelial cells in the brain, and in endocrine tissues, particularly the testes and adrenal [116]. In blood vessels it may be located in vascular cells other than the endothelium [122,123]. In most cells ACE appears to be located on the external surface of the cell membrane although there is some evidence that it may also be located intracellularly [124] (also see next section).

#### **ORGANISATION OF TISSUE RENIN-ANGIOTENSIN SYSTEMS**

The demonstration of the widespread expression of components of the renin-angiotensin system has led to a plethora of functions being ascribed to so called tissue renin-angiotensin systems (RASs) (see next section). In the rush to describe and define functions for tissue RASs, an important aspect, namely the organisation of such systems and the fate(s) of the locally derived components has been largely overlooked. This critical aspect is considered here first before proceeding to a discussion about possible functions of tissue RASs and their involvement in hypertension.

The three scenarios that are possible concerning the locally derived components are shown schematically in Figure 1.

**Figure 1. The three possible fates of locally synthesized RAS components. See text for explanation.**



First, locally synthesized renin and angiotensinogen could simply add to the extracellular pool of angiotensin generating capacity of a tissue, also contributed to by components derived from the circulation (see previously). Second, they could lead to distinct local renin-angiotensin systems, regulated separately and subserving functions that are independent of, or at best only partly complementary to, the system derived from the circulation or third, they could have functions not mediated via angiotensin II generation at all. At present, there is no conclusive evidence in favour of one or other of these scenarios. Indeed, the particular scenario may vary from tissue to tissue [125]. In terms of understanding the functions of tissue RASs there are important implications attached to each of the scenarios.

These implications, together with the evidence for each of the scenarios, are discussed in more detail below.

Scenario 1. Locally synthesized renin and angiotensinogen simply add to the pool of extracellular angiotensin II generating capacity of a tissue. Several lines of evidence suggest that at least in some sites locally produced renin and angiotensinogen are transported to the extracellular space. As discussed previously veno-arterial increases in prorenin have been demonstrated for the adrenal gland and the gonads [52] and high amounts of renin/prorenin have been found in several extracellular fluid compartments [44,126,127]. The persistence of circulating prorenin [52,128,129] and to some extent renin and angiotensins [130,131] (although this is controversial) following bilateral nephrectomy are also most in keeping with this scenario. Finally, cultured cells from several sites have been shown to secrete the renin they produce [56-59].

The main implication of this scenario is that in the end, the effects of the locally synthesized components are mediated through the same angiotensin II receptors that are responsive to angiotensin II produced either locally from components derived from the circulation or indeed to plasma derived angiotensin II. Thus in each case the locally derived components do not produce separate effects but add to the effects of the endocrine RAS on the tissue, their synthesis being regulated by locally perceived needs rather than systemic needs. The amounts of locally derived



components required to serve the above purpose (or indeed the other scenarios) is difficult to judge and may be quite small. On this basis, the argument sometimes put forward that tissue RASs do not exist in some sites because of the low levels of components detected [108] is not valid.

An important secondary implication that flows from the above and which distinguishes this scenario from the others (see below) concerns the effects of inhibitors of the RAS. The scenario predicts that agents capable of blocking the *interstitial* generation of angiotensin II or its actions would influence any effects of the locally-derived components to the same extent they would the effects of components derived from the circulation [125].

**Scenario 2. Locally synthesized renin and angiotensinogen lead to distinct local tissue renin-angiotensin systems.** The main alternative to the concept that locally synthesized renin and angiotensinogen add to the overall pool of tissue angiotensin generating capacity is that they lead to distinct local renin-angiotensin systems subserving functions independent of the system derived from the circulation. By definition, such systems would have to be sited in compartments inaccessible to components derived from the circulation. This can only occur in two situations : in an extracellular space with a barrier to blood such as the brain or in a site with a barrier to the extracellular space, namely the intracellular compartment.

Over the years considerable evidence has been accumulated for synthesis of all the components of the RAS within the brain and for an independent brain renin-angiotensin system subserving specialised functions [45]. Nevertheless, even in this relatively well studied site, the precise organisation of the system remains unclear with several topological mismatches apparent when the location of renin mRNA and activity, angiotensinogen mRNA and immunoreactivity, ACE, immunoreactive angiotensins and angiotensin binding sites are compared [45].

Recently, much interest has also focussed on the possibility of intracellular RASs. This has been particularly prompted by the observations of Re and co-workers, suggesting the presence of intranuclear receptors for angiotensin II [132]. They further found that exposure of isolated nuclei to angiotensin II led to changes in chromatin structure and increased RNA synthesis [133,134] although the precise nature of the genes transcriptionally stimulated were not identified. Recently Tang *et al.* [135] have confirmed the presence of specific nuclear angiotensin II binding sites although the effect of angiotensin II on RNA synthesis remains to be independently shown.

Despite these exciting findings, the evidence for intracellular generation of angiotensin II is limited and mainly comes from the co-localisation of immunoreactive renin and angiotensins in certain cell types [124,136-138]. In particular, although expression of the genes for both

renin and angiotensinogen occur in several tissues, whether co-expression occurs within the same cells remains to be shown (see previously). On the other hand, there is accumulating evidence [139-143] that enzymes other than renin and ACE, several located intracellularly, may physiologically generate angiotensin I and/or angiotensin II from angiotensinogen obviating the need for all components to be present together. Another question, regarding an intracellular RAS, concerns the mechanism by which the components are sequestered intracellularly. For instance, in the kidney renin is synthesized initially as preprorenin. The pre fragment directs the renin into the secretory pathway. An attractive hypothesis was proposed at one stage [144] that the use of alternative transcriptional start sites in some tissues could lead to the synthesis of renin lacking the pre fragment thus sequestering it intracellularly. However, evidence for the use of alternative renin promoters in extra-renal sites is lacking.

A major implication of this scenario relates to the effects of antagonists/inhibitors of the RAS. In direct distinction to the first scenario (see above), no prediction can be made in this scenario about the effects of such agents. Their effect on the local system will depend on their ability to gain access to the site of angiotensin II generation by the local system and/or the sites of action of the generated angiotensin II. Work in this potentially very important area is at an early stage. However, there is evidence that the various ACE inhibitors currently available penetrate the

blood-brain barrier to different extents and inhibit the brain RAS in specific locations to different degrees [145]. That this may be clinically important is suggested by the recent observation that two ACE inhibitors, captopril and enalapril, with different penetration of the blood-brain barrier had significantly different effects on quality-of-life parameters despite identical effects on blood pressure [146]. Effects on cognition and higher mental function have been proposed as one of the actions of a brain RAS [45]. As regards the pharmacological interruption of a putative intracellular system, the only currently available evidence is also indirect and comes from the observation of Mizuno *et al.* [147] that a more lipophilic ACE inhibitor (SA446) caused a greater inhibition of angiotensin II generation in a hindlimb vascular preparation than captopril. They suggested that this occurred because the former attained better access to an intracellular site of angiotensin generation. However, the area may become increasingly important if the hypothesis put forward by Kitami *et al.* [148] to explain their recent findings with a non-peptide renin inhibitor, ES-8891, proves correct. They found the drug to not only decrease plasma active renin when administered to marmosets for 7 days but also surprisingly to decrease plasma immunoreactive renin (PIR) and kidney renin mRNA level. Given the concomitant fall in circulating angiotensin II with inhibition of renin, one would normally have expected the latter two parameters (PIR and kidney renin mRNA level) to increase as seen for instance following ACE inhibition [78,80,91]. They suggested that being small

(M Wt = 795) and highly lipophilic ES-8891 may penetrate the cell membrane to form a renin-ES-8891 complex within the juxtaglomerular cell itself; the complex may then reduce renin secretion from the cell and signal a reduction in the transcription rate of the renin gene or an increase in the turnover rate of its transcript resulting in the observed change in the level of renin mRNA. As far as the endocrine RAS is concerned the ultimate effect of ES-8891 would be the same - a decrease in circulating angiotensin II. However, if in some extra-renal sites the action of locally derived renin is predominantly intracellular, renin inhibitors with varying access to the intracellular compartment may have markedly different effects on the synthesis and activity of such renin. In turn they may prove to be very useful tools for elucidating the role of extra-renal renin.

**Scenario 3. Locally synthesized renin and angiotensinogen subserve functions not mediated via angiotensin II generation.** This scenario has not attracted much attention and clearly there is not much direct evidence for it. Nevertheless the possibility that (pro)renin and/or angiotensinogen may have alternative functions certainly deserves consideration at least until much firmer evidence has been obtained for one of the other scenarios. Both, for instance, share considerable homology not only at the level of the gene but also in their amino acid sequences with proteins subserving other functions raising the possibility of other roles. In the case of renin, the homology is with the family of aspartyl proteases [1] and in the case of

angiotensinogen with the serine proteinase inhibitors such as alpha-1 anti-trypsin [149].

As discussed earlier, there is evidence that in some extra-renal sites the main product of renin gene expression may be prorenin rather than renin. Much effort has been spent on trying to identify alternate functions for prorenin but without success to date [128,129]. On the other hand, Sealey and co-workers [52] have recently proposed a mechanism through which prorenin may cause angiotensin generation within tissues without itself being irreversibly converted to renin. They have hypothesized the presence of a specific prorenin cell-surface receptor attachment to which leads to a temporary exposure of its active site and cleavage of angiotensin I from angiotensinogen. Evidence for the occurrence of such receptors however remains to be obtained. With regard to angiotensinogen, the large ratio of the size of the precursor (452 amino acids) to the product (10 amino acids) also encourages a teleologic argument favouring alternate functions [150]. It could also be argued that the different levels of expression of the two components in certain sites alluded to earlier (see Table 3, Pg 25) is as consistent with this scenario as with the others. A striking example of this is seen in the testes where renin is clearly expressed in substantial amounts [61] and has been localised to the interstitial Leydig cells [46] but there is no evidence for angiotensinogen expression [109,110]. As this is a site beyond the blood-tissue barrier one cannot easily

propose that such renin instead interacts with substrate derived from the circulation.

As in the case of the previous scenarios, this scenario has distinct implications. It implies a total lack of relationship between the locally-derived components and the endocrine RAS and predicts that most of the currently available inhibitors / antagonists of the system, perhaps with the exception of renin inhibitors (see above), should not influence the actions of the locally derived components.

Thus it is in this context of continuing uncertainty about the organisation of tissue RASs that the further work presented in this thesis concerning the expression of the renin gene in genetic and experimental hypertension (Papers 3-5) as well as work reported in this area in the literature needs to be viewed.

#### **TISSUE RASs : FUNCTIONS AND ROLE IN HYPERTENSION**

Despite the poor understanding of the organisation of tissue RASs a large number of putative roles have been proposed for such systems (Table 4) and the numbers grow as novel actions are described for angiotensin II. No unifying single role has been identified for all tissues. The expression of components of the RAS in several tissues in fetal life and ontogenic regulation of such expression [151-155] suggest that tissue RASs may play a fundamental role in development.

The finding of Fernandez et al. [156] that angiotensin II promotes angiogenesis may be relevant in this context.

**Table 4. Possible functions of some tissue RASs.**

Tissue	Functions
Kidney	Renal blood flow, glomerular haemodynamics, sodium absorption
Vascular wall	vascular tone, vascular structure arterial compliance
Heart	cardiac hypertrophy, contractility, cardiac conduction, coronary artery function
Brain	blood pressure, thirst, behaviour release of ACTH, LH and ADH neurotransmission, cognition
Adrenal	aldosterone secretion, catecholamine release
Ovary	ovulation, oestrogen production
Testes	androgen synthesis, seminiferous tubule contraction, epididymal fluid composition
Uterus	uterine blood flow, contractility
Chorion	placental blood flow
Gut	absorption of ion and water

The reproductive tract nicely illustrates many of the unresolved questions regarding tissue RASs (Table 4). Despite the clear documentation extending back many years of local synthesis of renin or at least prorenin in several



sites in both the male [46,47] and female [11,44,126] reproductive tracts, the organisation of tissue RASs in such sites and their functions remain largely obscure. The most convincing evidence is for an intrinsic ovarian RAS playing a role in ovarian follicular development [126,157].

While other functions of tissue RASs have attracted interest, most attention has focussed on their involvement in blood pressure regulation and particularly their role in hypertension. The effectiveness of ACE inhibitors in lowering blood pressure when circulating concentrations of renin are not raised or indeed low [33,34] and the disassociations observed more recently between the anti-hypertensive effects of renin inhibitors and their effects on circulating components of the system [158-160] have been advanced as indicating a role of "tissue" RASs in blood pressure regulation [161,162]. However, as discussed earlier, there is strong evidence that substantial generation of angiotensins from the endocrine RAS itself takes place outside the plasma compartment [42,163,164] and these findings therefore do not necessarily indicate an effect mediated through locally derived components. Indeed, the observation that in anephric humans or animals ACE [165-167] and renin [160] inhibitors do not consistently lower blood pressure could be interpreted as indicating a lack of effect of locally derived components on blood pressure at least via acute generation of angiotensins. Nevertheless, two recent exciting observations have in particular drawn attention to the role of tissue RASs in hypertension. First,

the finding that the renin gene locus is involved in the hypertension of several strains of genetically hypertensive rats in whom plasma renin activity is not elevated (see Pages 52-60). As discussed below, there is evidence that specific tissue systems may be over-active in some of these strains (also see Papers 3-5). Second, the finding of severe hypertension in association with low circulating active renin and angiotensin II levels but high renin expression in certain tissue sites in a transgenic rat strain incorporating a mouse renin gene [168]. This model developed by Mullins and co-workers has provided perhaps the most compelling evidence for an involvement of extra-renal renin gene expression in hypertension. Again, the involvement of specific tissue sites in this model is considered in more detail below when RASs in individual sites are discussed.

RASs in four tissues have attracted most attention in the context of hypertension - the vasculature, the brain, the adrenal gland and the kidney. Evidence for their involvement in hypertension has come primarily from studies of rat models of genetic and experimental hypertension (see Papers 3-5). Human data is singularly lacking. Below the evidence for overactivity of each of these RASs in hypertension and the mechanisms by which such overactivity may raise blood pressure is considered in more detail.

#### **Vascular RAS**

Despite the large literature on vascular renin, perhaps the most compelling evidence that increased local renin

synthesis in the vessel wall may contribute to hypertension has only come very recently through studies by Hilgers *et al.* [169] on the renin transgenic rats (TGpos) described above. Compared with transgene negative control rats, isolated hindlimb vasculature of these rats produced significantly more angiotensin I and II which was associated with an increase in perfusion pressure. Furthermore, while bilateral nephrectomy 24 hours prior to hindquarter perfusion greatly reduced angiotensin release from control rat hindquarters it did not alter angiotensin release from the hindquarters of TGpos rats suggesting that the renin in the latter rats was locally derived. In keeping with this, mRNA from the transgene (*Ren-2<sup>d</sup>*) was demonstrated in aortic and mesenteric arterial tissues of TGpos rats. Surprisingly, given the elegance of this approach, no similar studies seem to have been done that have compared angiotensin release by vascular beds of the spontaneously hypertensive rat (SHR) and control normotensive rats. However, several studies [19,26,29,170,171], although not all [18,172] have reported higher levels of renin in arterial tissues of the SHR compared with control Wistar Kyoto (WKY) rats. Both the source of this renin (i.e. local versus circulating) and in some cases the specificity of the activity measured have been questioned [173]. Further, in the only study that has reported on renin mRNA levels (Paper 3), no difference was seen in aortic renin mRNA levels of young (5 weeks old) SHR compared with WKY and if anything aortic mRNA activity was lower in older (12 weeks old) hypertensive SHR compared with WKY. This was in marked contrast to the finding of elevated

renin mRNA levels in several other tissues of the SHR at both ages. In experimental hypertension, the higher renin activity seen in arterial tissues of renovascular hypertensive rats appears to be predominantly renally-derived [27,174] although studies on renin mRNA levels have not been reported.

Only one study has reported on angiotensinogen synthesis by the vasculature in relation to hypertension. Shioto *et al.* [175] found aortic angiotensinogen mRNA levels to be fivefold higher in two-kidney, one-clip hypertensive animals 4 weeks after clipping compared with sham operated animals. Liver angiotensinogen mRNA was also increased. In both sites angiotensinogen mRNA levels returned towards sham levels by 12 weeks after clipping. The location of the angiotensinogen mRNA in the aortic tissue was not identified and it is possible that the change occurred predominantly in expression in the periadventitial adipose tissue as has been reported with other stimuli [176] than in expression within the aorta itself.

The strongest evidence to date that a locally derived component of the RAS cascade is increased in the vasculature in hypertension has actually been obtained for ACE. In a series of studies Miyazaki and colleagues have shown vascular ACE activity to be selectively increased in both genetic and experimental models of hypertension [175,177-179]. Recently, they have shown that in the two-kidney, one-clip model the increase in vascular ACE activity is

associated with an increase in ACE mRNA levels [175]. Studies by others support their observations in at least some models [180,181]. While these findings are exciting not only in terms of perhaps understanding the effects of ACE inhibitors in hypertension but also in relation to the recent discovery that a locus at or near the rat ACE gene may influence blood pressure in genetically hypertensive rats [182], some caution is necessary in their interpretation. The ACE activity in all the studies were measured in large vessels, the aorta or mesenteric artery, and it remains to be shown whether increased ACE activity also occurs in the resistance arteries which may be more relevant to hypertension. Also in most models increased vascular ACE activity was found after the rise in blood pressure had started or occurred suggesting that it was induced by the hypertension perhaps as a result of the associated cellular hypertrophy / hyperplasia in the vessel wall and was not itself a primary cause of the increased blood pressure. Nevertheless, as Shioto et al. [175] point out the increase in vascular ACE may set up a vicious cycle further increasing and sustaining the hypertension.

In addition to the well described direct vasoconstrictive effect of angiotensin II on vascular smooth cells several other mechanisms by which a vascular RAS may raise blood pressure have been proposed (Table 5).

**Table 5. Mechanisms through which a vascular RAS might influence blood pressure. See text for more details.**

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Direct vasoconstrictive effect on resistance vessel smooth muscle cells

Enhancement of sympathetic vascular tone

Modulation of endothelial function and its production of vasoactive substances

Effect on structure of resistance arteries

Effect on conduit artery compliance

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Most of the mechanisms do not necessarily distinguish between systemically derived angiotensin II and that locally produced but the emphasis in most discussions has been on the latter. Thus, it has been suggested that locally derived angiotensin II may modulate endothelial function and its production of vasoactive substances such as prostaglandins, endothelin and endothelium derived relaxing factor, which in turn would influence vascular tone [183]. Several studies have now convincingly shown that angiotensin II generated within the vessel wall influences the neural control of vascular tone [184-187]. As discussed previously the location of some of the locally derived components which may be important in vascular angiotensin II generation, e.g. angiotensinogen itself, and a chymotrypsin sensitive angiotensin generating enzyme described by Okunishi et al. [141], in the outer layers of the vessel wall are consistent with such a role for a vascular RAS.

Most interest, however, in recent years has focussed on the role of a vascular RAS in determining vascular structure. It is now clear that changes in resistance vessel structure and specifically a decrease in the lumen to wall ratio may be the main factor that chronically maintains increased vascular resistance in hypertension [188]. Undoubtedly, the level of blood pressure *per se* is an important determinant of vascular structure [189]. However, because (a) angiotensin II has been shown to promote vascular smooth muscle cell growth *in vitro* [190-192] (b) a chronic infusion of low dose angiotensin II has been shown to change resistance vessel structural parameters independent of any effect on blood pressure [193] and (c) some studies have found ACE inhibitors to reverse vascular hypertrophy better than other equipotent antihypertensive drugs [194-195] or more than expected on the basis of the amount of fall in blood pressure [196-197], it has been argued that the RAS and by implication, the local vascular RAS, may be an important independent determinant of vascular structural changes in hypertension. Indeed it has been suggested that an initial trophic change in vascular structure induced by the RAS may be the trigger factor that sets up a vicious cycle leading to hypertension [198]. Harrap and co-workers [199] have shown that treating the SHR with an ACE inhibitor for a relatively short period at a young age caused a permanent reduction in blood pressure in these animals. Similar treatment of older SHR, while reducing blood pressure significantly during treatment, had no effect on longer term blood pressure. Their conclusion from these

studies is that it may only be necessary for there to be a short time window in early life during which overactivity of the RAS, perhaps in selected vascular beds [200], induces permanent structural changes that in the longer term lead to increases in blood pressure. While these various observations have provoked much interest and have been seized upon by the pharmaceutical companies to promote the use of ACE inhibitors in hypertension, the role of the RAS in the vascular structural changes associated with hypertension remains far from being fully understood. As discussed earlier, although an increase at an earlier age cannot be excluded, we found no evidence for an increase in aortic renin mRNA in 5 weeks old SHR (Paper 3). Further, not all studies have found ACE inhibitors to be better than other agents at preventing or reversing vascular hypertrophy [201]; it is also becoming clear that the effect of angiotensin II on vascular smooth muscle cell growth is much more complicated than previously imagined and that it can act as a bifunctional growth factor that can activate both proliferative and antiproliferative pathways depending on the presence of other factors [202]; and finally, it is now even being questioned whether either hyperplasia or hypertrophy of vascular smooth muscle cells, processes believed to be influenced by angiotensin II, actually play any role in the structural changes in vessels seen in hypertension. It is being suggested that the main process is rearrangement of the wall constituents (remodelling) to produce a vessel with a smaller lumen for the same amount of



wall material rather than an actual increase in the size of the wall [203].

While most people have focussed on the effects of a vascular RAS on resistance vessel function and structure in hypertension, Safar and co-workers have drawn attention to the possible effects of an RAS in conduit vessels [204]. They found that ACE inhibitors improved the compliance of several large vessels in hypertensive subjects, an effect not seen in normotensive subjects (although serum ACE was inhibited to a similar extent) or with other types of antihypertensive drugs [204,205]. The improved compliance seemed to be due to effects on both functional and structural characteristics of the vessels [206]. They have interpreted their findings as indicating overactivity of a vascular RAS in the conduit vessels of hypertensive subjects leading, through one or more of the mechanisms previously discussed and possibly others, to a stiffening of such vessels. In turn this may contribute to both the elevation of blood pressure, particularly systolic, as well as to the complications of hypertension [204]. While their conclusions may be correct, to date they are indirect and based mainly on the observed effects of ACE inhibitors. These drugs influence several other systems apart from the RAS [207] and the effects on the various systems are not distinguished in their studies. Studies using the recently developed renin inhibitors and angiotensin II receptor antagonists may help identify the specific role of the RAS.

### Brain RAS

After the vascular RAS, the tissue RAS that has attracted most attention in terms of hypertension has been the one believed to be present in the brain. As in the case of the vascular RAS evidence has been sought for higher levels or activity of RAS components in the brain in various models of hypertension. Renin activity [208,209] and mRNA level (Paper 3) have both been reported to be increased in SHR brain compared with WKY. Likewise there is evidence that angiotensinogen may be increased in selected areas of the brain in various models of hypertension [209-212]. More recently, Kimura *et al.* [213] have reported the development of high blood pressure in transgenic mice carrying the rat angiotensinogen gene in association with high levels of transgene expression in the brain. However these animals also have increased levels of circulating angiotensinogen and angiotensin II which could account for the hypertension. Further studies on these mice are awaited with interest. Increased concentrations of immunoreactive angiotensin II, angiotensin II binding sites in specific areas of the brain, and ACE in the cerebrospinal fluid have also been reported in the SHR [214-216].

As discussed earlier (Page 30) important questions remain unresolved regarding the organisation of a brain RAS [45]. Nevertheless, apart from the finding of higher levels of components in various models of hypertension, the ability to introduce antagonists or inhibitors of the RAS directly into the brain beyond the blood-brain barrier by intracisternal

injection has provided a unique means for studying in isolation the role of the brain RAS in hypertension. Numerous studies have examined the effects of central administration of both ACE inhibitors and angiotensin II antagonists on blood pressure in several models of hypertension [217-225]. Despite some contradictions, these studies overall have shown significant reductions in blood pressure with both acute and chronic administration of agents and thus provided strong supportive evidence for an important role of a brain RAS in both genetic [217-220,223, 225] and experimental [221,222] forms of hypertension. However, the mechanisms involved have not been clearly defined. Several have been proposed : modulation of sympathetic control of peripheral vasomotor tone, desensitisation of the baroreflex, enhanced secretion of vasopressin, stimulation of salt and water appetite and neural enhancement of renal renin release [45,145]. The involvement of each of these mechanisms may vary from model to model.

#### **Adrenal RAS**

Adrenal renin activity has been found to be increased in both young and adult SHR/SHRSP compared with WKY [25,50]. As shown in Paper 3, this is associated with higher adrenal renin mRNA levels in the SHR. Interestingly, as reported in paper 5, a tissue-specific increase in adrenal renin mRNA also occurs in the chronic phase of two-kidney, one-clip hypertension at a stage when the involvement of the circulating renin in the hypertension is declining. This is

associated with an increase in biochemical renin activity localised to the zona glomerulosa layer of the gland [174]. In the DOC-salt hypertensive rat, kidney renin expression is markedly suppressed. On the other hand, the adrenal renin mRNA level is, if anything, slightly increased (Paper 5). Most recently, Mullins *et al.* [168] found that the fulminant hypertension in their transgenic rat carrying the mouse *Ren-2<sup>d</sup>* gene (TGpos rats) was associated with markedly elevated adrenal renin mRNA level and activity.

At face value, these findings together suggest an important role for an adrenal RAS in several forms of hypertension. However, as in the case of the other tissue RASs discussed the mechanisms remain obscure. Adrenal derived renin may contribute to circulating renin/prorenin [52,168] although the amount contributed, in terms of active renin, is much less than that by the kidney and seems unlikely to be physiologically important. There is good evidence that locally derived renin participates in adrenal angiotensin II production [50,59,226]. The adrenal gland has some of the highest levels of angiotensin II and substantial levels persist (together with adrenal renin) after nephrectomy [226]. The location of adrenal renin synthesis and activity predominantly in the outer zona glomerulosa layer of the adrenal cortex [25,50,69,74,174] points to a possible role in aldosterone production. Circulating angiotensin II is an important modulator of aldosterone secretion [1] but angiotensin II produced locally may provide additional regulation (Scenario 1). In support of this Doi and co-

workers have reported parallel changes in adrenal renin and aldosterone levels under various conditions in the rat [48]. More recently additional support for this function of an adrenal RAS comes from the finding of increased aldosterone levels in the TGpos rats (see above) despite a low circulating angiotensin II level [168]. On the other hand, most studies in SHR have not reported increased aldosterone levels [227-228] although there is evidence in this model of decreased adrenal responsiveness to angiotensin II at a receptor or post-receptor level which could account for the discrepancy [229,230]. While the adrenal RAS therefore probably plays a role in aldosterone production and secretion the overall evidence that mineralocorticoids participate in the hypertension of any of the above models is weak and other mechanisms need to be considered. One possibility is through increased catecholamine secretion [32] although the evidence for this is also very weak.

#### Kidney RAS

There is now considerable evidence that a local RAS also exists within the kidney. Both angiotensinogen [109,110,115] and ACE [116,117] are expressed in the kidney in addition to renin. A key location of an intrinsic renal RAS may be in the proximal tubule. Angiotensinogen mRNA has been localised to this site by *in situ* hybridization [115] and the brush border of proximal tubular cells contains high levels of ACE [116]. In keeping with this, proximal tubular fluid is characterised by angiotensin II concentrations that are 1,000 - fold higher than plasma concentrations [231]. By

affecting proximal tubular sodium absorption such a system could play a vital role in blood pressure homeostasis [7]. Another important location of a renal RAS could be in the renal arterial tree where modulation of renal vascular resistance and renal haemodynamics [200] could again significantly affect systemic blood pressure. In support of a RAS in this site, renin mRNA has been found to be expressed under certain situations in renal arterioles upstream of the JGA [152,232,233] and renin has been localised immunohistochemically in both afferent and efferent renal arterioles and in interlobular arteries [234].

The best evidence for overactivity of a renal RAS in hypertension again comes in young (5-6 weeks) SHR before the rapid rise in blood pressure. In these animals, higher renal renin mRNA level (Paper 3), renal renin content [235,236], angiotensin II content and tubular angiotensin II receptors [237], have all been reported compared with WKY rats. In keeping with a local intrarenal function of the increased renin, plasma renin activity in these animals is not elevated (238, Paper 3). In older hypertensive SHR all these parameters are the same or lower than in WKY rats [paper 3, 235-237] suggesting a modulating effect of the increased blood pressure. Nevertheless, it is possible that the overactivity of an intrarenal RAS in the SHR during a short time window at a young age sets off a chain of events leading to sustained elevation of blood pressure in this model [200]. Apart from renin, expression of angiotensinogen in

the SHR kidney also appears to show altered regulation compared with WKY which may be physiologically relevant [239].

In experimental renovascular hypertension, marked changes also occur in renal renin gene expression as shown in paper 4. Interestingly, in the 2-kidney, 1-clip model studied the increase in renin gene expression in the clipped kidney was found to persist beyond the acute rise in plasma renin into the chronic phase despite a return of plasma renin activity to sham levels. This has subsequently been observed by others as well [240]. The findings again illustrate, as in the case of the young SHR, the disassociation that can occur between kidney renin production and plasma renin levels. As discussed in paper 4, it is most likely that the continued higher expression of renin in the clipped kidney is a reflection of the role of a local RAS in maintaining glomerular haemodynamics in that kidney in the face of the decreased perfusion pressure. Whether the persistent stimulation of intrarenal renin in the clipped kidney contributes to the chronic phase hypertension in this model via mechanisms not involving a rise in plasma renin remains unclear.

#### CONCLUSIONS ON TISSUE RASs

Views on tissue RASs continue to evolve as different approaches provide new insights. The evidence for widespread

local synthesis of RAS components is now overwhelming and the involvement of tissue RASs in some sites in hypertension compelling. However crucial questions regarding the regulation and fate of locally derived components and the spatial organisation of tissue RASs remain to be answered. Innovative approaches are required to resolve these issues. Until they are resolved our understanding of the biological functions of tissue RASs, their role in various pathophysiological processes and our ability to manipulate such systems for specific clinical benefits will of necessity remain limited.

#### THE RENIN GENE LOCUS IN RODENT HYPERTENSION

The finding of higher renin mRNA levels in several tissues of young and adult SHR compared with WKY rats (Paper 3) led to a comparison of their renin genes by Southern blotting and the demonstration of polymorphisms with several restriction enzymes between the genes (Paper 6). Most of the polymorphisms were ascribed by further analysis to a difference in the size of a region in the first intron (intron A) of the gene made up of a tandemly repeating (minisatellite) sequence (Paper 6). Subsequently this region of intron A was also compared in the strains of the Milan and Lyon models of hypertension (Paper 7).



To date, seven rat renin alleles based on the size of the intron A minisatellite fragment flanked by *Bgl*III sites have been identified in inbred strains (two more with *Bgl*III fragment sizes of 2.9 and 1.3 kb respectively, in addition to the 5 alleles described in paper 6) [241]. It is likely that other alleles are also present or will emerge - minisatellites tend to be regions of high mutation rates because of asymmetric pairing and unequal crossing-over at meiosis of the tandemly repeating sequences and mutation rates up to 5% have been described [242]. Indeed, in a recent study of progenies from two backcrosses Mori *et al.* [243] found a mutation rate of 0.36% for the intron A minisatellite.

As indicated in paper 7, it seems unlikely that the tandem repeat region in intron A plays an indispensable role in renin gene function. Only two copies of the tandem repeat motif are present in the mouse *Ren-1<sup>d</sup>* gene and a significant sized minisatellite does not appear to be present in human DNA. However, tandemly repeated sequences, as small as dinucleotide repeats, are being increasingly recognised as playing an important role in gene function and regulation [244] and it remains possible that the rat renin gene minisatellite plays a regulatory role in that species. As alluded to earlier (Page 22), there is evidence that regulatory regions may be present in the renin gene downstream of the transcription start site. Implicating the minisatellite in the regulation of rat renin gene expression is difficult, although one possible means of say

investigating whether a variation in its size is responsible for the observed differences in renin mRNA levels between SHR and WKY, would be by isolating the respective fragments from SHR and WKY and studying their relative effects on transcription of a heterologous promoter/reporter gene cassette. To this end, before I finished in Professor Brammar's laboratory, I cloned by PCR amplification of genomic DNA, the 2.3 kb fragment from the WKY rat. The primers used flanked the minisatellite and incorporated restriction sites for cloning. For some unknown reason during the time available, it was not possible to clone the smaller 1.65 kb SHR fragment despite its satisfactory amplification and isolation and this work has not been taken further.

Notwithstanding uncertainty about its functional role, the intron A polymorphism has proved a very useful genetic marker in studying the role of the renin gene locus in various rodent models of hypertension. Rapp et al. [245] were the first to use it to show co-segregation of the Dahl salt-sensitive (Dahl S) rat renin allele with increased blood pressure in F<sub>2</sub> rats derived from a cross of this strain with the Dahl salt-resistant (Dahl R) rat. Despite the availability of several genetically hypertensive rat strains for over 10 and some for over 20 years, this seminal paper was the first description of an involvement of a specific gene locus in the hypertension of any of these strains and rightly aroused a lot of interest. Subsequently, using the oligonucleotide probe described in Paper 6 to

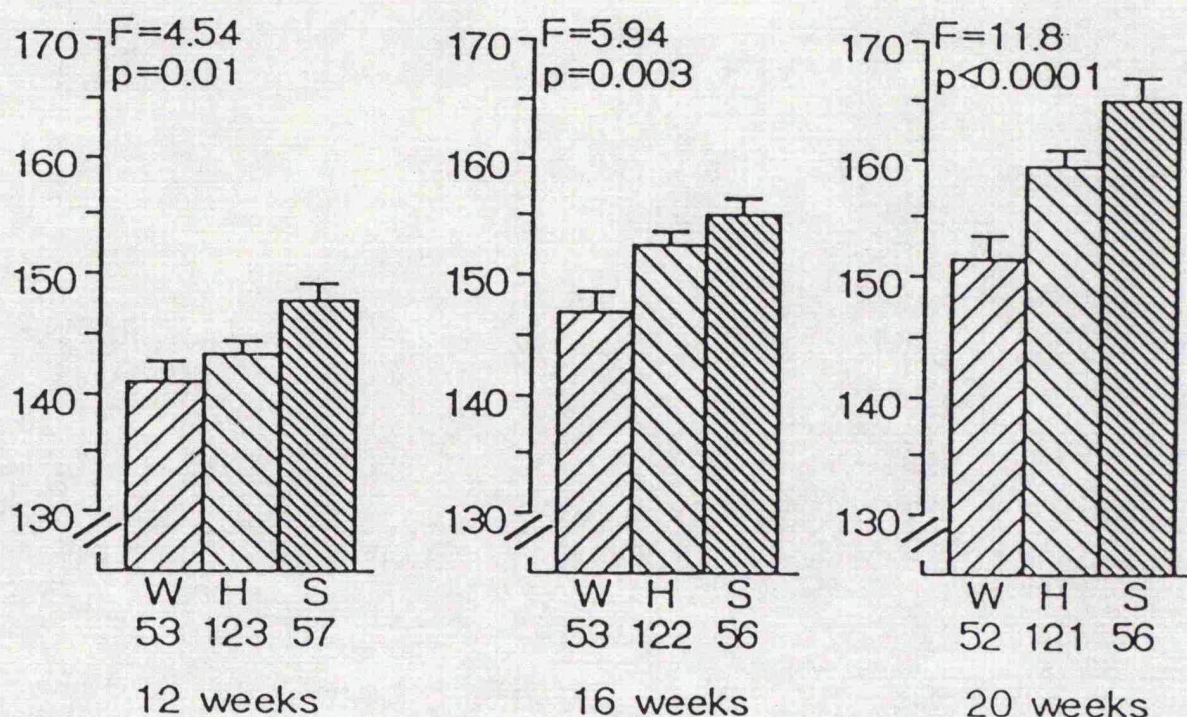
distinguish the alleles, Kurtz and co-workers showed cosegregation of the locus with blood pressure in an SHR x Lewis rat cross [246] and also in a panel of recombinant inbred (RI) rat strains derived from a cross of SHR with Brown Norway (BN) rats [247].

We have so far analysed the involvement of the locus in genetic hypertension in two crosses. First, as described in Paper 8, the locus was found to play a significant role in the hypertension of the Lyon model, despite, as discussed in paper 7, the Lyon hypertensive rat sharing the same sized intron A minisatellite fragment as the Lyon low-blood-pressure rat (LL). This work was carried out in collaboration with Prof Sassard and Dr Vincent in Lyon and Dr Lathrop in Paris. The locus was typed for both the intron A polymorphism using Southern blotting as well as, subsequently, for a microsatellite (CA repeat) polymorphism located in intron C of the gene (between base pairs 6976 and 7174) [1, 248], using the polymerase chain reaction (PCR). As expected, given the proximity of the two markers, there was 100% concordance between the results of the two typings. For brevity (and consistency with the rest of the data in the paper), only the results of the PCR genotyping were presented in the paper.

More recently, as shown in Figure 2 (unpublished data) we have also observed the genotype at the locus to have highly significant and increasing effects on indirect systolic blood pressure measured at 12, 16 and 20 weeks of age in our

own F2 rats derived from a cross of the SHR with the WKY rat. Description of the cross and results are not given in detail as they are to form part of the PhD thesis to be submitted by my research assistant, Mr Michael Kaiser.

**Figure 2. Longitudinal effects of the renin locus genotype on indirect systolic blood pressures (mm Hg) in male F2 rats derived from an SHR x WKY cross maintained on a normal salt diet. W, homozygous for WKY renin allele; S, homozygous for SHR renin allele; H, heterozygous. Numbers below each bar are the number of animals in group. F and p values are from analysis of variance.**



Overall the proportion of blood pressure difference between the parental strains segregating with the renin locus in the various crosses [245-247, Paper 8) has varied from 10 - 25% indicating, as anticipated [249], that it is only one of



several loci influencing blood pressure in these rodent models. The location of several other loci have also recently been elucidated [250]. One gene in particular,  $S_A$ , has aroused considerable interest [251-253]. Its function remains unknown. However, the findings that it is highly expressed in the SHR kidney (and liver), that its expression in some sites can be modulated by angiotensin-converting enzyme inhibitor treatment and that, at least in some strains, it can also be affected by changes in dietary salt intake [251], suggests a relationship with the renin-angiotensin system. In our SHR x WKY cross the effects of the renin locus and  $S_A$  locus [252] on blood pressure were additive (unpublished data).

Although in most crosses studied so far the renin locus has been found affect blood pressure to some extent, an important negative result needs comment. In a cross between SHRSP and WKY generated in Heidelberg, the locus was found to not to co-segregate with 16 weeks directly measured blood pressure [254] despite the co-segregation of other loci, including  $S_A$ , with blood pressure in this cross [182,253]. The SHRSP has the same sized intron A minisatellite fragment as the SHR and also shares other point polymorphisms at the locus with the latter suggesting that the two have identical renin alleles [241]. Thus the reason for its lack of co-segregation with blood pressure in the Heidelberg cross is perplexing, especially given the findings in our SHR x WKY cross (Figure 2). Environmental factors cannot be excluded. However, a more likely possibility, suggested by the data

presented in Paper 9, is that it is a consequence of genetic heterogeneity between WKY animals from different sources. In this paper we showed that DNA fingerprints of WKY rats from different sources, *including* animals from Heidelberg, are different. We proposed that such genetic heterogeneity may account to some extent for variable results obtained in comparisons of SHR and WKY in different laboratories. At the time we believed that results may vary because WKY from different sources may be different at the relevant loci. The findings of Lindpaintner *et al.* [254] extend this concept further and indicate that even if animals are identical at the locus being studied (as would appear to be the case for the renin locus in different WKY, see Paper 6), a difference in the genetic background may entirely mask the effect of the locus. The importance of the genetic milieu in influencing the effects of loci on blood pressure, even in these rodent models, is being increasingly recognised as further co-segregation studies are reported [255,256].

As an aside, another hope expressed in Paper 9 was that the anonymous markers for the rat genome identified through techniques such as DNA fingerprinting may provide useful markers for identifying the chromosomal location of genes involved in hypertension. This hope has been vindicated, not only from the use of a DNA fingerprint-type minisatellite to identify a locus on the rat X chromosome that may modulate blood pressure [182], but also by the development and application of an even more extensive panel of markers based on microsatellite sequences [182,248,257, Paper 8]. It is

likely that a set of such markers covering the entire rat genome at a distance of less than 10 centimorgans (i.e. suitable for linkage analysis) will be available in the not too distant future and that with appropriate crosses the chromosomal location of the main genetic factors involved in rat genetic hypertension will be identified. However, the nature of the genes involved and the molecular, cellular and physiological mechanisms through which they affect blood pressure will require much more work [258].

Returning to the renin locus, the mechanism by which it influences blood pressure likewise remains unclear. None of the hypertensive strains in which the locus has been shown to co-segregate with blood pressure show any consistent increase in plasma renin concentrations (PRC) (e.g. see Paper 3) compared with control strains and in several cases plasma and kidney renin are lower, certainly by adulthood. This suppression of PRC is almost certainly mainly the consequence of the effect of the high blood pressure on kidney renin synthesis mediated through the baroreceptor mechanism [7], although the findings in a recent study [259] on the SHR x BN RI strains described earlier (see Page 55) suggests that there may also be a direct effect of the renin genotype on kidney renin concentration independent of any effect on blood pressure. Even when a similar or lower plasma level of a pressor hormone such as renin is seen in hypertensive strains one is always faced with the possibility that it is "inappropriately" raised for the level of hypertension and thus still contributing to the

elevated blood pressure. Such an argument is difficult to refute [260], although given the findings on increased renin mRNA levels and/or activity in various tissue sites in hypertensive strains and the findings from the transgenic renin rat discussed earlier, it would seem more likely that, if the effect of the locus on blood pressure is due to an effect of specific renin gene alleles and not due to variation at another (linked) gene (see below), this is because of aberrant renin gene expression in one or more key tissue sites. The identification of the relevant tissue site(s), which could have important implications for our understanding of the role of the renin-angiotensin system in hypertension and the mechanisms through which anti-hypertensive drugs that influence the system act, is a key area for further investigation.

#### THE RENIN GENE LOCUS IN HUMAN HYPERTENSION

Since the celebrated debate more than 30 years ago between Platt and Pickering [261] on the genetic basis of human hypertension, interest in this area of hypertension research has continued, and has again particularly come to the fore in the last few years with the technological developments in the field of molecular genetics. It is now generally accepted that blood pressure, like height and weight, is a continuously distributed variable and that essential hypertension, however defined, simply represents one extreme of this distribution and not a distinct disease. Thus



hereditary influences are seen at all levels of blood pressure. Estimates of the degree of genetic influence on blood pressure vary but probably about 35% of the population variability in blood pressure is genetically determined, with a further 15% due to familial but non-genetic factors [249,262].

The main consequence of the above, which is also by and large generally accepted, is that it is highly unlikely that a mutation at a single gene locus will emerge as the cause of the vast bulk of essential hypertension. This is not to say that a single gene disorder may not by itself be sufficient to produce hypertension. Several examples of this already exist, most notably the recent demonstration that a chimaeric 11 $\beta$ -hydroxylase/aldosterone synthase gene causes glucocorticoid remediable aldosteronism (GRA) and hypertension [263] with a very high degree of penetrance. However, the more likely situation is that mutations at several gene loci can influence blood pressure. These can be regarded as *permissive* mutations for raising blood pressure. Whether in their presence blood pressure rises to hypertensive levels will depend not only on the strength of their effect, but also on the effects of and their interaction with other genes (the genetic background) and environmental factors. The main uncertainty presently facing researchers in the field is whether the magnitude of the effects of at least some of such permissive mutations are enough to identify them despite the diluting effects of the genetic background and environmental factors or whether

there are simply many genes with very small effects. The recent finding from sib-pairs analysis (see below) that mutations at the angiotensinogen locus may act as predisposing factors in 3%-6% of hypertensives with onset prior to age 60 [264] gives some cause for optimism.

As in the case of rodent genetic hypertension, since suitable probes became available [265,266], the renin gene locus has been an important target for investigations into the genetic basis of human hypertension. In an early study (Paper 10) we showed that aberrant expression of a duplicated renin gene was unlikely to be a cause of the hyper-reninaemia seen in, and pathologically important in, "primary" malignant hypertension.

Most subsequent studies looking at the role of the locus in the more common essential hypertension have also disappointingly proved negative [267-272]. However, they nicely illustrate the difficulties in carrying out genetic studies in human polygenic disorders and also the way the field had developed to try and overcome some of the problems. For discussion the studies can be grouped into two types : (a) association studies and (b) linkage studies.

An association study tests whether a marker, in this instance a DNA polymorphism (RFLP), occurs more frequently in cases versus controls. If positive it suggests that either the polymorphism itself or a *functional mutation with which it is in linkage disequilibrium* contributes to at

least some of the cases of the disorder being studied. In the first study of this type on renin, Morris and Griffiths [267] found no difference in the frequency of a *HindIII* RFLP in the human renin gene between hypertensives and control subjects. The study was small and the hypertensives were unselected. However, two subsequent studies of larger sizes, on Caucasian hypertensives chosen on the basis of a positive family history of early hypertension to increase the genetic contrast with the normotensive control group have also failed to find any association of several renin gene RFLPs and hypertension [269,270]. More recently, Barley *et al.* [271] chose slightly different, and perhaps more appropriate groups, for their comparison. Given that hereditary influences are present at all levels of blood pressure, rather than study subjects "arbitrarily" defined as hypertensive or normotensive, they compared renin RFLPs in subjects in the upper and lower quintiles of blood pressure for age and sex in a large cohort randomly sampled from their population. The subjects were also divided according to their ethnic background. In their white European group they again found no relationship between three renin gene RFLPs and blood pressure. However, in the black Afro-Caribbean group there was a significant association ( $p = 0.03$ ) of the *BglI* polymorphism, also located interestingly in the first intron of the gene (*vide rat polymorphism*), and blood pressure. The finding clearly needs to be confirmed in other black populations but could be relevant in light of the known differences in plasma renin levels between black

and white hypertensives and their differing responsiveness to angiotensin converting enzyme inhibitors [273].

The study of Barley *et al.* [271] also highlights an important issue that can critically affect the results of association studies, namely a difference in the genetic backgrounds of cases and controls. They found the relative frequencies of all the renin polymorphisms to be highly significantly different in their white European and black Afro-Caribbean populations, a finding that has also been reported for several other genes. Thus even small, and often unrecognised, differences in the racial admixture of cases and controls can give rise to spuriously positive (or indeed negative) results underscoring the importance of confirming results of association studies in several populations before they can be used with confidence. Likewise, it needs to be emphasised that a negative association study does not exclude an involvement of the locus, even an important one, in blood pressure regulation - it may simply be that the polymorphism(s) studied is simply not in linkage disequilibrium with the causative mutation(s) at the locus.

The other approach to identify genetic determinants of blood pressure, linkage studies, relies on the familial aggregation of hypertension. This is of course the classical approach which tests for departure from random segregation of traits and genetic markers in families which has been so successful in identifying loci responsible for several monogenic disorders. While inherently attractive, and

overall more reliable than association studies, the approach poses several difficulties in the analysis of a trait such as hypertension. Human pedigrees are in the main small. The late onset of hypertension makes it difficult to obtain informative multi-generational pedigrees for study which is a key requirement for this approach. Further, given the multifactorial nature of hypertension, the chances of both false positive and false negative assignment of subjects which will confound the analysis between the trait and the marker are likely to be high. Finally, while for many monogenic disorders, the problem of small pedigree sizes is circumvented by combining data from different pedigrees, the likely genetic heterogeneity of cause of hypertension in different pedigrees makes this a highly unreliable statistical technique in hypertension. Not surprisingly, so far few studies have reported this type of analysis in hypertension and only a couple of hypertensive pedigrees, of any size, have been analysed for linkage to markers at the renin locus. The results of both studies suggested that there was no linkage of the hypertension presumed to be segregating in the families with the renin locus [268,270].

To overcome some of the problems associated with multi-generational linkage analysis, an alternative family based approach which is being increasingly utilised in hypertension and in other polygenic disorders is sib-pair linkage analysis [274]. This approach is based on the premise that if a locus contributes to a trait, then sibs (or indeed other relatives) that are *concordant* for the

trait are more likely to have inherited and therefore share identical alleles at the locus than expected by random segregation of the trait and the marker. Apart from not needing large or multi-generational pedigrees, the other advantages of this approach include the greater likelihood of sibs being of similar ages obviating the problem of the late onset of hypertension, a lack of requirement of any *a priori* assumption regarding the model of inheritance of the trait, which is required in analysing classical linkage data, and the fact that the approach, unlike association studies, tests the involvement of the whole locus and not only a specific DNA polymorphism or a mutation with which it is in linkage disequilibrium (see previously). The main requirement for sib-pair studies as for all linkage analysis are polymorphisms that show a high degree of heterozygosity. This is being realised for many candidate genes in hypertension with the characterisation of highly polymorphic microsatellite sequences, e.g. CA repeats, within or near such genes [264,275]. Although such a polymorphism has not yet been described for the human renin, Jeunemaitre *et al.* [272] were able, by using renin gene haplotypes defined by combining information from three RFLPs, to achieve informativeness corresponding to a heterozygosity of 70%, in a study of 98 French hypertensive sib-pairs from 57 families. Although the analysis of their results was somewhat limited by the absence of parental haplotype information in 40 of the 57 sibships they did not find any excess sharing of renin alleles in their sib-pairs.

Therefore, given the mainly negative results to date, should we conclude that the renin gene does not play a significant role in the pathogenesis of essential hypertension ? Is its effect of the locus on blood pressure in rodent hypertensive strains simply a species-specific effect or indeed, as considered earlier, due to the presence of a *linked* blood pressure modulating gene which is located elsewhere in the human genome ? In my view, given the inherent limitations of the studies carried out (see above), it would be premature to reach these conclusions. Nevertheless, future studies, need to perhaps focus on selected groups of patients. As mentioned earlier, two kinds of influences tend invariably to decrease the observed effect, in both association and linkage studies, of a particular gene locus on blood pressure - environmental effects and genetic heterogeneity. In contrast to animal studies, it is unlikely that much can be done to overcome to any great extent the confounding effects of the environment in studies of the genetics of human hypertension particularly as these effects are probably accumulative over many years. On the other hand, control for genetic heterogeneity may be more feasible by identifying, through the use of intermediate phenotypes, subsets of the hypertensive population that are more likely to share the same genetic factors contributing to their hypertension. Intermediate phenotypes are manifestations of the molecular, cellular, tissue, organ and whole body processes through which a gene influences blood pressure [276]. Like the ultimate phenotype (blood pressure) intermediate phenotypes can also be affected by other

factors but the argument is that the lower one goes down the hierarchical scale (i.e. towards the gene) the less such influences, and therefore the more genetically homogeneous (at least in terms of genetic determinants of blood pressure) those populations selected on the basis of such traits. The power of detecting (and *parri passu* excluding) association / linkage is therefore enhanced. Given the vast literature on the renin-angiotensin system in hypertension, it is not difficult to identify intermediary phenotypes observed in subsets of hypertensives that may be useful in investigating the role of the renin locus. Two that immediately come to mind are (i) plasma renin level which shows a much wider spread in hypertensive subjects than normotensive controls [277-279]; thus hypertensives with high or low level can be specifically studied and (ii) non-modulation of the adrenal responsiveness to changes in dietary salt; in most subjects a low-salt diet increases the sensitivity of the adrenal gland to angiotensin II. However in a sub-set of essential hypertensives, estimated at around 30%, this responsiveness is blunted [280,281] but can be specifically reversed by converting enzyme inhibitors [282]. These so-called non-modulators tend to also have a strong family history of hypertension [280,281]. The mechanism underlying non-modulation is not clear but it is a trait shared by the SHR [283] and one possibility is that the blunted response is due to increased local angiotensin II production in the adrenal gland (see Pages 47-49).



Apart from its involvement in the susceptibility to the development of hypertension, the renin locus also deserves attention in another context given the recent report of Cambien *et al.* [284] that allelic variants at the locus for ACE may be a determinant of myocardial infarction (MI) risk. In a previous retrospective [277] and a more recent prospective study [285], Laragh's group have shown a strong association between high plasma renin levels and MI risk. While it is possible that the high plasma renin is a consequence of pre-existing but silent vascular disease it is equally tenable that it and the associated increase in vascular risk is a reflection of genetic variation at the renin locus. The ACE genotype associated with the increased MI risk is also associated with higher ACE activity [286] and an intriguing question that may need to be answered, if studies on the renin locus were to prove positive, is whether the association of the both genotypes with myocardial infarction is due to the same mechanism - an increase in tissue angiotensin II levels in specific sites ?

#### CONCLUSIONS ON THE RENIN LOCUS IN HYPERTENSION

Evidence for the involvement of the renin locus in several types of genetic hypertension in rats is now very strong. However both the nature of the mutation(s) at the locus responsible for the effects on blood pressure as well the intermediary phenotypes through which it affects blood pressure remain to be identified. The direct involvement of

the renin gene itself needs to be shown. While large scale backcrosses may allow the region at the locus responsible for the effect on blood pressure to be narrowed by identifying recombinants in the region of interest through the use of microsatellites [258], the approach likely to yield the most convincing answers, once technology is established in the rat, is homologous recombination [287]. Using this approach the involvement of specific differences in and around the renin gene between hypertensive and normotensive strains will become testable. By assessing the effects of the recombination on expression of the renin gene, such studies may also shed light on the mechanism by which the gene influences blood pressure and particularly on the involvement of specific tissue sites. Finally, while studies on the role of the renin locus in human hypertension have so far proved negative, there are inherent difficulties in conducting such investigation in man and it remains entirely possible, despite the current data, that variation at the renin locus plays an important role in the pathogenesis of human hypertension. The locus therefore continues to merit further investigation including any role it may play in increasing the susceptibility of individuals to other cardiovascular disorders such as myocardial infarction.

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# Expression of the renin gene in extra-renal tissues of the rat

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Expression of the renin gene in several rat organs is demonstrated by the detection of renin mRNA using a ribonuclease-protection technique. In two of these sites, the brain and the liver, renin mRNA levels are unaffected by changes in dietary salt which markedly affect renal renin mRNA levels. The findings provide the basis for an important ubiquitous local regulatory role for the renin-angiotensin system extending beyond the circulation.

## INTRODUCTION

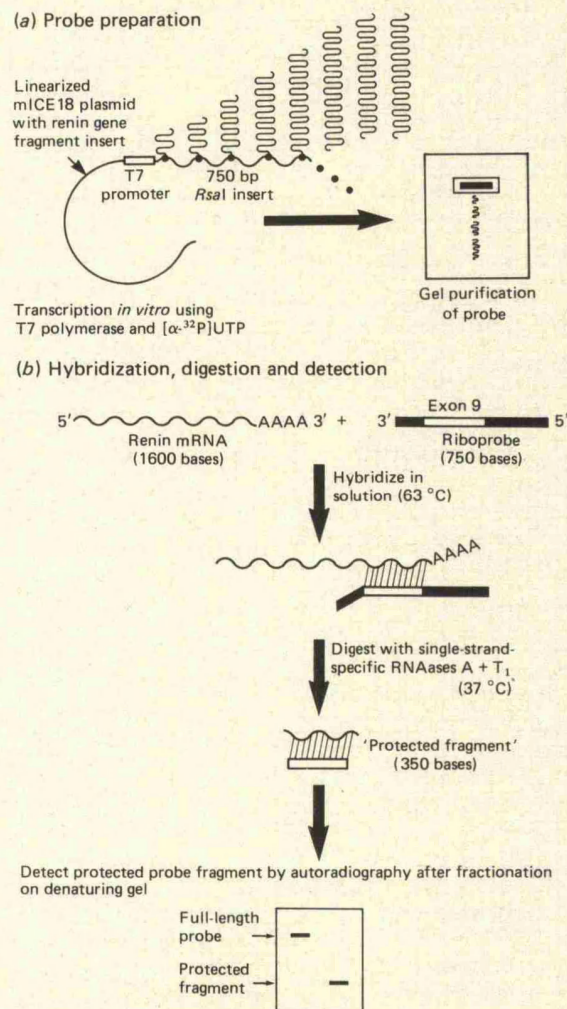
Renin is classically considered to be an enzyme that is synthesized by the juxtaglomerular cells of the kidneys and secreted into the circulation, where it cleaves angiotensinogen to release angiotensin I. Angiotensin I is converted into the octapeptide angiotensin II by converting enzyme, a ubiquitous peptidase. Angiotensin II is the active hormone of the renin-angiotensin (RA) system, contributing to cardiovascular homeostasis by causing arteriolar vasoconstriction and increased adrenal aldosterone biosynthesis [1].

Since the first description by Gould *et al.* [2], extra-renal renin-like activity has been demonstrated in a wide variety of tissues in several species [3]. Initial suggestions that such activity may wholly represent the action of other proteinases on renin substrate [4] have been refuted to some extent by better characterization of the physiochemical properties of the renin-like activity [5] and the demonstration of inhibition of the activity by renin-specific antibodies [6]. However, the source of the tissue renin and the physiological and pathological role(s) of the tissue RA systems remain uncertain. For instance, evidence has been produced for both uptake of circulating renin [7] and for local synthesis [8,9] to account for renin in arterial walls, a potentially important system with regard to both normal control of vascular tone and increased tone in hypertension. Although several functions have been attributed to the tissue RA systems [10], little is known about factors that regulate them and the relationship of these systems to the renal system.

With the cloning of the renin gene [11] and the development of highly specific and sensitive hybridization procedures [12], it has become possible to determine the extent of renin-gene expression outside the kidney. Using these techniques we now have evidence for widespread extra-renal renin-gene expression in the rat, with major differences between organs, providing the basis for an important ubiquitous local regulatory system the role of which may extend beyond the circulation.

## EXPERIMENTAL

The RNAase-protection technique used is shown in Scheme 1.



Scheme 1. An illustration of the RNAase-protection technique used to detect renin mRNA

## Preparation of RNA

Tissues were taken from 12-week-old female Wistar-Kyoto (WKY) rats immediately after they were killed, frozen in liquid N<sub>2</sub> and stored at -70 °C. Total



cellular RNA from each tissue was prepared by a modification of the method of Auffray & Rougeon [13] as previously described [14]. RNA concentrations were determined from absorbance measurements at 260 nm and checked by comparing ethidium bromide fluorescence of samples run on agarose gels.

#### Preparation of probe

A 0.75-kb endonuclease-*RsaI* fragment of rat genomic DNA containing the whole of exon 9 of the rat renin gene and flanking 5' and 3' sequences was inserted into the M13 derivative mICE18 which carries the T7 promoter [15].  $\alpha$ - $^{32}$ P-labelled anti-message-strand riboprobe was prepared by transcription *in vitro* [15] in the presence of T7 RNA polymerase (Boehringer) and [ $\alpha$ - $^{32}$ P]UTP (Amersham International, Amersham, Bucks., U.K.) and gel-purified [16] to remove prematurely terminated transcripts before use.

The *RsaI* fragment was derived from plasmid pRRnE4 containing 4.0 kb of rat renin genomic DNA kindly provided by Dr. Okhubo, University of Kyoto, Kyoto, Japan. Sequence analysis of the 0.75-kb fragment by him and independently by us had shown that it corresponded to the 3' end of the mouse *renI* gene [11] and spanned exon 9. The full rat renin-gene sequence has not been published.

#### RNAase protection

Hybridization of the probe to the RNA samples and RNAase digestion with single-strand-specific RNAases A and T1 were carried out as described by Melton *et al.* [12], except that the hybridization was carried out at 63 °C and digestion at 37 °C. These conditions had been determined empirically to maximize the signal-to-noise ratio with kidney RNA. The presence of renin mRNA is indicated by the protection of a 350-base fragment (corresponding to exon 9), which was detected by autoradiography after running the products on a 3.5% polyacrylamide/7M-urea gel.

#### Salt study

Female WKY rats (12 weeks old;  $n = 6$  per group) were put on a low-salt diet (0.007 mmol of NaCl/g; deionized water to drink), normal salt diet (0.112 mmol of NaCl/g; deionized water to drink) or a high-salt diet [0.112 mmol of NaCl/g; 1.5% (w/v) NaCl to drink] for 2 weeks. Organs were then removed and pooled for each group before RNA extraction. The low-salt diet was prepared by mixing Casilan (Farley Health Products Ltd., Nottingham, U.K.) with deionized water in a ratio of 25 g of Casilan to 75 ml of water. For the normal-salt and high-salt groups this was supplemented with 1.2 g of NaCl/100 ml of diet. All diets were supplemented with 1.7 g of KCl/100 ml of diet. Between days 12 and 13, 24 h urine samples were collected from three rats from each group, and sodium concentrations were analysed by flame photometry. The mean urine sodium concentrations were 1.3, 69 and 225 mmol/l for the low-, normal- and high-salt groups respectively.

#### RESULTS

Fig. 1 shows the result of an experiment where the protection technique was applied to RNA from a number of WKY-rat tissues. The presence of renin mRNA is indicated by the detection of a 'protected'

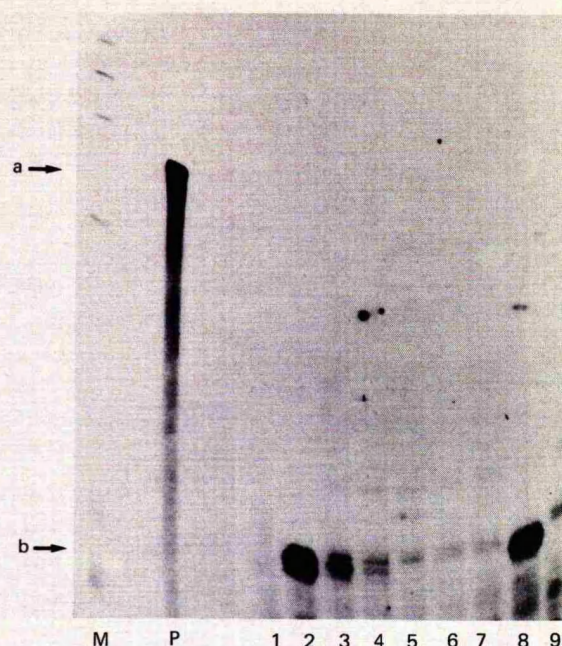


Fig. 1. Extra-renal renin-gene expression in the rat: detection of renin mRNA by RNAase protection: autoradiograph of 3.5%-polyacrylamide/7M-urea gel

See Scheme 1 and the Experimental section for details of the technique. a, RNA probe; b, protected fragment; M, marker track ( $^{32}$ P-5'-end-labelled phage- $\phi$ X174 *Hae*III fragments); P, undigested probe; lane 1, control *E. coli* RNA (250  $\mu$ g); lanes 2-9, RNA from WKY-rat tissues (250  $\mu$ g per sample unless otherwise stated): 2, kidney (40  $\mu$ g); 3, brain; 4, adrenal; 5, aorta; 6, heart; 7, lung; 8, liver; 9, spleen.

fragment (arrow b). As expected, renin mRNA was most abundant in the kidney. A relatively large amount (in terms of fraction of total RNA) of renin mRNA was also detected in the liver and brain. Renin mRNA was detectable in the other tissues examined as well, namely in adrenal, aorta, heart, lung and spleen, but in much smaller amounts. Although we have consistently detected low levels of renin mRNA in the adrenal, aorta and heart by the RNAase-protection method, renin mRNA is not always evident in RNA from lung and spleen. In these tissues the low levels are likely on occasion to be beyond the limits of sensitivity of the method.

Renin production in the kidney responds to changes in salt intake, being stimulated by low salt intake and inhibited by high-salt diets. By affecting aldosterone production in the adrenal, this plays a major role in maintaining extracellular fluid volume in response to changing salt intake [17]. The RNAase-protection assay was utilized to monitor the effect of variations in dietary sodium on renin mRNA levels in the kidney and in two extra-renal sites, namely liver and brain. The data (Fig. 2) clearly show the suppressive effect of increasing sodium intake on kidney renin mRNA levels and the contrasting absence of any such effect in the liver and brain. It should be noted that the level of renin mRNA in the liver in Fig. 1 is atypically high, the levels seen in Fig. 2 being more representative of what we consistently observe.



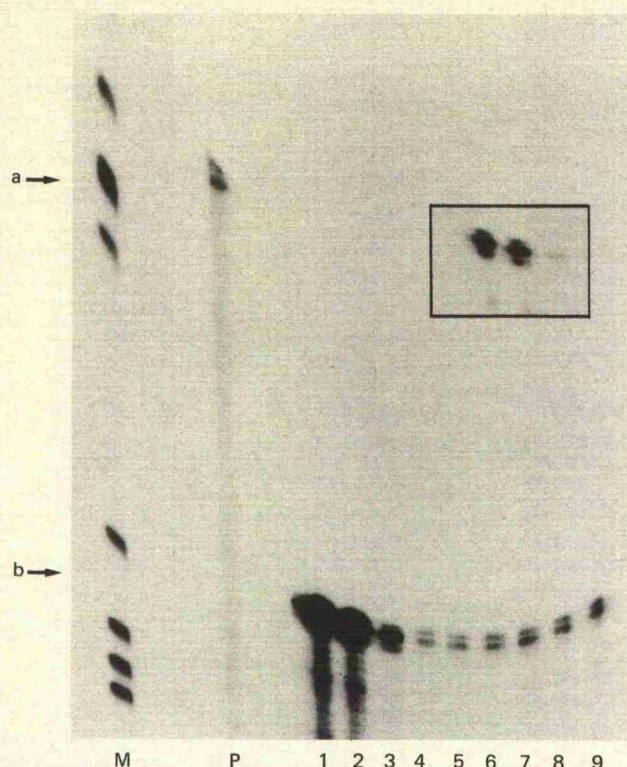


Fig. 2. Effect of changes in dietary salt on renin mRNA levels in rat kidney, brain and liver

a, RNA probe; b, protected fragment; M, marker track ( $^{32}$ P-5'-end-labelled phage- $\phi$ X174 *Hae*III fragments); P, undigested probe; lanes 1-3, kidney RNA (40  $\mu$ g per sample); lanes 4-6, brain RNA (250  $\mu$ g per sample); lanes 7-9, liver RNA (250  $\mu$ g per sample); lanes 1, 4 and 7, low-salt diet; lanes 2, 5 and 8, normal-salt diet; lanes 3, 6 and 9, high-salt diet (the inset shows the kidney tracks after a shorter exposure).

## DISCUSSION

Renin mRNA has previously been detected in mouse and rat brain [18] and mouse heart [19] by Northern blotting. By utilizing a more sensitive technique [12] we have extended the number of tissues shown to contain renin mRNA. The findings imply that at least some of the observed renin-like activity in these tissues is indeed due to locally produced renin. Data obtained by using primer extension on mRNA templates (K. Lilleycrop & W. J. Brammar, unpublished work) and RNAase protection (C. Miller & A. J. Carter, unpublished work) have demonstrated the presence of renin mRNA in the liver, testis and brain of mouse strains.

The data clearly show that the RNAase-protection assay detects renin mRNA in the kidney, brain, adrenal and liver. The yields of protected fragments obtained using RNAs from aorta, heart, lung and spleen are lower, but still readily detectable. Although we consistently find protected fragments with aorta and heart RNAs, the levels of renin mRNA in lung and spleen are on occasion beyond the limits of detection by the RNAase-protection technique in our hands.

It is quite normal for the RNAase-protection assay to

produce multiple clustered bands (see Figs. 1 and 2) at the position of the expected protected fragment as a consequence of the base-specific endonucleolytic cleavage of the single-stranded RNA. Although the relative intensities of the various protected species in the 350-base region are variable from one tissue to another, this variation is probably not significant, since it occurs to some extent with RNAs from the same tissue in different experiments [cf. brain RNA, Fig. 1, track 3, versus Fig. 2, tracks 4, 5 and 6]. Control *Escherichia coli* RNA subjected to the same manipulations never shows such bands in the 350-base region.

As plasma renin activity in anephric patients [20] and nephrectomized animals [21] is very low, it is unlikely that any extra-renal contribution to plasma renin concentration is of physiological importance. However, especially with the demonstration of the presence [22] and in some cases local synthesis [23] of other components of the RA system, several local autocrine and paracrine functions have been suggested, including regulation of vascular tone [8], facilitation of noradrenergic transmission [24], hormone secretion [25] and control of gene expression [26]. The human renin gene has three potential promoter elements ('TATA' sequences) where RNA polymerase might bind, and Morris [27] has suggested how use of two of the promoters might produce renin precursors with hydrophilic N-terminals incapable of entering the secretory apparatus and therefore locked within the cell to serve intracellular functions.

Vascular wall [28], brain [29] and adrenal [30] renin have all been implicated in the genesis of hypertension in experimental animal models. A particular controversy has existed concerning the source of vascular renin, with some studies [7] suggesting uptake of circulating renin. Although other evidence such as dissociation of vessel-wall levels from plasma levels [28], specific cleavage of synthetic renin substrate in perfused vascular preparations [8] and the demonstration of production of renin by cultured vascular endothelial [31] and smooth-muscle cells [9] have suggested local production of renin, this is the first demonstration of renin mRNA in the extra-renal vascular tree.

A surprising finding was the presence of renin-gene expression in the liver. Although some studies [32] had noticed renin-like activity in the liver, this was attributed to non-specific proteinases [33]. However, Dzau *et al.* [34] found significant renin activity in the mouse liver that was inhibitable by anti-renin antibodies. Taking our observation with the finding of Re *et al.* [35] of high-affinity receptors for angiotensin II in rat liver chromatin and the observation of increased RNA synthesis by isolated hepatic nuclei on exposure to angiotensin II [26], one can postulate a role for an intracellular RA system in the regulation of liver gene expression.

We have started to explore the physiological regulation of the tissue systems by examining the effect of changes in salt intake on renin mRNA levels in the kidney, liver and brain. The brain RA system has been attributed a role in the control of vascular tone, in inducing drinking behaviour and salt appetite, and in causing the release of adrenocorticotropin, vasopressin and lutropin [36]. This system, however, appears to be controlled differently from the renal system, as changes in salt intake which produced marked changes in kidney renin mRNA levels had no effect on brain levels. This is consistent with the observations of Dzau & Sands [37] that various physio-



logical states that markedly affected systemic renin activity had no effect on brain or pituitary activities. Likewise the liver system appears to be independently regulated.

Overall our studies therefore support the concept of a kidney-derived hormonal RA system intimately involved in circulatory homeostasis and independent ubiquitous tissue systems whose roles are likely to extend beyond the circulation. An important step in further defining the functions of the tissue systems will be the localization of precise cellular sites of expression in each tissue by the application of 'in situ' hybridization techniques.

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## Rapid communication

### Modulation of mouse renin gene expression by dietary sodium chloride intake in one-gene, two-gene and transgenic animals

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Jeanie I. Brooks and William J. Brammar

We have studied the effect of dietary NaCl loading on renin gene expression in one-gene, two-gene and transgenic mouse strains. By Northern blotting, we found an approximate twofold reduction in renin messenger (m) RNA in the kidneys of high-NaCl-treated compared with low-NaCl-treated animals. Using an RNase-protection assay designed to discriminate between the different renin gene transcripts, we have shown that renin mRNAs derived from the *Ren-1<sup>C</sup>* gene of one-gene strains and the *Ren-1<sup>D</sup>* and *Ren-2* genes of two-gene animals are all NaCl-responsive. Renin mRNA derived from a 19 kilobase *Ren-1<sup>D</sup>* transgene is also NaCl-responsive.

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**Keywords:** Renin genes, transgenic mice, dietary NaCl.

#### Introduction

The aspartyl protease renin catalyses the initial and rate-limiting step in the conversion of angiotensinogen to angiotensin II (AngII). AngII is both a potent vasoconstrictor and, through its ability to promote aldosterone release, a regulator of salt balance. Increased activity of the renin-angiotensin system has been implicated in the pathogenesis of certain forms of hypertension [1].

The primary site of renin biosynthesis is the juxtaglomerular apparatus of the kidney. The release of renin from the juxtaglomerular cells is influenced by changes in dietary NaCl intake. An increase in NaCl intake inhibits renin release [2]. Studies with isolated juxtaglomerular apparatuses have demonstrated that this NaCl-dependent renin release is mediated, at least partly, by the cells of the macula densa in the ascending loop of Henle [3]. Recent studies in the rat [4-7] have shown that kidney renin mRNA levels are also suppressed by chronic dietary NaCl loading, although whether this decrease is specific for renin mRNA, or simply reflects a general decrease in all mRNA species has not been clearly demonstrated.

Mice are polymorphic for the number of renin genes, with certain inbred strains harbouring one gene (*Ren-1*), and others containing two (*Ren-1* and *Ren-2*). In this report, we term the *Ren-1* gene of one-gene strains *Ren-1<sup>C</sup>* and that of two-gene animals *Ren-1<sup>D</sup>*. In addition, we have produced transgenic mice harbouring a *Ren-1<sup>D</sup>* transgene in a background strain containing the *Ren-1<sup>C</sup>* gene

[8]. The *Ren-1<sup>C</sup>*, *Ren-1<sup>D</sup>* and *Ren-2* genes display different tissue specificities, although all are expressed in the kidney [8,9]. Preliminary studies in two-gene Swiss mice have indicated that mouse renin gene expression, as in the rat, may also be modulated by NaCl intake [10,11]. It is not known which of the mouse renin gene transcripts are NaCl-responsive [11]. We have devised an RNase-protection assay that is capable of discriminating between the different mouse renin gene transcripts [8]. In this report, we have used the assay to study the effect of chronic dietary NaCl loading upon the individual renin gene mRNA levels in the kidneys of one-gene, two-gene and transgenic mice.

#### Materials and methods

Six 10-week-old male C57B1/6 and DBA2/J mice and six 9-12-week-old male F<sub>1</sub> transgenic line #59 mice [8] were placed on either a high-NaCl diet (0.112 mmol NaCl/g food, with 1.5% NaCl solution to drink) or a low-NaCl diet (0.007 mmol NaCl/g food, with deionized water to drink) for 13 days. The mice were then killed by cervical dislocation and the kidneys removed for RNA isolation. C57B1/6 mice possess the *Ren-1<sup>C</sup>* gene, while DBA2/J mice possess *Ren-1<sup>D</sup>* and *Ren-2*; the transgenic mouse line #59 carries both *Ren-1<sup>C</sup>* and approximately three copies of a *Ren-1<sup>D</sup>* transgene (*Ren-1\**) [8]. The *Ren-1\** transgene comprises the transcriptional unit plus approximately 5 kilo-

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base upstream DNA and 4 kilobase downstream DNA. In order to distinguish the transgene and its transcript from the endogenous *Ren-1<sup>C</sup>* gene, we inserted a synthetic deoxyoligonucleotide of 30 base pairs (bp) into exon-2 [8]. The transgene displays the same tissue specificity as that seen in DBA2/J mice, which is quite distinct from *Ren-1<sup>C</sup>* in C57B1/6 mice [8].

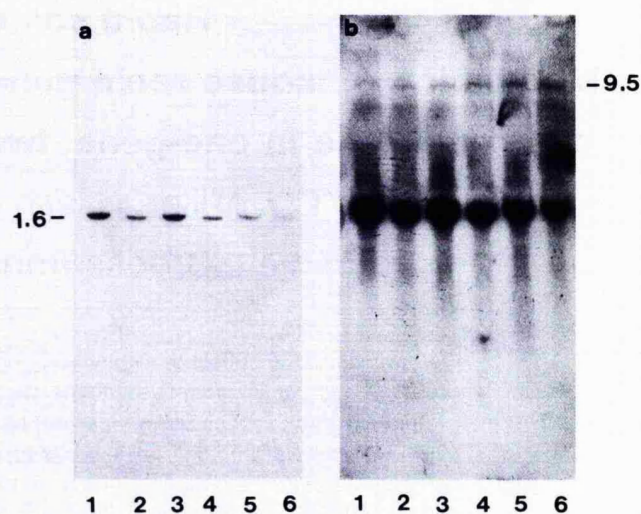
#### RNA isolation, Northern blotting and RNase-protection assays

Kidneys from each treatment were pooled, frozen in liquid nitrogen immediately after removal and their RNA isolated as previously described [8]. Absolute amounts were determined by spectrophotometric absorbance at 260 nm and the RNA integrity was checked by observing 28S and 18S ribosomal bands after electrophoresis. Total RNAs of 90 µg were separated by electrophoresis through agarose gels containing formaldehyde [12], transferred to Hybond-N filters (Amersham plc, Amersham, UK) and probed simultaneously with a renin complementary (c) DNA (pSMG5913) [13] and a mouse laminin cDNA [14]. Probes were labelled by random deoxyoligonucleotide priming of DNA polymerase in the presence of  $\alpha^{32}\text{P}$ -deoxycytidine triphosphate (dCTP) [15]. Labelled hybrids were detected autoradiographically and quantitated densitometrically using an LKB Ultrascan laser densitometer.

Transcripts from *Ren-1<sup>C</sup>* in C57B1/6 mice, from *Ren-1<sup>D</sup>* and *Ren-2* in DBA2/J mice and from *Ren-1<sup>C</sup>* and *Ren-1<sup>\*</sup>* in transgenic animals were discriminated from each other by RNase-protection analyses [8]. With these assays, a 150 bp protected fragment is diagnostic of *Ren-1<sup>C</sup>* expression in C57B1/6 mice. In DBA2/J mice, sequence mismatches between *Ren-1<sup>D</sup>* and *Ren-2* within exon 2 [13,16] give rise to a 150 bp protected fragment from the *Ren-1<sup>D</sup>* transcript and a 110 bp fragment from *Ren-2*. In transgenic animals, a 180 bp protected fragment is diagnostic of *Ren-1<sup>\*</sup>* expression and a 100 bp fragment is diagnostic of *Ren-1<sup>C</sup>* (see [8] for further details).

#### Results and discussion

Figure 1 shows a Northern blot of total RNA isolated from the kidneys of one-gene, two-gene and transgenic animals placed on either a high- or a low-NaCl diet. In all cases, decreased amounts of the 1600 base renin mRNAs were detected in the kidneys of mice placed on the high-NaCl diet. In contrast, we detected no change in the amount of laminin mRNA in the kidneys of these mice. Quantification of these results by densitometric analyses showed an approximate twofold decrease in renin mRNA in the high-NaCl-treated compared with the low-NaCl-treated animals. While similar results have been obtained in the rat [4-7], it has not been clearly demonstrated that these NaCl-dependent alterations are specific for renin mRNA. In this study we simultaneously monitored the level of a heterologous mRNA species (laminin) and have shown that the decrease in renin mRNA is not a consequence of a general decrease in all mRNA species. We cannot, however, eliminate the possibility that the concentrations of other mRNA species are affected by NaCl loading.

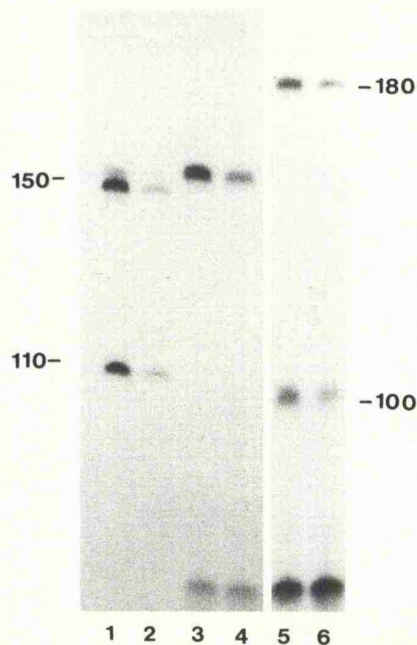


**Fig. 1.** Northern blot analysis of renin (1.6 kilobase) and laminin (9.5 kilobase) messenger (m) RNAs in the kidneys of mice placed on either a low- or a high-NaCl diet. Tracks 1 and 2 show RNA isolated from two-gene strain DBA2/J mice placed on a low-(track 1) or a high-(track 2) NaCl diet. Tracks 3 and 4 show RNA isolated from single-gene strain C57B1/6 mice placed on a low-(track 3) or a high-(track 4) NaCl diet. Tracks 5 and 6 show RNA isolated from transgenic mice placed on a low-(track 5) or a high-(track 6) NaCl diet. Figure 1b is an extended exposure of the autoradiograph to show the 9.5 kilobase laminin mRNA.

Of interest was our finding that basal renin mRNA levels in the transgenic animals (while still displaying NaCl effects) were lower than those in the C57B1/6 and DBA2/J strains (approximately 2.4-fold). At this stage, we do not know the mechanism underlying this phenomenon. It may be related to the multiple transgene copy number in these animals, or may be a consequence of the transgene product itself. The transgene contains a 30 bp tag in exon 2 which encodes an additional 10 amino acids at a position where prorenin is cleaved to produce active renin. We have yet to determine whether transgene-encoded renin can be activated. High levels of circulating inactive renin may suppress renin gene expression. We cannot, however, eliminate the possibility that these low renin mRNA levels are related to the slight age differences between the transgenics and the C57B1/6 and DBA2/J mice.

A 3 bp mismatch between *Ren-1<sup>D</sup>* and *Ren-2* in exon 2 [13,16] enabled us to design an RNase-protection assay capable of discriminating between the transcripts of these two genes [8]. Our results demonstrate that the levels of both *Ren-1<sup>D</sup>* and *Ren-2*-derived mRNAs in DBA2/J mice are NaCl-responsive (Fig. 2). It has previously been suggested that only *Ren-1<sup>D</sup>*, and not *Ren-2*-derived mRNAs are NaCl-responsive [11]. Likewise, a similarly designed RNase-protection assay showed that the levels of both *Ren-1<sup>C</sup>* and transgene mRNA in transgenic animals are also NaCl-responsive (Fig. 2). Although we do not know whether these NaCl-dependent changes are transcriptional effects, or are due to alterations in mRNA stability, the fact that *Ren-1<sup>C</sup>*, *Ren-1<sup>D</sup>* and *Ren-2* mRNAs are all modulated to a similar extent by NaCl suggests that the underlying control





**Fig. 2.** RNase-protection assays of renin messenger (m) RNAs in the kidneys of DBA2/J mice (tracks 1 and 2), C57B1/6 mice (tracks 3 and 4) and transgenic mice (tracks 5 and 6). Tracks 1, 3 and 5 show RNAs isolated from mice placed on a low-NaCl diet; tracks 2, 4 and 6 show RNAs isolated from mice placed on a high-NaCl diet. In DBA2/J and C57B1/6 mice, a 150 base pair (bp) protected fragment is diagnostic of *Ren-1<sup>D</sup>* and *Ren-1<sup>C</sup>* expression, respectively, while a 110 bp protected fragment is diagnostic of *Ren-2* expression in DBA2/J mice. In transgenic animals, a 180 bp protected fragment is diagnostic of transgene expression and a 100 bp fragment is diagnostic of *Ren-1<sup>C</sup>* expression. Tracks 5 and 6 are extended exposures of the autoradiograph to show clearly the protected species with transgenic RNAs.

mechanism is the same for all genes. Modulation of transgene mRNAs demonstrates that any transcriptional regulation involved in the NaCl response can be mediated by sequences contained within this 19 kilobase of *Ren-1<sup>D</sup>* DNA. Experiments designed to investigate further the basis of the NaCl-induced renin response are currently underway.

### Acknowledgement

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## A widespread abnormality of renin gene expression in the spontaneously hypertensive rat: modulation in some tissues with the development of hypertension

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### SUMMARY

1. Renin messenger RNA (mRNA) levels were compared in the kidneys, livers, brains, adrenals, aortae and hearts of spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats at 5 and 12 weeks of age using a ribonuclease-protection technique.

2. Relative levels of renin mRNA were increased in the kidney, liver, brain, adrenal and heart of the young SHR compared with the WKY. In the aorta, levels were similar in the two strains at 5 weeks.

3. In 12-week-old animals, while increased levels persisted in the liver, brain and adrenal of the SHR, the level in the kidney was now the same in the two strains and the levels in the heart and aorta were lower in the SHR compared with the WKY.

4. Renin mRNA levels in the kidneys of SHR and WKY were also compared by Northern blotting and confirmed the observations made with the ribonuclease-protection technique.

5. The findings indicate a widespread abnormality of renin gene expression in the SHR which is modulated in some tissues by the development of hypertension.

6. While the mechanism(s) for the abnormality remains to be determined, the increased renin mRNA levels in the SHR in several tissues concerned with blood pressure regulation suggests an important role for the renin-angiotensin system in the development and maintenance of hypertension.

7. However, the finding of increased renin mRNA in the liver also suggests abnormalities in other, as yet unknown, functions of the renin-angiotensin system in the SHR.

**Key words:** hypertension, Northern blotting, renal/extrarenal tissues, renin messenger RNA, ribonuclease protection.

**Abbreviations:** bp, base pairs; mRNA, messenger RNA; PRA, plasma renin activity; RAS, renin-angiotensin system; RNAase, ribonuclease; SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat.

### INTRODUCTION

The role of the renin-angiotensin system (RAS) in the pathogenesis of hypertension in the spontaneously hypertensive rat (SHR) remains unclear. Comparisons of plasma renin levels with those of control rats have yielded conflicting results, with increased, unchanged and decreased levels in the SHR all being reported both during the development phase of the hypertension and after hypertension is established [1-8]. On the other hand, inhibitors of the renin-angiotensin cascade, particularly the angiotensin-converting enzyme inhibitors, have clearly been shown both to lower the blood pressure in the older hypertensive rat [9, 10] and to prevent the development of hypertension if given to young rats [11, 12]. To explain the effects of these drugs in the absence of consistent evidence of an increase in the activity of the plasma RAS, it has been suggested that they may be acting on tissue RAS in key sites such as the vascular wall [13, 14], brain [15] and adrenal [14], where activity may be increased in the SHR and be involved in causing the hypertension.

Evidence for the presence of renin-like activity in several extra-renal tissues has accumulated over the last 20 years [16]. However, the biochemical measurement of 'true' renin activity in such tissues has been complicated by the presence of other proteases capable of generating angiotensins I and II from angiotensinogen [17]. In addition, debate has continued as to whether such renin is locally produced or taken up from the circulation [18]. With the availability of nucleic acid probes for renin gene sequences, it is now possible to examine tissues for renin messenger RNA (mRNA) using a variety of hybridization techniques. Using Northern blotting we have easily been able to detect renin mRNA in the kidney but have been

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unable to detect renin mRNA in most other tissues [19]. We have therefore utilized a more sensitive, solution-based hybridization technique to detect renin mRNA [19, 20] and have recently shown that the renin gene is expressed in several extra-renal tissues in the rat including the liver, brain, adrenal, aorta, heart, lung and spleen [20]. In the present study we compare the relative levels of renin mRNA in the kidney and several extra-renal tissues of SHR and Wistar-Kyoto (WKY) rats at 5 weeks of age, during the development phase of the hypertension in the SHR, and at 12 weeks, when hypertension is established in the SHR.

## METHODS

### Animals

Inbred colonies of SHR and WKY have been maintained in Leicester since 1981. Female animals obtained from the two colonies were raised under identical conditions with access to rat chow and tap water *ad libitum*. Experiments were carried out on animals aged 5 weeks and 12 weeks. Two days before the animals were killed systolic blood pressure was measured indirectly under light ether anaesthesia by the tail-cuff method [21] and a blood sample collected for plasma renin activity (PRA) measurement. Animals were killed by cervical dislocation and tissues were removed immediately thereafter, frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  before RNA extraction.

### PRA

A tail-vein blood sample ( $\sim 800\ \mu\text{l}$ ) was collected in pre-chilled tubes containing  $100\ \mu\text{l}$  of 10% (w/v) ethylenediaminetetra-acetate (potassium salt). Plasma was separated after spinning for 7 min at 3000 g in a refrigerated centrifuge and stored at  $-20^{\circ}\text{C}$ . PRA was measured by radioimmunoassay of generated angiotensin I, as described by Oldham *et al.* [22].

### RNA extraction

For each experimental group, kidneys, livers (1 g/animal), brains (including pituitaries) and hearts were collected from six animals and pooled before RNA extraction. Adrenal glands and aortae (dissected from the aortic arch to the iliac bifurcation) were pooled from 20 animals (including the core groups of six). Total cellular RNA was extracted by a modification of the method of Auffray & Rougeon [23] as previously described [19]. RNA concentrations were determined from multiple absorbance measurements at 260 nm and checked by comparing the ethidium bromide fluorescence of equivalent samples run on agarose gels. Comparison of the sizes of the RNA species in the electrophoresed samples and a check of the integrity of the ribosomal RNA bands also allowed us to exclude any significant degradation in any of the samples. RNA yields varied from  $\sim 500\ \mu\text{g/g}$  of tissue for aorta to over  $5000\ \mu\text{g/g}$  of tissue for liver and were very similar for SHR and WKY tissues.

### Ribonuclease (RNAase) protection assay of renin mRNA

The use of this technique to detect and compare renin mRNA levels in various tissues has been described in detail previously [20]. Briefly, identical amounts of total RNA from tissues to be compared were mixed in solution with a uniformly  $^{32}\text{P}$ -labelled anti-sense RNA probe to renin (arrow A, Figs. 1 and 2) synthesized by transcription *in vitro* from a 748 base-pair (bp) *RsaI* rat renin gene fragment (described below) cloned into the M13mp derivative, mICE18, carrying the T7 promoter. Hybridization was carried out at  $63^{\circ}\text{C}$  for 14–16 h and digestion with RNAases A and T1 for 1 h at  $37^{\circ}\text{C}$ . In the presence of renin mRNA in the total RNA sample, a 'protected' probe fragment of 350 bases (corresponding to exon 9 of the renin gene) remains (arrow B, Figs. 1 and 2), and is detected by autoradiography after running the products out on a 3.5% (w/v) polyacrylamide-7 mol/l urea gel. Since an excess of probe ( $5 \times 10^5$  c.p.m./assay) is used, the intensity of the signal obtained for the protected band indicates the amount of renin mRNA present in the RNA sample. Band intensities were quantified by densitometry using an LKB 2222-010 Ultrascan XL (LKB-Produkter AB, Bromma, Sweden) and for each comparison the SHR/WKY renin mRNA ratio was calculated. All samples were compared at least twice. For kidney samples, band intensities were quantified from shorter exposure autoradiographs than the ones shown.

### Northern blotting and hybridization

**Blotting.** RNA samples (50–100  $\mu\text{g}$ ) from kidneys were electrophoresed through 1.2% (w/v) agarose gels containing 2.2 mol/l formaldehyde [24] and transferred to Hybond N membrane (Amersham, Aylesbury, Bucks, U.K.) as recommended by the membrane manufacturer.

**Probes.** Renin. A 748 bp *RsaI* genomic fragment encompassing exon 9 of the rat renin gene was used. In the recently published rat renin gene sequence [25], this fragment comprises the sequence from base 10525 to base 11273.

**Laminin.** As an internal control the blots were reprobed for mRNA for the basement membrane protein laminin. A 675 bp *EcoRI-SalI* complementary DNA fragment derived from a mouse parietal endoderm library was used for this [26].

**Radioactive labelling of probes** was carried out using deoxy- $[\alpha\text{-}^{32}\text{P}]\text{CTP}$  (3000  $\mu\text{Ci}/\text{mmol}$ ; Amersham, Aylesbury, Bucks, U.K.) by the random oligo-primer method of Feinberg & Vogelstein [27].

**Hybridization and detection.** For both probes pre-hybridization (4 h) and hybridization (14–16 h) were carried out at  $45^{\circ}\text{C}$  in a buffer containing 50% (v/v) formamide,  $6 \times \text{SSPE}$  [ $20 \times \text{SSPE} = 3.6\ \text{mol/l NaCl}$ ,  $0.2\ \text{mol/l}$  sodium phosphate, pH 7.7,  $20\ \text{mmol/l}$  ethylenediaminetetra-acetate (sodium salt)],  $5 \times \text{Denhardt's}$  [ $100 \times \text{Denhardt's} = 2\%$  (w/v) of each of bovine serum albumin, Ficoll 400, and polyvinyl pyrrolidone], 0.5% (w/v) sodium dodecyl sulphate, 6% (w/v) polyethylene-glycol 6000 and denatured salmon sperm DNA (200  $\mu\text{g}/$

**Table 1. Characteristics of the 5- and 12-week-old SHR and WKY**

Values are expressed as means  $\pm$  SEM. Comparison of means was carried out by using Student's *t*-test. Statistical significance: \**P* < 0.01 compared with age-matched control (WKY) rats. Abbreviation: ANG I, angiotensin I.

	Age (weeks)	Weight (g)	Systolic blood pressure (mmHg) (kPa)	PRA (pmol of ANG I h <sup>-1</sup> ml <sup>-1</sup> )
SHR	5	89.3 $\pm$ 2.1	100.0 $\pm$ 2.9 (13.3 $\pm$ 0.4)	21.4 $\pm$ 2.0
WKY	5	91.7 $\pm$ 3.2	97.5 $\pm$ 2.1 (13.0 $\pm$ 0.3)	20.2 $\pm$ 1.9
SHR	12	214.0 $\pm$ 3.3	165.0 $\pm$ 3.7* (21.9 $\pm$ 0.5)	18.0 $\pm$ 3.6
WKY	12	219.7 $\pm$ 3.8	104.2 $\pm$ 3.0 (13.8 $\pm$ 0.4)	19.6 $\pm$ 1.8

ml). Membranes were then washed at 45°C for 2 h in 3  $\times$  SSPE and 0.1% (w/v) sodium dodecyl sulphate and for 1 h in 1  $\times$  SSPE and 0.1% (w/v) sodium dodecyl sulphate. Autoradiography was carried out at -70°C using Kodak X-Omat AR film (Eastman Kodak, Liverpool, U.K.) and intensifying screens. Before reprobing with the laminin probe, the renin probe was stripped from the filters by immersion for 90 min at 65°C in two changes of a solution containing 10 mmol/l Tris-HCl, pH 8.0, 1 mmol/l ethylenediaminetetraacetate (sodium salt) and 1  $\times$  Denhardt's. Molecular weights of the bands seen on the Northern blots were estimated from a concurrently run RNA molecular weight ladder (Gibco-BRL, Paisley, Scotland, U.K.).

## RESULTS

The characteristics of the four groups of animals studied are characterized in Table 1. At 5 weeks of age, the blood pressure of the SHR was not significantly different from that of the WKY, whereas at 12 weeks they had significantly higher blood pressure. No differences were observed at either age in the body weights or PRA levels between SHR and WKY. The extended groups of animals from which adrenal glands and aortae were obtained did not differ from the core groups with respect to either body weight or blood pressure. PRA was not measured in these animals.

Figs. 1(a) and 1(b) show the results of the RNAase-protection assay for renin mRNA for the 5-week-old animals. Higher relative levels (specific mRNA/total RNA) of renin mRNA were observed in the kidney ( $\times 2.5$ ), liver ( $\times 3.2$ ), brain ( $\times 2.5$ ) and adrenal gland ( $\times 3.5$ ) of the SHR compared with the WKY. The relative levels in the aorta were similar in the two strains ( $\times 1.05$ ). In the heart, although renin mRNA was detectable in the SHR, virtually none was seen in the WKY. It should be noted that much less total RNA was used in comparing kidney samples (40  $\mu$ g) than other samples (250  $\mu$ g).

Figs. 2(a) and 2(b) show the RNAase-protection results for the 12-week-old animals. While relative levels in the liver ( $\times 2.9$ ), brain ( $\times 2.1$ ) and adrenal ( $\times 3.1$ ) were still higher in the SHR, levels in the kidney were now the same

( $\times 1.0$ ) in the two strains, and the level in the heart slightly lower in the SHR ( $\times 0.8$ ). In the aorta, while renin mRNA was easily detectable in the WKY, very little was seen in the SHR.

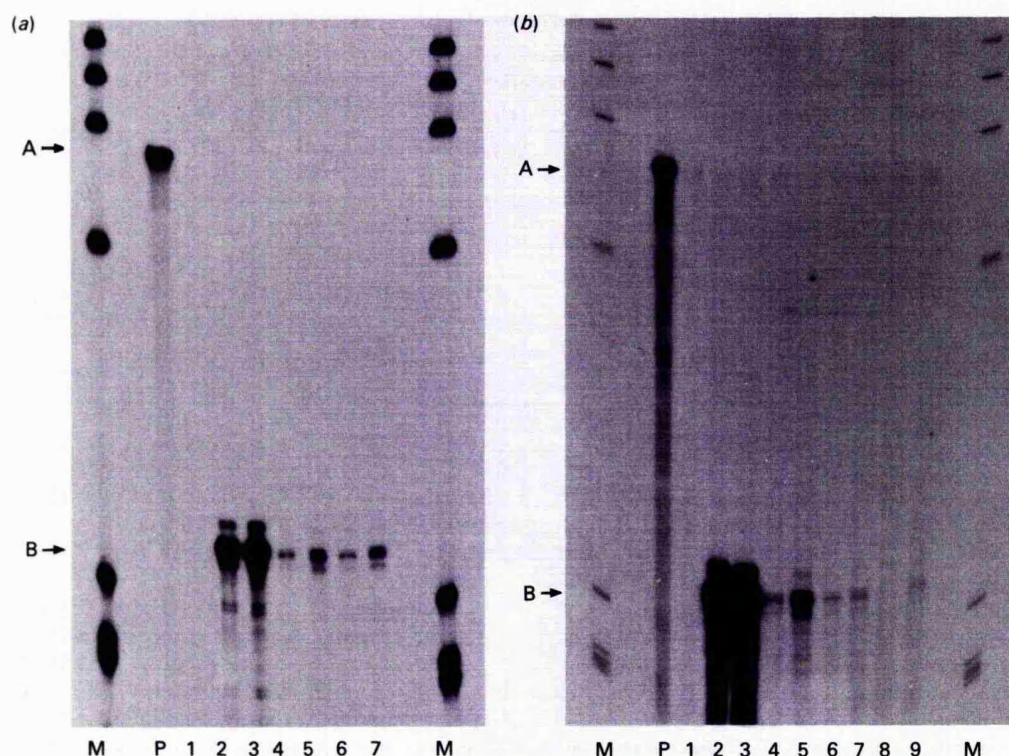
Each ratio given above for SHR to WKY renin mRNA is a mean of two separate comparisons. To estimate the variance between comparisons, associated with this method of quantifying differences between SHR and WKY renin mRNA levels, the difference in the results of each pair of comparisons was calculated and expressed as the percentage of the mean value for the two estimates. For two comparisons the difference was 16.0  $\pm$  11.8% (mean  $\pm$  sd).

Kidney RNA samples were also analysed for renin mRNA by Northern blotting. In these studies, as control, levels of laminin mRNA levels were also determined. Fig. 3 shows the results for both the 5- and 12-week-old rats. At 5 weeks the SHR kidney had a threefold higher level of renin mRNA compared with the WKY kidney, whereas levels of laminin mRNA were similar. At 12 weeks the levels of both renin and laminin mRNAs were about equal in the SHR and WKY kidneys.

## DISCUSSION

In this study we have used a solution-based hybridization technique (RNAase-protection) to compare renin mRNA levels in various tissues of SHR and WKY. The technique is more sensitive than Northern blotting [19, 28] and also has the advantage of allowing larger quantities of RNA to be assayed than is routinely possible with Northern blotting. Using this technique we have recently demonstrated the presence of renin mRNA in several rat tissues [20]. The stringency of the RNAase digestion step ensures that only renin mRNA (and not any related mRNA) results in the formation of the 350 base protected fragment. The presence of a cluster of bands around the position of the expected fragment (see Figs. 1 and 2) is common in such experiments and a consequence of the sequence-specificity of the RNAases [20]. Completeness of digestion was always checked for by the inclusion of a control sample of heterologous RNA (lane 1, Figs. 1 and 2). Kidney RNA samples were also analysed for renin





**Fig. 1.** Comparison of renin mRNA levels in tissues of 5-week-old SHR and WKY using an RNAase-protection technique. Autoradiographs of 3.5% (w/v) polyacrylamide–7 mol/l urea gels are shown. See the Methods section for details of the technique. A, Full-length RNA probe; B, protected probe fragment; M, marker track ( $^{32}\text{P}$ -5'-end-labelled phage  $\Phi$  X174 *Hae*III fragments); P, undigested probe. RNA samples (250  $\mu\text{g}$  per sample unless otherwise stated): (a) 1, control *Escherichia coli* RNA; 2, WKY kidney (40  $\mu\text{g}$ ); 3, SHR kidney (40  $\mu\text{g}$ ); 4, WKY liver; 5, SHR liver; 6, WKY brain; 7, SHR brain. (b) 1, control *E. coli* RNA; 2, WKY kidney (40  $\mu\text{g}$ ); 3, SHR kidney (40  $\mu\text{g}$ ); 4, WKY adrenal; 5, SHR adrenal; 6, WKY aorta; 7, SHR aorta; 8, WKY heart; 9, SHR heart.

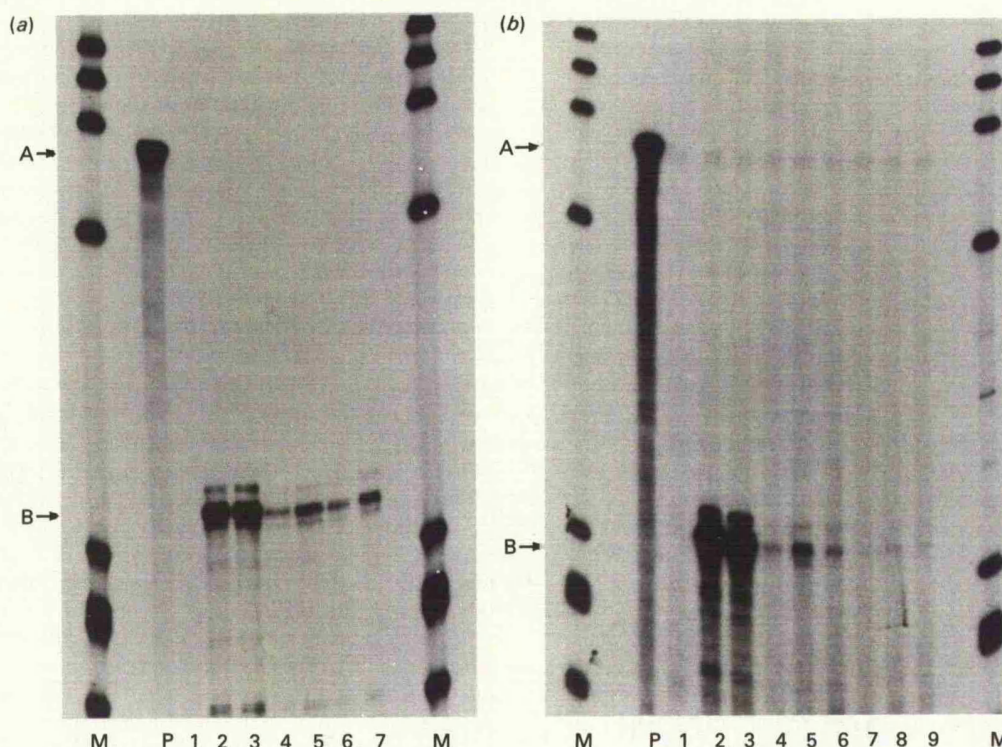
mRNA by Northern blotting and confirmed the results obtained using the RNAase-protection technique.

Within the limitations set by the analysis of pooled tissues (see below), our estimate of the level of interassay variability using the RNAase-protection technique, and the fact that in some tissues the amount of renin mRNA present was towards the limit of sensitivity of detection by this technique, our findings suggest a widespread abnormality of renin gene expression in the SHR with modulation in some tissues with increasing age and blood pressure. Although the pooling of tissues precluded us from analysing individual animals, the observation of similar differences in renin mRNA levels in several tissues of SHR and WKY at two different ages strongly indicates that the differences seen do not simply reflect individual variability between animals but genuine differences between the two strains.

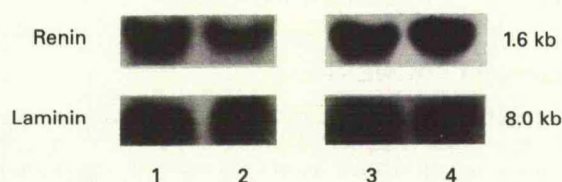
Our interpretation of the results needs two qualifications. First, as a comparison of relative levels of mRNA (specific mRNA/total RNA) does not distinguish differences arising as a consequence of increased gene transcription from those due to differences in rates of

degradation or indeed those due merely to changes in the proportion of other RNAs. One of these, rather than increased gene transcription, could be responsible for the differences in renin mRNA levels observed in SHR and WKY. The variability in response in the various tissues going from 5 to 12 weeks argues against a systematic abnormality in degradation of renin mRNA in the young SHR with reversal at 12 weeks, and since there is no evidence of large-scale variability in the proportion of other RNAs (e.g. laminin mRNA; Fig. 3) between SHR and WKY, it seems likely that the differences seen are primarily due to differences in levels of renin gene expression. However, until this is shown by direct measurement of gene transcription, e.g. by nuclear run-off studies, it remains possible that the differences seen in renin mRNA levels between SHR and WKY reflect alterations in mRNA stability. Secondly, the reason for the changes in the ratios from 5 to 12 weeks may not only be suppression, in absolute terms, of renin gene expression in the SHR tissue (as is probably the case in the aorta), but could also be due to inhibition in the SHR of a normal ontological increase in renin mRNA which





**Fig. 2.** Comparison of renin mRNA levels in tissues of 12-week-old SHR and WKY using an RNAase-protection technique. Autoradiographs of 3.5% (w/v) polyacrylamide-7 mol/l urea gels are shown. See the Methods section for details of the technique. A, Full-length RNA probe; B, protected probe fragment; M, marker track ( $^{32}\text{P}$ -5'-end-labelled phage  $\Phi$  X174 *Hae*III fragments); P, undigested probe. RNA samples (250  $\mu\text{g}$  per sample unless otherwise stated: (a) 1, control *E. coli* RNA; 2, WKY kidney (40  $\mu\text{g}$ ); 3, SHR kidney (40  $\mu\text{g}$ ); 4, WKY liver; 5, SHR liver; 6, WKY brain; 7, SHR brain. (b) 1, control *E. coli* RNA; 2, WKY kidney (40  $\mu\text{g}$ ); 3, SHR kidney (40  $\mu\text{g}$ ); 4, WKY adrenal; 5, SHR adrenal; 6, WKY aorta; 7, SHR aorta; 8, WKY heart; 9, SHR heart.



**Fig. 3.** Comparison of renin and laminin mRNA levels in the kidneys of 5- and 12-week-old SHR and WKY by Northern blotting. See the Methods section for details of the technique. RNA samples: 1, 5-week-old SHR (50  $\mu\text{g}$ ); 2, 5-week-old WKY (50  $\mu\text{g}$ ); 3, 12-week-old SHR (100  $\mu\text{g}$ ); 4, 12-week-old WKY (100  $\mu\text{g}$ ).

occurs in the WKY. This may indeed be the case in the heart. A better understanding of the ontogeny of extra-renal renin gene expression would help resolve these possibilities.

Several previous studies [1, 4, 5, 13, 15, 29-34] have compared renin enzyme activities in various tissues of the two strains and increased renin levels have been reported

in the adrenal [15], brain [29, 30], kidney [1, 4, 5, 31] and aorta [13, 34] of the SHR. While the results of many of these studies are therefore consistent with our findings on renin mRNA levels, a number of factors mitigate against a direct comparison of the results of the two types of study and may also provide an explanation for some of the discrepancies. In many cases animals of different ages to ours were studied and we have shown here that in some tissues there may be marked temporal changes in renin mRNA levels. In some, particularly older, studies, renin enzyme activity was not distinguished from that of other proteases [17] also capable of generating angiotensin I from angiotensinogen, making the interpretation of the results difficult. Thirdly, for some tissues, e.g. the vascular wall, there is evidence that renin may be taken up from the circulation [35] and therefore renin activity measurements will include this component as well as renin produced locally. Finally, we have no direct evidence that a difference in renin mRNA level is reflected in changes in the rate of synthesis of the protein.

To study the effects of the development of hypertension in the SHR on tissue renin gene expression we

examined animals at both 5 weeks, early in the development phase of the hypertension when our indirect blood pressure measurements showed no difference between the two strains, and at 12 weeks, when hypertension is established in the SHR. Our findings suggest that at least in those tissues directly exposed to the raised blood pressure, the kidney, aorta and heart, there is modulation of renin gene expression with the development of the hypertension. The observation in the kidney is not surprising as there is considerable evidence for a baroreceptor mechanism affecting renal renin production [36]. Data on the effect of hypertension on vascular wall or cardiac renin are much more limited. It is interesting, however, to note that in the study of Barrett *et al.* [33], showing a progressive decrease in aortic renin activity in the SHR going from the 'pre-hypertensive' phase to the hypertensive phase (not seen in the WKY), the decrease was only observed in those SHR that became hypertensive.

In our study, the aorta was a notable exception in not having a greater amount of renin mRNA in the 5-week-old SHR compared with the WKY. As modulation was seen in this tissue with the development of hypertension, it is possible that this was already taking place at 5 weeks and that at an earlier age the aorta shows the same abnormality seen in the other tissues of the 5-week-old animal. Several studies have demonstrated that important structural and functional differences, including changes in vascular wall structure, already exist between SHR and WKY by this age [37, 38], indicating that the hypertensive process is well underway.

Expression of the angiotensinogen gene has also been demonstrated in a wide variety of rat tissues [39]. The possible mechanisms by which local angiotensin production in several tissues may regulate systemic blood pressure have been reviewed recently [40–42]. The finding, therefore, in this study of increased renin mRNA levels in such sites in the SHR, both at an early stage and in some cases after the development of hypertension, suggests an important role for the RAS in the development and maintenance of hypertension and may also provide an explanation for the effects of inhibitors of the RAS in lowering the blood pressure in the SHR in spite of normal plasma renin levels. However, as with other characteristics that distinguish SHR from control rats, before any role in hypertension can be defined it will first be necessary to show that the abnormality in renin gene expression co-segregates with a tendency to hypertension.

Several other observations in the SHR could also be explained by abnormalities in its tissue RAS suggested by our findings. The SHR, like a substantial proportion of patients with essential hypertension (non-modulators), demonstrates a blunted aldosterone response to angiotensin II when sodium restricted [43]. It has been suggested that this is due to abnormal angiotensin receptor up-regulation in the adrenal gland in response to sodium restriction [44] which may be a consequence of increased activity of the local adrenal RAS. A similar blunting observed in the renal vascular response to angiotensin II in the SHR [45] may likewise be a consequence of an overactive intrarenal RAS. Despite the marked difference

in kidney renin mRNA levels between young SHR and WKY in this study we observed no difference in PRA levels between the animals. The implied disassociation between the renal production of renin and plasma levels in the young SHR may well reflect this local role for renin. Several studies have shown a superiority of angiotensin-converting enzyme inhibitors over otherwise equipotent antihypertensive drugs in preventing the development of or in reversing myocardial hypertrophy in the SHR [46, 47]. This indicates a specific role for the RAS in this process in the SHR and our finding of increased cardiac renin mRNA in the 5-week-old SHR may be relevant to this.

We have previously reported that the renin gene is expressed in the rat liver [20] and postulated, because of the finding of high-affinity receptors for angiotensin II on rat liver chromatin [48] and increased RNA synthesis by isolated hepatic nuclei on exposure to angiotensin II [49], that an intracellular liver RAS may play a role in the regulation of hepatic gene expression. An interesting finding in this study was that renin mRNA levels were also higher in this tissue of the SHR compared with the WKY. The significance of this observation is unclear and simply serves at this stage to emphasize our generally poor understanding of the functions of the tissue RAS. An important step in the elucidation of such functions will be the localization of the cellular sites of renin gene expression in the various tissues using hybridization techniques *in situ*.

Further studies will also be required to elucidate the cause(s) of the widespread increase in renin mRNA levels in the SHR. While it is possible, for instance, that it represents a generalized response to increased activity of the sympathetic nervous system in the SHR [50], a factor known to regulate renin production at least in the kidney [37], attention needs also to be focused on the renin gene itself and its regulatory elements, as our recent studies have shown that there are structural differences between the renin genes of SHR and WKY [51].

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## Kidney renin mRNA levels in the early and chronic phases of two-kidney, one clip hypertension in the rat

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The effect of clipping the left renal artery on left and right kidney renin mRNA levels during the early and chronic phases of two-kidney, one clip Goldblatt hypertension in the rat was studied. Renin mRNA levels were determined using northern and dot blotting. Four weeks after clipping, renin mRNA levels were sixfold higher in the left kidney and eightfold lower in the right kidney of the Goldblatt rats compared with the left kidney of the sham-operated rats. Similar analysis at 20 weeks after clipping showed a fourfold increase in the left kidney and a 16-fold suppression in the right kidney compared with age-matched sham-operated control rats. The study demonstrates the profound changes that occur in renin gene expression in the clipped and contralateral kidneys in this model of hypertension and shows that these changes persist into the chronic phase of the hypertension.

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**Keywords:** Goldblatt two-kidney, one clip hypertension, early and chronic hypertension, renin mRNA.

### Introduction

To date, most studies investigating the effects on the renin-angiotensin system of clipping the renal artery in the two-kidney, one clip Goldblatt model of hypertension have either measured plasma renin levels or analysed kidney renin content.

In the early phase in the rat (<6 weeks after clipping) most studies have found plasma renin levels to be raised [1-11]. In the chronic phase (>12 weeks after clipping), while some studies report normal or near-normal plasma renin [3,4,8,10], others have found it to remain elevated [5,9,11,12]. In the early stages of hypertension renal renin content has been shown to be increased on the clipped side and decreased in the contralateral kidney [2,6,11,13]. In the later stages, kidney renin content has been reported as being both normal [14] and increased [11,15] in the ischaemic kidney.

Plasma renin levels are determined not only by the rate of production and secretion by the kidney but may also be influenced by changes in hepatic clearance [16] and renal excretion [17]. It is also clear that the method of withdrawing blood and the use of anaesthesia can also influence the level measured [18]. The technical difficulties involved in the measurement of kidney renin content have been pointed out by Page [19].

With the use of recombinant DNA techniques it is now possible to study directly the effects of renal ischaemia on renin gene expression. In this study we have examined kidney renin messenger RNA levels in the early and chronic phases of two-kidney, one clip Goldblatt hypertension in the rat.

### Methods

Female Wistar rats ( $\approx 200$  g weight) were used. Two-kidney, one clip hypertension was produced by placing a silver clip (internal diameter 0.2 mm) around the left renal artery through a loin incision [9]. The right kidney was not disturbed. Control rats had a sham operation in which a clip was placed adjacent to the left renal artery. Blood pressure was monitored weekly by an indirect photoelectric method [20] under ether anaesthesia and animals with systolic blood pressures  $\geq 150$  mmHg by 4 weeks after clipping were used. The animals ( $n = 6$  per group) were randomly allocated to either the early group, studied at 4 weeks, or to the chronic group, studied at 20 weeks. In each case a matched sham-treated group was also studied.

#### Plasma renin activity

Two days before the rats were killed a tail-vein blood sample ( $\approx 800$   $\mu$ l) was obtained under light ether anaesthesia.

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sia in pre-chilled tubes containing 100 µl 10% K-EDTA. Plasma was separated after spinning in a refrigerated centrifuge and stored at  $-20^{\circ}\text{C}$ . Plasma renin activity (PRA) was measured by radio-immunoassay of generated angiotensin I as previously described [21].

#### RNA extraction

Both kidneys were removed immediately after the animal was killed by cervical dislocation and frozen separately in liquid nitrogen. The kidneys from each side were pooled for each group and stored at  $-70^{\circ}\text{C}$  prior to RNA extraction. Total cellular RNA was extracted by a modification of the method of Auffray and Rougeon [22] as previously described [23]. RNA concentrations were determined from absorbance measurements at 260 nm, and checked by comparing the ethidium bromide fluorescence of equivalent samples run on agarose gels. Comparison of the sizes of the RNA species in the electrophoresed samples and a check of the integrity of the ribosomal RNA species also verified the absence of significant degradation in any of the RNA preparations.

#### Blotting

##### Northern

RNA samples (60 µg) were electrophoresed through 1.2% agarose gels containing 2.2 mol/l formaldehyde [24] and transferred to Hybond N membrane (Amersham, Aylesbury, UK) as recommended by the membrane manufacturer. Size of renin mRNA was determined from a concurrently run RNA molecular weight ladder (BRL, Gibco-BRL, Paisley, UK).

##### Dot

Serial twofold dilutions of RNA from 20 µg to 0.625 µg were prepared in a buffer containing 50% formamide, 6% formaldehyde, 0.018 mol/l  $\text{Na}_2\text{HPO}_4$  and 0.002 mol/l  $\text{NaH}_2\text{PO}_4$  and dotted onto Hybond N membrane using a Hybri-Dot manifold (BRL, UK). Relative concentrations of renin mRNA in the samples were determined visually from the comparison of dilutions required to give spots of similar intensities.

#### Probes

Radioactive labelling of probes was carried out using  $\alpha^{32}\text{P}$ -dCTP (3000 µCi/mmol, Amersham, Aylesbury, UK) by the random oligo-primer method of Feinberg and Vogelstein [26] and specific activities of greater than  $1 \times 10^9$  counts/min per µg DNA were usually obtained.

##### Renin

A 750 bp *Rsa*I genomic fragment encompassing exon 9 of the rat renin gene was used. This was kindly provided by Dr Okhubo, Kyoto University.

##### Laminin

As an internal control the dot blots were reprobed for mRNA for the basement membrane protein laminin. A 675 bp *Eco*RI-*Sal*I cDNA fragment derived from a mouse parietal endoderm library [25] was used for this.

#### Hybridization and detection

For both northern and dot blots, prehybridization (4 h) and hybridization (16 h) was carried at  $45^{\circ}\text{C}$  in a buffer containing 50% formamide,  $6 \times$  SSPE ( $20 \times$  SSPE = 3.6 mol/l NaCl, 0.2 mol/l sodium phosphate, pH 7.7, 0.02 mol/l sodium EDTA),  $5 \times$  Denhardt's ( $100 \times$  Denhardt's = 2% w/v of each of bovine serum albumin, Ficoll 400 and polyvinyl pyrrolidone), 0.5% SDS, 6% PEG 6000 and 200 µg/ml denatured salmon sperm DNA. Membranes were then washed at  $45^{\circ}\text{C}$  for 2 h in  $3 \times$  SSPE, 0.1% SDS and for 1 h in  $1 \times$  SSPE, 0.1% SDS. Autoradiography was carried out at  $-70^{\circ}\text{C}$  using Kodak X-Omat AR film and intensifying screens. Prior to reprobing with the laminin probe, the renin probe was stripped from the filters by immersion for 90 min at  $65^{\circ}\text{C}$  in two changes of a solution containing 10 mmol/l Tris-HCl pH 8.0, 1 mmol/l sodium EDTA pH 8.0 and  $1 \times$  Denhardt's solution.

#### Statistics

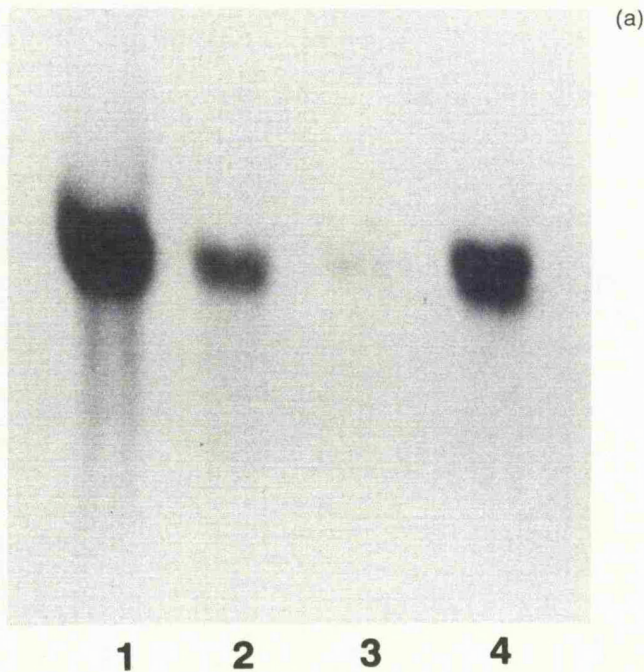
The animal weights, blood pressures and PRAs are expressed as mean values  $\pm$  s.e.m. Comparisons were carried out using Student's t-test. The PRA was transformed into logarithms before such comparisons were made since PRA is logarithmically and not normally distributed, but results in the text are expressed as the arithmetic means  $\pm$  s.e.m.

#### Results

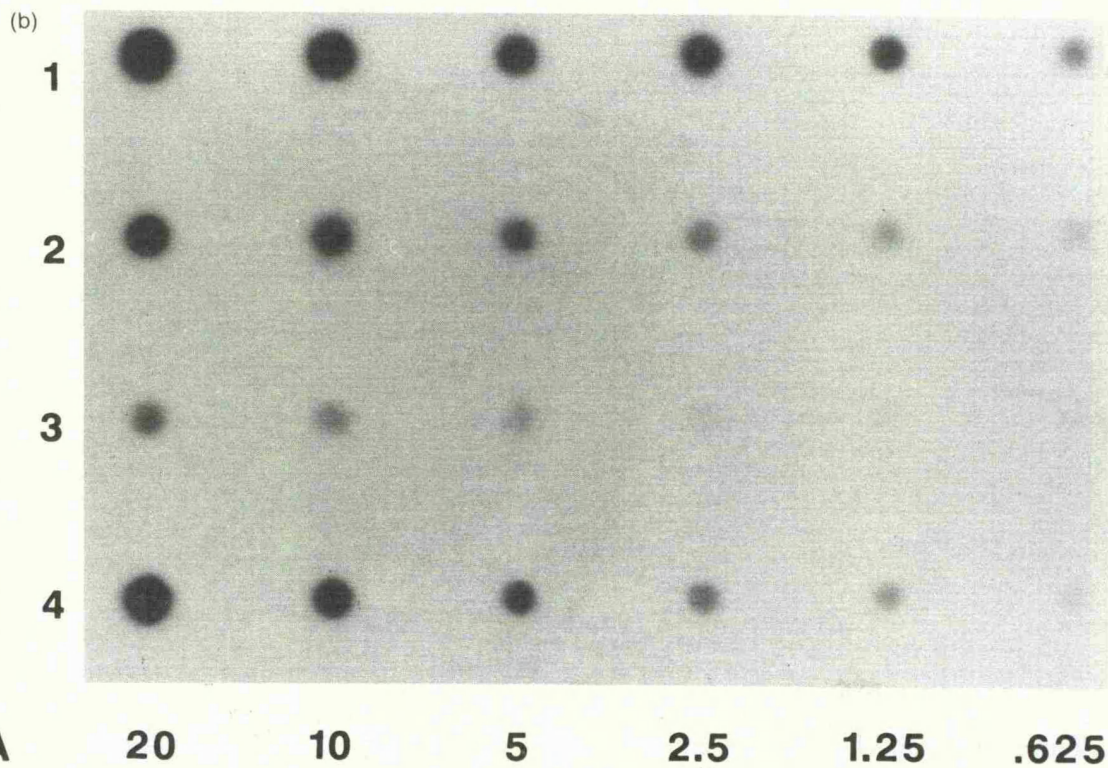
Table 1 summarizes the characteristics of the early and chronic Goldblatt groups and their respective controls. It also shows for each group the PRA (column 4), the kidney weights (column 5) and the RNA yields (column 6).

The weights of both the early and chronic Goldblatt rats (column 2) were not significantly different ( $P > 0.05$ ) from those of matched controls. The blood pressure (column 3) and PRA (column 4) of both experimental groups, however, were significantly greater ( $P < 0.01$ ) than those of their respective controls. The average weights of the left and right kidneys in the early Goldblatt group were similar and comparable to those of the control rats. In the chronic Goldblatt rats the average weight of the right kidney was considerably greater than that of the left. An increasing ratio between the weights of the clipped and contralateral kidney with time has also been observed by others [2,11]. The average RNA yield/kidney was greatest for the right hypertrophied kidney of the chronic Goldblatt rat (column 6) although, as a function of weight, the yields for all groups were much more similar ranging from  $\approx 1200$  µg/g to 1600 µg/g.

Figures 1a and 1b show the northern blot and dot blot results, respectively, for the left and right kidneys of the early Goldblatt rats and those of their controls hybridized with the renin probe. Figures 2a and b show the same data for the 20 weeks post-clipping Goldblatt rats and their controls. The probe was highly specific for renin mRNA, detecting in Northern blots a single species of 1.6 kb which is in agreement with previous estimates of the size of rat kidney renin mRNA [27,28]. Figures 1c and 2c show the dot blots after reprobing with the laminin probe. No significant difference is seen between the respective dilutions



**Fig. 1.** Northern (a) and dot (b) blots of RNA from kidneys of Goldblatt rats 4 weeks post-clipping and control rats probed with a renin probe. (c; overleaf) Reprobing of dot blot with laminin probe. Lanes: 1, left kidney, Goldblatt rat; 2, left kidney, control rat; 3, right kidney, Goldblatt rat; 4, right kidney, control rat.



in the various tracts. Repeat experiments gave the same results as shown.

On the basis of the serial dilutions we estimate a sixfold increase in renin mRNA (as a fraction of total RNA) in the left kidney and an eightfold suppression in the right kidney of the early Goldblatt compared with the left kidney

of the sham-operated control (column 7, Table 1). The right kidney of the control also had slightly greater renin mRNA (1.5:1.0) compared with the left. Similar analysis for the chronic group showed a fourfold increase in the left kidney and a 16-fold suppression in the right kidney compared with the left kidney of the sham-operated group. In



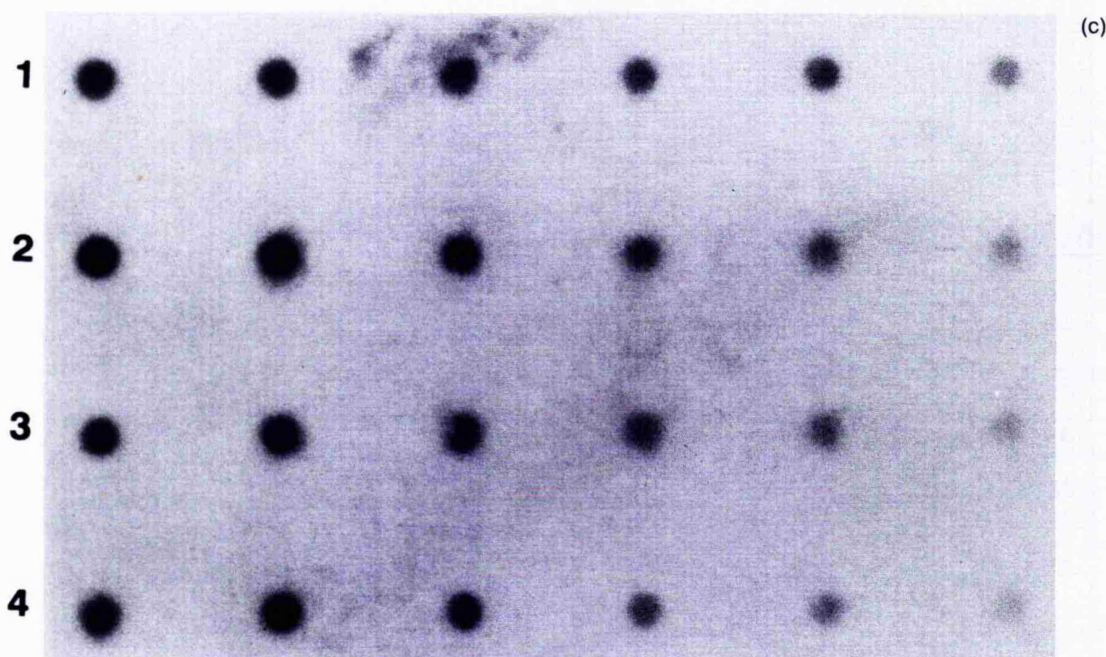


Fig. 1. (continued)

Table 1. Summary of the characteristics of and results obtained for the early and chronic Goldblatt rats and their respective controls.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Rat type	Average wt (g)	Systolic blood pressure (mmHg)	PRA (pmol Ang I /ml per h)	Total wt† kidneys (g)	Total RNA per kidney (μg)	Relative‡ amount of renin mRNA	Renin mRNA§ per kidney (arbitrary units)	Kidney renin mRNA per animal (arbitrary units)
Early Goldblatt	220.7 ± 7.1	164.2 ± 6.8*	83.0 ± 15.9*	L 5.35 R 5.75	1179 1550	6 0.12	7074 194	7268
Early Goldblatt control	228.3 ± 3.9	109.2 ± 3.0	18.6 ± 3.6	L 5.20 R 5.70	1143 1286	1.0 1.5	1143 1929	3072
Chronic Goldblatt	321.5 ± 15.4	170.8 ± 10.0*	27.8 ± 3.0*	L 5.55 R 8.0	1400 2138	4 0.06	5600 133	5733
Chronic Goldblatt control	348.0 ± 19.9	110.0 ± 5.3	9.4 ± 1.7	L 6.65 R 7.0	1335 1690	1.0 0.75	1335 1267	2602

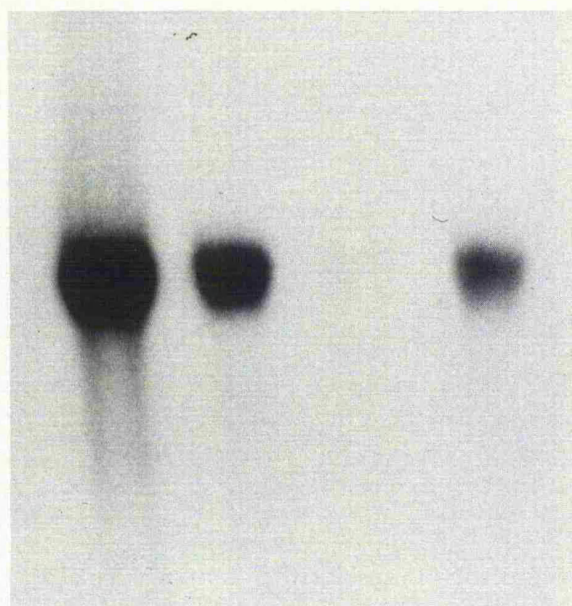
PRA, plasma renin activity; L, left; R, right. Six animals were used in each experimental group. Average weights, systolic blood pressure and PRA expressed as means ± s.e.m. \*Significantly raised compared to respective control  $P < 0.01$ . †Weights obtained for pooled kidneys before RNA extraction. ‡Ratios based on the signals observed in the dot blots (Figs 1b and 2b). The amount of renin mRNA for each group is expressed relative to the amount observed in the left kidney of the corresponding control rat. §Calculated from total RNA yield/kidney (column 6) and relative ratio of renin mRNA (column 7).

this case slightly less renin mRNA (0.75 : 1.0) was observed in the right kidney of the control compared with the left.

Assuming a steady state, on the basis of the above ratios

and the average RNA yield per kidney for each group (column 6, Table 1), we have calculated the relative amounts of renin mRNA in arbitrary units per kidney (column 8, Table

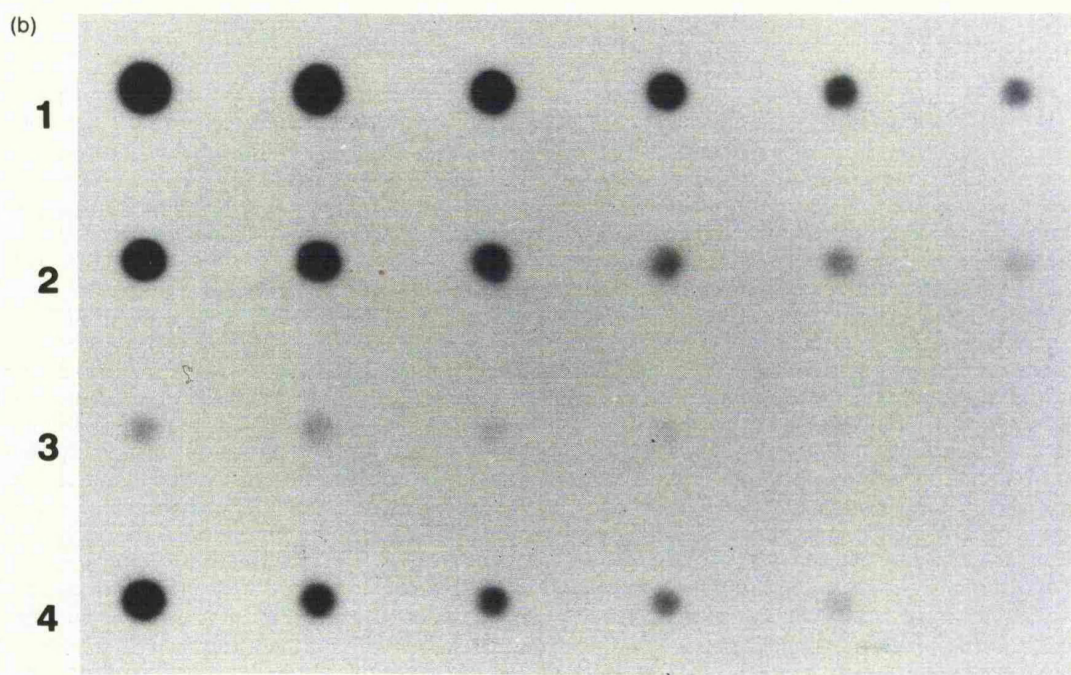




(a)

1 2 3 4

**Fig. 2.** Northern (a) and dot (b) blots of RNA from kidneys of Goldblatt rats 20 weeks post-clipping and control rats probed with a renin probe. (c; overleaf) Reprobing of dot blot with the laminin probe. Lanes: 1, left kidney, Goldblatt rat; 2, left kidney, control rat; 3 right kidney, Goldblatt rat; 4, right kidney, control rat.



(b)

$\mu\text{g}$  RNA      20      10      5      2.5      1.25      .625

1), total kidney renin mRNA per animal (column 9, Table 1) for each group, and a ratio of total kidney renin mRNA for each type of Goldblatt rat with respect to its control rat.

### Discussion

This study has used nucleic acid hybridization to assess the effects of clipping one renal artery on renin gene ex-



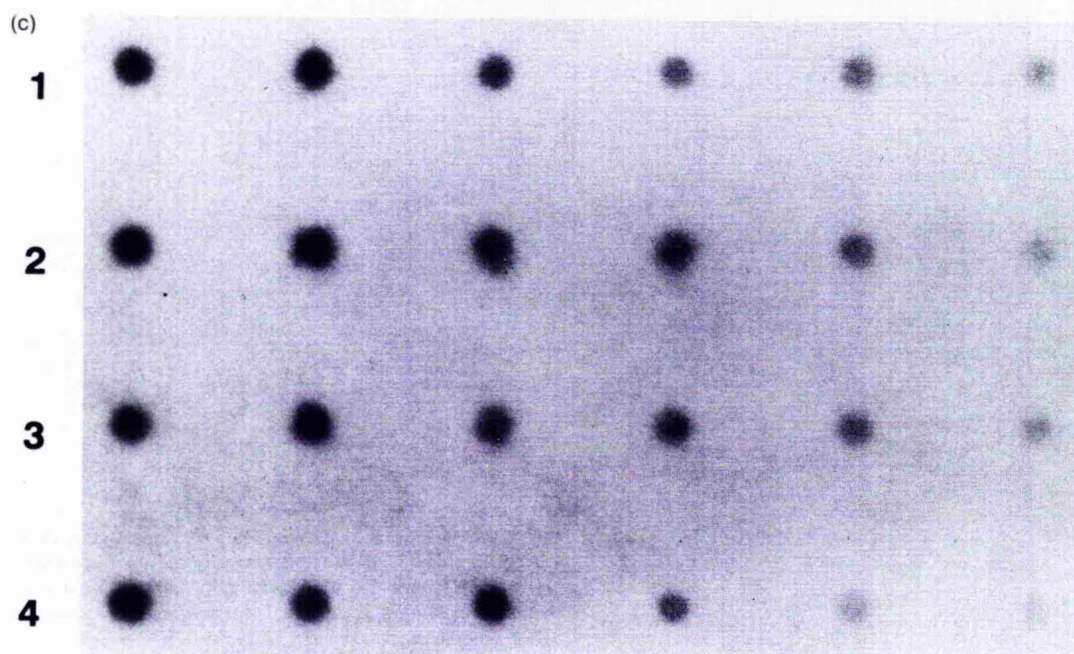


Fig. 2. (continued)

pression in both the ischaemic and contralateral kidney in the rat. The cDNA probe was shown to be specific for renin mRNA by northern blot analysis and then used in dot blot hybridizations to give a semi-quantitative comparison of the relative renin mRNA concentrations in control and experimental kidneys. Even with well characterized probes the dot blot procedure gives only an approximate assay for RNA concentrations, but it is preferred to northern blotting for this purpose because of the absence of an RNA transfer step, the efficiency of which can be variable.

Using these procedures, differences of approximately 50-fold in renin mRNA content are seen between the two kidneys of the Goldblatt rats. A major observation in this study is that the changes in kidney renin mRNA levels seen in the early (4 weeks post-clipping) two-kidney, one clip Goldblatt hypertensive rat persist to the chronic phase (20 weeks post-clipping). As the left and right kidneys in each group were pooled, detailed analysis of the relationship between blood pressure, PRA and renal renin mRNA content in individual animals is precluded. Despite this, the effects of the Goldblatt clipping procedure on renal renin mRNA are quite clear: there is an increase in total renal renin mRNA in the clipped animals in both the early and chronic phases (column 9, Table 1) and a gradient of renin mRNA content between the clipped and unclipped kidney is retained into the chronic phase. These results are unlikely to have been significantly influenced during the sacrifice of the animal and the freezing of the kidneys. The results are in agreement with the observations of Michel *et al.* [11] on renal renin content at 4 and 9 weeks after clipping. They found a persistent increase in renin content

in the clipped kidney and a decrease in the contralateral kidney compared with control rats, the differences being more marked if anything at 9 weeks than at 4 weeks.

The relationship of kidney renin mRNA levels to PRA needs to be carefully interpreted. In our study, although absolute PRA levels fell significantly from the early to the chronic phase, there was also a concomitant fall with age in the sham-operated control rats and the PRA in the chronic Goldblatt group was still significantly raised compared with its control group. This is in agreement with the findings in some studies [5,9,11,12] but not others [3,4,8,10]. As discussed previously, several factors, including the method of blood collection, can influence the PRA level measured. However, even if the higher renin levels observed by some groups in chronic Goldblatt hypertension are the result of stimulation during venesection, this itself would be an argument for a persistent abnormality in renin synthesis during the chronic phase suggested by our observations on renin mRNA levels. This is supported by the observation of Leenen *et al.* [7] that while there was no increase above control rats in basal PRA in moderately hypertensive two-kidney, one clip Goldblatt rats, after stimulation with pentobarbital anaesthesia PRA was significantly higher in the Goldblatt rats compared with controls. In the study by Michel *et al.* [11] no correlation was found between plasma renin concentration and renal renin indices.

Previous studies have examined the relationship between renal renin mRNA levels and plasma renin levels. Nakamura *et al.* [27] showed that 15 days of sodium depletion and captopril treatment in the rat increased PRC 46-fold, whilst renin mRNA content increased only threefold,

and suggested that post-transcriptional events are also likely to be important in determining plasma renin levels. Moffett *et al.* [13] found a 10- to 16-fold increase at 3 days in renin mRNA in the ischaemic kidney of rats made hypertensive by coarctation of the aorta, which was insufficient to account for the 30- to 50-fold increases in PRA. In this study, given the proviso discussed above regarding measurement of PRA, there was a 4.5-fold higher mean PRA in the early Goldblatt rat compared with its control and a 3.0-fold higher PRA in the chronic Goldblatt rat compared with its control. Even in the early group (4 weeks after clipping) the difference between the increases in PRA and increase in kidney renin mRNA levels was not as great as in the studies discussed above and our findings therefore suggest that whilst post-transcriptional factors may have an acute role in determining plasma renin levels, in the longer term renin gene transcription parallels plasma renin levels.

While it is generally agreed that the renin-angiotensin system plays some role in the early phase of two-kidney, one clip Goldblatt hypertension, its role in the chronic phase is much debated [29]. Our observation of a persistence of increased renin mRNA levels into the chronic phase of Goldblatt hypertension should not be interpreted as necessarily indicating a role in the maintenance of the hypertension. In addition to its role as a systemic hormonal system, the renin-angiotensin system also performs an important role locally within the hypo-perfused clipped kidney in maintaining glomerular filtration [30]. That the endogenous renal renin-angiotensin system is activated on the ischaemic side is shown by the marked increase in angiotensin II levels in renal venous plasma of that side compared with aortic plasma [31]. Inhibition of the intra-renal renin-angiotensin system may explain some of the deleterious effects of converting enzyme inhibitors on the morphology and function of the clipped kidney [11,32]. Therefore the persistent increased renal renin gene expression may perform an important local function within the kidney.

In summary, our results indicate that ischaemia produces increased renal renin gene expression in the clipped kidney both at an early stage and after several months renal artery constriction in the two-kidney, one clip Goldblatt rat. Likewise, there is concurrent persistent inhibition of renin gene expression in the contralateral kidney.

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## Renal and extra-renal levels of renin mRNA in experimental hypertension

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### SUMMARY

1. Using a ribonuclease-protection assay, renin mRNA levels were compared in the kidneys, livers, brains, hearts and adrenal glands of two-kidney, one-clip Goldblatt hypertensive rats with those of age-matched control rats at 4 weeks ('early') and 20 weeks ('chronic') after clipping, and in the kidneys and adrenal glands of rats treated for 3 weeks with deoxycorticosterone and salt (deoxycorticosterone-salt hypertension) with those of control rats.

2. While marked changes were observed in kidney renin mRNA levels in all three experimental groups compared with their respective controls, in most of the extra-renal tissue studied minimal, if any, difference was seen in renin mRNA levels between the hypertensive and control rats.

3. The findings suggest that in these extra-renal tissues renin gene expression is differently regulated from that in the kidney, and particularly that it is not profoundly affected by changes in the level of circulating angiotensin II.

4. An increase in renin mRNA was observed in the adrenal glands of the 'chronic' Goldblatt rats, which may be of relevance to the maintenance of hypertension in this model.

**Key words:** deoxycorticosterone-salt hypertension, gene expression, hypertension, kidney, renin, renovascular hypertension, tissue renin-angiotensin systems.

**Abbreviations:** ANG II, angiotensin II; DOC, deoxycorticosterone; PRA, plasma renin activity; RNAase, ribonuclease.

### INTRODUCTION

To date, studies on the effects of induction of experimental hypertension [e.g. Goldblatt, deoxycorticosterone

(DOC)-salt] on the renin-angiotensin system have in the main examined the kidney-derived system, primarily by measuring changes in plasma renin activity (PRA) or kidney renin content. Considerable effort has been directed towards elucidating the role of the changes observed in the hypertension, especially in Goldblatt hypertension [1]. Recent studies have established that the renin gene is also expressed in several extra-renal tissues [2–4]. Using a ribonuclease (RNAase)-protection technique, we have shown the presence of renin mRNA in the liver, brain, adrenal gland, heart, aorta, lung and spleen of the rat [3]. In this study, using this technique, we have compared the levels of renin mRNA in the kidneys, livers, brains, adrenal glands and hearts of 'early' and 'chronic' two-kidney, one-clip Goldblatt hypertensive rats with those of control rats. We have also studied the effects of treatment with DOC and salt (DOC-salt hypertension) on renin mRNA levels in the kidney and adrenal gland.

### METHODS

#### Animals

**Goldblatt hypertension.** Female Wistar rats (age 8–9 weeks, body weight ~200 g) were used. Two-kidney, one-clip hypertension was produced by placing a silver clip (internal diameter 0.2 mm) around the left renal artery through a loin incision. The right kidney was not disturbed. Control animals underwent a sham operation in which a clip was placed adjacent to the renal artery. Blood pressure was monitored weekly and animals with systolic blood pressure  $\geq 150$  mmHg by 4 weeks after clipping were used. The animals ( $n=6$  per group) were randomly allocated to either the 'early' group, studied at 4 weeks, or to the 'chronic' group, studied at 20 weeks. In each case a matched sham-treated group was also studied. All animals had their weight, blood pressure and PRA measured 2 days before being killed.

**DOC-salt hypertension.** Male Wistar rats aged 12 weeks were subjected to a right nephrectomy. The treated group of animals ( $n=8$ ) were then given DOC pivalate, 15 mg/kg body weight subcutaneously twice weekly for 3

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weeks (seven injections altogether), and 1% (w/v) NaCl to drink. Control animals ( $n=8$ ) were injected with an equal volume of 0.9% (w/v) NaCl subcutaneously and were given tap water to drink. Blood pressure was measured 2 days before the animals were killed. PRA was not measured in these animals.

#### Blood pressure measurement

Indirect systolic blood pressure was measured by a photoelectric method [5] under light ether anaesthesia.

#### PRA

About 800  $\mu$ l of blood was collected from the tail vein in pre-chilled tubes containing 100  $\mu$ l of 10% (w/v) EDTA (potassium salt). Plasma was separated after spinning in a refrigerated centrifuge and was stored at  $-20^{\circ}\text{C}$ . PRA was measured by radioimmunoassay of generated angiotensin I as described by Oldham *et al.* [6].

#### RNA extraction

Tissues were removed rapidly after the animals had been killed by cervical dislocation. The tissues were frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ . For each tissue all samples from a group were pooled before RNA extraction. Total RNA was extracted by a modification of the method of Auffray & Rougeon [7] as previously described [8]. RNA concentrations were determined from multiple absorbance measurements at 260 nm and were checked by comparing the ethidium bromide fluorescence of equivalent samples run on agarose gels. The latter also helped to exclude significant degradation of any of the RNA preparations.

#### RNAase protection assay of renin mRNA

This technique has been described in detail previously [3]. Briefly, identical amounts of total RNA from tissues to be compared were mixed in solution with a uniformly  $^{32}\text{P}$ -labelled anti-sense RNA probe to renin (arrow a, Figs.

1–4) synthesized by *in vitro* transcription from a 0.75 kb *Rsa*I rat renin gene fragment, encompassing exon 9 [9], cloned into the M13mp vector derivative mICE18 which carries a T7 promoter. After hybridization for 14–16 h at  $63^{\circ}\text{C}$ , digestion with RNAase A and RNAase T<sub>1</sub> was carried out for 1 h at  $37^{\circ}\text{C}$ . In the presence of renin mRNA in the sample being assayed, a 'protected' probe fragment of 350 bases (corresponding to exon 9 of the renin gene) remains (arrow b, Figs. 1–4), because of hybridization to renin mRNA [3], and is detected by autoradiography after electrophoresis of the products on a 3.5% (w/v) polyacrylamide/7 mol/l urea gel. Since an excess of probe is used, the intensity of the signal obtained for the protected band indicates the amount of renin mRNA present in the RNA sample. Band intensities, except for the heart samples from the Goldblatt rats (see the Results section), were quantified by densitometry using an LKB 2222-010 Ultrascan XL (LKB-Produkter AB, Bromma, Sweden), and for samples being compared (e.g. Goldblatt rat liver versus control rat liver) a ratio (Goldblatt/control) of relative renin mRNA levels was calculated. All samples were assayed twice. For those samples quantified by densitometry, a mean interassay variability of  $14.8 \pm 7.5\%$  was observed in the ratio of samples being compared.

#### RESULTS

The characteristics of the three experimental groups and their respective controls are shown in Table 1. Those for the Goldblatt rats have been reported previously [10]. In both 'early' and 'chronic' Goldblatt rats blood pressure was elevated compared with their controls. PRA was also elevated in both groups of Goldblatt rats compared with their controls, but less so in the 'chronic' Goldblatt rats. The blood pressure of DOC-salt-treated animals was significantly increased compared with control rats.

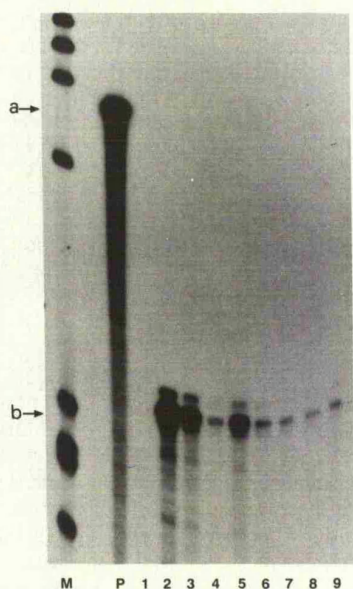
Figs 1 and 2 show the results of RNAase-protection assay for renin mRNA in the kidneys, livers and brains of the 'early' (Fig. 1) and the 'chronic' (Fig. 2) Goldblatt rats and their respective controls. Fig. 3 shows the results for the adrenal glands and hearts of both groups of Goldblatt rats. We have previously reported the changes in renin

**Table 1. Summary of the characteristics of the Goldblatt and DOC-salt-treated rats and their respective controls**

Values are means  $\pm$  SEM. Abbreviations: ANG I, angiotensin I; SBP, systolic blood pressure. Statistical significance (Student's *t*-test): \* $P < 0.01$  compared with corresponding control. Data for the Goldblatt groups have been published previously [10].

Rat type	Body wt. (g)	SBP (mmHg)	PRA (pmol of ANG I $\text{h}^{-1} \text{ml}^{-1}$ )
'Early' Goldblatt	220.7 $\pm$ 7.1	164.2 $\pm$ 6.8*	83.0 $\pm$ 15.9*
Control	228.3 $\pm$ 3.9	109.2 $\pm$ 3.0	18.6 $\pm$ 3.6
'Chronic' Goldblatt	321.5 $\pm$ 15.4	170.8 $\pm$ 10.0*	27.8 $\pm$ 3.0*
Control	348.0 $\pm$ 19.9	110.0 $\pm$ 5.3	9.4 $\pm$ 1.7
DOC-salt-treated	389.3 $\pm$ 9.6	139.4 $\pm$ 1.5*	—
Control	358.3 $\pm$ 11.3	107.8 $\pm$ 3.2	—

mRNA levels in the clipped and contralateral kidneys of these rats as assessed by Northern and dot blotting [10], and the results obtained by RNAase-protection assay (Figs. 1 and 2) were in agreement with these. In contrast to the several-fold changes observed in renin mRNA levels in the clipped and contralateral kidneys of both 'early' and 'chronic' Goldblatt rats compared with the kidneys of control rats [10], relative levels in the livers and brains of Goldblatt rats showed little, if any, change compared with controls (levels of  $\times 0.8$  to  $\times 1.2$  compared with controls) in both the 'early' and 'chronic' phases (Figs. 1 and 2). The relative level of renin mRNA in the adrenal gland in the 'early' Goldblatt rat also did not show any marked change compared with control, but an increase ( $\sim \times 3.5$ ) was observed in the adrenal gland of the 'chronic' Goldblatt rat (Fig. 3). Levels of renin mRNA in the heart were near the limit of sensitivity of detection of the technique. Weak 'protected' bands at 350 base pairs, not present in the heterologous RNA track (see below), were, however, clearly seen (Fig. 3), and visually



**Fig. 1.** Comparison of renin mRNA levels in the kidneys, livers and brains of 'early' (4 weeks after clipping) two-kidney, one-clip Goldblatt rats and control rats by using an RNAase-protection assay. The Figure shows an autoradiograph of a 3.5% (w/v) polyacrylamide/7 mol/l urea gel. For details of the technique, see the Methods section. a, Full-length probe; b, protected probe fragment; M, marker track ( $^{32}$ P-5'-end-labelled phage- $\phi$ X 174 *Hae*III fragments); P, undigested probe. RNA samples (40  $\mu$ g per sample for kidneys, 250  $\mu$ g per sample for other tissues): 1, *Escherichia coli* RNA; 2, left kidney, Goldblatt rat; 3, left kidney, control rat; 4, right kidney, Goldblatt rat; 5, right kidney, control rat; 6, liver, Goldblatt rat; 7, liver, control rat; 8, brain, Goldblatt rat; 9, brain, control rat.



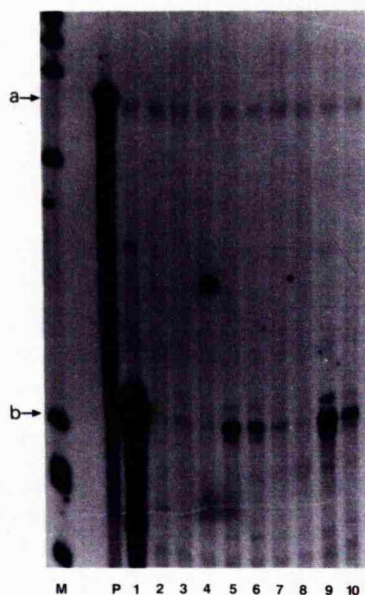
**Fig. 2.** Comparison of renin mRNA levels in the kidneys, livers and brains of 'chronic' (20 weeks after clipping) two-kidney, one-clip Goldblatt rats and control rats by using an RNAase-protection assay. The Figure shows an autoradiograph of a 3.5% (w/v) polyacrylamide/7 mol/l urea gel. For details of the technique, see the Methods section. a, Full-length probe; b, protected probe fragment; M, marker track ( $^{32}$ P-5'-end-labelled phage- $\phi$ X 174 *Hae*III fragments); P, undigested probe. RNA samples (40  $\mu$ g per sample for kidneys, 250  $\mu$ g per sample for other tissues): 1, *E. coli* RNA; 2, left kidney, Goldblatt rat; 3, left kidney, control rat; 4, right kidney, Goldblatt rat; 5, right kidney, control rat; 6, liver, Goldblatt rat; 7, liver, control rat; 8, brain, Goldblatt rat; 9, brain, control rat.

did not show any marked differences between Goldblatt and control rats in either the early or chronic stages.

In the DOC-salt-treated animals the relative renin mRNA level in the remaining kidney was on average four-fold lower than in the kidney of control rats (Fig. 4). In contrast, the level seen in the pooled adrenal glands of the treated animals was if anything slightly higher ( $\times 1.3$ ) than in the adrenal glands of control rats. As with the Goldblatt rats, the kidney RNA preparations from these animals were also analysed by Northern and dot blotting, and these studies confirmed the results seen with the RNAase-protection assay (data not shown).

It should be noted that much less RNA (40  $\mu$ g) was used when comparing kidney samples than when comparing other samples (250  $\mu$ g). Also, completeness of digestion of the probe in the absence of renin mRNA was always checked for by inclusion of a sample of heterologous RNA (lane 1, Figs. 1, 2 and 4; lane 2, Fig. 3).



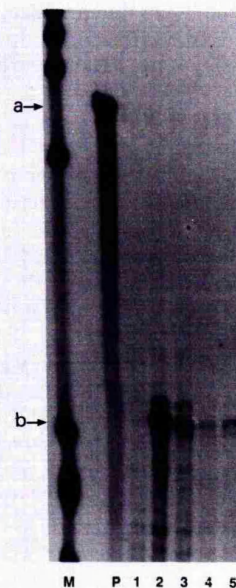


**Fig. 3.** Comparison of renin mRNA levels in the hearts and adrenal glands of 'early' and 'chronic' two-kidney, one-clip Goldblatt rats and control rats by using an RNAase-protection assay. The Figure shows an autoradiograph of a 3.5% (w/v) polyacrylamide/7 mol/l urea gel. For details of the technique, see the Methods section. a, Full-length probe; b, protected probe fragment; M, marker track ( $^{32}\text{P}$ -5'-end-labelled phage- $\phi\text{X}$  174 *Hae*III fragments); P, undigested probe. RNA samples (250  $\mu\text{g}$  per sample unless otherwise stated): 1, kidney (positive control, 40  $\mu\text{g}$ ); 2, *E. coli* RNA; 3, heart, 'early' Goldblatt rat; 4, heart, 'early' control rat; 5, adrenal gland, 'early' Goldblatt rat; 6, adrenal gland, 'early' control rat; 7, heart, 'chronic' Goldblatt rat; 8, heart, 'chronic' control rat; 9, adrenal gland, 'chronic' Goldblatt rat; 10, adrenal gland, 'chronic' control rat.

## DISCUSSION

An RNAase-protection assay was used in this study to compare renin mRNA in tissues of Goldblatt and DOC-salt hypertensive rats and their controls. The assay is highly specific for renin mRNA and provides increased sensitivity compared with other techniques such as Northern blotting, thus allowing detection of renin mRNA in extra-renal sites where the levels are much lower than those in the kidney [3, 4, 8, 11]. Comparison of the levels seen in the suppressed contralateral kidney, liver and brain of the Goldblatt rats (Figs. 1 and 2) indicates that, even in these sites with the highest relative levels of renin mRNA of the extra-renal tissues examined, levels are around 100-fold lower than those in a normal kidney.

We have previously reported and discussed the changes in kidney renin mRNA levels in the Goldblatt rats [10]. Two other studies have also analysed the changes in kidney renin mRNA in these animals. Moffett *et al.* [12] reported a transient increase in renin mRNA in the



**Fig. 4.** Comparison of renin mRNA levels in kidneys and adrenal glands of DOC-salt-treated and control rats by using an RNAase-protection assay. The Figure shows an autoradiograph of a 3.5% (w/v) polyacrylamide/7 mol/l urea gel. For details of the technique, see the Methods section. a, Full-length probe; b, protected probe fragment; M, marker track ( $^{32}\text{P}$ -5'-end-labelled phage- $\phi\text{X}$  174 *Hae*III fragments); P, undigested probe. RNA samples (40  $\mu\text{g}$  per sample for kidneys, 250  $\mu\text{g}$  per sample for other tissues): 1, *E. coli* RNA; 2, kidney, control rat; 3, kidney, DOC-salt-treated rat; 4, adrenal gland, control rat; 5, adrenal gland, DOC-salt-treated rat.

ischaemic kidney and a transient decrease to undetectable level of renin mRNA in the contralateral kidney, while Makrides *et al.* [13] found a threefold increase in renin mRNA in the ischaemic kidney and a 0.5-fold decrease in the contralateral kidney at 4 weeks after clipping. Changes in kidney renin mRNA in the chronic phase were not examined in these studies, but our finding of continued alteration in renin mRNA levels is consistent with the changes in kidney renin content observed by Michel *et al.* [14]. While the mechanisms leading to these changes in the renin mRNA levels remain to be elucidated, it is likely that in the clipped kidney the increase reflects the effect of decreased perfusion pressure on the afferent arteriole baroreceptor mechanism that controls renin production [15], and that the decrease in the contralateral kidney is at least partly the consequence of the feedback effect on the juxtaglomerular apparatus [15] of increased circulating renin and angiotensin II (ANG II) levels that occur in this model [16].

The decrease in kidney renin mRNA level in the DOC-salt-treated animals is likewise consistent with the observed changes in PRA and renal renin content in this



model [17–19]. To decouple the effects of DOC and salt on renin gene expression, Makrides *et al.* [13] studied rats on varying combinations of the two and showed that there was a synergistic effect of DOC and salt on kidney renin mRNA levels. However, as in the Goldblatt rats, the precise molecular mechanisms by which the observed changes in renin mRNA levels are actually brought about remain to be determined.

A major purpose of this study was to examine the effects on renin mRNA level in extra-renal sites of conditions known to profoundly affect kidney renin production. Allowing for the limitation set by the analysis of pooled tissues, the study clearly shows that in several of these sites renin mRNA levels are regulated differently from levels in the kidney. Specifically, the lack of an effect of the changes in plasma renin (and thus ANG II) levels that occur in the two models [16, 17] suggests that circulating ANG II does not profoundly affect the levels of renin mRNA in the extra-renal tissues, and therefore the extra-renal systems can be considered to be independent of the renal system. This is in agreement with our previous observation that changes in dietary salt intake, which had significant effects on kidney renin mRNA levels and circulating plasma renin levels [3], had little effect on renin mRNA levels in the liver or brain. Increasing evidence is accumulating for the concept that at least in some extra-renal tissues the local renin-angiotensin system may act intracellularly [20, 21]. This could provide one possible explanation as to why renin mRNA levels at such sites are not responsive to changes in ANG II concentrations at the cell-surface receptor.

Considerable effort has been directed towards defining the role of the renin-angiotensin system in the development and maintenance of experimental renovascular hypertension. Apart from measurement of plasma renin levels [16], such studies have primarily looked at the response to inhibitors of the system [22–25]. From these it is clear that in the early stages of the hypertension the renin-angiotensin system plays an important role [22, 23, 25]. In the more chronic phase, its role, however, is more controversial mainly because of the discrepancy observed between the continued response to inhibitors of angiotensin-converting enzyme and the developing failure of response to antagonists of ANG II [22–25]. Although this may reflect the additional properties of the former, such as the effects on the kinin system [26], it has also been suggested that the differences could reflect the varying accessibility of such agents to extra-renal sites of ANG II generation that are important in the maintenance of blood pressure [27]. Particular attention has been focused on the vascular wall renin-angiotensin system [28, 29]. Due to lack of material, we were unable to examine this tissue. An intriguing finding in our study, however, was that the adrenal renin mRNA level was considerably elevated in the 'chronic' Goldblatt rat compared with its control. Because of parallel changes in renin and aldosterone levels in the adrenal gland, it has been suggested that the adrenal renin-angiotensin system participates in the regulation of aldosterone production [30]. Direct evidence for this is, however, lacking, and we are unaware of any

studies showing an elevation of aldosterone levels in the chronic phase of Goldblatt hypertension. Recently, adrenal renin mRNA level and activity have also been shown to be raised in the spontaneously hypertensive rat [11, 31] and in hypertensive transgenic rats carrying the mouse *Ren-2* gene [32], suggesting that the adrenal renin-angiotensin system may contribute to the hypertensive process in various situations. This is an area that clearly merits further investigation.

Another site where the renin-angiotensin system has been considered to contribute to elevation of blood pressure in renovascular hypertension is the brain [33, 34]. In agreement with other studies [13] we did not observe any marked changes in total brain renin mRNA in either the early or chronic phases of Goldblatt hypertension. Although this suggests that the intrinsic brain renin-angiotensin system is not involved, some caution is needed in reaching this conclusion, as a lack of change in total brain renin mRNA may mask functionally significant changes in the renin-angiotensin system in different parts of the brain [35]. Methods allowing separate analyses of different regions of the brain, such as *in situ* hybridization [36], would have been of considerable help in this situation.

Several functions have been postulated for the tissue renin-angiotensin systems [27]. In the heart it has been suggested that the system may be causally involved in the cardiac hypertrophy associated with hypertension. This has mainly been based on the observation that angiotensin-converting enzyme inhibitors are more effective at preventing the development or causing the regression of myocardial hypertrophy in hypertension than otherwise equipotent antihypertensive agents [37, 38]. With the proviso that cardiac renin mRNA levels were near the limit of sensitivity of detection of the technique, we found no marked increase in cardiac renin mRNA levels in the Goldblatt rats, which argues against such a role in this model at least. As discussed earlier, it has been suggested that the adrenal renin-angiotensin system may participate in the regulation of aldosterone production [30]. In DOC-salt-treated rats aldosterone levels have been shown to fall [17]. It was therefore also surprising to find that, if anything, renin mRNA levels in the adrenal glands were slightly higher in the DOC-salt-treated animals than in the controls. However, as we have not measured adrenal renin activity (or aldosterone levels) and cannot rule out post-transcriptional changes regulating the level of renin enzyme in the adrenal glands, no firm conclusions can be reached from this observation regarding a disassociation between adrenal renin and aldosterone levels in this model.

In summary, we have examined the effects of induction of two types of experimental hypertension on renin mRNA levels in renal and extra-renal tissues. Marked differences were observed in the response of renin mRNA levels between the kidneys and extra-renal tissues to such manipulations. Identification of the factors regulating extra-renal renin gene expression are likely to provide important clues towards determining the pathophysiological roles of the tissue renin-angiotensin systems.

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## Rapid communication

### A major structural abnormality in the renin gene of the spontaneously hypertensive rat

Nilesh J. Samani, William J. Brammar and John D. Swales

The renin genes of the spontaneously hypertensive rat (SHR) and Wistar-Kyoto (WKY) rat were compared by Southern blotting using cDNA and oligonucleotide probes. A 'deletion' of ~650 base pairs was found in the first intron (intron A) of the SHR gene compared with the WKY gene. Our studies strongly suggest that this is due to a decrease in the number of copies of the tandemly repeated sequence present within intron A of the rat renin gene. In both SHR and WKY, this region of the gene was found to be different from that of the parent Wistar rat and those of other Wistar-based inbred strains. The functional significance of the abnormality and any role it may have in hypertension in the SHR remain to be determined.

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**Keywords:** Genetic hypertension in rats, renin gene, Southern blot analysis, cDNA/oligonucleotide probes, intron A, tandemly repeated sequence.

#### Introduction

The spontaneously hypertensive rat (SHR), extensively studied as a model of human essential hypertension has the potential to allow the identification of genes involved in that condition [1]. In the SHR, the hypertensive trait is largely genetically determined and cross-breeding studies have indicated that a limited number of loci (between one and four) are responsible for the raised blood pressure [1]. Despite the numerous phenotypic differences that have been described between the SHR and control strains, the nature of the genes involved remains unknown.

Several studies have examined the role of the renin-angiotensin system in the SHR. Comparison of plasma renin levels with those of control strains has yielded conflicting results, some of which may reflect methodological differences [2-9]. However, inhibition of the renin-angiotensin system with angiotensin converting enzyme (ACE) inhibitors, has clearly been shown to both lower the blood pressure in the hypertensive rat [10,11] and prevent the development of hypertension if given to the younger, pre-hypertensive rat [12,13]. Other investigators have shown increased activity of the renin-angiotensin system in the SHR in several tissues concerned with cardiovascular control, such as the blood vessel wall [14], brain [15] and adrenal gland [16], and suggested that these local systems may be the site of action of ACE inhibitors. More recently we have observed higher renin messenger

RNA (mRNA) levels in several tissues of young and mature SHR compared with WKY rats [17].

Such observations suggest abnormal regulation of renin gene expression which may be a consequence of alterations in the structure of the renin gene and/or its regulatory elements in the SHR. We have therefore compared, by restriction site mapping, the renin gene of the SHR with that of the WKY rat as well as with that of a number of other rat strains. This study shows that there is a major structural change within the renin gene of the SHR.

#### Methods

##### Animals

SH and WKY rats

Spontaneously hypertensive and WKY rats from the colony maintained in the University of Leicester from 1981, as well as rats obtained from four different commercial suppliers (Charles River Laboratories, Margate Olac Harlan, UK, Bicester, UK, Charles River Laboratories, Wilmington, Massachusetts, USA and Taconic Farms, Germantown, NY, USA) were analysed.

Other rat strains

The renin genes of the Wistar rat, a number of strains inbred from the Wistar rat (WAG, WAB, LEW, LOU/C, AS), and the Sprague-Dawley rat were also examined. These

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animals were obtained either from colonies maintained in the University of Leicester or from commercial suppliers. The characteristics of these strains have been described by Festing [18]. All these strains are normotensive apart from the AS rat which exhibits moderate hypertension [19].

#### Probes

##### cDNA probe

A mouse submandibular gland renin cDNA, pSMG 5913, containing a sequence from exon II to exon IX of the renin gene was used [20]. The probe was radioactively labelled with  $\alpha$ -<sup>32</sup>P-dCTP (Amersham, Aylesbury, UK) by the random oligo-primer method of Feinberg and Vogelstein [21]. The DNA polymerase (large fragment) used for this was obtained from Gibco-BRL, Paisley, UK.

##### Oligonucleotide probe

The sequence 5' AGTGCTCCACAGCCAGCCCATGGG 3', which is complementary to the most conserved area of the tandemly repeated sequence in intron A of the rat renin gene [22], was synthesized chemically by the solid phase phosphoramidite procedure [23] on an Applied Biosystems 380B DNA synthesizer (Applied Biosystems, Warrington, UK). 5' end-labelling of the oligonucleotide for use as a probe was carried out using  $\gamma$ -<sup>32</sup>P-ATP (Amersham, UK) and polynucleotide kinase (Pharmacia, Milton Keynes, UK) as described previously [24].

#### DNA analysis

Ten-microgram samples of DNA, isolated from the livers or tails of animals using standard protocols [25,26], were digested with 20 units of restriction endonuclease (obtained from Gibco-BRL, Pharmacia, or New England, Biolabs, Bishop Stortford, UK) under conditions recommended by the manufacturers. After size-fractionation on a 1% agarose gel, samples were denatured, transferred to Hybond N membrane (Amersham, UK), pre-hybridized (4 h) and hybridized (16 h) to probes at 64°C in buffers containing 6 × SSC (20 × SSC = 3.0 mol/l NaCl, 0.3 mol/l Na<sub>3</sub> citrate), 5 × Denhardt's solution (100 × Denhardt's = 2% bovine serum albumin, 2% Ficoll, 2% polyvinyl pyrrolidone), 0.5% SDS and 6% polyethylene glycol 6000. For both the cDNA and oligonucleotide probe post-hybridization washing stringencies were to 1 × SSC, 0.1% SDS at 64°C. Autoradiography was carried out at -70°C using intensifying screens and Fuji-RX film.

Molecular weights of fragments were calculated on the basis of concurrently run aliquots of *Hind*III digests of bacteriophage  $\lambda$ DNA and the BRL 1-kilobase DNA ladder (Gibco-BRL, UK).

## Results

In initial studies Southern transfers of WKY and SHR DNA digested with 24 different restriction endonucleases were probed with the renin cDNA probe pSMG 5913 (Figs 1a, b and c). Polymorphism was observed between the renin gene of the SHR and WKY rat with several of the enzymes. With a number of the enzymes a common pattern was observed, namely a smaller fragment in the SHR compared with the WKY rat DNA (see arrows Fig. 1), suggesting that

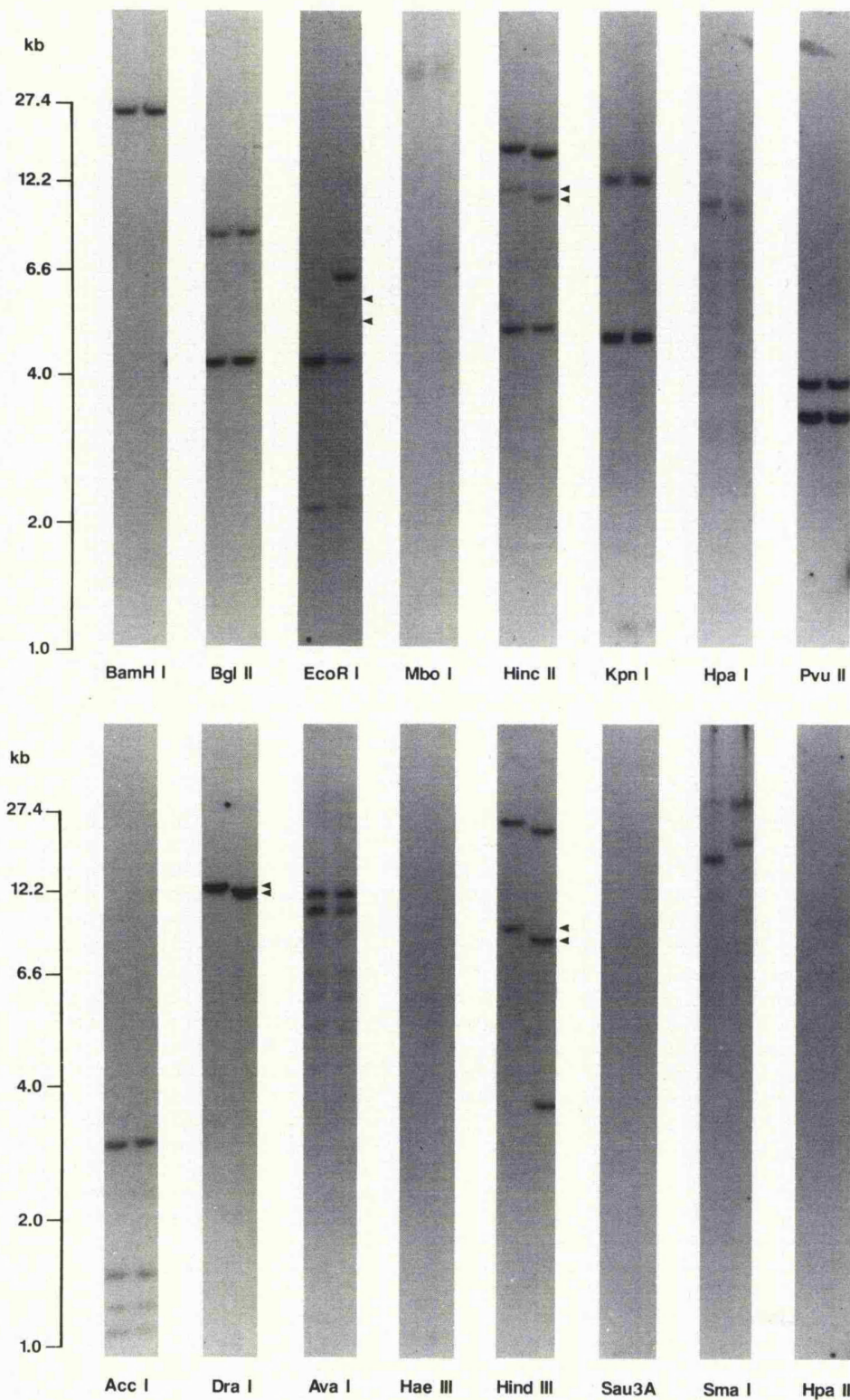
the same difference (i.e. a 'deletion' in the SHR compared with the WKY rat) was being detected in each case.

To localize the site of the 'deletion', the number of restriction fragments observed and their lengths were compared with those predicted on the basis of the published sequence of the rat renin gene [22]. In this analysis it was borne in mind that the pSMG 5913 cDNA does not contain sequence 5' of exon II and would also not detect any fragments consisting entirely of intron sequence. Although some discrepancies were observed between the sizes of fragments seen with the SHR and WKY rat DNAs and those predicted from the sequence of the Sprague-Dawley rat renin gene [22], with most enzymes the predicted fragments were observed. This allowed the site of the difference between the WKY rat and SHR genes to be localized to between based ~150 and 3090 of the gene, i.e. within intron A, with a 'deletion' in the SHR compared with WKY rat gene of about 650 base pairs. The patterns observed with *Bgl*I, *Bgl*II and *Taq*I were particularly useful in this analysis.

A striking feature of the rat renin gene within this region of intron A is a tandem repetition of a sequence with an average size of 38 bases which in the Sprague-Dawley gene occurs 46 times [22]. To localize the difference between the SHR and WKY genes more precisely a 25mer synthetic oligonucleotide, complementary to the most conserved region of the tandem repeat sequence, was used as probe. On genomic DNA digested with *Bgl*I or *Mnl*I the probe hybridized, under moderately stringent conditions (1 × SSC, 64°C), to a single fragment (Fig. 2). With both enzymes a difference of ~650 base pairs was again observed in the size of the fragment detected from the WKY and SHR allele. These results therefore confirmed that the difference between the two genes was due to an 'insertion/deletion' in intron A. Furthermore, analysis of the sequence published by Fukamizu *et al.* [22] indicates that while *Mnl*I does not cut within the tandem repeat sequence there are multiple *Mnl*I sites immediately outside this sequence on both sides. Thus, the observation that the difference between the two genes persists in DNA digested with *Mnl*I and probed with the repeated sequence strongly suggests that the difference between the two genes is due primarily to variation in the number of copies of the tandemly repeated sequence within intron A. This conclusion is supported by the finding that, despite similar DNA loadings, the intensity of the autoradiographic signal of the SHR fragment revealed by the oligonucleotide probe was consistently less than that of the WKY fragment (Figs 2 and 3).

To exclude the possibility that the difference observed was due to an aberration limited to animals from one source, we have examined the DNAs of SHR and WKY animals from several different commercial suppliers (see Methods). The DNAs from all SHR and WKY rats conformed to the patterns described above (data not shown).

To determine whether the observed difference in intron A of the SHR gene is specific to that strain, the renin genes of the Wistar rat (from which the SHR and WKY rat strains are derived), of other strains inbred from the Wistar and of the Sprague-Dawley rat were examined using the oligonucleotide probe (Fig. 3). The analysis showed that while the Wistar, Sprague-Dawley and several rat strains derived



**Fig. 1.** Comparison of the renin genes of the Wistar-Kyoto (WKY) rat and spontaneously hypertensive rat (SHR) by probing of Southern transfers of WKY and SHR DNA restriction endonuclease digests with the renin cDNA probe pSMG 5913. See Methods for details. For each enzyme, first lane is WKY DNA and second lane is SHR DNA. Although with some enzymes no hybridization was observed, the results obtained for all 24 enzymes used are shown for completeness. Pairs of arrows point to those WKY and SHR fragments detected with some restriction enzymes that showed a constant size difference between the WKY and SHR DNAs and which suggested that there was a 'deletion' in the renin gene of the SHR compared with the WKY.



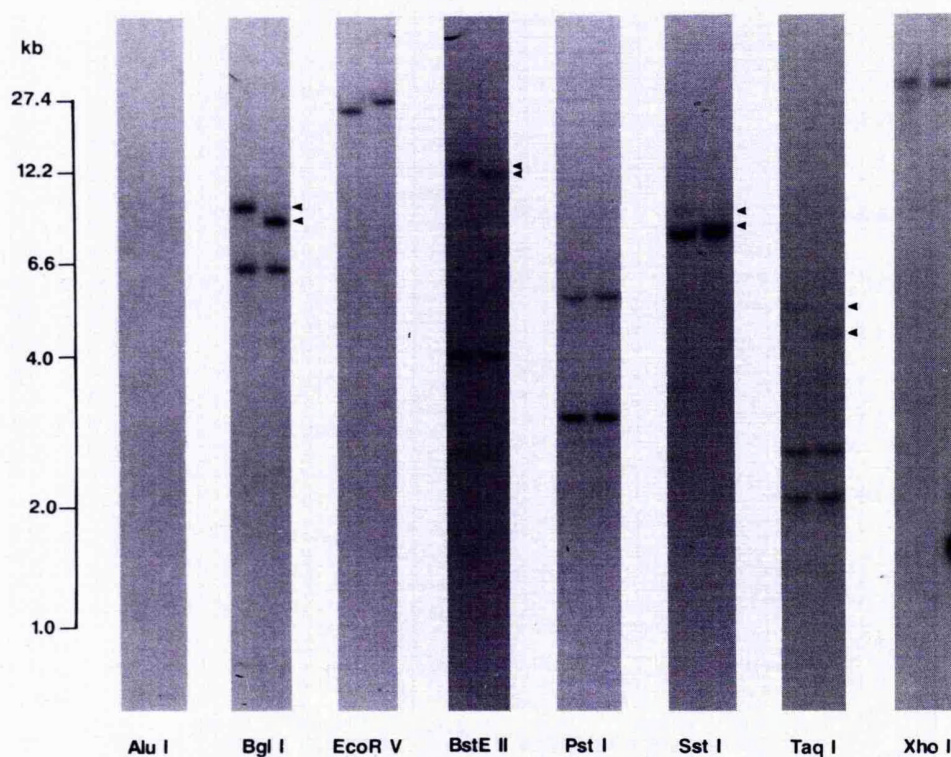


Fig. 1

from the Wistar had the same sized fragments (different from those seen in both SHR and WKY rats), variability in fragment size was also present in some of the other inbred strains (see Fig. 3), although none of the strains examined had the same sized fragment as the SHR.

On the premise that the difference in sizes of the Sprague-Dawley and SHR and WKY *Bgl*I fragments detected by the oligonucleotide probe (Fig. 3) is entirely due to differences in the number of copies of the tandemly repeated sequence, and given that the Sprague-Dawley renin gene has 46 copies of the repeat sequence with an average size of 38 bases [22], we have calculated that the WKY rat renin gene would have 34 copies and the SHR gene 17 copies of this sequence.

## Discussion

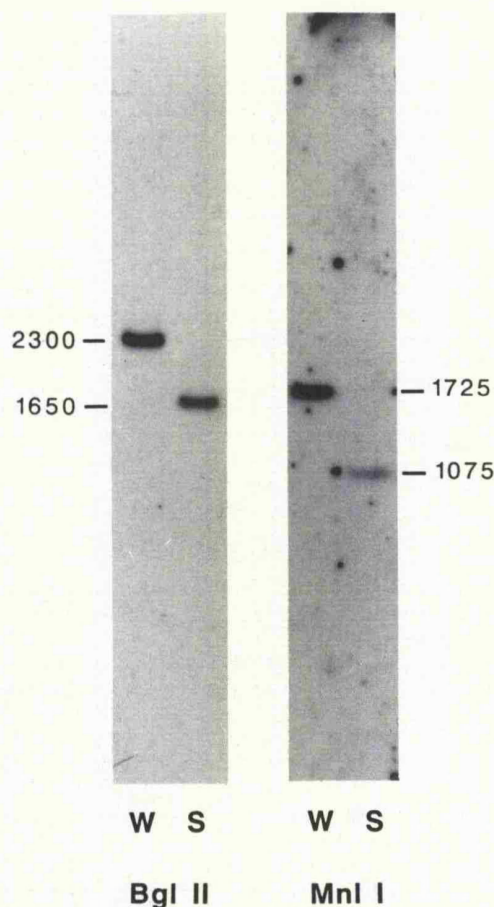
In this study using cDNA and oligonucleotide probes we have demonstrated and localized a major structural change within the renin gene of the SHR compared with the WKY rat. Initial studies using the cDNA probe suggested that the difference was due to a 'deletion' in the SHR gene of ~650 base pairs compared with the WKY gene. The data localized the deletion to part of intron A of the gene where the major feature is a large region of tandemly repeated sequence. In subsequent studies with the oligonucleotide probe based on the repeated sequence, the change was localized to within the repeated region. This was achieved because the enzyme *Mn*I does not cut within the repeated sequence but cuts at sites immediately flanking the tandem

repeat region. The observation that the difference of ~650 base pairs between the two renin gene alleles persists in their *Mn*I fragment strongly suggests that the difference is due primarily to variation in the number of copies of the repeated sequence within intron A.

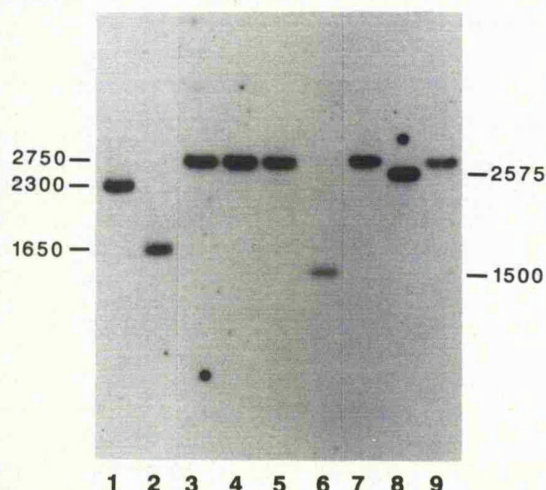
The finding that under conditions of moderate stringency ( $1 \times \text{SSC}$ ,  $64^\circ\text{C}$ ), the oligonucleotide probe hybridized to a single genomic fragment indicates that, unlike some other tandem repeat sequences [27], this is unique to the renin gene. However analysis of the sequence of the *Ren-1* renin gene of the two renin gene mouse strain DBA2/J [28] shows only two tandemly repeated copies of the relevant sequence within intron A, between nucleotides 3206 and 3281, and suggests that further tandem repetition occurred following the divergence of the mouse from the rat. As far as we are aware, the full intron A sequences of the other mouse renin genes (*Ren-2* in two gene strains and *Ren-1* in one gene strains) or the human renin gene have not been published.

We have analysed the intron A region containing the tandem repeat sequence in a number of different rat strains. Although DNAs from several strains derived from the Wistar had the same fragment size, some variability was observed and both the SHR and WKY DNAs had a different fragment size from that of the parent Wistar rat. Particularly intriguing was the finding that the homologous fragment in the AS rat is also smaller. The AS rat, which is widely used in transplantation research, exhibits moderate hypertension and is biologically closely related to the New Zealand genetically hypertensive (GH) rat [19,29]. As in the SHR, the role of the renin-angiotensin system in the





**Fig. 2.** Probing of Southern transfers of WKY (W) and SHR (S) DNAs digested with *BglII* and *MnlI* with a synthetic oligonucleotide probe complementary to the tandemly repeated sequence within intron A of the rat renin gene.



**Fig. 3.** Comparison of the renin gene intron A region containing the tandemly repeated sequence in a number of rat strains by probing of Southern transfers of DNAs digested with *BglII* with the oligonucleotide probe complementary to the tandem repeat sequence. 1, Wistar-Kyoto rats (WKY); 2, spontaneously hypertensive rat (SHR); 3, Wistar; 4, LOU/C; 5, LEW; 6, AS; 7, WAG; 8, WAB; 9, Sprague-Dawley.

hypertension of the GH rat is unclear, although plasma and renal renin levels have been reported as being decreased compared with control rats once the hypertension is established [29]. Recently Rapp and Wang [30] have also reported differences in the first intron of the renin gene of Dahl S (hypertensive) and R (normotensive) rats, the former having a 1.1-kb insertion compared with R rats. This contrasts with our observation of a deletion in intron A of the renin gene of the SHR compared with the WKY rat. While the exact nature of the difference in the Dahl rats and the relationship of their finding to our observations remains to be determined, it is clear that this region of the rat renin gene is highly polymorphic and that certain alleles have been selected and fixed together with elevation of blood pressure in a number of hypertensive rat models.

To investigate any relationship of the mutation in the renin gene of the SHR to hypertension we are currently examining its co-segregation with the tendency to hypertension in the  $F_2$  population following cross-breeding of SHR and WKY rats. If co-segregation with the hypertensive trait is observed it would indicate that either the mutation itself or a linked locus is a cause of hypertension in the SHR. It is interesting to speculate briefly at this stage on the possible functional significance of the deletion itself. It is now clear that regulatory sequences may lie within genes rather than necessarily upstream of the gene. It is also well established that several regulatory elements are composed of tandem repeat sequences and that to a certain extent the function of such elements depends on the number of copies of the tandem repeat [31,32]. It is therefore possible that the intron A tandem repeat segment in the rat renin gene acts as a silencer, for example, and that the mutation interferes with this function. This could provide an explanation for the observation of increased renin mRNA levels in several tissues of the SHR compared with the WKY rat (N.J. Samani, unpublished observations; [17,33]). Experiments to test this hypothesis are also now underway.

### Acknowledgements

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## Analysis of the renin gene intron A tandem repeat region of Milan and Lyon hypertensive rat strains

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Michael A. Kaiser, William J. Brammar\* and John D. Swales

The region of intron A of the rat renin gene containing a unique tandemly repeated sequence was analysed in the Milan and Lyon hypertensive rat strains and their controls, and in several Sprague-Dawley rats, using an oligonucleotide probe complementary to the tandemly repeated sequence and a renin complementary DNA probe. In the Milan rats, the size of the *Bgl* II DNA fragment encompassing the tandem repeat region was the same in the hypertensive (MHS) and normotensive (MNS) strains. In the Lyon model, a difference of 1.1 kilobase (equivalent to about 28 copies of the 38 basepair tandem repeat sequence) was observed in the size of the *Bgl* II fragment of the hypertensive (LH) and normotensive (LN) strains. However, the finding that the size of the fragment in the Lyon low-blood-pressure (LL) strain was the same as that in the LH strain rather than the LN strain suggests that the difference between the two latter strains is not by itself a major cause of the blood pressure difference between them in the intron A tandem region. An analysis of Sprague-Dawley rats, from which the Lyon strains are derived, showed that at least three different renin gene alleles, two with *Bgl* II fragments of the same size as those seen in the Lyon strains, are randomly segregating in this population.

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**Keywords:** Renin gene, intron A, tandemly repeated sequence, Milan rat, Lyon rat.

### Introduction

Recent interest has focused on the question of whether structural differences in the renin gene contribute to the tendency to hypertension in rat models of genetic hypertension [1-3]. We have shown that the rat renin gene is polymorphic within its first intron (intron A) and that this is probably caused by variation in the number of copies of a 38 basepair tandemly repeated sequence present within the intron [1]. A comparison of the renin gene of the spontaneously hypertensive rat (SHR) and the Wistar-Kyoto rat (WKY) revealed a difference of 650 basepair within this region, with a 'deletion' in the SHR allele compared with the WKY allele [1]. Wang and Rapp [2] have shown that a structural difference also exists in

Dahl salt-sensitive and salt-resistant rats in this region of the renin gene, and have demonstrated, using the polymorphism as a linkage marker, that the locus defined by this marker is responsible for some of the difference in blood pressure between the two strains [3]. Two other well defined rat models of genetic hypertension are the Milan strain, derived from the Wistar rat [4], and the Lyon strain, derived from the Sprague-Dawley rat [5]. In this study, using an oligonucleotide probe complementary to the tandemly repeated sequence as well as a renin complementary DNA (cDNA) probe, we have analysed the intron A region of the renin gene containing the tandemly repeated sequence in these strains and their controls, and in several Sprague-Dawley rats.

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Materials and methods

Animals

The Milan hypertensive (MHS) and normotensive (MNS) rats used were from a colony started in 1984 with breeding pairs donated by Professor Bianchi to the University of Sheffield, Sheffield, UK. The Lyon hypertensive (LH), normotensive (LN) and low-blood-pressure (LL) rats were obtained directly from the colony in Lyon, France. The Sprague-Dawley rats were obtained from Charles River Laboratory (Margate, UK).

Probes

Oligonucleotide probe

The sequence 5'AGTGCTCCACAGCCAGCCCATGGG 3', which is complementary to the most conserved area of the tandemly repeated sequence in intron A of the rat renin gene [6], was synthesized chemically by the solid-phase phosphoramidite procedure on an Applied Biosystems 380B DNA synthesizer (Applied Biosystems, Warrington, UK). The oligonucleotide was prepared for use as a probe by 5' end-labelling, carried out using  $\gamma$ -<sup>32</sup>P-adenosine triphosphate (ATP; Amersham, Aylesbury, UK) and polynucleotide kinase (Pharmacia, Milton Keynes, UK), as described [7].

cDNA probe

A mouse submandibular gland renin cDNA, pSMG 5913, containing the sequence from exon II to exon IX of the renin gene, was used [8]. The probe was radioactively labelled with  $\alpha$ -<sup>32</sup>P-deoxycytidine triphosphate (dCTP; Amersham) using the random oligo-primer method [9]. The DNA polymerase (large fragment) used for this was obtained from Gibco-BRL (Paisley, UK).

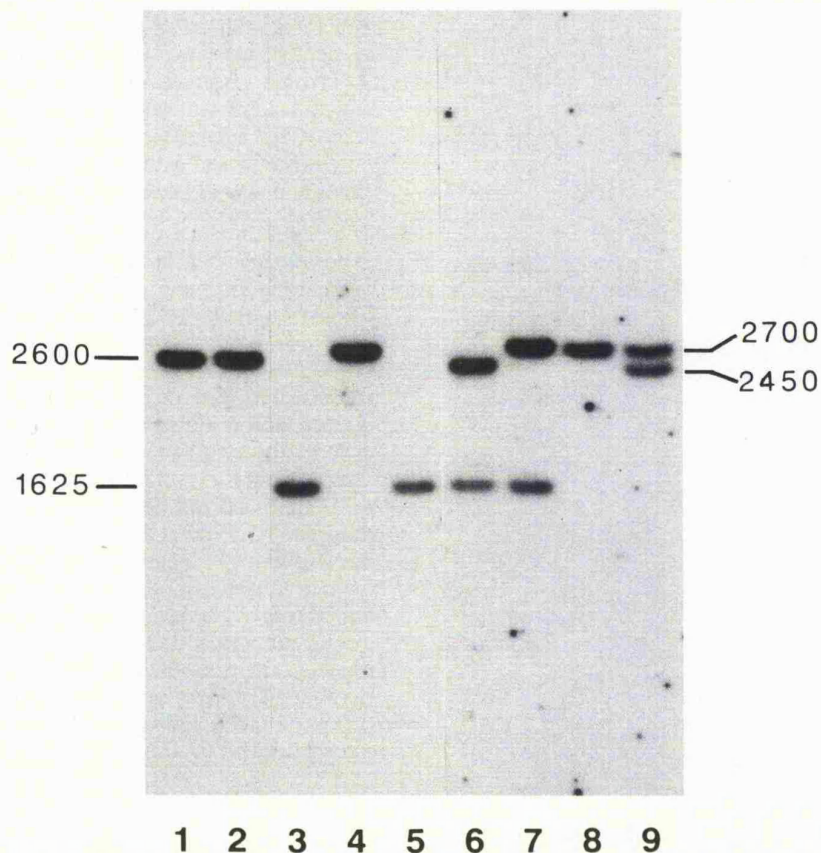
DNA analysis

Ten-microgram samples of DNA, isolated from a short fragment of the rats' tails using a standard protocol [10], were digested with 20 units of restriction endonuclease (Gibco-BRL) under conditions recommended by the manufacturer. After size-fractionation on a 1% agarose gel, the DNA samples were denatured, transferred to Hybond N membrane (Amersham), prehybridized for 4 h and hybridized for 16 h to probe at 64°C in buffer containing 6 × standard saline citrate (SSC; 20 × SSC = 3.0 mol/l NaCl, 0.3 mol/l Na<sub>3</sub> citrate), 5 × Denhardt's solution (100 × Denhardt's = 2% bovine serum albumin, 2% Ficoll, 2% polyvinyl pyrrolidone), 0.5% sodium dodecylsulphate (SDS), 6% polyethylene glycol 6000 and 200 µg/ml denatured salmon sperm DNA. For both the oligonucleotide and cDNA probes, posthybridization washing stringencies were to 1 × SSC, 0.5% SDS at 64°C. Autoradiography was carried out at -70°C using intensifying screens and Fuji-RX film (Fuji Photo Film Co., Tokyo, Japan). Molecular weights of fragments were calculated from concurrently run aliquots of *Hind*III digests of bacteriophage  $\lambda$  DNA and the BRL 1 kilobase (kb) DNA ladder (Gibco-BRL).

Results

Digestion of DNA with the restriction enzyme *Mnl* I and probing with the tandem repeat oligonucleotide probe most closely defines the size of the tandem repeat region in intron A of the rat renin gene and, therefore, the number of copies of the tandemly repeated sequence [1]. However, we have previously shown that digestion with *Bgl* II provides a convenient alternative for comparing this region of the renin gene in different rat strains [1]. We therefore used this much less expensive restriction enzyme in the present study. The results obtained are shown in Fig. 1. No difference was observed in the sizes of the *Bgl* II fragment detected in DNA from the MHS and MNS strains. In DNA from the Lyon strains, however, an interesting pattern was observed. While the LH and LN strains showed a difference of almost 1.1 kb in *Bgl* II fragment size (equivalent to a difference of about 28 copies of the 38 basepair repeat sequence), the DNA fragment from the LL strain was the same size as that from the LH rather than the LN strain. Two animals (from different breeding pairs) were analysed for each of these inbred strains and yielded identical results. The DNA prepared from four Sprague-Dawley rats was also analysed. *Bgl* II fragments of three different sizes were observed in this DNA and three of the rats were heterozygous (Fig. 1), indicating that there are at least three different alleles of the renin gene segregating in this outbred strain. Two of the Sprague-Dawley *Bgl* II DNA fragments were identical in size to those observed in the Lyon strains.

In order to support the findings with the oligonucleotide probe and, in particular, to confirm that the two fragments detected in some of the Sprague-Dawley rats both came from the renin gene, DNA was also analysed with a renin cDNA probe (pSMG 5913; see Materials and methods) following digestion with *Bgl* I. We had found previously in SHR and WKY that this analysis detects two fragments, with the larger fragment showing the same polymorphism as that detected using the oligonucleotide probe [1]. The results are shown in Fig. 2. As expected, while in the Milan strains both fragments detected were identical in size, in the Lyon strains a pattern of polymorphism similar to that found with the oligonucleotide probe was observed in the larger of the two fragments detected. In the Sprague-Dawley rats, an extra fragment was detected in those rats where two fragments were observed with the oligonucleotide probe, confirming that both fragments detected with the latter probe come from the renin gene. The similar fragment sizes of the two alleles of the fourth Sprague-Dawley rat (Fig. 1, lane 9) prevented their resolution in the above analysis with *Bgl* I (Fig. 2, lane 9), but other studies (data not shown) in which the DNA was probed with the renin cDNA probe after digestion with *Taq* I [1] confirmed that this rat was also heterozygous for the renin gene.



**Fig. 1.** Comparison of the renin gene intron A region containing the tandemly repeated sequence in Milan, Lyon and Sprague-Dawley rats by probing of Southern transfers of DNA digested with *Bgl* II with an oligonucleotide probe complementary to the tandem repeat sequence. 1, Milan hypertensive strain; 2, Milan normotensive strain; 3, Lyon hypertensive strain; 4, Lyon normotensive strain; 5, Lyon low-blood-pressure strain; 6-9, different Sprague-Dawley rats.

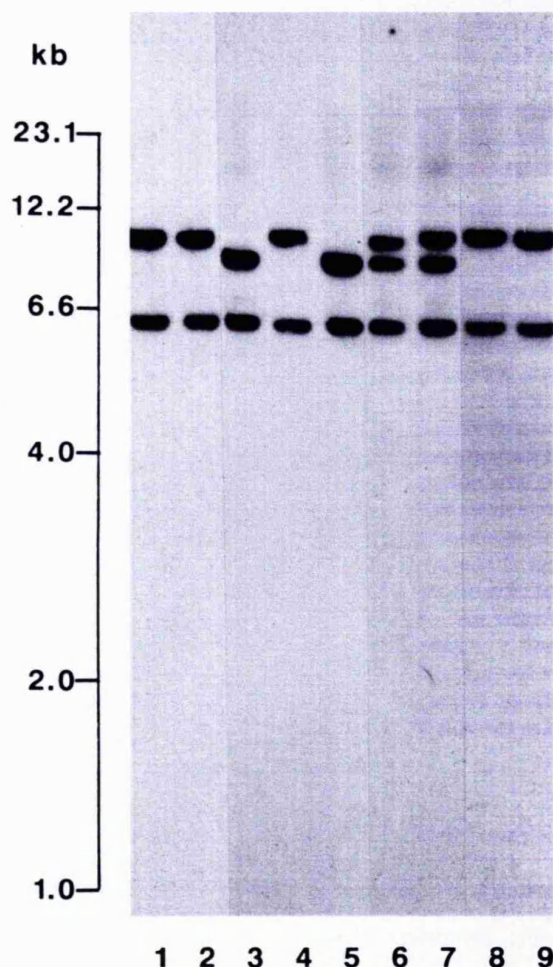
## Discussion

The basis of the hypertension in the various rat models of genetic hypertension remains poorly understood. In most strains, including the Milan [4] and Lyon [5] strains, genetic analysis has suggested that, while the trait is polygenic, a limited number of genes are involved. DNA restriction fragment length polymorphisms between hypertensive and control rats are providing a new source of linkage markers for defining the genes responsible [1,2,11,12]. Already, a polymorphism in the renin gene of Dahl rats has been shown to cosegregate with increased blood pressure [3]. The polymorphism used in that study was due to a 1.2 kb insertion in the first intron (intron A) of the renin gene of the salt-sensitive rat compared with the salt-resistant rat, giving rise to *Bgl* II fragments of 2.7 kb and 1.5 kb, respectively. We have analysed the homologous region in SHR and WKY and found a 'deletion' of 650 basepair in the renin gene of SHR compared with WKY [1]. This difference was localized to a region of intron A containing a 38 basepair tandemly repeated sequence [6], and our studies strongly suggested that it was due to a variation in the number of copies of the

repeat sequence [1]. In the present study we report our analyses of this region of the renin gene in the Milan and Lyon hypertensive rat strains and their respective control strains, and in several Sprague-Dawley rats.

The findings in the Lyon rat strains and their parental strain, the Sprague-Dawley rat, are particularly interesting. The results indicate that the differences between the Lyon strains are due to fixation (in the homozygous state) of one or other of the renin gene alleles randomly segregating in the founding population. This fixation may occur either by chance (genetic drift) or as the result of genetic selection for blood pressure differences [13]. In most instances, a cross-breeding study and cosegregation analysis would be required to distinguish between these possibilities [3,13]. The Lyon model has a unique advantage among rat models of genetic hypertension of having two control rat strains, LN and LL, simultaneously selected along with the hypertensive strain [5], and thereby increasing the chances of detecting differences that are due to genetic drift. Therefore, the observation that the LL strain has a *Bgl* II fragment of the same size as that in the LH strain strongly suggests that the difference in in-





**Fig. 2.** Analysis of the renin genes of Milan, Lyon and Sprague-Dawley rats by probing of Southern transfers of DNA digested with *Bgl* I with the renin complementary DNA probe pSMC 5913. 1, Milan hypertensive strain; 2, Milan normotensive strain; 3, Lyon hypertensive strain; 4, Lyon normotensive strain; 5, Lyon low-blood-pressure strain; 6–9, different Sprague-Dawley rats in the same order as in Fig. 1. kb, Kilobase.

tron A of the renin gene of the hypertensive and normotensive strains is not by itself a major cause of the blood pressure difference between the two strains. However, a small effect masked by other genes cannot be totally ruled out. It should also be emphasized that our findings do not exclude the renin gene as a locus coding for elevated blood pressure in either the Lyon or Milan strains, since the whole gene was not analysed for other differences between the hypertensive and control rats.

The observation by Rapp *et al.* [3] of cosegregation between blood pressure and a polymorphism in the tandem repeat region of intron A of the renin gene in Dahl rats has raised the important question of whether the polymorphism itself is causally responsible or whether it acts as a linkage marker for another abnormality at the lo-

cus which actually leads to an increase in blood pressure. The Dahl strains, like the Lyon rats, are derived from the Sprague-Dawley rat. An intron A *Bgl* II DNA fragment of 2.7 kb was observed in the Dahl salt-sensitive rat [2,3], which matches our observation in the Lyon normotensive strain. This suggests that the polymorphism in the Dahl rat probably acts as a linkage marker rather than being causally involved, although further studies are required.

The significance of the tandemly repeated sequence remains unclear. The finding that, under conditions of moderate stringency, the oligonucleotide probe does not hybridize to multiple DNA fragments indicates that this sequence is unique to the renin gene. Analysis of the sequence of the mouse *Ren-1* gene shows only two tandemly repeated copies [14]. The full intron A sequence of the human renin gene has not yet been published. However, we have probed several human DNA samples digested with *Bgl* II with the oligonucleotide probe and did not detect a hybridizing fragment under the same conditions as those used for rat samples (data not shown). This suggests that the tandemly repeated sequence is not essential for renin gene function. Its potential role in the regulation of renin gene expression in the rat remains to be investigated. In any case, polymorphisms occurring as a consequence of variation in the number of copies of the tandem repeat sequence are providing useful linkage markers for studying the role of the renin gene in rat models of genetic hypertension.

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# Genetic determinants of diastolic and pulse pressure map to different loci in Lyon hypertensive rats

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Several genetic loci involved in blood pressure regulation have recently been localized in experimental models of hypertension, but the manner in which they influence blood pressure remains unknown. Here, we report a study of the Lyon hypertensive rat strain showing that different loci are involved in the regulation of steady-state (diastolic pressure) and pulsatile (systolic – diastolic, or pulse pressure) components of blood pressure. Significant linkage was established between diastolic blood pressure and a microsatellite marker of the renin gene (*REN*) on rat chromosome 13, and between pulse pressure and the carboxypeptidase B gene (*CPB*) on chromosome 2. These findings show that two independent loci influence different haemodynamic components of blood pressure, and that pulse pressure has a specific genetic determination.

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The pathological elevation of blood pressure is a haemodynamic abnormality that results from an inappropriate balance between blood flow and vascular resistance. The disorder has a heterogeneous origin that is likely to involve multiple genetic factors<sup>1</sup>. Recent studies of genetically hypertensive rats have led to the localization of several loci that are involved in blood pressure regulation<sup>2-7</sup>, but the manner in which these affect the phenotype is still unknown. The complexity of the phenotype has led to a number of propositions to study traits that may be directly related to single genes affecting blood pressure (so-called "intermediate phenotypes")<sup>8-11</sup>, but the loci that are hypothesized to underlie these characters have yet to be identified.

An alternative approach to the resolution of genetic heterogeneity and to the identification of the specific effects of different loci may be to consider different haemodynamic components as sub-phenotypes of blood pressure. Here, we have applied such a dissection to study genetic factors underlying blood pressure regulation in the Lyon hypertensive (LH) rat<sup>12</sup>. The LH rat has moderately elevated blood pressure compared to models such as the stroke-prone hypertensive rat (SHRSP)<sup>3</sup> or the Dahl rat<sup>4</sup>, but its phenotype values are significantly higher than those in the Lyon normotensive (LN) and Lyon low blood pressure (LL) control strains. In the LH strain, hypertension is associated with increased plasma lipids, greater body weight, and insulin-resistance. Here, we show that diastolic blood pressure is linked to *REN* on rat chromosome 13, whereas pulse pressure is linked to the marker *CPB* on rat chromosome 2. These results suggest that the dissection of blood pressure into sub-phenotypes through precise haemodynamic measurements may

contribute to the resolution of genetic heterogeneity in other animal models and human hypertension.

## Crosses and markers

Continuous blood pressure measurements were obtained on 16 LH, 14 LN, 15 F<sub>1</sub> and 345 F<sub>2</sub> males at 29–31 weeks of age via precisely positioned intra-aortic catheters (Table 1). Systolic and diastolic blood pressures differed by 38 mm Hg and 25 mm Hg, respectively, in LH and LN animals. Although blood pressure was shifted towards the LN values in the F<sub>1</sub>, the systolic and diastolic means were intermediate between LH and LN in the F<sub>2</sub> cohort. The degree of genetic determination (ratio of genetic to total variance) in the F<sub>2</sub> cohort was estimated as 28% for systolic blood pressure, and as 13% for diastolic blood pressure.

DNA samples from LH and LN animals were screened

**Table 1 Blood pressure phenotypes in LH, LN, F<sub>1</sub> and F<sub>2</sub> cohorts**

Cohort	No.	Systolic (mm Hg)	Diastolic (mm Hg)	Pulse pressure (mm Hg)
LH	16	174.1 ± 2.1	118.4 ± 1.7	55.6 ± 0.7
LN	14	136.4 ± 1.3	93.1 ± 1.2	43.3 ± 0.9
F <sub>1</sub>	15	145.8 ± 2.0	92.0 ± 1.5	51.1 ± 0.9
F <sub>2</sub>	345	149.5 ± 0.4	99.6 ± 0.3	49.9 ± 0.2
Estimated degree of genetic determination		0.28	0.13	0.51

Table 2 Linkage of *REN* and *CPB* to blood pressure phenotypes

	Genotype	No.	Systolic	Diastolic	Pulse pressure (mm Hg)
<i>a</i> <i>REN</i>	LH/LH	81	151.6 ± 0.9	101.6 ± 0.7	50.0 ± 0.5
	LH/LN	165	150.0 ± 0.8	99.8 ± 0.5	50.2 ± 0.3
	LN/LN	82	146.2 ± 0.8	96.9 ± 0.5	49.3 ± 0.5
	Lod score statistic*		3.4	5.6	0.5
	P-value (ANOVA)		0.0001	<0.0001	0.39
<i>b</i> <i>CPB</i>	LH/LH	159	152.1 ± 0.7	100.6 ± 0.6	51.5 ± 0.3
	LH/LN	112	149.0 ± 0.7	99.3 ± 0.5	49.7 ± 0.4
	LN/LN	57	145.8 ± 0.9	98.3 ± 0.8	47.6 ± 0.6
	Lod score statistic*		4.4	1.3	7.0
	P-value (ANOVA)		<0.0001	0.04	<0.0001

\*Base 10 logarithm of the likelihood ratio statistic for the test of linkage.

with microsatellite markers representing 241 distinct loci, and allelic differences in the LH and LN strains were found for 26 (11%) of these. The frequency of microsatellite allelic differences is lower than that we have observed in other crosses with these markers<sup>8,13</sup>. This probably reflects the close genetic relationship between the LH and LN strains, which were expected to contain many regions of identity-by-descent since they were selected for blood pressure differences from a small Sprague-Dawley colony. Two of the informative markers were found to have allelic variants within the LH or LN lines, but had stable transmission (that is, no mutation) within F<sub>2</sub> families. As discussed below, lack of complete inbreeding at these loci did not affect the results of our study since complete genealogies of all F<sub>2</sub> animals were available.

#### Genetic linkage

Two of the markers were significantly linked ( $p < 0.0001$ ) to blood pressure phenotypes (Tables 2 and 3). The first is contained in the renin gene (*REN*) on rat chromosome 13 (ref. 3). *REN* gave lod scores of 3.4 with systolic blood pressure, and 5.6 with diastolic blood pressure (Table 2a). Renin is a candidate gene in hypertension, and it may be directly implicated in the pathophysiology of the disease. Under the hypothesis that the renin gene is directly responsible for blood pressure variation, we estimated that this locus could account for 6% of the total systolic blood pressure variance (21% of the estimated genetic variance), and 8% of the total diastolic blood pressure variance (62% of the estimated genetic variance). Alternatively, *REN* may be linked to another gene that is responsible for blood pressure differences, and in this instance it could account for a larger proportion of the F<sub>2</sub> genetic variance.

A second microsatellite marker contained in the carboxypeptidase B gene (*CPB*) showed significant linkage to blood pressure (Table 2b). *CPB* has been assigned to a linkage group on rat chromosome 2 (refs 13, 14). Although *CPB* was one of the two markers that were not completely

homogenous in the parental lines, the lack of complete inbreeding does not affect our conclusions with respect to linkage as described below. *CPB* was significantly linked to systolic pressure ( $p < 0.0001$ ), but only weakly correlated with diastolic blood pressure ( $p = 0.043$ ). When multivariate analysis of variance was applied, we found that the linear combination of systolic blood pressure (SBP) and diastolic blood pressure (DBP) that gives the best discrimination of genotypes at the *CPB* locus was proportional to 1.1 SBP - 0.9 DBP, which is very close to the definition of pulse pressure. This suggested that pulse pressure would be the best phenotype for obtaining evidence of linkage to *CPB*. The estimate of genetic determination of pulse pressure (51%) was higher than for other phenotypes, providing further evidence that this is an important genetic parameter in the Lyon model (Table 1).

The lod score statistic for linkage of pulse pressure with *CPB* was 7.0 compared to a value of 4.4 for systolic blood pressure. The 1-lod-unit confidence interval for the recombination estimate between *CPB* and pulse pressure was 0-0.27, and a linked gene within this recombination range could account for 10-29% of the total variance of the pulse pressure in the F<sub>2</sub> (20-57% of the estimated genetic variance). In contrast, we found no evidence of linkage between *REN* and pulse pressure ( $p = 0.39$ ). For diastolic blood pressure and *REN*, the mean associated with LH/LN differed significantly from the LN/LN mean ( $p < 0.01$ ) and LH/LH ( $p < 0.1$ ) using the Scheffé method. For pulse pressure and *CPB*, similar comparisons were significant ( $p < 0.01$ ). Thus, we concluded that the effects of the linked loci were codominant for these phenotypes. No evidence for interaction of the loci was found for any of the phenotypes in two-way ANOVA of *REN* and *CPB*.

The distribution of genotypes at the *CPB* locus deviated significantly from mendelian proportions ( $p < 0.0001$ ). After examination of the genealogies, we found no LN/LN homozygous animals amongst all 67 F<sub>2</sub> progeny that were descended from one pair of LH × LN progenitors. Genotype frequencies in 261 F<sub>2</sub> animals descended from other progenitors were in mendelian proportions (66 LN/LN, 129 LN/LH and 66 LH/LH genotypes), and all F<sub>2</sub> animals that contributed to this part of the cross could be deduced to have LH/LN genotypes. Although DNA samples from most progenitors were not available for study, these results suggest that a single LN progenitor had an LH/LN genotype and that the other F<sub>0</sub> animals that had contributed to the cross were of the predominant parental types. When DNA samples from 16 other LH or LN animals were tested, one animal was found to be a LH/LN heterozygote. Only one other marker exhibited similar heterogeneity in the parental strains. Mendelian segregation was observed at other loci characterized in the cross. Thus, recent contamination of the LH or LN strains seemed unlikely, and an experimental error leading to the confusion of LN and LH animals in the formation of the



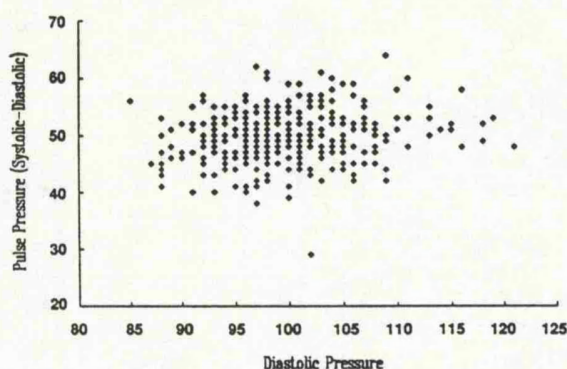


Fig. 1 Scatter plot of pulse pressure and diastolic blood pressure in the  $F_2$ . The two variables show little correlation (0.18).

cross could be excluded.

Linkage to *CPB* was confirmed by the fact that systolic and pulse pressure were correlated with LH/LN and LH/LH genotypes at the *CPB* loci in both sets of  $F_2$  progeny, and that the phenotypic means associated with these genotypes did not differ statistically in the two parts of the pedigree. It should be noted that the genetic determination of the phenotypes given in Table 1 may be underestimated if the parental populations are not completely inbred at the loci responsible for blood pressure variation.

# Discussion

We have obtained accurate phenotype values for systolic and diastolic pressures through continuous blood pressure measurements by intra-aortic catheter in a large  $F_2$  cohort. This allowed separate tests of linkage between genetic markers with the steady-state haemodynamic component (diastolic pressure) and the pulsatile component (pulse pressure). Although pulse pressure varies along the arterial tree, we obtained comparable data for all  $F_2$  animals by assuring that measurements were taken at the same arterial location (Table 1). Diastolic blood pressure and pulse pressure have a relatively weak correlation in the  $F_2$  (0.18 compared to 0.84 between diastolic and systolic blood pressures; Fig. 1). This suggests that these two sub-phenotypes are modulated independently through different physiological mechanisms. The hypothesis that different genetic loci influence the two blood pressure components is confirmed by the strong linkage between diastolic blood pressure and *REN* on chromosome 13, and between pulse pressure and *CPB* on chromosome 2.

An increase in diastolic blood pressure is primarily a consequence of increased peripheral vascular resistance<sup>15</sup>, caused by a decrease in the calibre of small arteries due to vasoconstrictor agents, and secondary structural changes

of the arterial wall<sup>16</sup>. If *REN* is directly involved in LH hypertension, an increase in angiotensin II generation due to activation of the renin-angiotensin system might be responsible for the chronic elevation of diastolic blood pressure<sup>17</sup>. On the other hand, one of several mechanisms could be responsible for the elevation of pulse pressure<sup>15,18</sup>: (i) decreased distensibility of large arteries; (ii) increased volume or velocity of ventricular ejection; or (iii) modification of the reflected waves, arising from changes in calibre or elastic properties along the arterial tree. Although further data are needed to distinguish between the latter possibilities, we can conclude that at least two genetic mechanisms are important for the regulation of blood pressure in the LH rat.

Previously, linkage of *REN* with blood pressure was described in crosses involving the Dahl rat-model<sup>2,7</sup>, and weaker evidence for linkage has also been found in the spontaneously hypertensive rat (SHR)<sup>3</sup>. Our finding that *REN* is also linked to blood pressure in an  $F_2$  cohort from LH  $\times$  LN suggests that the same locus is implicated in hypertension in the Lyon model. If the marker is linked to the same haemodynamic parameter in the Dahl and Lyon crosses, this would represent a shared sub-phenotype characteristic of the two models. Since linkage has not been found between *REN* and blood pressure in a cross involving SHRSP<sup>19</sup>, this suggests that at least one genetic difference exists between hypertension in SHRSP, and that in the Lyon or Dahl models (and possibly SHR). On the other hand, linkage to the angiotensin-converting enzyme (*ACE*) gene on chromosome 10 exists in both the SHRSP and Dahl strains<sup>5-7</sup>. In our SHRSP data<sup>5</sup>, chromosome 10 markers exhibited linkage to systolic and diastolic blood pressure, but not to pulse pressure (unpublished result). Markers from this region were not informative in the Lyon cross, and no differences were found in more than 1,000 base-pairs of the non-coding region of the *ACE* gene that we sequenced in LH and LN. Therefore, the *ACE* region may be identical-by-descent in LH and LN, and not responsible for any of the genetic variance in blood pressure.

Linkage between blood pressure and another locus on chromosome 2 (guanylyl cyclase A or *GCA*) has been described in crosses involving SHRSP<sup>6</sup> and Dahl<sup>7</sup> strains. We did not detect differences between LH and LN at the *GCA* locus. In other crosses, *GCA* and *CPB* are not closely linked, and *CPB* is not correlated with blood pressure in the SHRSP  $\times$  WKY crosses we have characterized (unpublished results). Thus, it is likely that at least two distinct loci are involved in blood pressure variation in different crosses.

It is likely that other genetic factors influence blood pressure differences between the LH and LN strains. The comparison of blood pressures in LH, LN and the  $F_1$  hybrid cohorts suggests that inheritance of these phenotypes may be partially recessive. In particular, diastolic blood pressure is similar in the  $F_1$  and LN cohorts, whereas it is significantly higher in the LH cohort (Table 1). Since the LH gene at the *REN*-linked locus appears to have a codominant or dominant effect on diastolic blood pressure (Table 2), other genes may have an influence on this phenotype. Pulse pressure and the locus linked to *CPB* are both compatible with codominant inheritance, but we estimate that other loci could account for 43–80% of the genetic variance of pulse pressure in the  $F_2$ .

The present work extends the mapping of genetic loci

Table 3 Primer data for markers linked to blood pressure

Locus	Primer Sequence (5' to 3')
<i>REN</i>	AAGAAACACTGTCCTCCACTG GGCTATGGGTAGTATGATTAGAAGA
<i>CPB</i>	GTGCTAGTAGACAATAAGATAGATG CTCTCCAACCTATTCTTCATGAG



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involved in blood pressure by showing that it is possible to localize genes related to specific haemodynamic parameters. These parameters may constitute sub-phenotypes that provide new insights into the different physiological mechanisms that combine to produce hypertension. The design of new  $F_2$  studies will be orientated towards the understanding of the mechanisms by which this locus affects pulse pressure in Lyon rats, but will also include measurements of haemodynamic parameters such as cardiac output, aortic impedance and total peripheral resistance in order to find linkages with these sub-phenotypes involved in blood pressure modulation. This approach is clearly not possible in a large population of human hypertensive patients, but results obtained in the rat model can orientate clinical investigations in humans and could guide therapeutic management. In the present case, genetic linkage to pulse pressure provides an interesting clue in the search for major gene effects in human by segregation analysis. Such a finding could represent a first step towards the classification of hypertension into genetically determined sub-phenotypes.

# Methodology

**Blood pressure measurements.** Seven male LH and 11 female LN rats from the 36th–38th generation of our inbred colonies were randomly selected and mated to produce  $F_2$  hybrids. From this cross-breeding, 25 male and 34 female rats were chosen at random and mated to produce an  $F_2$  cohort. The animals were housed under controlled conditions (temperature  $21 \pm 1^\circ\text{C}$ , humidity  $60 \pm 10\%$ , 12-h day/night cycle), fed standard rat chow (UARAO3, Villemoisson sur Orge, France) containing 0.3% Na, and given free access to tap water. Direct blood pressure measurements were obtained on 345 male  $F_2$  rats at 29–31 weeks of age using a modification of our computerized technique<sup>20</sup>. Under anaesthesia, a catheter was inserted via the femoral artery into the lower abdominal aorta, and the rat was placed into an individual recording cage and allowed to recover for 24 h. The arterial catheter was connected to a blood pressure transducer (Statham P23ID, Gould Inc., Cleveland) via a rotating swivel which allowed the animals to move freely. Calibration of the instrument was verified before each measurement. Blood pressure measurements were recorded beat-by-beat for 3 consecutive hours during the day. The data were processed off-line as described<sup>21</sup>.

**Genotype determinations.** Genotypes for  $F_2$  animals were determined by the observation of length differences in PCR amplification products on denaturing sequencing gels. PCR amplification was performed by 29 cycles of:  $92^\circ\text{C}$  for 1 min,  $60^\circ\text{C}$  for 1 min, and  $72^\circ\text{C}$  for 30 s, preceded by a step in which a pre-prepared polycarbonate 96 well micro-titre plate (Technie) containing 35  $\mu\text{l}$  pre-mix (1  $\times$  PCR Buffer (NBL), 125  $\mu\text{M}$  dNTP, 50 ng DNA) covered with mineral oil is heated to  $94^\circ\text{C}$  for 4 min, at the end of which time 15  $\mu\text{l}$  of primer start (1  $\times$  PCR Buffer (NBL), 1  $\mu\text{M}$  each primer, 1 U *Taq* Polymerase I (NBL)) is added, and the reaction is brought to  $60^\circ\text{C}$  for 1 min, and  $72^\circ\text{C}$  for 1 min. PCR products were resolved on standard sequencing gels (6% polyacrylamide, 8 M urea, 135 mM Tris-HCl, 45 mM boric acid, 2.5 mM EDTA). Products were visualized by capillary transfer to positively charged nylon membrane (PALL), which were fixed in 0.4 M NaOH, neutralized in 5  $\times$  SSC, and hybridized in a 10% SDS/7% PEG solution for 3 h at  $42^\circ\text{C}$  with a 3' radio-labelled (Terminal Transferase Kit, Boehringer) PCR primer used in amplification. Membranes were then washed twice in 2  $\times$  SSC/0.1% SDS for 10 min at room temperature, and exposed to autoradiography.

**Linkage analysis and statistical methods.** A panel of 94  $F_2$  animals was selected for initial screening with the 26 informative marker loci. The panel was composed of equal numbers of animals from the upper and lower thirds of the blood pressure distribution, and a non-parametric test (Kruskal-Wallis test) was applied to determine if markers were potentially linked to blood pressure. Markers were characterized in all  $F_2$  animals when the initial test gave a  $p$ -value  $<0.05$ . In the total panel, the evidence for linkage was judged to be significant if  $p < 0.0001$  in an ANOVA test. Robust and non-parametric tests<sup>22</sup> gave similar results. Test statistics were also obtained by maximum likelihood linkage analysis with the LINKAGE programs<sup>23</sup> when the ANOVA test was significant. In the linkage analysis, a mixture of normal distributions with three trait locus means corresponding to LH/LH, LH/LN and LN/LN genotypes, and a common variance are estimated, in addition to a recombination rate between the marker and trait loci. Estimates for the other parameters were calculated for recombination values ( $\theta$ ) within a 1-lod-unit support interval of the maximum likelihood estimates ( $\theta = 0$  for both markers). These results were used to obtain upper limits for the proportion of phenotypic variance that could be attributed to linkage to the markers. Linear discriminant analysis was applied using the BMDP statistics package<sup>23</sup>. The degree of genetic determination was estimated as  $(V_g - V_e)/V_g$ , where  $V_g$  was the total variance of the phenotype in the  $F_2$  cohort, and  $V_e$  was a pooled estimate of the phenotype variance in the LN, LH and  $F_1$  cohorts.

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## DNA fingerprinting of spontaneously hypertensive and Wistar-Kyoto rats: implications for hypertension research

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Probes to hypervariable minisatellite regions of DNA identify multiple loci scattered over the autosomal chromosomes and produce a complex Southern blot pattern of fragments termed a DNA 'fingerprint'. As concern has been raised that different stocks of spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) may not be biologically identical, we have compared the DNA of SHR and WKY from several sources using two such probes which identify different sets of minisatellite sequences. While the DNA fingerprints of SHR from the various sources were identical, variability was observed in those of WKY, indicating genetic heterogeneity between different WKY stocks. In animals from one of the commercial suppliers even inter-rat variability in DNA fingerprints was seen, suggesting genetic heterogeneity within that single colony. These observations indicate that experimental results obtained using WKY from different sources may not be directly comparable and could provide an explanation for some of the conflicting data that exist on the comparative characteristics of SHR and WKY. In separate studies, direct comparisons both of the DNA fingerprints of SHR and WKY and of SHR and stroke-prone spontaneously hypertensive rats (SHRSP) showed multiple differences between the strains. The polymorphisms seen could provide useful linkage markers in locating the chromosomal sites of the genetic loci responsible for raised blood pressure in the SHR and the propensity to strokes in the SHRSP.

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**Keywords:** Spontaneously hypertensive rats, Wistar-Kyoto rats, stroke-prone spontaneously hypertensive rats, DNA fingerprinting, genetic heterogeneity, linkage markers.

### Introduction

The SHR [1] and its derivative, the SHRSP [2], have been extensively studied as models of human essential hypertension. As a normotensive control strain in such studies, the WKY, which was derived from the same outbred Wistar stock as the SHR, has been widely employed.

In the vast literature that has accumulated on the comparative characteristics of SHR and WKY there are several areas in which data from different laboratories are in conflict [3,4] and this has considerably slowed progress in the elucidation of the mechanism(s) of hypertension in the SHR. While many of these discrepancies probably reflect differences in method between studies, concern has been

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raised that some of the differences may be due to biological variability between different stocks of SHR and in particular WKY [4]. To date, evidence for biological variability between different stocks of SHR or WKY has primarily been based on the demonstration of differences in phenotypic characteristics among the different stocks [5–10]. With the development of recombinant DNA techniques that allow direct analysis of genomic DNA, it is now feasible to address more directly this important question. In this paper we present such an analysis of SHR and WKY, using two probes that identify distinct sets of hypervariable minisatellite sequences of DNA [11]. These probes (33.6 and 33.15), each consisting of tandem repeats of different versions of a core sequence, identify multiple loci scattered over the autosomal chromosomes in humans and other species [11,12] and therefore allow several genetic loci to be screened for variability (polymorphism) at the same time. The complex Southern blot pattern produced in such an analysis is termed a DNA 'fingerprint' [11,12]. Since DNA fingerprints show substantial germ-line (and somatic) stability [12], they should be identical for all animals in inbred strains, and differences would indicate genetic variability within such strains.

Major advances in our understanding of several single-gene and polygenic disorders have been made possible by the use of polymorphisms in DNA, detected by nucleic acid probes, as genetic linkage markers to locate disease loci. In this work, we therefore also assess the extent of differences in the DNA fingerprints of SHR, WKY and SHRSP which may help in the identification of the genetic loci responsible for raised blood pressure in the SHR and the propensity to strokes in the SHRSP.

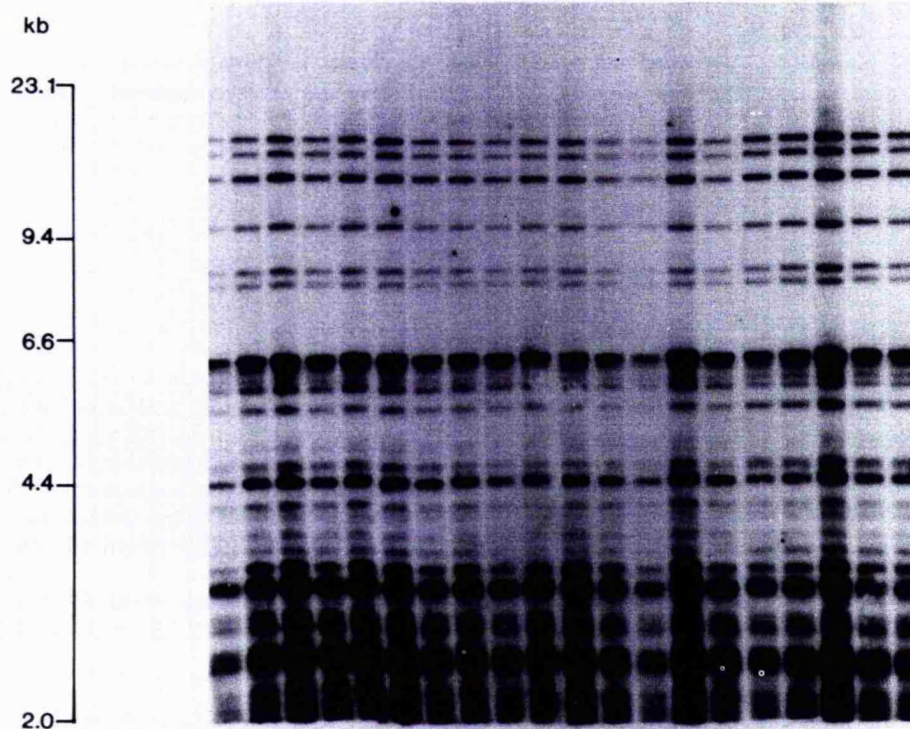
## Methods

### Animals

SHR and WKY from the following sources/suppliers were studied: (1) colonies maintained at the University of Leicester, Leicester, UK, since 1981 (derived from inbred colonies maintained by ICI Pharmaceuticals Ltd, Macclesfield, UK); (2) Charles River Ltd (Margate, UK); (3) Harlan Olac Ltd (Bicester, UK); (4) Taconic Farms (Germantown, New York, USA), and (5) Charles River Breeding Laboratories (Wilmington, Massachusetts, USA). SHRSP were from the colony maintained at the University of Heidelberg, Heidelberg, Federal Republic of Germany. WKY from this source were also studied.

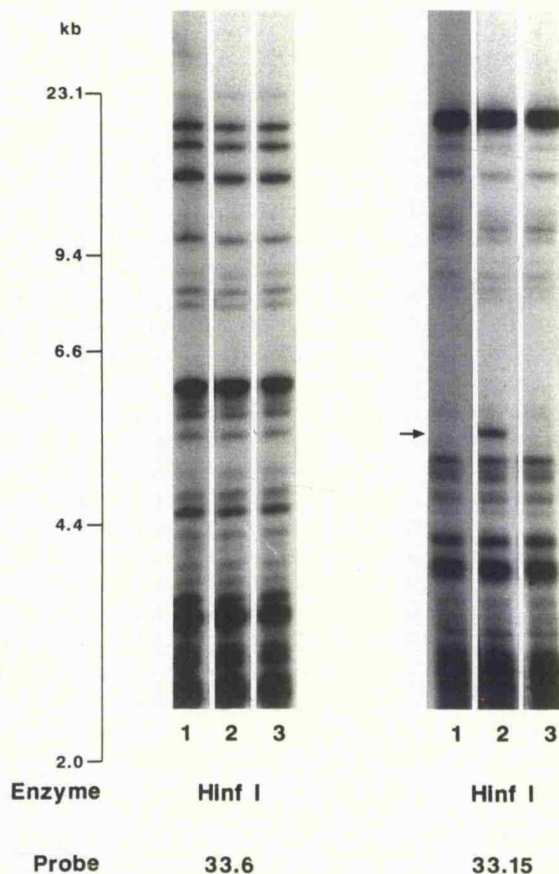
### DNA analysis

Ten-microgram samples of DNA, isolated from the livers or tails of animals using standard protocols [13,14], were digested with 20 units of the restriction enzyme *Hinf* I, under conditions recommended by the manufacturer (Gibco-BRL, Paisley, UK), and then ethanol-precipitated and size-fractionated on 20 cm-long 0.8% agarose gels as described previously [15]. Following denaturation, samples were transferred to nitrocellulose membranes (Hybond C, Amersham, Aylesbury, UK) by Southern blotting [16] and hybridized to  $\alpha$   $^{32}$ P-labelled human minisatellite probes 33.6 and 33.15 prepared by primer extension of single-stranded M13 templates as described elsewhere [11]. Hybridization and washing stringencies were  $1 \times$  SSC (equal to 0.15 mol/l NaCl and 0.015 mol/l  $\text{Na}_3$  citrate) at  $64^\circ\text{C}$ . Autoradiography was carried out at  $-70^\circ\text{C}$  using intensifying screens and Fuji-RX film (Fuji Photo Film Co.



**Fig. 1.** DNA fingerprints of 20 individual Wistar-Kyoto rats (WKY) from four different litters of rats from the Leicester WKY colony, detected by Southern blot hybridization of *Hinf* I digests of DNA with  $^{32}$ P-labelled human minisatellite probe 33.6. kb, kilobase.





**Fig. 2.** Comparison of DNA fingerprints of Wistar-Kyoto rats from the University of Leicester, UK (lane 1); Charles River Ltd, UK (lane 2), and Harlan Olac Ltd, UK (lane 3). kb, kilobase.

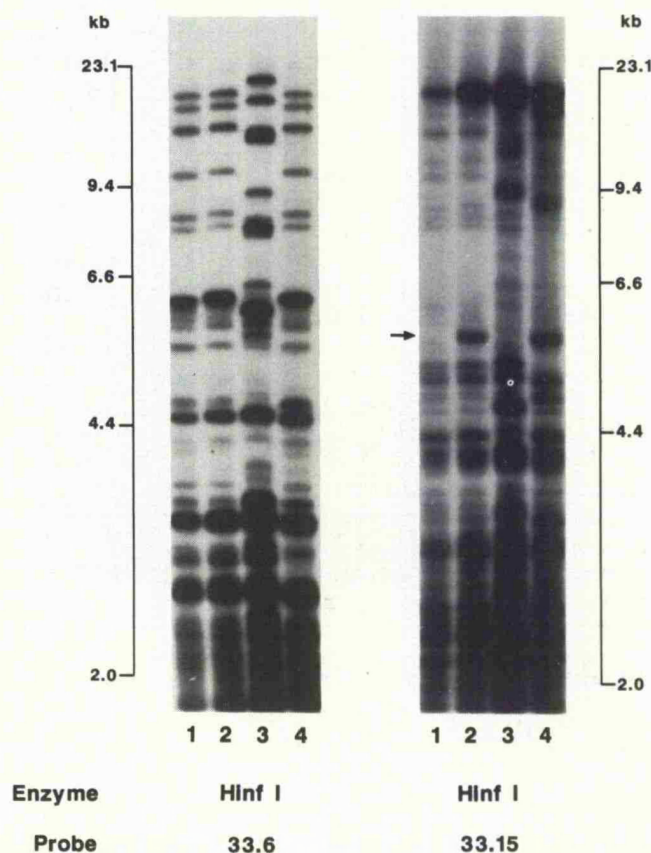
Ltd, Tokyo, Japan). Bacteriophage  $\lambda$  DNA digested with *Hind* III (Gibco-BRL) was used during electrophoresis as a molecular weight marker.

## Results

The probes used in this study, 33.6 and 33.15, contain tandem repeats of different versions of the human core sequence and detect different sets of human minisatellites [11,12,15]. Our results (Figs 1–8) show that both probes hybridize to multiple rat DNA fragments and produce a pattern of comparable complexity to that obtained with human DNA. As with human DNA, probes 33.6 and 33.15 produce different rat DNA fingerprints (Figs 2–8).

Figure 1 shows the DNA fingerprints obtained with probe 33.6 for 20 individual WKY from four different litters from the colony maintained in Leicester. The identical pattern observed for the different rats is indicative of genetic homogeneity in the rats of this colony and consistent with the strict brother-sister mating carried out. It also suggests

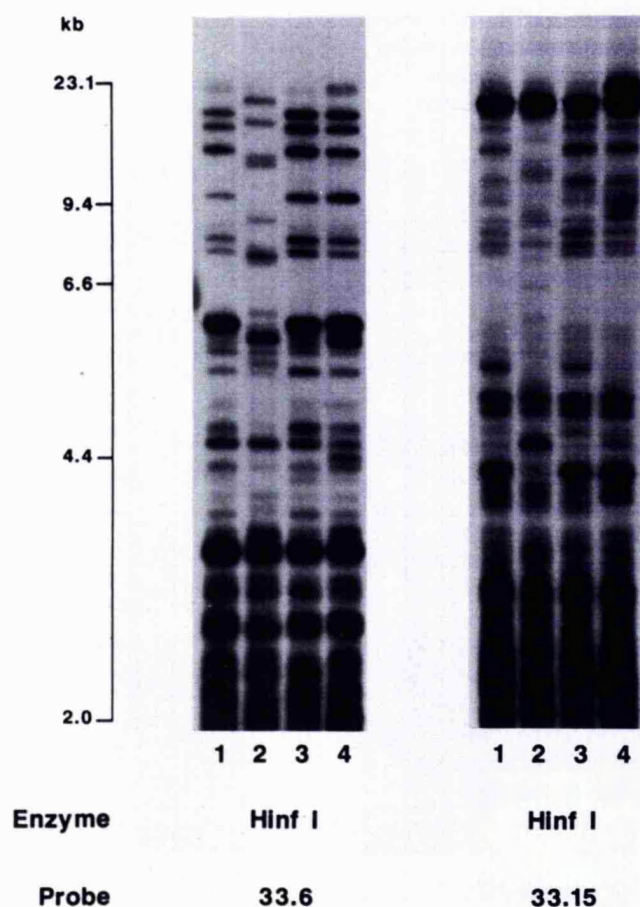
that most or all of the loci detected in the DNA fingerprints show substantial germ-line stability. The analysis included both male and female rats and no Y chromosome-specific fragments were observed.



**Fig. 3.** Comparison of DNA fingerprints of Wistar-Kyoto rats from the University of Leicester, UK (lane 1); Charles River Breeding Laboratories, USA (lane 2); Taconic Farms, USA (lane 3), and the University of Heidelberg, Federal Republic of Germany (lane 4). kb, kilobase.

Figures 2–4 show the DNA fingerprints of WKY from different sources. In Fig. 2, WKY from different UK sources have been compared. While the DNA fingerprints of WKY from Leicester (lane 1) and from Harlan Olac Ltd (lane 3) were identical (which is consistent with the fact that both colonies are derived from an inbred colony which was maintained at ICI Pharmaceuticals), at least one difference (see arrow, Fig. 2) was present in the DNA fingerprint of WKY from Charles River Ltd compared with these rats. Figure 3 shows a comparison of the DNA fingerprints of WKY from Leicester, from the two USA suppliers, and from those maintained at the University of Heidelberg. Multiple differences were present in the DNA fingerprint of the Taconic Farms WKY (lane 3) compared with the others. While the DNA fingerprints of the Leicester rat (lane 1), the Charles River Breeding Laboratories rat (lane 2) and the Heidelberg rat (lane 4) were very similar with probe 33.6, some differences were seen with probe 33.15. It is interesting to note that a fragment of similar size ( $\sim 5.8$  kilobase) to the strongly hybridizing fragment seen in the DNA fin-





**Fig. 4.** Comparison of DNA fingerprints of two different Wistar-Kyoto rats from each of Charles River Breeding Laboratories, USA (lanes 1 and 3), and Taconic Farms, USA (lanes 2 and 4). kb, kilobase.

gerprint of WKY from Charles River Ltd, but not the other UK rats (see arrow, Fig. 2), was also present in the DNA fingerprints of WKY from both the Charles River Breeding Laboratories and the University of Heidelberg (see arrow, Fig. 3). In order to confirm the observation regarding the Taconic Farms WKY, further animals from the two USA suppliers were analysed. Figure 4 shows the DNA fingerprints of two different WKY from each of Charles River Breeding Laboratories and Taconic Farms (DNA in lanes 1 and 2 are the same as those compared in Fig. 3, lanes 2 and 3). Somewhat surprisingly, differences were observed not only between the rats from the two sources (lanes 1 and 3 versus lanes 2 and 4) but also between the two Taconic Farms rats (lanes 2 and 4), suggesting genetic heterogeneity within the WKY colony maintained by this supplier. The DNA fingerprints of the two Charles River Breeding Laboratories rats were identical.

Figures 5 and 6 show a comparison of SHR from different sources. In contrast to the situation with WKY, the DNA fingerprints observed with both probes were identical for the SHR examined from the various sources.

In Fig. 7, the DNA fingerprints of SHR and WKY are directly compared. While the two strains have several common hybridizing fragments, multiple differences are also

present in their DNA fingerprints. The potential use of the differences in identifying the chromosomal sites of the genetic loci responsible for hypertension in the SHR is discussed below.

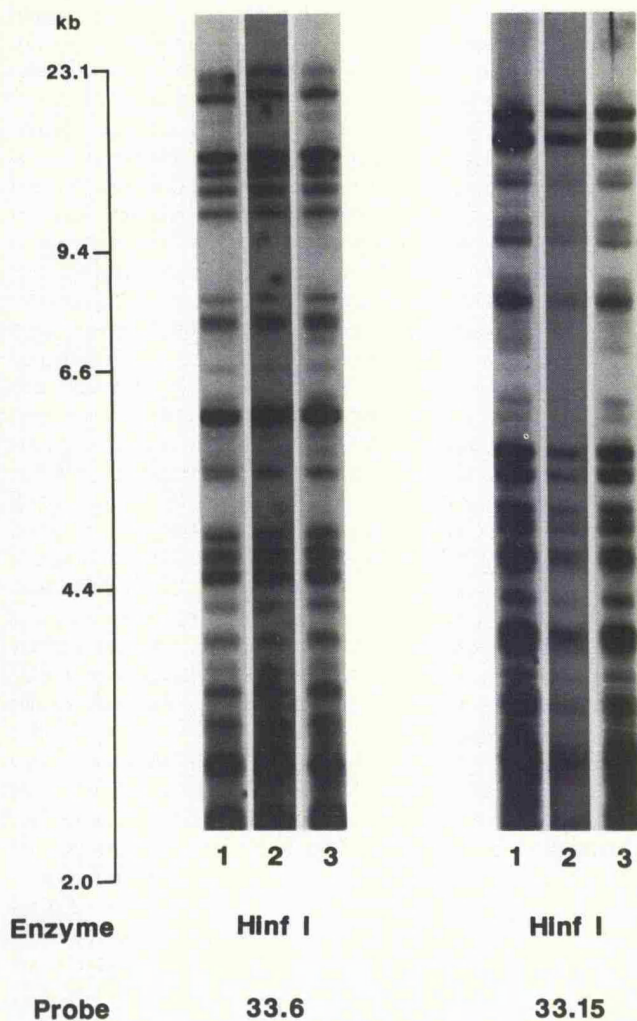
Figure 8 shows a comparison of DNA fingerprints of SHR and SHRSP. In a preliminary study we had confirmed that the DNA fingerprints of several different SHRSP (Heidelberg) rats were identical (data not shown). The possible significance of the differences observed in the DNA fingerprints of SHR and SHRSP is also discussed below.

In Figs 1–8, we show DNA fingerprints obtained following digestion of DNA with the restriction enzyme *Hinf*I. Enzymes which have a 4-base-pair-recognition sequence and are therefore likely to cut DNA frequently (randomly, there should be sites every 256 bases) are used with the DNA fingerprinting probes to maximize the resolution of the polymorphic minisatellites [11,12,15]. Apart from *Hinf*I, other enzymes such as *Alu*I and *Hae*III (both from Gibco-BRL) can also be used [12]. While the minisatellites recognized are the same as those seen following digestion with *Hinf*I, the DNA fingerprint patterns observed are different because of the generation of different sized fragments containing the minisatellites following digestion with *Alu*I and *Hae*III. We have also compared the DNA fingerprints of SHR and WKY from different sources following digestion with *Alu*I and *Hae*III, and these comparisons have confirmed the observations made with *Hinf*I (data not shown).

## Discussion

The ability to detect variability at the level of genomic DNA in the form of restriction fragment length polymorphisms (RFLPs) following the digestion of DNA with restriction endonucleases and the use of such RFLPs as genetic markers has led to enormous advances in the understanding of a wide variety of conditions. RFLPs can arise as a consequence of a number of different mechanisms: they commonly occur as a result of change in the DNA sequence (often a single base) resulting in the gain/loss of a restriction endonuclease cleavage site; they may also occur as a result of a deletion or insertion of DNA between two restriction endonuclease cleavage sites. The above two mechanisms often result in dimorphisms whose usefulness as genetic markers is limited by their low heterozygosity. Dispersed throughout the genome of human and other vertebrates there are also areas (minisatellites) consisting of tandem repeats of a short nucleotide sequence. These sites can provide multiallelic RFLPs as a consequence of variability in the number of repeats. Cloned human minisatellites capable of detecting single hypervariable regions (HVRs) have been developed [17,18] but are only of use with human DNA. In contrast, the probes used in this study (33.6 and 33.15) detect simultaneously multiple HVRs in the DNA of many species and produce the complex Southern blot pattern that has been termed a 'DNA fingerprint' [11,12,15]. Since its first description in 1985 [11], DNA fingerprinting has found several applications in humans not only because of the individual specificity of DNA fingerprints but also because it is possible to trace the inheri-



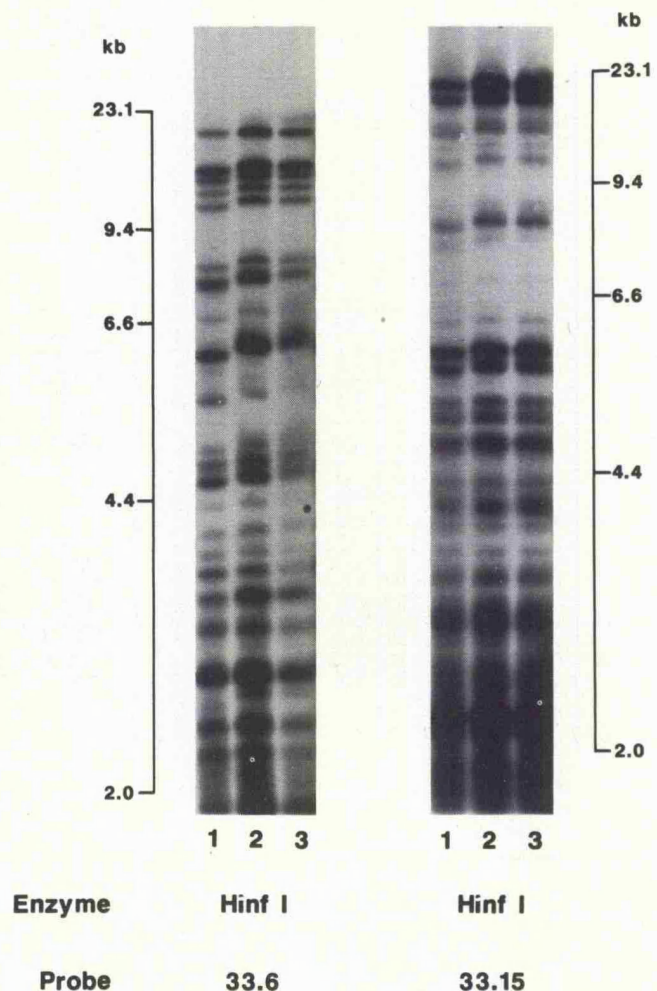


**Fig. 5.** Comparison of DNA fingerprints of spontaneously hypertensive rats from the University of Leicester, UK (lane 1); Charles River Ltd, UK (lane 2), and Harlan Olac Ltd, UK (lane 3). kb, kilobase.

tance of DNA fingerprints [12]. Both probes 33.6 and 33.15 have also been shown to hybridize to multiple variable DNA fragments from a wide variety of animals, suggesting several other potential areas of application [12].

In this study we have utilised probes 33.6 and 33.15, which detect different sets of HVRs, to study the genetic homogeneity of SHR and WKY from different sources. When data on SHR and WKY from different investigators have been compared there has usually been a tacit assumption that the two strains are fully inbred and that animals from different sources are genetically homogeneous. Such an assumption has been challenged by several studies that have shown phenotypic differences between WKY or SHR from different sources [4–10], including some of those used in this study [4,10]. We have now shown that there are differences in the DNA fingerprints of WKY from different sources at a level not attributable to *de novo* mutation of the variable loci detected. In particular, the DNA of WKY obtained from Taconic Farms showed several differences from that of WKY from other sources. An added

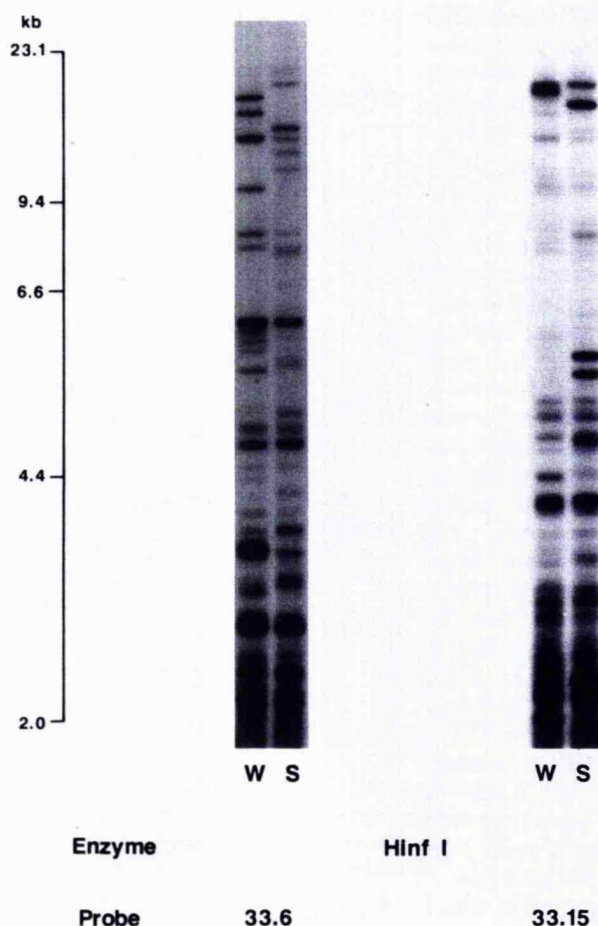
observation was that the two WKY from Taconic Farms also showed marked differences in their DNA fingerprints.



**Fig. 6.** Comparison of DNA fingerprints of spontaneously hypertensive rats from the University of Leicester, UK (lane 1); Charles River Breeding Laboratories, USA (lane 2), and Taconic Farms, USA (lane 3). kb, kilobase.

There are several possible explanations for the differences observed. It is possible that some of the rats analysed were not WKY, but some other strain. We consider this to be very unlikely, as the extent of the variability observed was much less than we have observed between different strains of rats (data not shown). Another possibility is that there has been genetic contamination of the WKY from one source by another strain. Such contamination has been observed with a number of commercially available inbred strains [19,20]. While we cannot exclude this possibility, especially that of a contamination in the distant past, we also consider this to be unlikely because of the limited number of differences seen between WKY from separate sources compared with those seen between different rat strains. A third possible explanation is that of spontaneous mutation in the WKY from one source which was then propagated within that colony, independent of





**Fig. 7.** Comparison of DNA fingerprints of Wistar-Kyoto rats (W) and spontaneously hypertensive rats (S). Animals were obtained from the University of Leicester colonies. kb, kilobase.

other colonies. While most of the minisatellites detected by probes 33.6 and 33.15 show germ-line stability, an appreciable rate of mutation to new-length alleles has been observed at some hypervariable loci in human pedigrees [21], and a locus showing marked instability has been demonstrated in the mouse [22]. However, the most likely explanation for the differences observed in the DNA fingerprints of WKY from different sources is that these rats were distributed to different suppliers before they were fully inbred [4,6] and hence the differences observed are at those loci that were still segregating at the time of the animals' distribution. A possible explanation for the differences observed between the DNA fingerprints of the two Taconic Farms WKY could be a combination of the above and the fact that their WKY colony has not been maintained by strict sib mating [4]. While inter-rat variability was not observed in the DNA fingerprints of the two WKY examined by Charles River Breeding Laboratories, too few rats were examined from this and the other sources (except the Leicester colony, as seen below) to adequately assess their DNA fingerprint homogeneity.

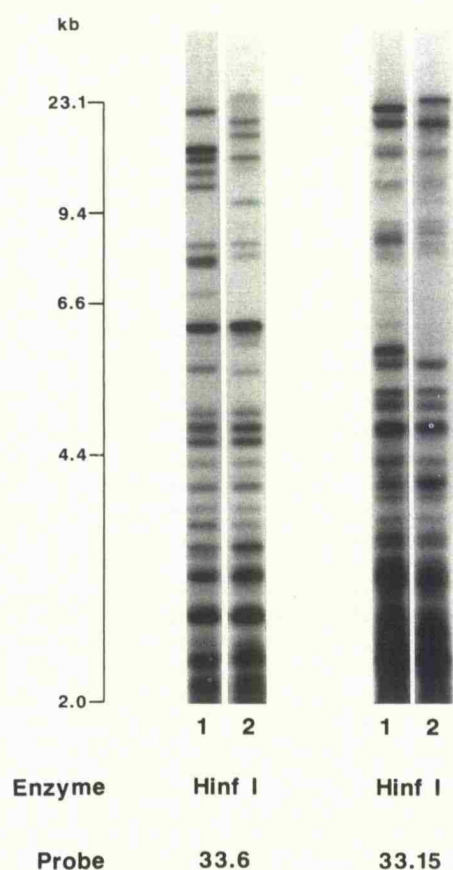
In contrast to the WKY, we found the DNA fingerprints of SHR from different sources to be identical. This is consistent with the fact that the SHR were fully inbred before they were widely distributed [4]. The identity of DNA fingerprints of SHR reared in different colonies again emphasizes the germ-line stability of DNA fingerprints. However, it has to be stated that we have examined a very limited number of rats (one or two) from the different sources and it is possible that differences exist which have been missed. A study of larger numbers of animals from the different sources is currently underway. It is also very important to emphasize that identity of DNA fingerprints does not necessarily imply genetic homogeneity. Although the probes detect multiple loci, only a fraction of the genome is covered. Several differences have been reported between SHR from different sources [5,8-10] and some of these may well reflect genetic variability not detected by DNA fingerprinting. In this study, we were particularly interested in comparing the DNA fingerprints of SHR from Charles River Breeding Laboratories and Taconic Farms, since a recent study [10] has shown that the rats from these two sources differ in their hypertensinogenic response to NaCl administration. In the animals we studied, however, we did not observe any differences in the DNA fingerprints which might have provided a clue to the basis of this differential response to NaCl.

Recently, Kurtz *et al.* [23] have reported on a comparison of SHR and WKY from Taconic Farms and Charles River Breeding Laboratories using an oligonucleotide probe corresponding to the core sequence of 33.6. They found, as we did, that there were differences in the DNA fingerprints of WKY from the two sources and that there was inter-rat variability in the animals from Taconic Farms. No differences were observed in the DNA fingerprints of the SHR. Thus their findings in these animals are in agreement with ours.

In this study we have also shown the usefulness of DNA fingerprinting in monitoring the genetic homogeneity of an inbred colony by demonstrating that the DNA fingerprints of rats from such a colony (the WKY colony at Leicester) are identical. DNA fingerprinting is particularly likely to quickly identify any genetic contamination of an inbred strain, as this will result in substantial changes in the pattern observed. At the moment, such monitoring relies on the laborious screening of a large panel of immunological, morphological and biochemical markers, each exhibiting very limited polymorphism [24].

Despite intensive study, the nature of the genetic abnormalities which predispose the SHR to hypertension remains unclear. Several studies [25-28] have shown that a limited number of genetic loci (between one and four) code for the hypertension found in the SHR. Co-segregation of a number of traits with blood pressure at the biochemical/physiological level has been observed in the SHR [29-32], which allows some inferences about the genetic causes of the high blood pressure. A new source of markers to study the genetics of hypertension in the SHR is now becoming available in the form of RFLPs. Differences between SHR and WKY are being defined in several genes [33-35] which will be useful as markers in linkage analysis. In this study we have shown that differences exist be-





**Fig. 8.** Comparison of DNA fingerprints of spontaneously hypertensive rats (SHR; lane 1) and stroke-prone SHR (SHRSP; lane 2). SHR were obtained from the University of Leicester and SHRSP from the University of Heidelberg. kb, kilobase.

tween the DNA fingerprints of SHR and WKY which should provide an additional source of such linkage markers.

Although multiple differences were observed between the DNA fingerprints of SHR and WKY, it is not possible to define at this stage how many independent markers are available, as not all of the fragments may necessarily come from different loci [12,15,22]. Breeding studies are currently underway to determine the number of independent loci being detected. Another aspect which also needs to be resolved is the chromosomal distribution of the HVRs detected by probes 33.6 and 33.15. While in the mouse at least eight out of 13 loci detected by these probes were found to be interstitial and dispersed in location [22], in humans it is currently estimated that ~70% of minisatellites are clustered in the proterminal regions of chromosomes [36]. If a large population of the minisatellites detected in the rat show a similar preferential localization to one region of the chromosomes, this would limit their usefulness as linkage markers. The ideal method of mapping the loci responsible for raised blood pressure in the SHR would be to have a complete RFLP linkage map for the rat and to carry out the sort of analysis described by Lander and Botstein [37]. Unfortunately, the linkage map currently

available in the rat is far from complete, although the loci detected by probes 33.6 and 33.15 and those detected by probes to other minisatellite sequences [11,12] may help considerably towards achieving this aim.

During the inbreeding of the SHR by Okamoto and colleagues, substrains with differing tendencies to cerebrovascular lesions were observed, and selection for those animals that developed strokes allowed the strain of the SHRSP to be established [2]. Studies in SHRSP have shown that the incidence of strokes in these animals is closely related to the severity of the blood pressure developed and to a more rapid rise in blood pressure at a younger age compared with SHR [38]. The involvement of genetic factors other than those that regulate blood pressure in the tendency to strokes has not been clarified [38]. In this study we have shown that differences exist between the DNA fingerprints of the SHRSP (from the colony in Heidelberg) and the SHR. While these differences may have little to do with the peculiar characteristic (a propensity to strokes) of the SHRSP and may simply reflect the random fixation of other segregating alleles during its selection, or even subsequent changes within the Heidelberg SHRSP colony itself, it is possible that they are linked to the genetic sites that define the propensity to strokes in the SHRSP and therefore merit further genetic and molecular analysis.

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## The renin gene in patients with malignant hypertension and raised plasma renin activity

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### SUMMARY

1. We have examined the hypothesis that the raised plasma renin activity in patients with malignant hypertension without an underlying cause is the consequence of expression of a duplicate renin gene.

2. DNA extracted from leucocytes of patients with malignant hypertension and of normotensive controls was digested with the restriction endonuclease *Pst*I and hybridized with a radioactively labelled human renin complementary DNA probe. As an internal control the DNA was concurrently hybridized with a human *c-myc* proto-oncogene probe.

3. The signals for each subject from the two probes were quantitatively compared by densitometry.

4. There was no evidence of duplication of the renin gene in the patients with malignant hypertension.

**Key words:** gene duplication, malignant hypertension, renin gene.

**Abbreviations:** cDNA, complementary DNA; PRA, plasma renin activity.

### INTRODUCTION

Malignant hypertension is defined by very high blood pressure in association with a rapid deterioration in renal function, characteristic fundal changes and a poor prognosis if untreated [1]. Pathologically, the hallmark of the condition is a fibrinoid necrosis affecting the arterioles [2].

Although malignant hypertension can occur in secondary forms of hypertension, the largest group comprises patients in which no underlying cause can be found [3]. A common feature not only in those cases complicating renovascular disease but in cases of 'primary' malignant hypertension is a raised plasma renin activity (PRA) [4-6], and the role played by the renin-angiotensin system in the pathogenesis of malignant hypertension has been much debated [7, 8]. In those cases without an underlying cause,

the mechanism by which PRA is elevated has not been elucidated, although it is commonly attributed without conclusive proof to be a consequence of hypertensive vascular damage to the microcirculation of the kidneys [7]. It is proposed that a vicious circle is set up by the renin causing a further increase in blood pressure.

In this study we examine an alternative hypothesis, namely that the raised PRA in such cases is a consequence of a duplication of the renin gene and therefore that the primary event leading to malignant hypertension in these cases is the 'switching on' of expression of a duplicate renin gene. The study has been prompted by two recent observations: first, the finding that certain strains of mice have a duplication of the renin gene on chromosome-1 [9], and secondly that in some patients with another sporadic condition, Alzheimer's disease, there is duplication of the  $\beta$ -amyloid gene on chromosome-21, which may be of crucial importance to the pathogenesis of that condition [10].

### MATERIALS AND METHODS

#### Patients and controls

Patients attending the hypertension clinic at Leicester Royal Infirmary were used in this study. All had presented with malignant hypertension for which no secondary cause had been found at initial investigation or subsequent follow-up of several years.

Control patients, matched for age and sex, were chosen from patients with non-cardiovascular disorders attending a general medical clinic. All had had blood pressure readings measured in the normal range on several occasions, and none had a family history of hypertension. All patients and controls were caucasian.

#### PRA

PRA had been measured by radioimmunoassay of generated angiotensin I in all the hypertensive patients before the start of treatment as part of a previous study [6].

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### DNA analysis

**DNA extraction.** Ten millilitres of venous blood were collected in 0.1% (w/v) potassium-ethylenediaminetetraacetate and stored at  $-70^{\circ}\text{C}$ . Samples were thawed at  $37^{\circ}\text{C}$ , mixed with 1 vol. of  $1\times\text{SSC}$  (150 mmol/l NaCl, 15 mmol/l trisodium citrate, pH 7.0) and centrifuged at 10 000 g for 15 min to pellet leucocytes and nuclei. DNA was then prepared from the leucocytes by the method described by Jeffreys & Morton [11]. All the patients and controls had a normal total and differential white cell count.

**Probes.** Renin. A 640 base-pair *EcoRI*-*SacI* human kidney renin complementary DNA (cDNA) fragment comprising the sequence from base 700 to 1340 of the full-length human renin cDNA [12] was used. The fragment had been isolated by Dr John Mullins, University of Leicester, from a human kidney cDNA library constructed in the vector M13mp11 [13] (J. Mullins, personal communication). The fragment spans the portion of the human renin gene coding for exons 7–10 [14].

*c-myc*. As an internal control (see the Results section) a 770 base-pair *Clal*-*AhaIII* genomic fragment in the region of exon III of the human *c-myc* proto-oncogene [15] was used.

Both probes were radioactively labelled using deoxycytidine 5'-[ $\alpha$ - $^{32}\text{P}$ ]triphosphate (3000  $\mu\text{Ci}/\text{mmol}$ , Amersham, U.K.) by the random oligo-primer method of Feinberg & Vogelstein [16]. For each probe, 10 ng of DNA fragment was used per reaction and incorporation in excess of  $1\times 10^9$  c.p.m./ $\mu\text{g}$  was usually obtained. The whole of the reaction product for each probe was used per hybridization.

**Southern blotting, hybridization and autoradiography.** About 5  $\mu\text{g}$  of DNA from each subject was digested with the restriction endonuclease *PstI*. Digested samples were electrophoresed on a 0.8% (w/v) agarose gel and the DNA transferred to Hybond N membrane (Amersham, U.K.) by Southern blotting [17]. Pre-hybridization, hybridization to probes, and post-hybridization washing-off of excess probe were carried out as recommended by the membrane manufacturer. Autoradiography was performed at  $-70^{\circ}\text{C}$  using Kodak X-Omat AR film and intensifying screens.

**Analysis of signals.** Quantification of autoradiographic signals was carried out using an LKB laser densitometer (LKB 2202; LKB Produkter AB, Bromma, Sweden). Each signal was scanned at five positions along its length and the resulting curves were merged and averaged. The intensity of each signal was calculated (in arbitrary units) as the area under the curve.

## RESULTS

### Patients and controls

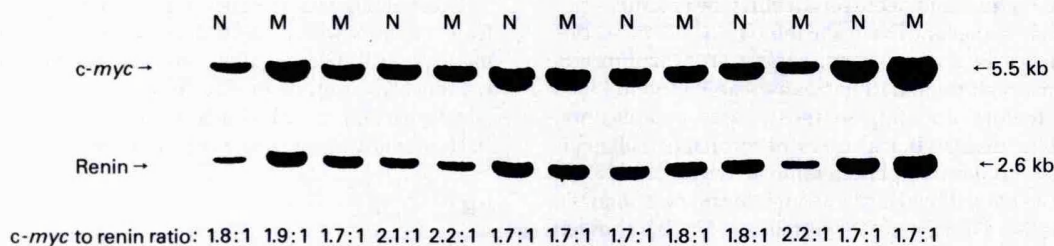
Seven hypertensive patients (five males, two females) and six controls (four males, two females) were investigated (the same control was used for two hypertensive males of the same age). The age range was from 40 to 65 years (mean  $\pm$  SD for whole group,  $55.8 \pm 7.8$  years). The mean ( $\pm$  SD) supine blood pressure at presentation for the hypertensive group was  $232 \pm 20.2$  mmHg ( $30.9 \pm 2.7$  kPa) systolic and  $143 \pm 9.9$  mmHg ( $19.1 \pm 1.3$  kPa) diastolic, and for the control group was  $127 \pm 17.0$  mmHg ( $16.9 \pm 2.3$  kPa) systolic and  $76 \pm 8.0$  mmHg ( $10.1 \pm 1.1$  kPa) diastolic.

### PRA levels

The mean PRA for the hypertensive patients was 8.67 pmol of angiotensin I  $\text{h}^{-1} \text{ml}^{-1}$  with a range of 2.5–19.1 pmol for angiotensin I  $\text{h}^{-1} \text{ml}^{-1}$ . This compares with a mean PRA of 1.54 (range 0.15–3.5) pmol of angiotensin I  $\text{h}^{-1} \text{ml}^{-1}$  in a group of unselected normotensive subjects and a mean PRA of 1.67 (range 0.15–9.1) pmol of angiotensin I  $\text{h}^{-1} \text{ml}^{-1}$  in patients with essential hypertension measured in the same laboratory [18]. Apart from one patient, all the others had a PRA above the upper limit for the normotensive group.

### DNA analysis

Experiments were first carried out to identify the restriction endonuclease most suitable for this study by hybridization of DNA digested with a number of enzymes with both the renin and *c-myc* probes. Digestion with *PstI* was considered the most suitable as it gave a single band



**Fig. 1.** Southern blot of DNA from patients with malignant hypertension (M) and normotensive controls (N) digested with *PstI* and hybridized concurrently to a human renin cDNA probe and a human *c-myc* probe. Abbreviation: kb, kilobases.

of 2.6 kilobases with the renin probe and a single band of 5.5 kilobases with the *c-myc* probe. No polymorphisms have been described at either locus with this enzyme ([19], J. Varley, personal communication).

Fig. 1 shows the results of the hybridizations with the two probes using DNA from the subjects of this study. For each subject a ratio of *c-myc* to renin signal strength was calculated from the areas under the curve for the two peaks. For all subjects the ratio lay between 1.7:1 and 2.2:1. The mean ratio for the control group was 1.8:1. Assuming that all the normal controls had a single renin gene on each of their two chromosomes, a single gene duplication on only one of the two chromosomes in any patient would have resulted in a ratio around 1.2:1 in this particular study. More copies of the renin gene would have further decreased the *c-myc* to renin ratio. The findings therefore indicated that there was no amplification of the renin gene in any of the patients.

To assess the precision of the methods employed we have isolated DNA from blood collected from the same subject on different occasions and analysed the different DNA isolates with the *c-myc* and renin probes as described above. These studies have shown that when run together the different isolates vary by less than 10% in their *c-myc* to renin ratios.

It must be emphasized that comparison of signals from different subjects in such a study as ours is only valid for samples that have been hybridized together with the same labelled probes. This is because the efficiency of radioactive labelling of each probe may vary considerably from one occasion to the next and the relative signals obtained with the two probes and hence the ratio will vary.

## DISCUSSION

As a mechanism of creating evolutionary diversity and change, gene duplication followed by modification of the duplicate gene is well established [20]. A major role for somatic gene amplification of oncogenes as a determinant of tumour prognosis and resistance to cytotoxic drugs is also becoming increasingly clear [21]. Pertinent to the present study is the recent demonstration of duplication of part of chromosome-21 containing the  $\beta$ -amyloid gene in leucocyte DNA of patients with sporadic Alzheimer's disease and also those with karyotypically normal Down's syndrome [10]. Premature deposition of  $\beta$ -amyloid protein in the brain is considered to be an important pathogenetic feature of Alzheimer's disease as well as Down's syndrome [22], and it is argued that this may be a consequence of the excess gene dosage [10].

This study was also prompted by the finding that certain strains of mice have a duplication of the renin gene on chromosome-1 [9]. The duplicate gene (*Ren-2*) is expressed with a different tissue specificity from that of the primary gene and is under hormonal influence. Levels 100-fold higher than in the kidney are seen in the submandibular glands of the two-gene mouse strains as a consequence of androgen-regulated expression of *Ren-2* [23].

The mechanism(s) leading to the development of malignant hypertension remains unclear. A particular controversy surrounds the role of the renin-angiotensin system in the pathogenesis of the characteristic vascular lesions. One school of thought implicates the height of the arterial pressure alone as the dominant factor accounting for damage to the arterial wall, and suggests that any role that renin may have is exclusively through further raising blood pressure [7]. The other argues for a more fundamental role and proposes that humoral factors and in particular the renin-angiotensin system has a direct vasculotoxic effect and may produce the vascular changes seen in malignant hypertension independently of effects on the blood pressure [1, 8].

In this study we have looked at patients with malignant hypertension without an underlying cause. What triggers the hyper-reninaemia in this situation is unclear. Some have argued that it is a consequence of damage to the microcirculation of the kidneys [7]. Fibrinoid necrosis can be demonstrated in the afferent glomerular arteriole in malignant hypertension [2] and it is possible that this lesion changes the functional properties of the juxtaglomerular apparatus although there is no direct evidence on this point. An alternative possibility that has been suggested [1, 8] is that renin secretion is stimulated by a negative sodium balance that occurs as a result of perfusion pressure natriuresis and diuresis at the onset of malignant hypertension.

Given the observations in Alzheimer's disease and in the mouse, and particularly in view of the proposed direct vasculotoxic effect of renin, we felt that it was important to investigate the possibility that the hyper-reninaemia in such patients is a consequence of the expression of a duplicate renin gene. On the basis of this hypothesis the triggering event leading to development of malignant hypertension would be a 'switching on' of expression of the duplicate gene. The increased renin would then lead to the changes of malignant hypertension by one or other of the mechanisms discussed above. In the patients studied no evidence of duplication was seen. Although a much larger group of patients with malignant hypertension would need to be studied to ensure that there is no subset of patients in whom renin gene duplication is the underlying mechanism, our findings exclude the possibility that it is the universal (or even a common) cause of the hyper-reninaemia in these patients.

Such an explanation could remain consistent with the sporadic occurrence of malignant hypertension, since not all bearers of any heritable amplification of the renin gene would necessarily develop the disease; this would depend on the effect of environmental and other genetic factors on the expression of the additional renin gene(s). On treating malignant hypertension renin levels fall [24] and since conventional wisdom has been that this is due to a reversal of intrarenal vascular damage this may also appear to be against the hypothesis considered here. However, there is no direct proof for this and indeed in malignant hypertension complicating experimental renovascular hypertension Möhring *et al.* [25] have shown that saline repletion, which lowered PRA, led to reversal of

the changes of malignant hypertension without affecting blood pressure.

We used the *c-myc* gene as an integral control to guard against the problem of different DNA loadings from different subjects. Although somatic amplification of the *c-myc* gene has been observed in several tumour types [26, 27], there is no evidence from examination of the leucocyte DNA of patients with such tumours of an inherited variability in the number of *c-myc* genes. There was no history of malignant disease in any of the patients or controls in this study.

Studies in twins have revealed strong heritable influences affecting PRA under a variety of fluid balance conditions [28]. It has been shown recently for another circulating product, fibrinogen, that genetic variation at the fibrinogen locus is important in determining plasma concentrations [29]. One therefore needs to consider whether any alterations in the renin gene are responsible for some of the observed variability in PRA in the population [18]. In the absence of evidence for gene amplification ([19, 30] and this study), the relationship of PRA to the variations in the renin gene as defined by the restriction fragment length polymorphisms that have been described [19, 30] needs to be investigated. This may have particular relevance to essential hypertension as patient groups with high, normal and low PRA have been defined in this condition and attempts made [6, 18, 31, 32] to attach pathogenetic, prognostic and therapeutic significance to these groupings.

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