Highly Regulated Cell Type-restricted Expression of Human Renin in Mice Containing 140- or 160-Kilobase Pair P1 Phage Artificial Chromosome Transgenes*

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Patrick L. Sinn, Deborah R. Davis, and Curt D. Sigmund‡

From the Departments of Internal Medicine and Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242

We generated transgenic mice with two P1 artificial chromosomes, each containing the human renin (HREN) gene and extending to –35 and –75 kilobase pairs, respectively. HREN protein production was restricted to juxtaglomerular cells of the kidney, and its expression was tightly regulated by angiotensin II and sodium. The magnitude of the up- and down-regulation in HREN mRNA caused by the stimuli tested was identical to the endogenous renin gene, suggesting tight physiological regulation. P1 artificial chromosome mice were mated with transgenic mice overexpressing human angiotensinogen to determine if there was a chronic compensatory down-regulation of the transgene. Despite a 3-fold down-regulation of HREN mRNA, plasma angiotensin II and blood pressure was modestly elevated in the double transgenic mice. Nevertheless, this elevation was significantly less than a different double transgenic model containing a poorly regulated HREN transgene. The increase in blood pressure, despite the decrease in HREN mRNA, suggests that the HREN gene can partially, but not completely, compensate for excess circulating angiotensinogen. These data suggest the possibility that increases in circulating or tissue angiotensinogen may cause an increase in blood pressure in humans, even in the presence of a functionally active servo-mechanism to down-regulate HREN expression.

Renin provides only one known function, to cleave the 10-amino acid precursor peptide angiotensin I (Ang-I), from the N terminus of mature angiotensinogen (AGT). Ang-I is further cleaved by angiotensin-converting enzyme into the physiologically active hormone, angiotensin II (Ang-II). Ang-II exerts a wide variety of systemic effects but classically serves to cause vasoconstriction, stimulate aldosterone release, enhance renal sodium transport, regulate renal blood flow, and increase thirst. This, along with the clinical observation that inhibitors of the renin-angiotensin system are effective antihypertensive treatments, strongly supports the role for the renin-angiotensin system in blood pressure regulation and makes it a likely candidate as a primary mediator in the pathogenesis of hypertension (1, 2). Indeed, genetic evidence supports the hypothesis that abnormalities in the regulation of the renin-angiotensin system may cause hypertension, a potent risk factor for cardiovascular diseases such as stoke, atherosclerosis, and myocardial infarction (3, 4).

In most mammals, renin is thought to be the rate-limiting step in the production of Ang-II. However, this process is tightly regulated as Ang-II elicits negative feedback on both primary renin synthesis and renin secretion in juxtaglomerular (JG) cells, thereby providing a means of autoregulation (5). In addition, renin production and secretion are tightly regulated in response to changes in arterial pressure and plasma sodium levels (6, 7). Therefore, gaining a clear understanding of renin gene regulation in response to physiological cues such as Ang-II is essential if we are to understand how perturbations in this homeostatic system lead to cardiovascular disease.

Numerous in vivo and in vitro studies designed to reveal DNA sequences required for regulation of the renin gene have been reported (reviewed in Ref. 8). For example, we have previously described two in vivo model systems to examine HREN gene regulation. The first, 900-HREN, contains a complete HREN genomic sequence spanning a region from 896 bp in the 5′-flanking region to 400 bp in the 3′-flanking region of the gene (9, 10). The second model, 140-HREN, also contains the complete HREN genomic sequence; however, this construct is truncated at –149 bp in the 5′-flanking region of the gene (11). While both models exhibited JG cell-specific expression of the transgene in kidney, they were generally ubiquitously expressed in most extrarenal tissues. In particular, high levels of HREN expression were evident in lung and adipose tissue, neither of which is considered a bona fide site of renin expression. In addition to ectopic expression, a paradoxical increase in HREN mRNA was observed when hypertensive mice were obtained from breeding 900-HREN transgenic mice with mice expressing human angiotensinogen (HAGT) (10). This suggests that there may be a complex arrangement of regulatory elements controlling both cell-specific expression and regulation in response to physiological cues and that the 900-HREN and 140-HREN transgenes may contain some, but not all, signals required for appropriate regulation. Moreover, the 140-HREN transgene was particularly sensitive to position artifacts upon expression (11). Therefore, position effects, arising from random transgene integration into the genome, will probably be a complicating factor when interpreting experiments such as these.

Recent studies have identified a cell-specific enhancer of transcription upstream of the mouse renin gene (12), and a
homologous sequence is also present upstream of the HREN gene (13). Interestingly, the HREN enhancer is located approximately 10 kb further upstream than the mouse renin (MREN) enhancer, suggesting that important regulatory elements controlling HREN expression may lie far upstream of the transcription start site. In addition, a second transcriptional enhancer, active in chorionic cells, was identified in the HREN gene (14). A mouse homolog to this enhancer has yet to be found, suggesting that the HREN and MREN may exhibit aspects of differential regulation, utilize different regulatory sequences, or both.

Because of the previous failure to accurately emulate HREN expression and regulation, we attempted to create a valid in vivo model of HREN expression by generating two new transgenic mouse models consisting of P1 phage artificial chromosomes (PACs), containing a large genomic fragment centered around the HREN gene. We hypothesized that such models would contain all of the regulatory regions necessary for proper gene expression and regulation within the transgene and would pave the way for future experiments in which the physiological significance of specific regulatory regions may be ascertained without the influence of position artifacts.

MATERIALS AND METHODS

Creation of Transgenic Mice, Animal Husbandry, and Experimental Conditions—Two PAC clones containing HREN were obtained from Genome Systems using a previously described HREN-specific primer set to screen a PAC library using the pAD10SacII vector backbone (9). The original address of the PAC clones was PAC-28-13A for PAC140 (Genome Systems control number 4916) and PAC-111-11L for PAC160 (Genome Systems control number 4917). PAC transgenic mice were generated by microinjection of closed circular PACs using standard techniques as described previously (15). Four of 218 PAC140 live offspring were identified as positive founders by HREN-specific PCR (9). Each founder also tested positive by PCR using PAC vector primers. Moreover, 100 randomly selected negative offspring were also rescreened using the vector primer set, and 99 were confirmed as nontransgenic. The remaining one was positive for the PAC vector but negative for the HREN gene. Three independent founder lines of each construct were bred for study. Southern blot analysis was utilized to confirm genotype using standard blotting techniques after digestion with HindIII and NotI.

Captopril (Sigma) was dissolved in the drinking water (0.5 mg/ml) to give approximately 100 mg/kg/day of captopril for 10 days. Microosmotic pumps (Alzet; model 1007D) were utilized to administer saline and subpressor (200 ng/kg/min) and pressor doses (1000 ng/kg/min) of Ang-II. Systolic blood pressure was determined on a Visitech Systems BP-2000 tail cuff apparatus. Mice were trained for 7 consecutive days prior to initial measurements. Base-line pressure was measured over 3 days, the pumps containing saline or Ang-II were implanted under metaphase anesthesia, and finally measurements were taken over the next 5 days. High salt diets (5.0% NaCl, Teklad TD92102) or low salt diets (0.23% NaCl, Teklad TD96207) were administered for 14 days before mice were killed and plasma and kidneys were collected.

Double transgenic mice were created by mating PAC140 line 6680/2 to HAGT line 204/1 or PAC160 line 6407/1 to HAGT line 204/1 as described previously (16). Catheters were implanted into the carotid artery under ketamine/xacpromazine anesthesia, and BP was first recorded under anesthesia. Mice were then allowed to recover for 48 h before mean arterial blood pressure was determined in conscious unrestrained mice. The blood pressure was defined as an average pressure of recordings made on 3 consecutive days. Captopril (50 mg/kg) or losartan (10 mg/kg) was infused through a venous catheter while blood pressure was concurrently recorded through the arterial line. In a separate experiment, double transgenic and control mice were acclimated to the tail cuff apparatus for 7 days, tail cuff systolic pressure was recorded for 5 consecutive days, and then carotid catheters were implanted as above. After a 48-h recovery period, mean arterial pressure was directly recorded in conscious, unrestrained mice for 2–3 days.

All experimental mice were fed standard mouse chow and water ad libitum unless otherwise indicated and were killed by CO₂ asphyxiation. Care of mice met or exceeded the standards set forth by the National Institutes of Health (37). Procedures were approved by the University of Iowa Animal Care and Use Committee.

RESULTS

Two independent PACs obtained from Genome Systems were initially only known to contain the HREN gene based on PCR amplification of a region encompassing exons 3 and 4. Therefore, in order to identify the size of the cloned inserts, the location and orientation of HREN, and the extent of 5'-flanking DNA present in the constructs, FGE was utilized (Fig. 1, A and B). The PAC vector is known to have NoI sites flanking the insert; therefore, the data reveal that both PAC constructs contain an internal NoI site. Approximate insert sizes of 140 kb (clone A, PAC140) and 160 kb (clone B, PAC160) were determined by adding the sizes of the two nonvector NoI fragments (Fig. 1A, lanes 2–4). Southern blot analysis using an HREN cDNA probe revealed that the HREN gene was present on an identical 40–45-kb NoI–SfiI fragment in both PAC140 and PAC160 (Fig. 1B, lanes 2–4). This along with additional Southern blots using both HREN cDNA and vector end probes allowed us to elucidate the orientation of the transgenic element of HREN and develop a long-range restriction map of the PAC140 and PAC160 constructs (Fig. 2). PAC140 contains approximately 35 kb of 5'-flanking region and 90 kb of 3'-flanking region, and PAC160 contains approximately 75 kb of 5'-flanking region and 70 kb of 3'-flanking region.

Both PAC constructs were microinjected into mice and three

Field Inversion Gel Electrophoresis (FGE)—PAC construct analysis was conducted on a Bio-Rad FGE Mapper Electrophoresis System. Purified PAC DNA or genomic mouse DNA (purified from spleen tissue) was digested with NotI or NoI plus SfiI and was electrophoresed on the field inversion apparatus using the manufacturer’s protocols and conventional electrophoresis was performed for HindIII and EcoRI digests. Ethidium bromide-stained gel images were digitally recorded with a Stratagene Eagle Eye II Photographic System. Southern blot analysis was conducted using standard blotting techniques and a HREN cDNA randomly labeled probe unless otherwise indicated. Stripping of Southern blots was achieved by boiling the nitrocellulose in 0.1% SDS, 0.1× SSC for 5 min.

RNase Protection Assay—HREN and MREN mRNA levels were determined with an RNase protection assay as described previously (11). HREN and MREN probes were partial cDNA sequences cloned into pCRII (Invitrogen) and pGEM-4 (Promega), respectively. The mouse β-actin cDNA and 18 S templates were obtained from Ambion (Austin, TX). The full-length probe for HREN, MREN, 18 S, and mouse actin were 410, 430, 125, and 315 bp, respectively, and the expected protected fragment sizes were 300, 326, 80, and 245 bp, respectively. Quantifications of RNase protection assays and Southern blots were performed using a Molecular Dynamics Storm 620 PhosphorImager System and the ImageQuant software provided by the manufacturer.

Radioimmunoassay and Immunohistochemistry—Plasma renin activity (PRA) and renin concentration were determined using a Pro-I 125I radioimmunoassay kit (NEN Life Science Products) and human angiotensinogen substrate (Scrivs Laboratories) as described previously (11). The trypsin-based prorenin activation protocol was conducted as described previously (17). Plasma Ang-II concentration was determined using an Ang-II 125I radioimmunoassay kit (Peninsula Laboratories, Inc.) and the manufacturer’s protocol as described previously (18).

Visualization of HREN localization in the renal cortex was achieved using immunohistochemistry and a HREN-specific antibody (R12) as described previously (11, 19). The human renin antibody was a generous gift from Drs. Pierre Corvol and Florence Pinet. Sections were examined from six mice from line 6680/2 and five mice from line 6407/1. At least three sections per kidney were examined. Mouse renin antibody was provided by Drs. Craig A. Jones and Kenneth W. Gross and has been previously described (20). Serial sections from two PAC160 line 6407/1 mice (32 pairs) were alternated on different slides and separately reacted with the antisera to human or mouse renin. This goat anti-mouse renin antibody was detected using a rhodamine-labeled rabbit anti-goat secondary antibody. The images were captured with a Bio-Rad MRC-1024 Hercules Laser Scanning Confocal Microscope equipped with a krypton/argon laser.

Statistics—All numerical data are presented as mean ± S.E. Statistical analysis was performed using a two-tailed, unpaired Student’s t test using the SigmaPlot or Systat software package.
numbered lanes spleen DNA and subjected to FIGE. The 5-kb ladder flanks the gel (M). B, the identical FIGE gel shown in A was Southern blotted with a randomly labeled HREN cDNA probe. C, Southern blot was performed with HindIII-digested genomic DNA isolated from spleen. Purified PAC160 construct was also digested as a positive control (+ lane). Representative mice from three PAC160 lines (6407/1 (lane 1), 6919/1 (lane 2), and 7217/2 (lane 3)) and three PAC140 lines (6293/1 (lane 4), 6680/2 (lane 5), and 7120/1 (lane 6)), along with a nontransgenic negative control (− lane) were run. D, Southern blot was performed with NotI-digested genomic spleen DNA and subjected to FIGE. Numbered lanes represent the same lines identified in C. The positive controls are purified PAC160 and PAC140 constructs diluted into genomic DNA from nontransgenic DNA (−) to ensure identical mobility. For esthetic purposes, an attempt was made to equalize band intensity by varying the levels of transgenic DNA loaded by dilution with nontransgenic DNA. All lanes contain 10 μg of total DNA. The molecular sizes are indicated in kb.

Pulsed field and conventional Southern blot analysis. A, the purified PAC constructs PAC140, PAC160, or the parent PAC Vector (V) were digested with NotI, SalI, or both NotI and SalI and subjected to FIGE. A 5-kb ladder flanks the gel (M). B, the identical FIGE gel shown in A was Southern blotted with a randomly labeled HREN cDNA probe. C, Southern blot was performed with HindIII-digested genomic DNA isolated from spleen. Purified PAC160 construct was also digested as a positive control (+ lane). Representative mice from three PAC160 lines (6407/1 (lane 1), 6919/1 (lane 2), and 7217/2 (lane 3)) and three PAC140 lines (6293/1 (lane 4), 6680/2 (lane 5), and 7120/1 (lane 6)), along with a nontransgenic negative control (− lane) were run. D, Southern blot was performed with NotI-digested genomic spleen DNA and subjected to FIGE. Numbered lanes represent the same lines identified in C. The positive controls are purified PAC160 and PAC140 constructs diluted into genomic DNA from nontransgenic DNA (−) to ensure identical mobility. For esthetic purposes, an attempt was made to equalize band intensity by varying the levels of transgenic DNA loaded by dilution with nontransgenic DNA. All lanes contain 10 μg of total DNA. The molecular sizes are indicated in kb.

A schematic representation of the PAC140 and PAC160 constructs. NotI (N) and SalI (S) sites are shown, and circled sites represent those known to be contained within the vector, directly flanking the insert. The cross-hatched box represents the location of the proposed chorionic enhancer, and the gray box represents the location of the proposed kidney enhancer (13, 14). The localization of HindIII restriction sites used in conventional Southern blotting are indicated with open arrows. The arrowsheads indicate the distance from the beginning or end of the HREN gene and vector arms. Closed circles indicate distance between restriction sites.

Independent founders from each construct were obtained and bred to establish lines. Southern blots of genomic DNA digested with HindIII (Fig. 1C) or NotI (Fig. 1D) and probed with a HREN cDNA probe identified bands in genomic DNA from transgenic mice that comigrated with purified PAC DNA, indicating the absence of any gross rearrangements of the transgene. Additional Southern blots using EcoRI and NotI plus SalI digests also revealed appropriately sized restriction fragments (data not shown).

We next determined the tissue distribution of HREN expression across all lines using RNAse protection assay (RPA). High relative expression was observed in the kidneys of line 6680/2 mice but not in the extrarenal sites tested (Fig. 3). Moreover, differential primer extension analysis indicated approximately equal levels of MREN and HREN expression in the kidney of this line (data not shown). As summarized in Table I, high levels of HREN mRNA were consistently localized in the kidney of all six lines of mice. In addition, HREN expression was consistently observed in placenta, another appropriate site of renin production (21). The highest level of extrarenal expression was observed in lines 7217/2 and 6919/1. This is presumably due to the high transgene copy number identified in these lines (see below). However, it is important to note that, in comparison with the kidney, extrarenal expression of HREN in these mice was substantially lower.

Within the kidney, appropriate JG cell-specific expression was confirmed using immunohistochemistry with a HREN-specific polyclonal antibody (Fig. 4, A and B). No significant extra-JG expression was observed in the two lines tested, and no expression was observed in any kidney from nontransgenic mice (Fig. 4C), thereby demonstrating the specificity of the antibody. These data suggest appropriate cell-specific expression of the transgene within the kidney. Interestingly, careful examination of the tissue sections revealed that approximately 50% of the JG apparatuses expressed HREN. This contrasts with only 10% in another transgenic model of HREN expression in which a minimal HREN promoter (149 bp) drove ubiquitous extrarenal, but JG cell-specific intrarenal expression (11). To determine if mouse and human renin were co-expressed in JG cells, we next examined serial kidney sections with antisera specific for each protein. Like human renin, mouse renin was only detected in JG cells (Fig. 4D). Serial analysis revealed mouse and human renin to be colocalized (Fig. 4, E–H). JG apparatuses co-expressing both mouse and human renin were found approximately 75% of the time (Fig. 4, compare G with H). The 25% remaining JG apparatuses expressed either mouse or human renin but not both, due either to differences in cell-specific expression or, more likely, to differences in localization of JG cells in the 10-μm serial sections.

In our initial studies, we observed an apparent correlation between transgene copy number and renal HREN expression. Therefore, to formally determine if the PAC transgenes are expressed in a copy number-dependent fashion, we repeated Southern blots from multiple animals from each line (n = 3), first probing for HREN and then stripping blots and probing for mouse actin as a loading control (Fig. 5A). RPAs probed simultaneously for HREN and mouse actin mRNA were performed on kidney RNA from the same mice (Fig. 5B). The lowest copy numbers were observed in lines 6293/1, 6680/2, and 7120/1. Line 6407/1 had an intermediate copy number, and the highest...
copy numbers were observed in lines 6919/1 and 7217/2. A strong correlation between transgene copy number and renal HREN expression was observed (\( R^2 = 0.91, p < 0.0001 \), Fig. 5C). This strongly suggests that renal HREN expression is proportional to copy number and is therefore immune from position artifacts, caused by random integration in the genome, normally experienced by smaller transgenes.

We next tested if HREN protein is appropriately translated, processed, and released into the plasma of these mice (Table II). HREN is processed into two forms, prorenin (an inactive zymogen) and active renin. Interestingly, despite differences in transgene copy number, all six transgenic lines secreted approximately equal amounts of active renin and total renin (active renin plus prorenin) into the plasma. Nontransgenic animals had only background levels of plasma human renin, confirming the specificity of the assay, and there was no significant difference in endogenous mouse active and total plasma renin activity between transgenic and nontransgenic mice.

We next examined if the transgenes are physiologically regulated. To accomplish this, studies were performed in PAC140 line 6680/2 and PAC160 line 6407/1 mice. These lines were chosen because they have a relatively low transgene copy number and are therefore most likely to express the gene at physiologically relevant levels. The renin gene is normally feedback-inhibited by Ang-II. Therefore, relief of feedback inhibition by blocking Ang-II production should cause an induction of renin mRNA. Mice were treated with either captopril (an inhibitor of angiotensin converting enzyme) or vehicle in their drinking water for 10 days. An approximately 6-fold induction of renal HREN mRNA was observed in response to captopril treatment (Fig. 6, top panels), which closely paralleled the increase in endogenous MREN mRNA (Fig. 6, bottom panels).

In order to determine if HREN mRNA was appropriately down-regulated in response to direct Ang-II infusion, mice were implanted with micro-osmotic minipumps containing saline (vehicle), a dose of Ang-II that does not elevate blood pressure (nonpressor), or a dose of Ang-II that elevates blood pressure by approximately 20 mm Hg over 5 days (pressor). Whereas the vehicle and nonpressor doses did not raise blood pressure, the pressor dose caused a moderate increase in blood pressure (20.7 ± 5.4 mm Hg in PAC140, 25.2 ± 1.9 mm Hg in PAC160,
and 17.1 ± 0.7 mm Hg in control mice; Table III). Importantly, both the nonpressor and pressor doses of Ang-II resulted in a significant attenuation of renal HREN mRNA (Fig. 7, top panel). As with the captopril treatment, the extent of down-regulation again closely mirrored the level of attenuation seen with the endogenous MREN gene (Fig. 7, bottom panel). Together, these data clearly demonstrate that this transgene responds appropriately to Ang-II-mediated feedback regulation.

In addition to Ang-II, dietary sodium is also a potent regulator of renin synthesis. Therefore, to test if the transgene is appropriately responsive to salt, mice were fed either a high salt (5.0% NaCl) or a low salt (0.23% NaCl) diet for 14 days. Both HREN and MREN mRNA levels were significantly lower in mice fed a high salt diet (Fig. 8). Moreover, plasma levels of human renin were attenuated in mice fed high salt (data not shown; \( p = 0.061 \) in PAC140, \( p = 0.014 \) in PAC160).

There is a strict species specificity in the enzymatic reaction between renin and angiotensinogen, such that human renin cannot catalytically cleave mouse angiotensinogen to form Ang-I (22). However, providing a source of homologous substrate, HAGT, would allow Ang-I production, which, because the Ang-I peptide is completely conserved, would be quickly converted to Ang-II by mouse angiotensin-converting enzyme. This provided an opportunity to determine if the PAC transgenes responded appropriately to Ang-II-mediated feedback regulation.

Previous transgenic models of HREN gene regulation have had severe limitations due to ectopic expression, inconsistent expression among lines, and inappropriate responses to physiological stimuli (9, 11, 24). Presumably, these models lack regulatory elements necessary for proper regulation or are strongly subject to position effects inherent with most standard transgenic approaches. In an attempt to overcome these limitations, we have constructed transgenic mice with P1 phage artificial chromosomes containing the HREN gene and consisting of large amounts of 5'- and 3'-flanking sequences. We report highly restricted expression in JG cells of the kidney and tight physiological regulation in response to Ang-II and salt.

The inserts in the PAC clones employed in this study are 140 and 160 kb in length. Therefore, a potential concern may arise from unpredictable rearrangements or truncations of the transgene when using high molecular weight constructs such as these. PCR analysis revealed that all mouse positive for the HREN portion of the transgene were also positive for the PAC vector. Moreover, Southern blots of both conventional and pulsed field gels indicated that the HREN gene in all lines was intact both locally (in the vicinity of the HREN coding region) and long range (distant 5'- and 3'-flanking sequences). This is particularly important, since these constructs were microinjected as closed circular plasmids and not as linearized DNA. Therefore, a random break must have occurred during the integration process into the genome. Indeed, a screen of DNA from 100 negative founders of the microinjections revealed one sample that was positive for the PAC vector but negative for the HREN gene, suggesting the potential for some transgene breaks during integration. It is interesting to note that all six PAC lines expressed renal HREN mRNA in a copy number-dependent fashion. This suggests that a random break in the circular construct caused by its insertion into the genome may have been “repaired” by the formation of tandem repeats of the transgene at the insertion site or that the break occurred in a region of the transgene that does not effect HREN expression.

**DISCUSSION**

Previous transgenic models of HREN gene regulation have...
Human Renin Regulation in Transgenic Mice

Plasma human renin concentration (PRC) and mouse plasma renin activity (PRA) were determined as described under "Materials and Methods" using either purified human angiotensinogen substrate (for human renin) or endogenous substrate (for mouse renin). Total renin was determined using a prorenin activation assay and represents the sum of active renin and prorenin. PRC reflects the use of saturating levels of exogenous substrate, while PRA reflects the use of endogenous substrate already present in plasma.

In addition, since the pattern of HREN expression was replicated in all lines, it is likely that the transgene was insulated from position effects caused by its insertion near other genes. Indeed, the fact that the gene exhibited copy number proportional expression suggests that all regulatory elements needed for its expression are present in the construct. Previous transgenic models using large transgenes encoded on cosmids, bacterial artificial chromosomes, and yeast artificial chromosomes appear to exhibit copy number-proportional expression due to the presence of locus control regions that contain DNase I-hypersensitive sites having the ability to manipulate chromatin at the local level (25, 26). It is interesting to note that, although expression of HREN mRNA appears proportional to copy number, the level of active human renin and human prorenin released into the circulation was not different among the six lines tested, suggesting the possibility that production of renin is under active translational or post-translational control.

The PAC transgenics reported herein displayed a more restricted tissue distribution of HREN expression than previously reported HREN mice. Previous models exhibited quantitatively high levels of HREN mRNA in the lung and adipose tissue that markedly surpassed its expression in kidney (9). In mice with low copies of the PAC transgene, HREN expression was limited to kidney, whereas in the high copy number mice, expression in some ectopic sites such as lung became detectable. Importantly however, HREN mRNA levels in the lung of these mice were only a small fraction of its level in kidney. We were surprised to find that HREN expression in the PAC mice was more restricted than in another transgenic model containing a 45-kb construct. The 45-kb construct extends from approximately 25 kb 5′ to 8 kb 3′, and HREN expression in the lung exceeded the level of HREN expression in the kidney (27). These data suggest the possibility that regulatory elements or a locus control region outside the 45-kb region but included in the PAC constructs (extending from 35 kb 5′ to 70 kb 3′ of the gene) is necessary before a completely restricted pattern of expression is observed.

Two lines of PAC transgenics each displayed JG cell-specific expression within the renal cortex. Indeed, the one striking similarity among all HREN transgenic models thus far reported is JG cell-specific expression in kidney under basal conditions. Remarkably, this is true irrespective of the size of the construct. For example, we have recently demonstrated that a HREN transgene containing only a minimal promoter (149 bp of 5′-flanking sequence) directs JG-specific expression of a genomic construct (11). One hypothesis consistent with this observation is that regulatory elements needed for JG cell-specific expression are located within the body of the gene. This hypothesis is supported by studies demonstrating that various 5′-flanking regions (without the coding region) of the HREN gene cannot support the expression of a reporter gene in the JG cells of transgenic mice. Alternatively, it is possible that baseline expression of HREN in JG cells is permissive and regulated at the post-transcriptional level. Indeed, this is supported by studies suggesting that the renin gene is post-transcriptionally regulated (28, 29). Whatever the explanation, it is clear that despite this similarity, previous HREN transgenic models differ from the PAC mice in two important respects. First, the HREN mRNA response to physiological challenge was generally abnormal in mice containing shorter transgenes (149- and 896-bp promoters) but normal in mice containing the 45-kb and PAC transgenes (10, 11, 30, 31). Second, the proportion of JG apparatuses within the kidney expressing HREN was markedly higher in the PAC transgenics than the 149-bp promoter transgenics (approximately 50 versus 10%). Importantly, most JG apparatuses in the kidney of the PAC mice expressing HREN also expressed endogenous MREN. This finding is particularly interesting, since recent studies suggest that one mechanism to regulate the level of renin mRNA in the kidney is to modulate the number of JG apparatuses and afferent arteriolar cells expressing renin (32). Although it remains unclear what molecular and physiological signals stimulate this recruitment of cells to express renin, it may involve the recapitulation of a developmental program of renin expression throughout the renal vasculature (33).

Angiotensin II or high dietary salt has repeatedly been dem-

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Table II

| Construct | Line | n  | Active human PRC | Total human PRC | Active mouse PRA | Total mouse PRA |
|-----------|------|----|------------------|----------------|-----------------|----------------|
| PAC140 6292/1 | 4 | 15.1 ± 5.4 | 14.6 ± 2.4 | 23.8 ± 6.2 | 45.5 ± 14.0 |
| PAC140 6680/2 | 4 | 15.1 ± 2.2 | 22.4 ± 2.4 | 21.6 ± 2.2 | 47.1 ± 17.2 |
| PAC140 7120/1 | 4 | 13.1 ± 4.1 | 23.1 ± 3.8 | 23.2 ± 3.0 | 34.0 ± 7.8 |
| PAC160 6407/1 | 4 | 11.2 ± 3.6 | 17.6 ± 2.7 | 19.2 ± 3.6 | 37.4 ± 18.6 |
| PAC160 7217/2 | 4 | 10.7 ± 3.1 | 16.0 ± 3.8 | 20.5 ± 5.3 | 52.6 ± 14.0 |
| PAC160 6919/1 | 4 | 9.9 ± 1.8 | 20.7 ± 0.9 | 18.9 ± 2.2 | 50.6 ± 11.1 |
| Control  | 4 | 2.4 ± 1.0 | 4.3 ± 2.8 | 19.5 ± 2.9 | 42.1 ± 7.8 |

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Fig. 6. Kidney renin mRNA response to captopril. The HREN (top panels) and MREN (bottom panels) response to captopril treatment is shown for representative PAC140 (n = 4) and PAC160 (n = 4) transgenic lines as indicated. HREN and MREN mRNA were normalized to the β-actin signal, and quantification was performed as described under "Materials and Methods." A representative RPA is shown at the right of each row. The presence of captopril in the drinking water and the presence or absence of the transgene in the genome are indicated. *, p < 0.05 versus untreated mice.
human renin mRNA levels were determined by RPA. The quantification results are shown in the upper panel, and representative RPAs are shown in the lower panels. B, the same mRNAs were probed with a MREN-specific RPA probe. *, p < 0.05 versus single transgenics.

Due to the strict species specificity of HREN for HAGT and MREN for mouse AGT, HREN transgenic mice exhibit no observable blood pressure phenotype (22). However, double transgenic mice containing an overexpressed HAGT gene and a poorly regulated HREN transgene (900-HREN) exhibit chronic hypertension and elevated circulating Ang-II (16). In order to test whether the PAC transgenes would be chronically down-regulated, we mated PAC lines 6680/2 and 6407/1 to the same physiological cues and to the same extent as the endogenous renin gene.

Systolic blood pressure (SBP) was measured using an automated tail cuff apparatus as indicated under “Materials and Methods.” Base-line SBP is the average of measurements taken on days 3, 4, and 5 postimplantation. ∆SBP = peak SBP – base-line SBP. All values are means ± S.E.

**TABLE III**

| Construct | Line | Ang-II dose | n  | Base-line SBP | Peak SBP | ∆SBP | p versus saline |
|-----------|------|-------------|----|--------------|---------|-------|----------------|
| PAC140    | 6680/2 | Saline      | 4  | 122.0 ± 3.5   | 124.3 ± 1.9 | 2.3 ± 4.4 | 0.40           |
|           |       | Nonpressor  | 4  | 123.2 ± 2.6   | 128.1 ± 3.6 | 4.9 ± 1.3 | 0.00004        |
|           |       | Pressor     | 4  | 112.2 ± 3.9   | 132.8 ± 2.9 | 20.7 ± 5.4 | 0.58           |
| PAC160    | 6407/1 | Saline      | 4  | 129.1 ± 7.2   | 130.0 ± 6.3 | 0.7 ± 6.1 | 0.038          |
|           |       | Nonpressor  | 4  | 122.0 ± 3.7   | 126.8 ± 2.7 | 4.9 ± 2.1 | 0.54           |
|           |       | Pressor     | 4  | 117.5 ± 0.9   | 142.7 ± 2.1 | 25.2 ± 1.9 | 0.009          |

Mouse renin mRNA response to dietary salt intake. Mice from lines 6680/2, 6407/1, and nontransgenic controls were fed high salt (H, n = 4) or low salt (L, n = 4) chow for 14 days as indicated under “Materials and Methods.” A, kidney HREN mRNA levels were then quantified by RPA and normalized to β-actin. The quantification results are shown in the upper panel, and a representative RPA is shown in the bottom panel. B, the same mRNAs were probed with an MREN-specific RPA probe. *, p < 0.05 versus saline-infused mice.

**FIG. 8.** Kidney renin mRNA response to dietary salt intake. Mice from lines 6680/2, 6407/1, and nontransgenic controls were fed high salt (H, n = 4) or low salt (L, n = 4) chow for 14 days as indicated under “Materials and Methods.” A, kidney HREN mRNA levels were then quantified by RPA and normalized to β-actin. The quantification results are shown in the upper panel, and a representative RPA is shown in the bottom panel. B, the same mRNAs were probed with an MREN-specific RPA probe. *, p < 0.05 versus high salt.
remain unclear as both transgenes (PAC and 45 kb) were similarly down-regulated in the double transgenics. However, one possible explanation stems from methodology used to measure arterial pressure in the mouse. Our studies demonstrate an increase in blood pressure when measured directly through an indwelling catheter in a conscious and freely moving mouse housed in its home cage, but not when measured either under anesthesia or via tail cuff. Although we observed no difference in tail cuff systolic pressure in any group, when the same mice were measured in the conscious, freely moving state, we reproducibly observed a modest elevation of blood pressure in the PAC140/HAGT and PAC160/HAGT and a marked increase in blood pressure in the 900-HREN/HAGT double transgenic groups but not in the control mice. We have observed that blood pressure in some hypertensive mouse models can be accurately measured via tail cuff, while others cannot, suggesting that results from different laboratories may be difficult to directly compare unless the methods for recording blood pressure are identical. Although Catanzaro et al. (31) indicate that the blood pressure in a sampling of mice was confirmed by direct measurements in the conscious state, those measurements were either made on the same day as the catheter implantation surgery or the following day. We have found that it is necessary to allow 48 h for the blood pressure-lowering effects of anesthesia to wear off. We then routinely record pressures for a minimum of 3 consecutive days, the last day being 5 days after the initial administration of anesthesia.

In conclusion, our data suggest that the PAC transgenes can partially, but not completely, compensate for the increased production of Ang-II and increased pressure in double transgenic mice. Presumably, this is due to the presence of elevated plasma angiotensinogen in the HAGT model, such that even a 3-fold decrease in HREN would still leave sufficient enzyme in the circulation to interact with excess HAGT. Consequently, it is tempting to speculate that an elevation in plasma or tissue levels of AGT in humans may cause a predisposition to hypertension. Genetic evidence suggests that some patients carrying a variant of the AGT gene may have elevated circulating AGT and exhibit hypertension (4). Other studies have implicated one haplotype of the AGT gene in causing higher transcription of AGT mRNA in vitro (34), and the mRNA from this same haplotype is present in higher levels in decidual spiral arteries of pregnant women heterozygous for the normal and altered allele (35). Similarly, we have recently demonstrated that an elevation of tissue angiotensinogen production can cause hypertension in an animal model without altering plasma levels of Ang-II (18). From a practical standpoint, it is interesting to note that inhibitors of angiotensin-converting enzyme (such as captopril) are effective antihypertensive agents not only in patients with elevated plasma renin but also in patients with normal or even low plasma renin levels, who likely have intact “servo-control” (feedback) mechanisms regulating renin synthesis and release (2).

Finally, the use of high molecular weight DNAs (>100 kb) for the construction of transgenics will undoubtedly play a more prominent role in the investigation of gene regulation in the years to come. Using PACs, bacterial artificial chromosomes, or potentially yeast artificial chromosomes to create transgenic mice has many advantages as exemplified by our study. First, large native gene flanking regions probably insulate the transgene from position effects, which have always been a concern and limitation in transgenic studies. Second, large transgenes presumably lead to low copy number, which can have advantages if the goal of the study is to investigate proper gene expression and not overexpression per se. Third, high molecular weight transgenes are more likely to have all of the regulatory regions necessary for tissue-specific expression and physiological regulation. The development of a technique to manipulate PAC and bacterial artificial chromosome clones by homologous recombination in bacteria will undoubtedly provide an important new tool for engineering more useful transgenes (36).

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Patrick L. Sinn, Deborah R. Davis and Curt D. Sigmund

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