Brief Review

Structure, Expression, and Regulation of the Murine Renin Genes

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It has long been known that the renin-angiotensin system plays an integral role in the regulation of blood pressure and electrolyte and fluid balance in mammals. The advent of molecular biologic techniques has afforded new insights into the genes regulating blood pressure. Laboratory mice and rats have been used as experimental models to examine the structural organization and expression of the renin gene. It is now well established that some mice, unlike rats and humans, contain a duplicated copy of the renin locus, which accounts for the high level of renin activity long known to be found in the submandibular gland of some mice. Indeed it is this fortuitous observation that facilitated the isolation of the first complementary DNA clones for renin and ultimately the many species-specific probes now available to analyze mammalian tissues for evidence of primary renin expression. The use of complementary DNAs as probes for primary renin expression helped confirm and further clarify earlier studies demonstrating the presence of renin activity in a number of extrarenal tissues. Although expression in some of these tissues is evolutionarily conserved, their significance has still been elusive. In this report we review the impact of molecular biology on our current understanding of renin gene structure and organization, tissue- and cell-specific expression and regulation, and the changes in renin expression throughout ontogeny. In addition, we describe how new developments in gene transfer technology have added important tools to our arsenal for examining renin gene regulation and how these technologies can be used to develop new tools for renin and hypertension research. (Hypertension 1991;18:446-457)

For several decades the renin-angiotensin system (RAS) has been the subject of intensive investigation (see Reference 1 for review). This interest stems from its role in regulating blood pressure and electrolyte balance and its potential involvement in at least some forms of hypertension. Renin is an aspartyl protease whose only known substrate is angiotensinogen. Early attempts to purify renin from the kidney, its classic site of synthesis, were hampered due to its instability in the presence of copurifying proteases. Furthermore, kidney homogenates contained only small amounts of renin. Nevertheless, several laboratories had early success in partially purifying renin.2-8 Werle et al9 and Cohen et al10 reported the complete purification of reninlike enzymes from the mouse submandibular gland (SMG) that eventually were positively identified as renin based on a number of biochemical criteria.11,12 (See Reference 13 for review.) With the purification of mouse SMG renin, new methodologies were developed that led to the purification of true renal renin from a variety of species.14-21

Part of the success in purifying SMG renin was due to the fortuitous finding that some but not all mice contain copious amounts of SMG renin. Bing and colleagues22,23 demonstrated that SMG renin levels in mice are controlled genetically. Two allelic designations for what was termed the renin regulatory locus (Rnr) were assigned to mice based on their SMG renin levels: Rnr a (SWR) and Rnr b (C57BL/6) for strains with high and low SMG renin, respectively.24 Basal renin levels in both types of strains are inducible with androgen25 and the control of renin activity and synthesis was shown to be genetically indistinguishable.26 Genetic analysis revealed that the renin structural genes also resided on chromosome 1 and were tightly linked to the Rnr locus.26-28 Chirgwin et al29 also demonstrated the localization of the renin locus to chromosome 1 by somatic cell genetics.

Evidence for a gene duplication at the Rnr locus began to resolve the difference in SMG renin activity between Rnr a and Rnr b mice.26-31 With the molecular cloning and subsequent sequence analysis of mouse SMG and kidney renin complementary DNA clones for renin and ultimately the many species-specific probes now available to analyze mammalian tissues for evidence of primary renin expression. The use of complementary DNAs as probes for primary renin expression helped confirm and further clarify earlier studies demonstrating the presence of renin activity in a number of extrarenal tissues. Although expression in some of these tissues is evolutionarily conserved, their significance has still been elusive. In this report we review the impact of molecular biology on our current understanding of renin gene structure and organization, tissue- and cell-specific expression and regulation, and the changes in renin expression throughout ontogeny. In addition, we describe how new developments in gene transfer technology have added important tools to our arsenal for examining renin gene regulation and how these technologies can be used to develop new tools for renin and hypertension research. (Hypertension 1991;18:446-457)
DNAs (cDNAs), issues pertaining to the specificity of renin gene expression in these tissues began to be resolved (see below). Moreover, analysis of the SMG renin cDNA sequence permitted the first demonstration of colinearity between the predicted amino acid sequence and purified SMG renin. With this cDNA as probe, other cDNAs and genomic clones for interspecific renin genes were identified and are present in strains of mice which contain a single renin locus (DBA/2J). Only a single renin gene exists in rats and humans.

Both the mouse and rat renin genes stretch over a span of approximately 13 kb and contain nine exons and eight introns. Both Ren-1 alleles and Ren-2 share similar overall sequence organization within the exon-intron region of the gene. The sequences of the coding regions (cDNAs) of all three murine renin genes, regions of the 5' and 3' flanking regions of the Ren-1 and Ren-2 genes as well as the sequence of the entire Ren-1 genomic region extending approximately 1,200 basepairs 5' and 400 basepairs 3' of the coding region have been reported.

An examination of the coding regions of Ren-1d and Ren-1e revealed them to be 99% identical, and a comparison of Ren-1 against Ren-2 revealed them to be 97% identical. Several restriction fragment length polymorphisms have been identified that can be used to differentiate between the genes at the DNA level. Significant homology also exists between the mouse and rat renin genes (=88%) and between the mouse and human renin genes (=78%). Interestingly, the mouse and rat renin genes lack an additional exon (exon 5A) encoding three amino acids in mature human renin.

At the amino acid level all three mouse genes exhibit approximately 97% identity. The most significant differences are at positions in the renin-2 protein that correspond to three potential asparagine-linked glycosylation sites in the renin-1 polypeptide. It is probable that renin-2 is a nonglycosylated form of renin, and these glycosylation differences may account for the differences in the thermostability of the renin proteins although the contribution of other minor structural differences between the isoforms has not been examined.

Significant differences in structural organization, identified by comparative DNA sequence analysis and fine structural physical mapping, exist outside the actual coding regions of the three inbred murine genes (summarized in Figure 1). Two highly repetitive elements have been mapped within the renin locus; a B2 repetitive element unique to the 5' region of the Ren-2 gene and a B1 element within the 5' flanking region of all three mouse genes. The B1 element has been conserved throughout evolution as evidenced by its presence at homologous positions upstream of the rat and human renin genes. When the renin loci in mice and rats were examined with increased scrutiny, several unique insertions within the mouse genes (but not rat) were detected. A 500 base pair (bp) insertion flanked by five nucleotide direct repeats, designated M3 (Mouse insertion 3), resides within 80 bp of the transcription start site of all three mouse genes. Interestingly, the B2 repetitive element described above resides within the M3 insertion. The presence of the B2 element and another 143 bp insertion designated M2 can be used to distinguish between Ren-2 and either Ren-1 or Ren-1e. The identification of these sequences resolves the disturbing lack of sequence homology first noted by Soubrier et al in this region when compared with the rat and human renin genes.

Three insertions have been mapped between Ren-2 and Ren-1d in DBA/2J mice. A partial intracisternal A particle (IAP) genome lies 1 kb 3' of exon 9 in Ren-2 but is absent from the equivalent positions in either Ren-1 allele. A small 300 bp insertion designated M4 lies further 3' and is only associated with Ren-1d 3' flanking sequences (Figure 1). The largest insertion is M1, a 7.2 kb sequence flanked by short direct repeats found between Ren-2 and Ren-1d. M1 is repetitive in the mouse genome and lies very close to the duplication junction between Ren-2 and Ren-1. Most interestingly, a wild-derived mouse species, Mus hortulanus, which also harbors the duplicated renin gene lacks M1, M2, M4, and the IAP suggesting that insertion or deletion events took place after the ancestral duplication of the Ren-1 locus.
Table 1. Differential Expression of the Murine Renin Genes

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cell type</th>
<th>Genes</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>Juxtaglomerular</td>
<td>Ren-1=Ren-1^f=Ren-2</td>
<td>Cell-specificity developmentally regulated</td>
</tr>
<tr>
<td>Submandibular gland</td>
<td>Granular convoluted tubule</td>
<td>Ren-2=S&gt;Ren-1^f</td>
<td>Androgen regulated, expression maximum</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>puberty in males</td>
</tr>
<tr>
<td>Fetal adrenal</td>
<td>Throughout developing cortex</td>
<td>Ren-2=S&gt;Ren-1^f</td>
<td>Cell-specificity developmentally regulated,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ren-1^f decreases near birth</td>
</tr>
<tr>
<td>Adult adrenal</td>
<td>X-zone, zona fasciculata</td>
<td>Ren-2=S&gt;Ren-1     ^f</td>
<td>Cycling phenotype in DBA/2J mice</td>
</tr>
<tr>
<td>Testes</td>
<td>Interstitial Leydig cells</td>
<td>Ren-1^f&gt;Ren-2=S&gt;Ren-1</td>
<td></td>
</tr>
<tr>
<td>Ovary</td>
<td>Theca, corpus luteum</td>
<td>Ren-2=S&gt;Ren-1^f, Ren-1^f</td>
<td>No comparison made between levels of Ren-1^f</td>
</tr>
<tr>
<td>Anterior prostate</td>
<td>Glandular epithelium</td>
<td>Ren-1^f&gt;S&gt;Ren-1=S&gt;Ren-1</td>
<td>Ren-2 and Ren-1^f not detectable</td>
</tr>
<tr>
<td>Fetal subcutaneous</td>
<td>Specific population of fibroblasts</td>
<td>Ren-1^f=S&gt;Ren-1=S&gt;Ren-2</td>
<td>Developmental restriction, expression not</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>directly detectable in adults</td>
</tr>
</tbody>
</table>

Genetic analysis first suggested that the Ren-1 and Ren-2 structural genes were tightly linked to each other and coincidentally with Rvr. To more precisely map the junction between the two loci Abel and Gross examined the region spanned by both genes by pulsed field gel electrophoresis. They demonstrated that both genes were separated by a distance of approximately 21 kb and shared the same transcriptional polarity with Ren-2 upstream of Ren-1^f. In addition the precise boundaries of the duplicated segment have now been reported. Sequence analysis of a genomic clone containing the junction between Ren-2 and Ren-1^f suggests that the duplication arose as a result of a recombination event within regions of poor homology. The same precise recombination junction is present in M. hortulanus.

Expression of the Murine Renin Genes

Sites of Renin Expression

Major progress has been made in determining the tissue-specific and cell-specific expression profile for the murine renin genes over the past several years. It has now become clear that the expression patterns of renin in the mouse are far more complicated than originally anticipated. In addition to the kidney, renin or renin messenger RNA (mRNA) has been detected in a considerable number of extrarenal tissues. For the purposes of this review only tissues in which primary expression of renin mRNA has been reported will be discussed in detail. Among these sites are kidney, SMG, testes, ovary, heart, anterior prostate, and adrenal gland (References 36, 60–74, and C.D. Sigmund and K.W. Gross, unpublished observations). Renin transcripts are relatively abundant in these tissues although expression on a per-cell basis has been shown to differ over at least two orders of magnitude. In addition renin transcripts have been reported by Ekker et al to be present in liver, whole brain, hypothalamus, spleen, thymus, lung, and prostate when examined by the polymerase chain reaction (PCR), an assay capable of detecting but not accurately quantitating extremely small amounts of renin mRNA.

Differential and Cell-Specific Expression

In mice with a single renin gene, as in rat or human tissues, detection of renin at the organ level can be easily performed by in situ hybridization or Northern blot analysis of total RNA. However, additional techniques had to be devised to examine the specificity of expression of renin mRNA in strains containing both Ren-1 and Ren-2. Several assays have been developed to differentiate among Ren-1^f, Ren-1^f, and Ren-2-specific messages in a mixed population of total mRNA. The primer extension technique described by Field and Gross have demonstrated that the mouse renin genes are differentially expressed in a number of extrarenal tissues. This data is summarized below and in Table 1.

Kidney. Field and Gross have demonstrated that all three renin genes in inbred mice are expressed approximately equivalently in the kidney. Immunocytochemical and in situ hybridization techniques designed to detect total renin or renin mRNA have localized the renin-expressing cell population to juxtaglomerular (JG) cells, the modified smooth muscle cells of the afferent arterioles. Renin expression and release from these cells is physiologically regulated.
Adrenal gland. As with kidney, expression of Ren-1\(^\text{c}^\) and Ren-2 occur at equivalent levels in the adrenal gland of adult female DBA mice.\(^{71}\) Surprisingly, no renin mRNA is detectable in adrenal glands of adult C57BL/6 mice or other inbred mouse strains containing a single renin gene.\(^{69,74}\) It is now apparent that Ren-1\(^\text{c}^\) undergoes a developmental restriction in its expression pattern (see below). A genetic study of adrenal renin mRNA in F\(_1\) hybrid mice revealed that the expression profile differences in adults are encoded in \textit{cis} and not strictly dependent on the genetic background of the mice per se.\(^ {71}\)

The cellular basis of adrenal renin expression has been examined in detail in our laboratory. Unlike rats and humans where renin has been detected in the outermost cortical zone, the zona glomerulosa, mouse renin exists in two distinct inner cortical zones, the zona fasciculata and X-zone.\(^ {69,74}\) Most interestingly, in DBA/2J mice expression of renin in these zones is entrained to hormonal regulation and exhibits a cycling phenotype in females corresponding to the estrous cycle.\(^ {69,74}\)

Submandibular gland. SMG is one of the few tissues unique to the mouse with respect to expression of renin.\(^ {78}\) Even with PCR renin mRNA has not been detected in the rat SMG.\(^ {73}\) Using the primer extension assay described above, Field and Gross\(^ {62}\) reported that expression of Ren-2 is the predominant species of SMG renin message in DBA mice. In fact, Ren-1\(^\text{c}^\) has been undetectable in the SMG of DBA mice by our primer extension analysis, although it has been detected at very low levels by RNase protection.\(^ {70}\) Comparing SMG Ren-2 expression in DBA mice to Ren-1\(^\text{c}^\) expression in C57BL/6 mice revealed that Ren-2 expression was elevated by two orders of magnitude. When measured on a per-cell basis, the level of steady-state Ren-2 mRNA approximates levels of renin message in renal JG cells.

In the SMG, renin expression is restricted to the granular convoluted tubule (GCT) cells, the glanular epithelium of the SMG. These cells make up approximately 20% of the cellular mass of the SMG, which makes renin approximately 2% of total SMG protein, a fact that certainly helped in the early purification of SMG renin. Expression of the gene in males becomes evident in the GCT cell population near puberty and is regulated by androgen. Treatment of female mice with testosterone increases the expression of Ren-2 to male levels.\(^ {25}\) SMG renin levels are also regulated by thyroxine.\(^ {79-81}\)

Reproductive tissues. Expression of renin and accumulation of prorenin has been observed in the reproductive tissues of several species (see Reference 82 for review). In testes renin expression is apparently limited to interstitial Leydig cells.\(^ {61,69}\) Although the differences in renin mRNA levels are not very marked, locus-specific and allele-specific primer extension assays have confirmed that Ren-1\(^\text{c}^\) is present in slight excess over Ren-2 or Ren-1\(^\text{c}^\).\(^ {71}\)

Ekker et al\(^ {73}\) have reported that expression of both Ren-2 and Ren-1\(^\text{c}^\) were approximately equal in total RNA from ovary of DBA/2J mice using differential PCR. In the rat, renin mRNA has been detected in cells of the corpus luteum.\(^ {65}\) However, ovarian renin appears to be produced by theca, stromal, and luteal cells in humans,\(^ {83}\) and human theca cells in culture.\(^ {84}\) Low levels of renin mRNA are visible by Northern blotting of total RNA from C57BL/6 ovaries (C.D. Sigmund and K.W. Gross, unpublished observation).

Fabian et al\(^ {71}\) have recently reported the high level expression of the Ren-1\(^\text{c}^\) allele in the mouse (but not rat) anterior prostate (a coagulating gland). No expression was detected from Ren-1\(^\text{c}^\) or Ren-2 in DBA/2J or congeneric mice in which these genes are contained on a Balb/C genetic background. This indicates the ability to express renin in this tissue is encoded in \textit{cis}. Like the SMG, expression appears to be limited to the glandular epithelium (J. Fabian, C. Kane, and K.W. Gross, unpublished observation).

Subcutaneous tissue. Recently, we demonstrated that renin is expressed in subcutaneous tissue during fetal development.\(^ {85,86}\) Transcripts from each Ren-1 allele were present in excess of Ren-2 transcripts demonstrating yet another site in which the mouse renin genes are differentially expressed. This expression was limited to a specific population of mesenchymal cells (fibroblasts) near the developing skeletal muscle of the body wall. The localization of these cells was intriguingly similar to the localization of angiotensin II receptors reported by Millan et al\(^ {87}\) and Zemel et al\(^ {88}\) raising the question of a possible role in the development of this tissue. Renin mRNA was also found in fetal rat subcutaneous tissue suggesting that expression at this site may be more widespread.\(^ {86}\)

Developmental Aspects of Renal and Adrenal Renin Expression

Renin expression in the kidney exhibits an interesting developmental phenomenon first observed through the use of immunocytochemistry\(^ {89-92}\) and more recently confirmed by in situ hybridization.\(^ {94-97}\) During fetal development, foci of cells containing renin mRNA are first detectable at 14.5 days of gestation. By 15.5 days, renin mRNA was detected in cells surrounding the lumens of the first histologically identifiable large intrarenal arteries. As the arterial tree begins to branch, renin expression is progressively restricted to smaller blood vessels and finally to JG cells at or around birth. In adults, renin expression is generally limited to JG cells. However, under certain pathophysiological conditions and during blockage of the RAS by angiotensin converting enzyme inhibition, renin-expressing smooth muscle cells can be detected farther up the afferent arterioles and interlobular arteries suggesting a recruitment of these cells to the renin-expressing population and an apparent recapitulation of the fetal pattern of renin expression.\(^ {91,94}\) This suggests that renal vascular smooth muscle cells retain the potential to express renin. Whether vascular cells in other arterial beds express renin or have the ability to
express renin is still under intensive investigation (see References 98 and 99 for review).

In the adrenal gland, expression of renin first becomes detectable around 14.5 days of gestation; however, unlike renin expression in DBA/2J mice, the levels of Ren-1 mRNA in C57BL/6 or Balb/C mice decrease with subsequent development and become undetectable by in situ hybridization or Northern blotting near birth.69,70 In DBA/2J mice at 14.5 days of gestation, adrenal renin mRNA can be seen throughout the entire gland. This is a time before completion of the migration of the neuroendocrine cells that make up the adrenal medulla. As the medulla takes form, the renin-expressing cells become concentrated into concentric rings that make up the zona fasciculata and X-zone of the adrenal cortex.69

Transcriptional Regulation of the Murine Renin Genes

Transcriptional Start Sites

Like some other mammalian genes, transcription of renin has been shown to occur at several distinct sites. In the mouse several transcription start sites have been detected by S1 nuclease mapping and primer extension.50 Some of these sites do not contain recognizable TATA boxes and the renin genes of all species examined lack classical CAAT boxes at the -80 position.100 In the kidney and adrenal gland transcription starts primarily at a single site. However, in SMG and anterior prostate, for example, a significant proportion of the transcripts are initiated at sites further upstream (Reference 50 and J. Fabian and K.W. Gross, unpublished observation). The significance of the multiple transcription start sites remains unclear. Perhaps such an organization allows the renin gene to be differentially regulated by different intracellular signals or transcription factors.

Hormonal Regulation of Murine Renin Expression

Renin expression in renal and several extrarenal tissues is hormonally responsive. Expression of renin in the SMG is positively modulated by androgen and thyroid hormone.24-27,79,81 Tronik and Rougeon80 have reported that the effects of the hormone are at the level of transcription with an increase in basal transcription rate twofold after thyroxine treatment and fourfold after testosterone treatment. Both Ren-1 and Ren-2 contain two regions of 10 and 12 bases each within their 5′ flanking regions which share homology with other thyroxine-responsive genes and these sequences contain discrete thyroxine responsive elements.80 In addition, Burt et al53 postulated the presence of six potential estrogen-responsive elements in Ren-1. Renin expression may be hormonally modulated during pregnancy101,102 and ovarian renin levels change throughout estrus.103 Similarly, cell-specific adrenal renin mRNA levels vary substantially during estrus in the mouse strongly suggesting they are hormonally regulated.74

In the kidney, renin mRNA has been shown to be regulated over a range of two orders of magnitude, increasing with sodium depletion and angiotensin converting enzyme inhibition and decreasing under conditions of high salt or arterial pressure. Some of these effects are consistent with a role of angiotensin II as a negative regulator of renin expression (short-feedback loop104-108). However, there is no heretofore demonstration of a direct effect of angiotensin II on JG cell renin expression.

Role of cis-Acting Sequences Regulating Murine Renin Gene Expression

In the following sections, we review this recent data and the approaches taken to identify cis-acting regulatory elements in the mouse renin genes. The results of transfection experiments are summarized in Figure 2 and the results of transgenic experiments are summarized in Figure 3.

Transfection Analysis

One of the most common techniques used to identify regulatory elements is transfection analysis. In these experiments, putative transcriptional regulatory elements are fused to an easily detectable reporter gene such as chloramphenicol acetyl transferase (CAT). The activity of the promoter element is then assessed by examining the level of CAT activity in extracts of transiently transfected cells. By examining a series of constructs containing different sequences or progressive deletions of a single sequence, important regulatory elements can often be identified within short stretches of DNA.

Ekker et al109 have transfected constructs containing the promoter region of the mouse Ren-1 (−449 to +30) and Ren-2 (−2,500 to +7) genes fused to CAT (Figure 2) into five cell lines that do not express renin (baby hamster kidney, L cells, Chinese hamster ovary, JEG-3, and a human mammary tumor cell line). They report that the promoter regions of these genes are inactive in noncognate cells unless a functional enhancer (SV40) is coupled to the constructs. The function of a promoter is to direct correct and accurate use of a transcriptional initiation site. Since correct transcription initiation was achieved when the promoters were coupled to SV40 enhancers, this suggests the promoter elements themselves functioned properly. Therefore, the authors concluded that the promoter elements themselves do not act tissue-specifically because they will cooperate with any functional enhancer to direct accurate transcription initiation. In addition, progressive deletions of the upstream sequences did not cause any significant increase in transcriptional activity in these cells109 or in myeloma or 3T3 cells,110 and the region between −2,500 and −118 (Ren-2) could not stimulate transcription when fused to the thymidine kinase promoter.109 From this data the authors further suggested it is unlikely major negative regulatory elements (transcriptional repressor) exist in the 5′ flanking sequence used in the constructs. Therefore, accurate
transcription must be dependent on one or more specific enhancer elements, and the absence of transcription without enhancers in noncognate cells is due to the absence of trans-acting factors required to interact with sequences in the 5' flanking region of the gene. The renin promoters could become tissue specific, however, in B-cells when coupled to a functional enhancer and a conserved decanucleotide with the ability to confer expression in B-cells but not non-B-cells.\textsuperscript{110,111} This appears to be the property of the decanucleotide sequence, a functional TATA element, and a B-cell specific transcription factor, since all other renin sequences were dispensable for this activity. One can conclude, therefore, that the renin promoter acts cell-specifically only when the correct combination of cis-acting sequences and transcription factors are present.

In an independent set of experiments Nakamura et al.\textsuperscript{112} have identified several potential negative regulatory elements upstream of Ren-1\textsuperscript{4} but not Ren-2. These experiments used promoterless 5' flanking regions from Ren-1\textsuperscript{4} (~707 to ~367) and Ren-2 (~1055 to ~571) fused to the thymidine kinase promoter and CAT (Figure 2) and were transfected into AtT-20 and JEG-3 cells. The postulate for a repressor binding sequence upstream of Ren-1\textsuperscript{4} was based on two observations: 1) sequence analysis comparing Ren-1\textsuperscript{4} with Ren-2 revealed the presence of a Ren-2-specific insertion upstream of the gene (M2, see above) and 2) Ren-2 expression is at least 100-fold in excess of Ren-1\textsuperscript{4} in SMG.\textsuperscript{62} Therefore, the authors proposed that the M2 sequence insertion has disrupted a putative repressor binding site upstream of Ren-2 and that the inactivation of this site is responsible for high level expression of Ren-2 in the SMG. Their results indicate that when this Ren-1 5' flanking region is placed in its normal forward orientation upstream of the TK promoter and CAT, promoter activity becomes attenuated compared with a control lacking the renin sequence. No repression was observed, however, when the Ren-1 5' flanking region was placed in the reverse orientation or when the Ren-2 5' flanking region was in either orientation suggesting the presence of a silencer element within Ren-1\textsuperscript{4}. Potential repressor binding sequences upstream of Ren-1 were identified based on a sequence comparison with other genes containing silencer elements and by binding of nuclear proteins from SMG and JEG-3 cells. These sequences have been termed negative regulatory elements. Consistent with this conclusion is more recent data demonstrating that 1) when a negative regulatory element from Ren-1\textsuperscript{4} is removed, synthesis of CAT in JEG-3 cells no longer is repressed and 2) removal of the M2 element from the Ren-2 construct causes attenuated CAT synthesis (Figure 2) (Reference 113 and R. Pratt and V. Dzau, personal communication).

Interestingly, the wild-derived mouse strain \textit{M. hortulanus}, which is interfertile with inbred mice, contains an allelic form of Ren-2 (Ren-2\textsuperscript{2}) that lacks M2 and therefore has a regional structure similar to Ren-1\textsuperscript{4}. Interestingly, Ren-2\textsuperscript{2} expression is still high and Ren-1\textsuperscript{4} (the Ren-1 allele in \textit{M. hortulanus}) is still low in SMG\textsuperscript{57} suggesting an SMG expression pattern similar to \textit{M. domesticus} and \textit{M. musculus} mice. Although these results apparently contrast the transfection results described above, several important points should be noted: 1) JEG-3 cells generally do not express renin; 2) although both JEG-3 cells and whole SMG contain a 72 kd nuclear protein that binds to the negative regulatory element sequence, JEG-3 cells, but not SMG, contain an additional nuclear protein that can also bind to the negative regulatory element sequence, JEG-3 cells, but not SMG, contain an additional nuclear protein that binds to the negative regulatory element sequence; 3) the Ren-1\textsuperscript{4} and Ren-2\textsuperscript{2} loci in \textit{M. hortulanus} mice reside in their normal chromosomal context, whereas the transfected constructs certainly do not; and 4) the level of depression in the transient transfections is only 60\%,\textsuperscript{112,113} whereas the depression of Ren-1\textsuperscript{4} in vivo is greater than 99\%.\textsuperscript{62,70} There are

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**Figure 2.** Schematic diagram of summary of transfection studies performed to date shows a portion of the Ren-2 (upper left) and Ren-1 (upper right) 5' flanking regions. Both genes are drawn to the same scale. Regions of the Ren-2 gene not present in Ren-1 are denoted by blank regions and are labeled as either \textDelta{}B2 or \textDelta{}M2 (\textdelta{} standing for deletion). Extent of the 5' flanking region used in transfection analysis is shown below each gene along with the major finding of the report. For details see the text and specific references. NRE, negative regulatory element; CRE, cyclic AMP-responsive element.
Experiments in Transgenic Mice

Methods are currently available for introducing cloned copies of genes into the mouse genome and germ line to form transgenic mice. Under appropriate conditions the introduced genes exhibit regulated expression characteristics of the endogenous gene.

Introduction of genes affords novel opportunities for studying gene function and dissecting the mechanisms regulating complex gene interactions, such as those which typify physiological homeostasis. Although more labor intensive than transient transfection, transgenic mice allow investigators to address specific questions in vivo regarding tissue- and cell-specific expression, hormonal regulation, and developmental aspects of gene expression. In addition, transgenic mice can be reasonably cost effective when one considers that all tissues and cell types of the organism can be examined over time. For a detailed review of transgenic mice along with a discussion of their advantages and disadvantages and their use in cardiovascular research, see Field and Mockrin et al. Technical aspects of producing transgenic mice are comprehensively described in Hogan et al.

The first set of reports describing the use of transgenic mice for renin research characterized large Ren-2 and Ren-1 genomic constructs. In Tronik et al. and Mullins et al. phenocopies of “two renin gene” mice were made by integrating a copy of the Ren-2 gene into a genetic background containing only the Ren-1 gene. Tronik et al. reported correct tissue-specific expression of a Ren-2 genomic transgene containing 2.5 kb and 3.0 kb of 5' and 3' flanking sequence, respectively, including all exon and intron sequences (Figure 3). In their experiments, the transgene was quantitatively expressed as expected for a Ren-2 gene and responded to androgens and thyroxine in the SMG. Qualitatively (but not quantitatively) correct tissue- and cell-specific expression of a Ren-2 transgene containing an additional 2.8 kb of 5' flanking and an additional 6.5 kb of 3' flanking sequence (Figure 3) was reported by Mullins et al. and included the characterization of an estrus-dependent cell-specific adrenal Ren-2 expression pattern (cycling phenotype, see above). This cycling phenotype apparently is dependent on an unlinked locus present in the genetic background of DBA/2J mice. In the absence of this locus, adrenal renin mRNA is still evident but expression does not cycle.
Miller et al.\textsuperscript{70} interested in investigating the differences between \textit{Ren-1}\textsuperscript{c} and \textit{Ren-1}\textsuperscript{d} expression, created transgenic mice with a slightly altered \textit{Ren-1}\textsuperscript{d} genomic construct on a genetic background containing only \textit{Ren-1}\textsuperscript{c}. The construct contained 5 kb of 5' flanking sequences, 4 kb of 3' flanking sequence along with the \textit{Ren-1}\textsuperscript{d} coding sequence containing a synthetic oligonucleotide within exon 2 for purposes of differentiating the two \textit{Ren-1} alleles. The aim of their experiment was to determine if the differences in expression between the two alleles (see above) are due to differences in trans-acting factors or cis-acting sequences linked to each gene. Their results strongly suggest that the different patterns of tissue-specific expression exhibited by the two genes are primarily due to sequence differences linked in cis and are in agreement with those of Fabian et al.,\textsuperscript{71} who examined regulation of endogenous \textit{Ren-1}\textsuperscript{c} and \textit{Ren-1}\textsuperscript{d} genes.

To further define the regulatory region of \textit{Ren-2} with respect to tissue- and cell-specific expression, Sola et al.\textsuperscript{121} and Sigmund et al.\textsuperscript{122} have constructed transgenic mice containing fusions between putative \textit{Ren-2} controlling regions and SV40 T antigen (Figure 3). In these experiments, T antigen provides a multifaceted reporter function because 1) the T antigen sequence itself can be used to detect renin-T-antigen fusion transcripts on Northern blots and tissue sections, 2) monoclonal and polyclonal antibodies to T antigen can be used to detect large T antigen immunologically, and 3) T antigen is a potent oncogene that can cause tumorigenic amplification of renin-expressing cells and lead to the identification of previously unidentified sites of renin expression. The latter reporter function can be used as a sensitive detector and has been used to help identify renin expression in subcutaneous tissues of the mouse and rat (References 85 and 86, see above).

Sola et al.\textsuperscript{121} examined the tissue-specific expression profile of a \textit{Ren-2} promoter-T antigen fusion gene containing approximately 2.5 kb of 5' flanking sequence. No transgene expression was detected in JG kidney cells or GCT SMG cells, and when tumors formed in their mice, they formed in non-renin-expressing tissues that did not themselves express the endogenous renin gene \textit{Ren-1}\textsuperscript{c}. This result by itself suggests that sequences necessary for tissue-specific expression of \textit{Ren-2} either lie farther upstream, within the structural gene itself or in the 3' flanking region. Several mammalian genes contain important regulatory sequences within introns or 3' of the gene.\textsuperscript{123–128} However, when these results are examined in the context of their previous report, showing that a genomic construct containing the same proximal 2.5 kb of 5' flanking sequence\textsuperscript{119} exhibits tissue-specific expression, it strongly suggests that sequences within the structural gene or 3' flanking region must be present in conjunction with the 2.5 kb upstream region to establish a tissue-specific phenotype.

We have also examined a similar T antigen fusion construct containing an additional 2.1 kb of \textit{Ren-2} 5' flanking sequence (a total of 4.6 kb\textsuperscript{122}). We demonstrated that this construct exhibits the tissue- and cell-specific expression phenotype characteristic of \textit{Ren-2}. Furthermore, expression of the transgene in the SMG of male mice was greater than in female mice suggesting androgen sensitivity. We concluded from these studies that 4.6 kb of \textit{Ren-2} 5' flanking sequence contains all the necessary cis-acting regulatory sequences needed to confer a tissue- and cell-specific expression profile on an exogenous reporter gene in vivo. Interestingly, when comparing our results with those reported by Sola et al.\textsuperscript{121} and Tronik et al.,\textsuperscript{119} it appears that important tissue- and cell-specific regulatory elements may lie within the −4.6 to −2.5 kb region of \textit{Ren-2}. Since 4.6 kb of 5' flanking sequence was sufficient to direct tissue- and cell-specific expression on its own, this suggests the potential of redundancy in control elements, perhaps within the structural gene or 3' flanking region and in the −4.6 kb to −2.5 kb upstream region. Such redundancy in regulatory elements will have to be tested experimentally.

Development of New Tools for Renin Research

Isolation of a Renin-Expressing Cell Line From Transgenic Mice

Some of the inherent problems and limitations with interpreting transient transfection studies stems from the use of non-renin-expressing cells as hosts. Presumably, the cells do not express their endogenous renin gene because they lack the correct complement of trans-acting factors required to initiate transcription of the gene.\textsuperscript{109} The murine renin genes exhibit a complicated pattern of differential extra-renal expression (Table 1). Expression in these tissues can be examined in transgenic mice or in culture with a wide spectrum of cell types derived from each of the renin-expressing tissues. In a report published by our laboratory,\textsuperscript{115} we describe a strategy to develop a library of renin-expressing cells with different tissue- and gene-specificities. The approach is based on the specific targeting of SV40 T antigen to renin-expressing cells by directing expression of the oncoprotein by a tissue- and cell-specific renin regulatory region. We have been successful in the isolation of tumoral renal cells that maintain expression of renin over past 30 passages in culture. More recently, we have observed that the levels of renin mRNA could be modulated by the extracellular environment (i.e., composition of the media, C.D. Sigmund and K.W. Gross, unpublished observation). A more detailed examination of the kinetics of renin secretion, the response to intracellular calcium, the presence of angiotensin receptors, and the usefulness of the cells as hosts in transfection analysis are all ongoing in our laboratory. This data should allow us to assess whether these cells will provide an in vitro model for JG cells. We believe that the strategy used to isolate...
these cells can be used to derive renin-expressing cell lines from other renin expression tissues such as SMG, adrenal gland, testes, and ovary.

**Transgenic Models of Hypertension**

Recent years have seen an increased use of transgenic technology in cardiovascular research (See References 116 and 117 for review). Not only is this tool being used to examine gene regulation (see above) but attempts are being made to exploit it to develop animal models of human disease. Certainly excellent examples of how this technology is used are exemplified in recent reports by Mullins et al.,129 Ohkubo et al.,130 Steinhelper et al.,131 Field et al.,132 Sigmund et al.,133 and Jacob et al.,134 where expression of an inserted gene product results in a pathophysiologically measurable response. As transgenic animals allow investigators to measure the contribution from one or several genes, other informative transgenic models will be forthcoming.

The technology to eliminate ("knock-out") expression of genes is at the forefront of biomedical research and represents a particularly exciting frontier. The ability to delete or make precise changes to genes by homologous recombination in embryonic stem cells along with the ability to implant these cells and have them contribute to the germ lines of mice opens up a virtually unexplored arena in which the power of genetics can be applied to whole mammalian systems (see Reference 135 for review). Studies are currently underway in this and other laboratories to knock out the renin gene in mice. Although we can not anticipate the results, we believe the ability to address questions concerning the role of renin during fetal development, the significance of determination of renin as an essential gene are feasible with this approach. Certainly, we can look forward to many fascinating discoveries in the coming years using these new technologies.

**Acknowledgments**

We thank Mary Kay Ellsworth, Colleen Kane, Chuanzhen Wu, and Frank Pacholec for technical assistance with some of the work performed in our laboratory and with some of the work performed in our laboratory and research center. We are also indebted to Drs. Craig Jones, John Fabian, Kenneth Abel at R.P.C.I., and Dr. Richard Pratt at Stanford University for their critical comments on the manuscript and to Cheryl Mrowczynski for excellent secretarial assistance.

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Hypertension. 1991;18:446-457
doi: 10.1161/01.HYP.18.4.446

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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