Long-Range Control of Renin Gene Expression in Tsukuba Hypertensive Mice

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Long-Range Control of Renin Gene Expression in Tsukuba Hypertensive Mice

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Abstract

Renin, a rate-limiting enzyme in the renin–angiotensin system, is regulated to maintain blood pressure homeostasis: renin gene expression in the kidney is suppressed in a hypertensive environment. We found that expression of a 15-kb human RENIN (hREN) transgene was aberrantly upregulated (>4.2-fold), while the endogenous mouse renin (mRen) gene was suppressed (>1.7-fold) in Tsukuba hypertensive mice (THM), a model for genetically induced hypertension. We then generated transgenic mice using a 13-kb mRen gene fragment that was homologous to the 15-kb hREN transgene and found that its expression was also upregulated (>3.1-fold) in THM, suggesting that putative silencing elements of the renin genes were distally located in the loci. We next examined the possible role of a previously identified mouse distal enhancer (mdE) located outside of the 13-kb mRen gene fragment. Deletion of the mdE in the context of a 156-kb mRen transgene did not affect its transcriptional repression in THM, implying that although the silencing element of the mRen gene is located within the 156-kb fragment tested, it is distinct from the mdE. Consistent with these results, deletion of the 63-kb region upstream of the mdE from the endogenous mRen gene locus abrogated its transcriptional repression in THM. We finally tested whether dysregulation of the short renin transgenes also occurred in the fetal or neonatal kidneys of THM and found that their expression was not aberrantly upregulated, demonstrating that aberrant regulation of short renin transgenes commences sometime between neonate and adult periods.

Introduction

The renin–angiotensin system is a vasopressor signaling cascade that plays a pivotal role in blood pressure regulation and electrolyte homeostasis. The first and rate-limiting reaction in this cascade is catalyzed by renin, which is predominantly synthesized in the juxtaglomerular (JG) cells of the kidney. Renin cleaves its unique substrate, angiotensinogen (AGT), to generate angiotensin I. By the action of the angiotensin-converting enzyme (ACE), angiotensin I is further converted to angiotensin II, which increases blood pressure through vasoconstriction and
aldosterone secretion by binding to angiotensin receptors. Because renin activity is reflected in blood pressure changes, renin expression is strictly regulated through a feedback mechanism initiated by various physiological stimuli to maintain blood pressure homeostasis [1]. For example, renin gene transcription is activated and suppressed in hypertensive and hypotensive environments, respectively, in vivo [2–4].

JG cells are located in the walls of the afferent arterioles of the glomeruli, and because of this unique spatial configuration, renin genes can sense blood pressure changes through renal baroreceptors, macula densa signals, renal sympathetic input, and angiotensin II receptor-mediated feedback signaling [1, 5]. Through cell-surface G protein-coupled receptors, these extracellular stimuli are converted into changes in cellular cAMP levels, which then play a pivotal role in the regulation of renin gene transcription [6, 7]. Although physiological stimuli leading to altered renin gene transcription have been intensively investigated [8–10], cis-DNA sequences and trans-factors involved in these processes are not well understood.

To date, while numerous proximally located cis-regulatory elements of renin have been identified using cultured cells (such as immortalized mouse renin-producing renal tumor As4.1 cells [11, 12]), their in vitro and in vivo significance sometimes appears to differ [13, 14], and such discrepancies may reflect the difficulty in reconstituting a complex physiological microenvironment in a culture dish. In addition, because of the size limitation of DNA fragments that can be introduced into plasmid vectors for reporter assays, it is likely that only a fraction of cis-regulatory elements, primarily those located close to the coding region, have been preferentially identified.

Recent advances in genome-wide analysis have revealed that gene transcription is frequently regulated by distal regulatory elements through loop interactions with target promoters [15]. Examination of transgenic mice (TgM) carrying human RENIN (hREN) gene fragments of various sizes suggested the existence of long-range regulation in renin gene transcription [16, 17]. Sigmund and colleagues generated TgM using 140-kb or 13-kb hREN gene fragments, carrying 5’-flanking sequences of 35 kb or 900 bp, respectively, and tested their angiotensin II-induced hypertension responsiveness [3, 4, 17]. They found that although hREN gene expression from the 140-kb transgene (Tg) as well as the endogenous mouse renin (mRen) gene was appropriately suppressed in their hypertension model animals, the 13-kb hREN’Tg expression was inappropriately upregulated. This suggested that a silencer element for the hREN’gene is located outside of the 13-kb fragment but within the boundaries of the 140-kb Tg. However, because only one Tg line was investigated in this analysis, it may have been subjected to position-of-integration site effects thereby generating aberrant regulation. It is also possible that the result was the mere reflection of a species-specific difference in regulation of the two homologues.

In the mRen and hREN genes, distal enhancer elements have been identified 3 and 12 kb upstream, respectively, of their transcription start sites in in vitro experiments [18–20]. The mouse distal enhancer (mdE) is comprised of multiple binding sites for transcription factors, including a cyclic AMP-responsive element (CRE) [18, 20]. Using transgenic and knockout mice, we and others have shown that these enhancers are essential for basal transcriptional activity of the genes [21, 22]. Although its essential role in full induction of the mRen gene transcription in ACE inhibitor-induced hypotensive status has been established [22, 23], its total role in the regulation of renin–angiotensin system, hREN and mRen selectively catalyze hAGT and mAgT, respectively, to generate angiotensin I [25]. Therefore, a TgM carrying
hREN alone exhibits normal blood pressure (systolic blood pressure [SBP], 97.0 ± 7.3 mmHg; [24]). However, because the amino acid sequences of angiotensin I are identical in human and mouse, TgM carrying both hREN and hAGT genes reproducibly exhibit chronic hypertension, at least in adults (SBP, 129.1 ± 7.1 mmHg; [24]), as a result of overproduction of angiotensin II by murine ACE. On the other hand, because renin gene transcription is strictly regulated to maintain blood pressure homeostasis, it is quite intriguing that THM exhibits severe hypertension.

We therefore hypothesized that the 15-kb hREN Tg in our disease model might be also subject to aberrant regulation, as was the case for the 13-kb hREN Tg [3, 4, 17], and that distal regulatory elements of both human and mouse renin genes might be essential for their transcriptional repression in THM. To test this hypothesis, we generated TgM carrying the 13-kb mRen gene fragment that was homologous to the 15-kb hREN Tg, as well as mRen knock-out mice carrying a 63-kb deletion in its 5’ upstream region, and these and other mutant renin gene alleles were subjected to the THM environment to examine their transcriptional responses in vivo. These studies demonstrated that distal silencer element of the mRen gene was essential for its proper regulation in adult, but not in fetal and neonatal THM.

**Materials and Methods**

**Construction of the mRen gene fragment and generation of TgM**

A BAC clone carrying the mouse Ren-1c gene (RPCI23-240p23, GenBank accession no. AC068906) was obtained as described elsewhere [14]. To facilitate long-range structural analysis of the Tg and discriminate the transcripts of transgenic from endogenous mRen loci, SfiI and PvuII restriction sites as well as flip recombinase recognition target (FRT) sequences, as a tag were introduced into the 3’-untranslated region of the gene (RPCI23-240P23+ FRT, [14]). The transcriptional start site of the Ren-1c gene [26] corresponded to the nucleotide (nt) position 89,006 of this clone or chr1:135,247,251 (NCBI37/mm9), and all of the nt positions hereafter are expressed relative to this site (designated as +1).

The 13-kb mRen gene fragment was subcloned from this BAC clone using a prophage-recombination system [27]. To facilitate retrieving vector construction, the following oligonucleotides were annealed (generating KpnI-SfiI-Spel-Xhol-MluI-AflII-SfiI-BssHII-ScaI sites) and replaced with the KpnI-Sacl portion of the pBluescript II KS (+) to generate pBS-MCS: 5’-CGGCCAAAAAGGCCACTAGTCTCGAGAAACGACGGCTGACACTTAAAGGCCAAA AAGGGCAGGCGGCTAGCT-3’ and 5’-CGGCCGCGGCTTTTGGGCTTAAGTTCGACG CGTCTGTTCTCGAGACTAGTGCCCTTTTTGCGGTAGCT-3’.

The MluI-Xhol region (nucleotides 94,530–105,069) of the BAC was subcloned into the MluI/Xhol sites of pBS-MCS. The plasmid was then treated with AfeI/Xhol, blunt-ended, and

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**Fig 1. Expression of endogenous mouse Renin and human RENIN transgenes in THM. (A) Schematic representation of chimeric renin-angiotensin system in Tsukuba hypertensive mice. mRen, mouse Renin; mAgt, mouse Angiotensinogen; hREN, human RENIN; hAGT, human ANGIO TENSINOGEN; ACE, Angiotensin-converting enzyme. (B) Breeding strategy for obtaining normotensive (control; ctrl) and hypertensive mice (Tsukuba hypertensive mice; THM). hREN, human RENIN; hAGT, human ANGIO TENSINOGEN; Tg, Transgene. (C and D) Total RNA was isolated from the kidney of normotensive (control; ctrl) or hypertensive (THM) TgM (8-week old). Levels of endogenous mouse Ren (endogenous; C) or human transgenic REN (hREN; D) gene expression were analyzed by qRT-PCR. Each value represents the ratio of endogenous mRen or hREN Tg gene expression to that of Gapdh. The expression value of male control animals in each group was arbitrarily set at 100. qPCR analyses were repeated three times. Number of animals analyzed is shown in parentheses below each panel and mean ± SD are shown. Statistically significant differences between the control animals and THM were determined using an unpaired t-test (##, P < 0.01).

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self-ligated to obtain pmRen-3’fr, which carries a 3’-homology sequence (MluI [94,530]-AfeI [100,425]). The 5’-homology sequence was PCR-amplified using the following primer set and the BAC DNA as a template: 5’-AAAGGCCGCAGGTAGTACTGAGGTACTTTTCA-3’ and 5’-TTTCGACCGGTCCAGACTAGGTATTAGGATAAGCA-3’ (BssHII and MluI sites underlined).

Following MluI/BssHII digestion, the fragment was cloned into the MluI site of pmRen-3’fr to derive the retrieving vector. The plasmid was linearized with BpiI/MluI and used to transform Escherichia coli cells (strain EL250; a gift from N.A. Jenkins, National Cancer Institute, Frederick, Maryland, USA) harboring the 240P23+FRT BAC [14]. After selection on the basis of ampicillin resistance, transformants that underwent accurate recombination were identified by restriction enzyme digestion and DNA sequencing.

The 13-kb mRen gene fragment was released from the retrieving plasmid by digestion with BssHII, gel purified, and microinjected into the pronuclei of fertilized eggs of ICR mice (Charles River Laboratories Japan, Kanagawa, Japan). Tail DNA from founder offspring was screened first by PCR and then by Southern blotting.

For structural analysis of Tgs, agarose-embedded thymus DNA was treated with SfiI and fractionated by pulsed-field gel electrophoresis. For copy number analysis, the DNA was digested with PvuII and fractionated by conventional agarose gel electrophoresis. Following capillary transfer onto nylon membranes (PerkinElmer, Waltham, MA), blots were hybridized with a [α-32P]-labeled DNA probe (nt 98,147–98,713 [AC068906]). Tg copy numbers were determined by comparison with a standard curve generated by spiking non-Tg mouse genomic DNA with varying amounts of plasmids containing the mRen gene sequence. Signal intensities of the bands were quantified by phosphorimager and ImageQuant software (GE Healthcare, Princeton, NJ).

**Generation of mutant alleles by CRISPR/Cas9 genome editing**

The following pairs of oligos were annealed, phosphorylated and ligated to BbsI site of pX330 (a gift from Feng Zhang; Addgene plasmid # 42230, [28]) for generating hCas9/gRNA expression vectors. 5’-large-del allele: 5’-CACCGATAGAATGCAGCTCATGTCT-3’/5’-AAACACATGAGCTGCATTCTATC-3’ and 5’-CACCGAGGGAGAAATAA GTAGGTG-3’/5’-AAACCACCTACTTTATTTCTCCCTCA-3’. pseudo-WT allele: 5’-CACCGCTTGGCCTAGGGTTACTGGG-3’/5’-AAACCCCAAAGCTACCTAGGCCAAGC-3’. The CRISPR/Cas9 plasmid (and the donor DNA fragment described below in case for generating pseudo-WT allele) was microinjected into the pronuclei of fertilized eggs of C57BL/6J mice (Charles River Laboratories Japan, Kanagawa, Japan). The donor DNA fragment was PCR amplified by using following primer set and the plasmid bearing 13-kb mRen and the FRT in its 3’-untranslated region as a template: 5’-GGCTGGGATTTAGGATAGTACTTTACTAACAACTCCCATC-3’. Tail DNA from founder offspring was screened first by PCR and then by Southern blotting.

For structural analysis of mutant alleles, tail (5’-large-del) or thymus (pseudo-WT) DNA was treated with EcoRV and PvuII, respectively, and fractionated by conventional agarose gel electrophoreses. Following capillary transfer onto nylon membranes, blots were hybridized with [α-32P]-labeled DNA probes corresponding to nt 86,373–86,793 and nt 98,147–98,713 sequences, respectively [AC068906].

**Animal procedures**

Mice were housed in a pathogen-free barrier facility in a 12-hour light/12-hour dark cycle, and fed standard rodent chow. Adult (8-week old) and neonatal (1-day old) mice were sacrificed.
by cervical dislocation or beheading, respectively, and the kidneys were immediately removed and flash-frozen in liquid nitrogen. For fetal studies, female mice were sacrificed at 17.5 days of gestation (detection of the vaginal plug was designated day 0.5), and fetuses were removed from the uterine sacs. Fetal kidneys were immediately flash-frozen in liquid nitrogen.

Animal experiments were performed in a humane manner under approval from the Institutional Animal Experiment Committee of the University of Tsukuba. Experiments were performed in accordance with the Regulation of Animal Experiments of the University of Tsukuba and the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Northern blot analysis
Total RNA was isolated from mouse kidneys using ISOGEN (Nippon Gene, Tokyo, Japan) and analyzed as described elsewhere [14]. A DNA fragment (KpnI-NcoI), corresponding to exons 3 to 9 of the Ren-1c cDNA (nt 303–1,123; GenBank accession no. NM031192) was [α-32P]-labeled and used as a probe. Mouse Gapdh gene expression analyzed by a mouse cDNA (nt 565–1,017; GenBank accession no. M32599) probe was used as the internal control.

qRT-PCR
Total RNA from kidneys was converted to cDNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). Quantitative amplification of cDNA was performed with the Thermal Cycler Dice (TaKaRa Bio, Shiga, Japan) using SYBR Premix EX Taq II (TaKaRa Bio). PCR primer sequences are as follows: endogenous mouse renin gene (5’-GC CCTTCTGCCACCCCCAGTAA-3’ and 5’-CAAAGCCAGACAAAAATGGCCC-3’), mRen Tg (5’-CATCCACCGGATCTAGATAAC-3’ and 5’-CAAAGCCAGACAAAAATGGCCC-3’), hREN Tg (5’-GCTTTTCTCGACAGCACATC-3’ and 5’-TGCCAATGGGCTGTATCAATG-3’), and mouse Gapdh gene (5’-AAAATGGTGAAGGTCGGTG-3’ and 5’-TGAGGTCAATGGGGTCGT-3’).

Measurement of blood pressure
Systolic blood pressure was measured by a programmable sphygmomanometer (BP-98A; Softron, Tokyo, Japan) using the tail-cuff method, as previously described [2].

Statistical analyses
Values are expressed as mean±SD. Number of animals analyzed is shown in each panel. All data were analyzed using an unpaired t-test. Results with P<0.05 were considered statistically significant.

Results
Expression of a 15-kb hREN Tg in THM
We first tested whether renin gene expression in THM appropriately regulated the maintenance of blood pressure homeostasis. To this end, we crossed female homozygous hREN TgM with male heterozygous hAGT TgM and obtained progeny carrying either hREN Tg alone (control; ctrl) or both hREN and hAGT Tgs (THM, Fig 1B). Total RNA was prepared from the kidneys and expression of the hREN Tg as well as the endogenous mRen gene were analyzed by qRT-PCR. As anticipated, endogenous mRen gene expression was significantly lower (female, 2.5-fold; male, 1.7-fold) in THM than in control mice (Fig 1C). In the same
hypertensive environment, however, hREN Tg expression was upregulated (female, 4.2-fold; male, 6.0-fold; Fig 1D). These results indicated that the hREN Tg, but not the endogenous mRen gene, was inappropriately regulated in THM from the perspective of blood pressure homeostasis.

**Generation of TgM carrying a mRen gene fragment that is homologous to the 15-kb hREN Tg**

Two possible explanations for dysregulation of the hREN Tg in THM seemed most likely. First, species-specific differences in their transcriptional regulatory mechanisms may account for the divergent phenotypes such that the regulatory elements contained in the hREN Tg are not able to function properly in the mouse environment. Second, the 15-kb hREN Tg fragment may lack a putative cis-element(s) that is required for its proper regulation in THM. In possible accord with the latter hypothesis, it has been reported that a 140-kb hREN P1 artificial chromosome Tg was appropriately downregulated in a hypertensive environment [17].

To ask where the mouse ortholog of this putative human transcriptional regulatory sequence is located, as well as whether the hREN and mRen genes share common regulatory mechanisms in maintaining blood pressure homeostasis, we generated TgM carrying a mRen gene fragment that was homologous to the 15-kb hREN Tg. The homologous sequence was determined using the Ensembl genome browser (http://www.ensembl.org, [29]) and defined as a 13-kb fragment containing approximately 1 kb and 1.8 kb of 5'- and 3'-flanking sequences, respectively, of the mRen gene (Fig 2A, bottom). The DNA sequences were retrieved from a modified BAC (RPCI23-240p23+FRT [14]) by defective prophage λ-Red recombineering (Fig 2B [27]). In this modified BAC, FRT sequences (133 bp with artificial SfiI and PvuII recognition sites) were inserted into the 3'-untranslated region of the mouse Ren-1c gene, which allows distinction between the endogenous and transgenic mRen genes (Fig 2C). The 13-kb DNA fragment, released from the retrieving vector by BssHII digestion, was injected into the pronuclei of fertilized mouse eggs. Tail DNA from offspring was analyzed by PCR and Southern blotting to screen for transgenesis (data not shown), and two TgM lines (244 and 179) were established. The integrity of the Tg was confirmed by the long-range structural analysis of high-molecular-weight thymic DNA (Fig 2D). Quantification of the signal intensities of Southern blot bands was used to estimate that the Tg copy numbers of lines 244 and 179 were 84 and 7, respectively (Fig 2E).

**Basal level Tg expression in the kidney of 13-kb mRen TgM**

Multiple copies of the mRen transgene in the TgM suggested that it could be overexpressed. Therefore, we conducted Northern blot analysis using mRen cDNA probe to roughly quantify the overall (endogenous plus transgenic) mRen gene expression (Fig 3A). Unexpectedly, mRen mRNA levels did not differ significantly between non-TgM and TgM lines. To precisely quantify the expression levels of the Tg, we performed qRT-PCR analysis. Tg-specific primers were designed based on the FRT sequences present only in the 3'-untranslated region of the mRen Tg (Fig 3B). The results in Fig 3C revealed that the Tg was in fact expressed, but as shown in Fig 3A, the levels were much lower than those of the endogenous gene. This result was rather beneficial for our studies because blood pressure elevation due to overexpression of mRen Tg alone in the basal state (non-THM situation) was not suitable for the analysis of its regulation in hypertensive environment. We then measured the systolic blood pressure of non-Tg and Tg animals and found their levels did not differ significantly, as expected from the Tg expression level (Table 1). Therefore, we decided to use these 13-kb mRen TgM as reporter lines in gene expression analyses in vivo.
**Fig 2.** Generation of mouse renin transgenic mice. (A) Homology comparison of the human and mouse Ren gene loci determined by the Ensembl genome browser (http://www.ensembl.org, [29]). Conserved regions are indicated by shaded lines. Human RENIN (top, 15 kb) and mouse renin (bottom, 13 kb) Tg fragments used for microinjection are shown with their exon–intron organization. Lengths of the gene body, and the 5′ and 3′ flanking regions are shown in bp. (B) The RPCI23-240p23-FRT BAC carries the mouse Ren gene locus with the FRT sequences inserted at the 3′-untranslated region of the gene (top, [14]). Restriction enzyme sites with their positions relative to the transcriptional start site (+1) are shown. The 5′ (nt +962 to −683 relative to the transcription start site) and 3′ (nt +5,525 to +11,552) homology fragments were prepared by PCR amplification and restriction enzyme digestion (MluI/AfeI), respectively, of the BAC clone and subcloned into the retrieving vector (middle). Following the retrieving reaction (bottom), the BstBII fragment was released and used for microinjection. Gene body and flanking regions are shown as solid and open rectangles, respectively. (C) Partial restriction enzyme map of the mouse endogenous and Tg Ren gene loci. Knock-in of the FRT sequences (gray rectangle) generated artificial SfiI and PvuII sites in the Tg locus, which were used to discriminate the endogenous and Tg loci. The positions of restriction enzyme sites and expected restriction enzyme fragments with their sizes are shown beneath each map (top SfiI, bottom PvuII). The probe used for Southern blot analysis in D and E is indicated by a gray rectangle. (D and E) DNAs from thymic cells of non-Tg and Tg animals were digested with SfiI (D) or PvuII (E) in agarose plugs, separated by electrophoresis, and hybridized to the probe shown in C. On the right of each panel are expected bands with their sizes (in kb). A partial digestion product is marked by an asterisk. Signal intensities of the bands in (E) were quantified by phosphorimager, and the Tg copy numbers were estimated by calculating Tg:endogenous. ratios (beneath the panel). Tg, transgene. Lines in D and E indicate that lanes were run on the same gel but were noncontiguous.

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13-kb mRen Tg expression in the kidneys of THM

To examine its regulation in THM, the 13-kb mRen Tg allele was bred onto a normotensive or hypertensive (THM) environment (Fig 4A). Total RNA was extracted from the kidneys of the mice, and the abundance of mRen Tg (Fig 4B and 4C), endogenous mRen (Fig 4D and 4E), and hREN Tg (Fig 4F and 4G) transcripts was determined by qRT-PCR. In the THM, while endogenous mRen gene expression was suppressed, mRen Tg (female, 3.1~5.1-fold; male, 22.2~30.3-fold) and hREN Tg (female, 2.6~5.6-fold; male, 3.5~5.5-fold) expression was significantly upregulated in the same RNA samples. These results suggest that the dysregulation of hREN Tg in THM was not due to species-specific differences in transcriptional regulation, but rather to the lack of putative silencing elements in both the 13-kb mRen and 15-kb hREN Tgs.

Assessment of a possible role for the mdE enhancer in the kidney of THM

Because a putative silencing element of the mRen gene in THM was inferred to be located outside of the 13-kb gene fragment that was used for transgenic analysis, we focused on mdE, an enhancer sequence that resides approximately 12 kb upstream of the hREN transcriptional start site and is not contained in the 13-kb mRen fragment (Fig 2A). Its human ortholog is present approximately 12 kb upstream of the hREN transcriptional start site, and the 15-kb hREN Tg fragment did not bear this sequence (Fig 2A). To assess the role of mdE in the kidneys of THM, we employed previously generated 156-kb mRen BAC TgM lines [22]. This set of TgM lines carry either wild-type (wt) or mdE-null (mut) mRen BACs, both integrated at the identical chromosomal site. These alleles were subjected to a normotensive or hypertensive (THM) environment, as was the case for the 13-kb mRen Tg (Fig 4A), and their expression levels were determined by qRT-PCR analysis of total kidney mRNAs (Fig 5A). As shown in Fig 5B, the expression of mdE-null (mut) mRen Tg was significantly lower than that of mdE-wt mRen Tg when analyzed in the normotensive environment, which was consistent with our previous results [22]. In the hypertensive environment (THM), the expression of the mdE-wt mRen Tg was significantly suppressed, demonstrating that 156-kb mRen sequences carried sufficient information to confer appropriate regulation. The expression of the mdE-mut mRen Tg was further suppressed in the hypertensive environment (THM), demonstrating that the mdE sequence is dispensable for the observed transcriptional downregulation in the THM. Endogenous mRen (Fig 5C) and hREN Tg (Fig 5D) expression levels in these RNA samples were similar to the previous results (Figs 1 and 4). These results revealed that the 156-kb mRen fragment
Table 1. Comparison of systolic blood pressure (SBP) between non-TgM and mRen TgM.

|        | Female |          |          | Male     |          |
|--------|--------|----------|----------|----------|----------|
|        | n      | non-TgM  | mRen TgM | non-TgM  | mRen TgM |
|        | n      | line 244 | line 179 | line 244 | line 179 |
| SBP (mmHg) | 6 | 109.5 ± 4.3 | 114.3 ± 5.5 | 115.4 ± 16.7 | 109.2 ± 5.5 | 104.3 ± 2.6 | 106.0 ± 10.8 |

n, total number of mice analyzed in each group.

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Fig 3. Ren gene expression in TgM. (A) Northern blot analysis of the TgM. Total RNA samples (20 μg) from the kidney of non-Tg and Tg animals (8-week old) were electrophoresed on a 1.2% agarose gel and subjected to Northern blot analysis with the mouse Ren (top) and Gapdh (middle) probes. The KpnI/NcoI fragment (exons 3–9) from the mouse Ren-1 cDNA was used for simultaneous expression analysis of the endogenous and Tg Ren genes. Ethidium bromide staining of the gel is shown at the bottom (the positions of 28S and 18S rRNA are indicated). Lines indicate that lanes were run on the same gel but were noncontiguous. (B) Sequences of the mouse Ren Tg (Ren+FRT). Hatched region is the FRT sequence. Positions of primers used for qRT-PCR in C are underlined. (C) Expression levels of the mouse Ren Tg were analyzed by qRT-PCR. Each value represents the ratio of mRen Tg expression to that of Gapdh. Each sample was analyzed at least three times, and mean ± SD are shown.

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possesses sequences conferring proper regulatory information in THM, and that mdE is dispensable for this function.

Fig 4. Expression of the mouse Ren Tg in THM. (A) Breeding strategy for introducing mouse Ren Tg (mRen Tg) into normotensive (ctrl) or hypertensive (THM) mice. (B-G) Total RNA was isolated from the kidney of normotensive (ctrl) or hypertensive (THM) animals (8-week old) and subjected to qRT-PCR analyses. Expression levels of mouse Ren Tg (mRen Tg; lines 244 and 179 in B and C, respectively), endogenous Ren (endogenous, D and E), human REN Tg (hREN Tg; F and G), and Gapdh (data not shown) were determined. Each value represents the ratio of mRen Tg, endogenous, or hREN Tg expression to that of Gapdh. Expression value of male control animals in each group was arbitrarily set at 100. qPCR analyses were repeated twice. Number of animals analyzed is shown in parentheses below each panel and mean ± SD are shown. Statistically significant differences between ctrl and THM were determined using an unpaired t-test (#, P < 0.05; ##, P < 0.01).

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Fig 5. Expression of the mouse Ren BAC Tg in THM. (A) Breeding strategy for introducing the mouse Ren BAC Tg (mdE wild-type, wt, or mutant, mut) into normotensive (ctrl) or hypertensive (THM) mice. (B–D) Total RNA was isolated from the kidney of normotensive or hypertensive animals (8-week old) and subjected to qRT-PCR analyses. Expression levels of mouse Ren BAC Tg (BAC-Tg; B), endogenous Ren (endogenous; C), human REN Tg (hREN Tg; D), and Gapdh (data not shown) were determined. Each value represents the ratio of BAC-Tg, endogenous, or hREN Tg expression to that of Gapdh. Expression value of wt control animals in each group was arbitrarily set at 100. Number of animals analyzed is shown in parentheses below each panel and mean ± SD are shown. Statistically significant differences between ctrl and THM were determined using an unpaired t-test (#, P < 0.05; ##, P < 0.01).

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Generation of 5'-large-deletion mutant and FRT-knock-in pseudo WT alleles of endogenous mRen gene by genome editing

The previous results implied that putative silencing element(s) resided within the 156-kb region of the locus examined, yet outside of the 13-kb region of the mRen gene. We therefore decided to delete the 5'-upstream region of the endogenous mRen gene (63-kb; 5'-large-del; Fig 6A) using CRISPR/Cas9 genome editing [30]. Guide RNAs were designed to target 5'-ends of the 156-kb BAC Tg and of the mdE to remove intervening sequence between these sites (Fig

![Diagram](https://example.com/diagram.png)

Fig 6. Generation of endogenous mouse renin mutant alleles by genome editing. (A) Schematic representation of the 156-kb mRen Tg, endogenous 5'-large-del and endogenous pseudo-WT alleles. Positions of mdE and FRT sequences are indicated by gray rectangles. (B) Partial restriction enzyme maps of the mouse endogenous WT and mutant alleles. The Cas9 target sites for generating 5'-large-del and pseudo-WT alleles are shown by solid and open arrowheads, respectively. Targeting at two upstream sites removes 63-kb sequence from the 5'-upstream region of mRen gene, generating a 2.4-kb EcoRV restriction fragment in the mutant allele. Knock-in of the FRT sequence at a downstream site introduces artificial PvuII site, generating 4.0- and 3.6-kb PvuII restriction fragments in the mutant allele. Probes used for Southern blot analysis in C are indicated by gray rectangles. (C) DNAs of WT and mutant animals were digested with EcoRV or PvuII, separated by electrophoresis, and hybridized to the probes shown in B. Shown on the right of each panel are expected bands with their sizes (in kb). (D) Sequence alignment of WT (reference) and 5'-large-del alleles confirmed the 63-kb sequence deletion in the 5'-large-del allele of mRen gene. PAM and g(uide)RNA sequences are shaded and underlined, respectively. Cleavage sites predicted from PAM locations are indicated by arrowheads.

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Following microinjection of two targeting plasmids into pronuclei, correct recombination events in the founder mice were confirmed by Southern blot and sequencing analyses (Fig 6B–6D). Aside from this mutation, the 3’-untranslated region of the endogenous, wild-type mRen allele was marked by FRT insertion to generate a pseudo-WT allele (Fig 6A, bottom), to discriminate its expression from that of the 5'-large-deletion allele in the same sample. CRISPR/Cas9-mediated genome editing was used for the mutagenesis, and correct recombination was confirmed by Southern blot and DNA sequencing analyses (Fig 6B and 6C and data not shown).

5’-large-deletion mRen gene expression in the kidneys of THM

5’-large-del allele in combination with pseudo-WT allele was bred onto a normotensive or hypertensive (THM) environment (Fig 7A). Total RNA was extracted from the kidneys of the mice, and the abundance of endogenous 5’-large-del mRen (Fig 7B), endogenous pseudo-WT mRen (Fig 7C), and hREN Tg (Fig 7D) transcripts was determined by qRT-PCR. Expression of pseudo-WT mRen gene was suppressed in THM (Fig 7C), as was seen in the true wild-type allele (Figs 1C, 4D, 4E and 5C), while that of hREN Tg was upregulated in THM (Fig 7D). In the same set of samples, however, expression of 5’-large-del mRen gene in THM did not differ.

Fig 7. mRen gene expression in the 5’-large-del mutant allele. (A) Expression of mRen gene in the 5’-large-del mutant allele was analyzed in normotensive (ctrl) and hypertensive (THM) mouse environments. (B–D) Total RNA was isolated from the kidney of normotensive or hypertensive animals (8-week old) and subjected to qRT-PCR analyses. Expression levels of endogenous 5’-large-del mREN (B), endogenous pseudo-WT mREN (C), hREN Tg (D), and Gapdh (data not shown) were determined. Each value represents the ratio of renin genes expression to that of Gapdh and mean ± SD is shown. Values of male control animals in each group was arbitrarily set at 100. Number of animals analyzed is shown in parentheses below each panel. Statistically significant differences between ctrl and THM were determined using an unpaired t-test (N.S., not significant; #, P < 0.05; ##, P < 0.01).

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significantly from that of the control animals (Fig 7B). These results clearly demonstrated that putative silencing element(s) in THM reside within the 63-kb upstream region of the mRen gene that was deleted in the 5’-large-del allele.

13-kb mRen Tg expression in the fetal and neonatal kidneys of THM

We previously reported that mating of male hREN TgM (15 kb) and female hAGT TgM produced a severe hypertension phenotype during pregnancy of the mother (pregnancy-associated hypertension, PAH). It was therefore predicted that fetoplacentally-produced hREN enters the maternal circulation where it catalyzes maternally produced hAGT to cause blood pressure elevation [31]. We therefore tested the hypothesis that the lack of a silencing element led to dysregulation of the 15-kb hREN Tg expression in the fetus by examining renin gene expression in the fetal kidney of THM. To obtain control and THM fetuses in the same litter, we set up the mating as shown in Fig 4A. Kidneys were collected from 17.5 days post-coitum (dpc) fetuses, and renin gene expression was analyzed by qRT-PCR. Expression of the endogenous mRen gene was suppressed in THM when compared with that in the control littersates (Fig 8C and 8D). Although the expression of both 13-kb mRen and hREN Tgs was upregulated in the kidneys of adult THM (Fig 4B, 4C, 4F and 4G), mRen Tg expression did not differ significantly between THM and control animals (Fig 8A and 8B), and hREN Tg expression was even downregulated appropriately in THM (Fig 8E and 8F).

We then collected kidneys from newborn (1-day-old) THM, analyzed renin gene expression by qRT-PCR (Fig 8G and 8H), and found that expression of both endogenous mRen and hREN Tg was appropriately suppressed in THM. These results demonstrated that dysregulation of the short renin Tgs in THM commenced sometime within the two months after birth.

Discussion

Thanks to recent advances in locus-wide (3C, [32]) and even genome-wide (Hi-C, [33]) methodologies for identifying interactions between distinct genomic DNA segments, a number of long-range interactions between promoter and distal regulatory elements have been reported [34, 35]. Although it has been implied that the hypothetical silencing element is located far away from its protein-coding region of the hREN gene [17], equivalent findings in the mouse have not been documented. In this study, we showed that a putative silencing element of the mRen gene was also distally located to its protein-coding region, suggesting that transcriptional mechanisms, at least in adult kidney, are conserved between the two species (Table 2).

Both the human distal enhancer (hdE) and mdE are distally located to the renin genes [18, 19] and bear multiple transcription factor binding sites including a cAMP-responsive element (CRE). Desch et al. reported that CREs in hdE and proximal promoter region of the hREN gene were essential for its correct transcriptional regulation in response to administration of β-adrenergic receptor agonist and low sodium diet in TgM [36]. Although we have discovered an indispensable role of mdE in basal mRen gene transcription [22], its possible role in the hypertensive environment has not been tested. Therefore, they were possible candidates for cis-elements responsible for transcriptional repression of renin genes in THM (Fig 2A). Previous [37] and our current results (Fig 5) demonstrated that both hdE and mdE are dispensable for transcriptional suppression of renin genes in THM. In other words, novel distal elements confer appropriate regulation of the hREN and mRen genes in THM. This notion was further supported by the fact that deleting the 63-kb upstream region of the endogenous mRen gene abrogated its transcriptional repression in THM. This result not only demonstrated the existence of a putative silencing element within the mRen gene locus, but also ensured that we could ultimately identify the element by CRISPR/Cas9-mediated mutagenesis of the locus. It
must be noted that upregulation of the 5'-large-del mRen gene was not observed in THM, which was the case for short renin Tgs. Deductively, it was suggested that additional cis-element, possibly located 3' to the gene, may be suppressing such an aberrant activation. In addition, what kind of physiological stimuli in THM (e.g. pressure overload, high concentration of AII) is in fact modulating renin gene transcription in THM remains to be determined.

We inferred that aberrant regulation of the 15-kb hREN Tg is the basis for pathogenesis of hypertension in adult THM. Furthermore, we hypothesized that the same dysregulation may be associated with the expression of short renin genes in the fetal kidney, which may be, at least in part, a cause of the maternal hypertension in PAH model animals [31]. To test this hypothesis, we analyzed the kidney RNA of fetuses with THM genotype (17.5 dpc) and found that the expression of neither hREN (15 kb) nor mRen (13 kb) Tgs were upregulated (Fig 8A,
The expression of hREN Tg was rather downregulated appropriately in THM (Fig 8E and 8F, Table 2). As far as we know, method to measure fetal blood pressure in mice has not been developed. However, because of abundant expression of hAGT Tg in the liver of fetal mouse (17.5-dpc, [38]) and reduced endogenous mRen gene transcription in fetuses (Fig 8C & 8D), we assume hypertensive condition and/or high concentration of plasma angiotensin II exists in the mouse fetus with THM genotype. In support of this notion, it has been reported that direct infusion of angiotensin II into a fetal lamb raised its blood pressure and suppressed plasma renin activity [39]. Therefore, it is predictable that 15-kb hREN Tg expression in the fetal kidney (Fig 8E and 8F) was suppressed by increased angiotensin II production or hypertensive stimulus.

We previously reported an overexpression of the 15-kb hREN Tg in the placenta of THM during late gestation [31], while it was reported that endogenous hREN mRNA levels are higher during early gestation than those during term in human placenta [40]. It is therefore possible that a lack of distal regulatory elements in the 15-kb hREN Tg caused its aberrant expression in the placenta and pathogenesis of PAH. Germain et al. identified a cis-regulatory element between −5.8 and −5.5 kb upstream from the transcription start site of hREN in primary human chorionic cells [41]. A lack of such an element in the 15-kb hREN Tg may account for the dysregulation.

In the same way as in THM fetuses, hREN Tg expression was appropriately suppressed in the kidney of THM neonates (1-day-old), demonstrating that regulation of short renin Tg expression in THM changed during two months period after birth. Alteration in transcriptional environment of the kidney or epigenetic change in proximal promoter region of renin genes by long-term exposure to high concentration of AII and/or high blood pressure, in combination with a lack of distal sequences may account for the phenotype. Although exact reason for developmental difference of the renin gene regulation in THM remains to be determined, it is certain that distal silencing element is essential for proper regulation of renin genes in the adult THM, because expression of the endogenous mRen gene in THM was properly regulated throughout life.

In summary, our results demonstrate that putative silencing elements of both hREN and mRen genes in the kidney of adult THM are located at long distance from their protein-coding regions. In the mRen gene, mdE was dispensable for the repression in THM and approximate location of a putative silencing element was determined, by genome editing, to be upstream of mdE and inside a 156-kb mRen gene locus. Meanwhile, transcriptional responses of the short renin transgenes in the fetal/neonatal vs adult kidneys were apparently different, although

Table 2. Expression of human and mouse renin Tgs in adult and fetal THM.

| Renin gene expression in hREN/hAGT double TgM (THM) | Adult | Fetus |
|----------------------------------------------------|-------|-------|
| 13-kb Tg human                                     | ↑     | N.D.  |
| 15-kb Tg human                                     | ↑     | ↓     |
| 140-kb Tg human                                    | ↓     | N.D.  |
| 13-kb Tg mouse                                     | ↑     | ↓     |
| 156-kb Tg mouse                                    | ↓     | N.D.  |
| endo. wild-type                                    | ↓     | ↓     |
| endo. 5'-large-del                                  | →     | N.D.  |

Tg, transgene; endo., endogenous; ↑, induced; ↓, suppressed; →, not changed; N.D., not determined.

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endogenous mRen gene behaved similarly at all these stages. We therefore conclude that distal silencing element of the mRen gene (and probably that of hREN gene, too) was essential for proper regulation of renin genes in adult THM.

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