Physiological significance of two common haplotypes of human angiotensinogen using gene targeting in the mouse

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Cvetkovic, Branimir, Henry L. Keen, Xiaoji Zhang, Deborah Davis, Baoli Yang, and Curt D. Sigmund. Physiological significance of two common haplotypes of human angiotensinogen (AGT) was the first gene to be genetically linked to hypertension in humans. Analysis of the gene sequence identified a number of polymorphisms, several of which were reported associated with increased blood pressure (BP) or other cardiovascular diseases. One haplotype of the human AGT (hAGT) gene consisting of an allele at the −6 (A vs. G) position in the promoter and the sequence encoding amino acid 235 (Thr vs. Met) attracted the most attention and has been the subject of numerous association studies. In this report, we addressed the physiological relevance of alleles at these two positions using an experimental mouse model system. Transgenic mice were generated by targeting each haplotype [−6G/235Met (GM) and −6A/235Thr (AT)] as a single copy transgene to the mouse hypoxanthine phosphoribosyl transferase locus, allowing direct comparison of the two transgenes in vivo. Our results indicate that both transgenes exhibit the same transcriptional activity and produce similar levels of hAGT protein in the plasma of the transgenic mice. BP analysis was performed in double transgenic mice generated by breeding each hAGT line to mice expressing a human renin gene. A small but significant increase in BP and relative heart weight was demonstrated by mice carrying the GM haplotype. Moreover, compensatory downregulation of endogenous renin expression was more pronounced in mice containing the GM variant. Our findings suggest that the AT and GM haplotypes of the hAGT gene have no effect on gene expression, but may affect the cardiovascular system and the regulation of BP differently.

renin-angiotensin system; hypertension; genetics

THE RENIN-ANGIOTENSIN SYSTEM (RAS) has long been known to play a major role in blood pressure (BP) regulation. Ever since angiotensinogen (AGT) was shown to be genetically linked to hypertension a decade ago, a number of laboratories have attempted to identify and characterize mutations in the AGT gene that could be responsible for the development of hypertension and preeclampsia in humans (25, 44). To date, a number of single nucleotide polymorphisms in the human AGT (hAGT) gene have been reported, but two haplotypes of the gene consisting of allelic variants at two positions have attracted the most attention: −6A/235Thr (AT) and −6G/235Met (GM) (25, 38). These two variants exhibit strong linkage disequilibrium (22).

The initial study describing linkage of AGT to hypertension in populations in Salt Lake City, US, and Paris, France (25), was confirmed by examining European (6) and African Caribbean (7) populations, but was refuted in a study examining a European cohort consisting of 350 families (4). Since then, most of the effort at establishing the significance of AGT in the development of hypertension and hypertension-related disorders has come from association and case-control studies performed in numerous diverse populations. However, due to the uneven distribution of the hAGT variants among different races, the complex nature of the mechanisms regulating BP, and the well-established influence of environmental factors contributing to the development of hypertension, the results were largely inconsistent and often contradicting. For example, several investigators have found positive correlation between hAGT variants and BP (2, 8, 17, 19, 20, 23, 24, 26, 27, 34, 39, 40), whereas others have failed to find any association (3, 13, 21, 28, 33) in studies done on white and Asian populations in North America, Australia, Japan, Europe, and Taiwan. Mainly negative associations were found in black populations in the Caribbean, US, and Nigeria (14, 36, 37). The association between the 235T allele and high BP was most reproducible in the Japanese but absent in most of the black populations studied, despite the observation that the AGT gene itself is linked to hypertension in blacks (7). In general, when a positive association was reported, it was generally with the AT haplotype. In vitro analyses of hAGT transcriptional activity suggest that the A-to-G change at the position −6 leads to decreased hAGT promoter activity, indicating that this could be a phenotype-causing mutation (22). Supporting this is
the observation that the −6A allele is expressed at a higher level in decidual spiral arteries in women heterozygous for both alleles (32).

The confusion generated by the conflicting association study results underscores the importance of assessing the physiological significance of sequence variation in the AGT gene (or any other potential polygenic disease-causing gene) using an experimentally amenable system that has the potential to elucidate the mechanisms by which genetic variations can cause functional abnormalities leading to hypertension. Such a system should minimize the confounding influences observed in complex human populations. Therefore, to investigate the physiological significance of the two most common haplotypes of the hAGT gene, we generated transgenic mice by targeting a single copy of each hAGT haplotype to the mouse hypoxanthine phosphoribosyl transferase (HPRT) locus. This method of developing transgenic mice by gene targeting, in contrast to traditional pronuclear injection technique, results in controlled transgene insertion into the mouse genome, that is, at a single site and in a single copy, and thus eliminates copy number and position effects on transgene expression, allowing a direct comparison between lines. We previously reported that targeting the hAGT gene to the mouse HPRT locus does not affect transgene expression and that the transgenic mice developed using this method generate a fully functional human protein that is expressed and secreted at physiological levels (10). In this report we describe the development and characterization of independently derived transgenic mouse lines that carry either the AT or GM haplotype of the hAGT gene.

MATERIALS AND METHODS

Generation of targeting constructs. To generate the targeting constructs, we used a 13.8-kb segment of genomic DNA containing all five exons and accompanying introns of hAGT gene along with 1.5 kb of 5′ and 0.3 kb of 3′ untranscribed region. As part of the cloning strategy, the whole hAGT sequence was first isolated as an NheI fragment, and its ends were modified using oligonucleotide linker fragments containing NotI restriction enzyme sites by in vitro ligation. The modified fragment was then subcloned into a pBluescript SKII vector (pBlueAGT). To introduce mutations in the two positions of the gene, the hAGT sequence was separated into an ∼5-kb BamHI fragment containing exon 2 and an ∼8-kb fragment containing the remainder of the hAGT sequence. Mutations were performed using a Chameleon mutagenesis kit (Stratagene), and the complete genomic hAGT constructs were reconstituted by reinserting the BamHI fragments. The correct orientation of the internal BamHI fragment encompassing exon 2 was determined by restriction enzyme digestion of the final constructs using suitable restriction enzyme sites asymmetrically positioned within and outside the BamHI fragment. In a final cloning step, the hAGT from each construct was cloned into a pMP8SKB targeting vector, resulting in two targeting vectors with approximate size of 27 kb (5).

Embryonic stem (ES) cell electroporation. BK4 ES cells, 1 × 10^7, were electroporated in ES cell medium [DMEM-H medium with 1-glutamine and glucose (4,500 mg/l)] with 10 µg of PvuI linearized plasmids using voltage and capacitance settings of 250 V and 500 µF, respectively. ES cells were plated 1:4 on plates containing a layer of fibroblast feeder cells and were grown in ES cell culture media containing LIF (1,000 U/ml) and 20% FCS. After 24 h, the media was substituted with selective HAT media [ES cell media containing LIF, FCS, hypoxanthine (0.016 mg/ml), aminopterin (0.175 µg/ml), and thymidine (0.0048 mg/ml)], and the cells were grown for 1 wk. HAT-resistant colonies were isolated and expanded, and a portion were used for DNA analysis. Chimeric mice were generated using routine procedures.

Experimental mice. The generation of transgenic mouse models used in this study was achieved by breeding mice from the hAGTGM1 and hAGTAT2 transgenic lines with PAC-hREN transgenic mice (42). All mice were fed standard mouse chow (LM-485; Teklad Premier Laboratory Diets, Madison, WI) and had access to water at libitum. Care of the mice used in the experiments met or exceeded the standards set forth by the National Research Council in their Guide for the Care and Use of Laboratory Animals. All procedures were approved by the University Animal Care and Use Committee at the University of Iowa. The genetic background of the hREN transgenic mice was used in each assay. Mice (B6SJL × SJL/J F2) but have since been backcrossed for at least three generations prior to breeding with hAGT mice. The ES cells used to generate the hAGT mice were from 129 mice; chimeras were bred to C57BL/6 mice and then successively backcrossed to C57BL/6 for at least three generations. Given the number of backcrosses, the mouse renin gene in the background must be the Ren-1 allele.

Genetic analysis. Genomic DNA preparations from ES cells or mouse tails were used in a PCR reaction with oligonucleotides that specifically amplify 550 bp of hAGT sequence (5’TGGTGCTAGTCGCTGCAAAACTTGACACCG-3′ and 5′-CAGGGAGCAGCCACTTCTCTCTGCAGC-3′) as described previously (45). For Southern blots, ∼10 µg of genomic DNA from individual targeted ES cell clones, and/or control BK4 and R1 ES cells, was digested with BamHI enzyme overnight and resolved on a 0.8% agarose gel. Membranes were probed overnight with a radioactively labeled DNA probe which hybridizes to 250 bp sequence located in the third intron of the endogenous HPRT gene. After washing, the membranes were analyzed using a Storm 820 PhosphorImager system (Molecular Dynamics). For genotyping of transgenic animals, genomic DNA was purified from tail biopsy samples as previously described (41). Human REN and hAGT oligonucleotide primer sets specific for human sequences were used to assay the presence or absence of the transgenes (31). The mouse AGT (mAGT) locus was genotyped by Southern blot analysis as previously described (29). Briefly, XbaI-digested tail DNA was separated on a 0.8% agarose gel, transferred to nitrocellulose membranes, and hybridized with a radioactively labeled DNA probe that spans the area of the targeted disruption, exon 2. The locus harboring the deletion is identified as a smaller DNA fragment following detection of the hybridized probe.

Northern blot analysis and RNase protection assay. Tissues were harvested following CO2 asphyxiation and snap-frozen in liquid N2. RNA was prepared using Tri-Reagent (MRC) as suggested by the manufacturer. For tissue-specific expression, 25 or 30 µg of individual RNA preparation (or the whole RNA preparation in case of tissues such as aorta and adrenal gland) was used in each assay. Lane samples were resolved on a 3% agarose gel. Membranes were probed overnight with radioactively labeled RNA probe spanning nucleotides 302–931 of the hAGT cDNA sequence. After washing, the signals were detected using X-ray film (Kodak) or Storm 820 PhosphorImager system (Molecular Dynamics). RNase
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Catheters were filled and flushed twice daily with sterile dilute heparinized saline solution (200 U/ml). After a 2-day recovery period, during which mice remained in their home cages, BPs were recorded using a PowerLab 4SP system (AD Instruments). After connection to the recording equipment, the mice were allowed a 15- to 30-min accommodation period before data acquisition. Mice were allowed to move freely. BP and HR were recorded continuously for 45–90 min, and the data from the whole measurement session averaged. Only results from 2–5 consecutive days of measurements were used in the final data analysis for each group of mice. Statistical analysis was performed using SigmaStat program.

Measurement of organ weights. Relative organ weights were determined by measuring whole organ wet tissue weight and whole body weight immediately following CO2 asphyxiation.

Statistical analysis. Data are expressed as mean ± SE. Group comparisons were either made with t-tests (Figs. 2, 4, and 6B) or with ANOVA followed by the Student modified t-test with Bonferroni correction (Figs. 5, 6A, and 7). A value of P < 0.05 was considered statistically significant. In each case the data set were determined to exhibit a normal distribution and therefore nonparametric analysis was deemed unnecessary.

RESULTS

We used a transgenic mouse model to analyze the effects of the two hAGT variants, AT and GM, on hAGT gene behavior and RAS function in vivo. First, site-directed mutagenesis was performed to generate two hAGT constructs that were identical in sequence except at two positions representative of the allelic variants found in human populations: position –6 of the promoter (A-to-G substitution); and mutation in the coding region of the gene (C-to-T substitution of a middle base in codon 235, changing the coding from Thr to Met). Each hAGT construct was then subcloned into a pMP85KB vector, resulting in two targeting vectors, pMPAT and pMPGM. The sequence difference between the final targeting vectors was confirmed by DNA sequencing. Transgenic ES cells were generated from both targeting vectors, and integration of the hAGT transgenes into the mouse genome was determined by the hAGT-specific PCR and a Southern blot assay designed to identify a single recombination event at the HPRT locus (10). We determined that both pMPAT and pMPGM vectors were both equally efficient at generating single transgene insertions into the ES cell genome, identified in 82% and 96% of the ES cell lines, respectively. Several ES cell lines transgenic for either the hAGTAT or hAGTGM gene variant were used to derive chimeric mice. From the chimeras, we successfully developed four lines of transgenic mice: one line harboring the hAGTGM variant (described previously in Ref. 10) and three lines transgenic for the hAGTAT variant.
To determine whether sequence variation between the hAGT transgenes has an effect on tissue-specific distribution of transgene expression, hAGT mRNA expression was analyzed by Northern blot in both male (Fig. 1) and female mice (not shown). We found no qualitative difference in expression between the hAGTGM and hAGTAT transgenes, indicating that the spatial pattern of expression is unaffected by the two substitution mutations. It is important to note that all three independently derived transgenic lines harboring the AT variant were phenotypically identical in this assay, confirming that identical genetic modifications result in the same phenotype of the transgenic animals. Independent analysis of all three hAGTAT lines showed virtually identical phenotypes in all assays presented in this report.

RNase protection analysis of hAGT mRNA expression demonstrated that both variants are expressed at almost identical levels in liver, kidney, brain, and aorta (Fig. 2). Immunocytochemical analysis of tissue sections derived from either the GM or AT lines showed that the expression of both hAGT protein variants was restricted to the appropriate cell type in the kidney (Fig. 3). The same hAGT-specific antibody used for protein detection in the kidney was employed in a Western blot assay to identify the presence of the hAGT protein in plasma (Fig. 4A). Identical migration of a characteristic hAGT doublet in all samples tested indicated no gross perturbations in protein structure or posttranslational processing of the hAGT protein derived from either variant. Moreover, similar band intensities indicated equal levels of hAGT in the plasma of all four mouse lines. This was also confirmed in the AT2 line, the AT line used to generate double transgenic mice (see below), by quantifying total hAGT plasma protein levels by radioimmunoassay (Fig. 4B). Although there was a trend toward increased plasma hAGT levels in the GM mice, the difference did not reach significance (P = 0.056).

Thus far, analysis of the two hAGT transgenic mouse models allowed only molecular characterization of the hAGT variants since mouse renin (mREN) does not recognize hAGT as a valid substrate. Indeed, there is a strict species specificity in the interaction between renin and AGT (18). Consequently, to test whether the two hAGT variants affect the cardiovascular system differently, we bred the hAGTGM1 and hAGTAT2 lines to PAC-hREN mice that carry a human renin (hREN) gene. In these mice, the hREN gene is inserted in the mouse genome in the context of 160 kb of human sequence, as part of a P1 artificial chromosome (PAC).

Expression of hREN is restricted to kidney; is expressed at physiological levels; and is tightly regulated in response to physiological cues (42). The resultant double transgenic hREN/hAGT mice represent the most physiologically relevant model of the human RAS developed to date, as both transgenes exhibit appropriate tissue-specific expression and at physiological lev-
els. It is important to note that the physiological analyses described below were performed on the mice from the F1 generation of the transgenic times transgenic cross, which can generate double transgenic mice that are haploid for both transgenic loci (one harboring hREN, and one the hAGT gene). Since the hAGT transgene is positioned on the X chromosome, the location of the HPRT gene, its expression can be affected by random X inactivation (10). Thus male and female mice were analyzed separately in each double transgenic line. We also reconfirmed the hAGT genotypes in our double transgenic lines by sequencing the PCR-amplified segment of the hAGT promoter encompassing the mutation at position 6.

BP measurements were performed on male and female double transgenic mice (RA) from both PAC-hREN/hAGTGM1 and PAC-hREN/hAGTAT2 lines, using single or nontransgenic littermate mice as controls (Fig. 5). No difference in the BP phenotype was observed in female mice from either double transgenic line. We also reconfirmed the hAGT genotypes in our double transgenic lines by sequencing the PCR-amplified segment of the hAGT promoter encompassing the mutation at position 6.

In males, PAC-hREN/hAGTGM1 mice exhibited a significant increase of $9.7 \pm 0.9$ mmHg ($P = 0.049$, $n = 8$) compared with their control littermates. This BP phenotype was specific for the mice carrying the GM variant, as male mice from the PAC-hREN/hAGTAT2 had BP levels similar to their control littermates. Although we usually consider it the most valid to compare each transgenic to their age-matched littermate controls, we recognize the importance of pointing out that there was no statistically significant difference in BP between male PAC-hREN/hAGTGM1 and PAC-hREN/hAGTAT2 mice ($P = 0.85$) or when the two negative control groups were directly compared ($P = 0.1$).

Interestingly, despite no difference in BP in the females, there was an increase in heart weight-to-body weight ratio in female GM double transgenic mice compared with control and AT2 mice ($P = 0.022$, Fig. 6A). In contrast, there was no difference when comparing male double transgenic to control mice ($P = 0.14$). To determine whether this difference was due to enhanced expression of one hAGT allele over the other in heart, samples from female AT2 and GM mice were directly compared. However, no difference in AT2 or GM hAGT expression was evident in the heart (Fig. 6, B and C).

Additional support for a differential effect of the GM allele is supported by our observation that expression of the endogenous mREN mRNA in the kidney is significantly downregulated in double transgenic GM mice ($P = 0.01$, Fig. 7). Although there was a trend for renal mREN mRNA to be decreased in the AT double transgenic mice, this difference did not reach statistical significance ($P = 0.08$). A trend toward a decrease
in PAC-hREN mRNA was also observed in the male GM and AT mice which did not reach significance.

**DISCUSSION**

We generated a new model system which allowed us to specifically test the physiological effects of common allelic variation in the AGT gene in vivo. To this end, we generated two different hAGT transgenes, representative of the two most common haplotypes observed in humans, by site-directed mutagenesis, and inserted both transgenes in a single copy to the identical location in the mouse genome. The identical genetic environment in both genetic background and transgene location allowed us to directly compare the transcriptional and functional activity of each haplotype and to link any significant difference in phenotype(s) to the sequence differences of our hAGT transgenes.

The molecular analysis of the two hAGT haplotypes showed that variation at nucleotide −6 and amino acid 235 positions have no effect on the tissue- and cell-specific expression pattern in either males or females and that both variants are transcribed with equal efficiency in liver, kidney, brain, and aorta. This result was unexpected since previous reports indicated that the −6 variant affects baseline transcriptional efficiency both in vitro, in the context of a reporter construct, and that it may influence the steady-state level of AGT mRNA in vivo in decidual spiral arteries of first trimester pregnant women and consequently perhaps in other tissues as well (22, 32). There are several possibilities for this disparity. As was previously shown by the work in our laboratory, the hAGT gene can behave differently when studied in vitro and in vivo (46). Whereas deletion mutations in the promoter and a 3′ enhancer of the hAGT gene were shown to have an obvious effect on the transcription of the gene in HepG2 cells, no effect on gene regulation was detected in vivo when these mutations were studied in transgenic mice. The reason for this remains elusive, although it is conceivable that the HepG2 cell culture model is lacking all of the factors involved in the normal regulation of the hAGT expression, since it is an immortalized cell line.

We also have to consider the possibility that the environment of a mouse cell does not allow for the effect of the hAGT variants to be revealed. This could be either due to the absence of cellular factors that can recognize specific elements of the hAGT promoter or that those factors are significantly different from their human counterparts so that they cannot serve the same function. Indeed, the AGT gene is only 60% identical between humans and mice, a level of homology which is rather low, considering the evolutionary importance of the RAS pathway. Presumably this low homology reflects the species specificity of the renin-AGT reaction itself, thus affecting enzyme-substrate
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Fig. 7. Expression of mouse and human renin mRNA in kidney. Relative mRNA expression levels were determined by RPA in control single transgenic mice PAC-hREN (CON) and double transgenic PAC-hREN/hAGT\textsuperscript{GM1} (GM) and PAC-hREN/hAGT\textsuperscript{AT2} (AT) mice, using mouse actin as an internal control. Mouse renin (mREN) mRNA exhibited apparently reduced expression in all double transgenic mice reaching statistical significance only in male PAC-hREN/hAGT\textsuperscript{GM1} mice ($P\text{=0.01}; n=6$). The reduction of mREN expression in male PAC-hREN/hAGT\textsuperscript{AT2} mice failed to reach significance ($P\text{=0.08}; n=4$). Human renin (hREN) mRNA expression was unaffected in female mice from either double transgenic line, while it exhibited moderately reduced expression in the male mice. However, the apparent decrease did not reach statistical significance in either group (male PAC-hREN/hAGT\textsuperscript{GM1}, $P\text{=0.06}; n=6$; male PAC-hREN/hAGT\textsuperscript{AT2}, $P\text{=0.35}; n=4$).

kinetics. The level of homology in the renin genes between human and mouse is also relatively low, whereas the homology for angiotensin converting enzyme (ACE) and AT\textsubscript{1} receptor are very high. We therefore cannot rule out the possibility that important regulatory sequences in the human and mouse AGT could be dissimilar. Such differences might prevent appropriate responsiveness of the available transcriptional control machinery to the variations in the cis regulatory elements of the hAGT gene. An analysis of the -6 position of AGT in eight different mouse strains reveals 100\% conservation of the A at that position, thus making it unlikely that there is a natural variation in sequence at that position in the mouse gene. Despite all these possibilities, it is important to point out that expression of hAGT in the mouse appears in the correct spectrum of cells and tissues, and expression remains responsive to the same set of hormones as in humans (45).

It is also important to recognize that we have specifically analyzed the effect of only two of the reported variants present in the hAGT gene. It is possible that the -6 and 235 variants are simply genetic markers in linkage disequilibrium with the real phenotype-causing mutation(s). It has recently been reported that the -6 and 235 variants are in close or complete linkage disequilibrium with several other variants in the regulatory region (at positions -532, -152, -20, and +31) and within the coding region (amino acid 174) (24, 38). The C-to-T change at position -532 has been shown to be present only in a subgroup of individuals carrying the A variant at position -6 and was the only mutation associated with increased plasma levels compared with mutations at position -6 and codon 235 (35).

In addition, although a trend toward a higher level of hAGT was observed in GM mice, this difference did not reach statistical significance. This raises the question of whether hAGT\textsuperscript{235M} and hAGT\textsuperscript{235T} act similarly biochemically. Along these lines it is interesting to note that although the reaction between renin and AGT appears identical, subtle differences in protein structure caused by sequence variation at position 235 can be distinguished with monoclonal antisera (9). Similarly, other hAGT variants with identical kinetic constants for cleavage by renin appear to have different immunoreactivities to specific monoclonal antisera (16). Interestingly, the cysteine at position 232 (Cys232) of hAGT was reported to be involved in the interaction between hAGT and eosinophil major basic protein (proMBP)(15), a complex that occurs during pregnancy (15). Given the proximity, Cys232 may interact with Met235 or Thr235 and thus modulate the association between AGT and proMBP. Although there was no difference in cleavage of hAGT\textsuperscript{235M} and hAGT\textsuperscript{235T} by renin, cleavage of the proMBP-bound AGT was seven times slower than unbound AGT, and more hAGT\textsuperscript{235M} is found complexed with proMBP than hAGT\textsuperscript{235T}. Therefore, there may be some conditions under which variation at position 235 may influence the rate of ANG II production.

To test whether hAGT variants have a differential effect on BP, we developed double transgenic mice expressing both the hAGT and hREN genes. The hREN gene we chose is derived from a large transgene encoded on a 160-kb PAC. This transgene exhibits a highly restricted tissue-specific expression profile and is tightly regulated in response to physiological cues which normally regulate the renin gene (42). Elevated BP (BP) was only observed in the male mice from the PAC-hREN/hAGT\textsuperscript{GM1} line and only compared with their non-double transgenic littermates. There was no difference in BP comparing male mice from the PAC-hREN/hAGT\textsuperscript{AT2} line to either control mice or the PAC-hREN/hAGT\textsuperscript{GM1} mice. Similar to BP, an increase in heart weight was observed in both male and female PAC-hREN/hAGT\textsuperscript{GM1} mice. Moreover, the decrease in endogenous mREN expression in kidney only reached
statistical significance in the male PAC-hREN/hAGT\textsuperscript{GM1} mice.

The difference in BP phenotype between male and female PAC-hREN/hAGT\textsuperscript{GM1} mice is likely due to the different magnitude of expression of the RAS genes in these two groups of mice. The level of hAGT production in the male transgenic mice is higher than in females due both to random X inactivation in females (X chromosome harbors the hAGT transgene) and to the potentialization of hAGT expression by testosterone. It is possible that the smaller increase in ANG II in female double transgenic mice may be within their ability to compensate and thus retain a normal BP. In females, the decrease in endogenous mREN expression was not as great as in males, and there was no attempt to compensate by reduction in hREN expression. We can, therefore, hypothesize that the 0.5-copy equivalent of the hAGT transgene in female double transgenic mice produces increased ANG II levels that are within the compensatory range of the mouse RAS, whereas the 1-copy equivalent of the hAGT transgene present in the male mice is sufficient to produce elevated ANG II levels that can overcome the compensation. Our results are in agreement with the data derived from the work by Smithies and coworkers (29), who showed that one additional copy of hAGT gene leads to a 8.3 ± 2.3 mmHg increase in BP.

The phenotypes associated with hypertension (i.e., increased BP and heart weight) that were found specifically in the mice carrying the GM variant of the hAGT gene were unexpected for two reasons. First, when an association between an allele of AGT and a cardiovascular phenotype was reported, it was generally with the AT haplotype (38, 43). In contrast, only one study reported an association of the GM variant with hypertension (30). Secondly, the molecular analysis of the two hAGT variants in our model showed no difference in the distribution and levels of expression of the mRNA or protein in all tissues tested and in plasma. Previous studies suggested that the AT variant may be associated with higher expression of AGT (22). It is interesting to note that the AT haplotype is conserved in nonhuman primates, suggesting that it is the ancestral allele. It has been hypothesized that the AT allele is the “thrifty” allele of AGT in humans because it leads to increased AGT production, resulting in increased ANG II levels and increased salt and water retention. From an evolutionary sense, this may have been necessary to protect against salt and water loss when they were in short supply. The GM haplotype may have appeared more recently under conditions where water and salt are replete, as the favorable “thrifty” AT allele became unfavorable (or “hypertension-causing”) due to augmented salt and water retention that could not be compensated by the BP-regulating mechanisms. Despite this supposition, why does the GM variant yield higher BP in the mouse? First, we must be cautious in this conclusion, as the GM mice exhibited higher BP when compared with their nontransgenic controls but not when compared with the AT mice. It is possible that this can be an anomaly of the sampling used. Arguing against this is the phenotype of increased heart weight and the enhanced down-regulation of endogenous renin mRNA which accompanied the increased BP in the GM mice. One possibility that should be considered is that we used a tightly regulated hREN construct. It is possible that the effects of the AT variant may become unmasked only in the context of poorly or dysregulated renin expression. Indeed, several studies point to a relationship between AGT genotype and plasma renin activity, suggesting a potential interaction between the two loci (1, 12). We are currently breeding mice containing each AGT haplotype with transgenic mice expressing a dysregulated human renin gene. We also plan on transferring both genes onto a mouse AGT-deficient genetic background to eliminate effects of the endogenous RAS.

In conclusion, development of transgenic mice using gene targeting at the HPRT, or some other defined and selectable locus, should allow investigators to analyze the impact of small genetic variations in the genes involved in BP regulation, including the hAGT gene, on the development of hypertension. The high efficiency of this method allows a high-throughput generation of several transgenic lines carrying different forms of the transgene of interest, whose function can be directly compared on both molecular and physiological level. This should expedite the evaluation of a number of potential functional mutations identified in these genes and help determine the mechanisms by which small genetic variations impact their activity. Although one must be cautious when interpreting the results obtained from the evaluation of human genes in the mouse system, we feel that this approach will be invaluable in advancing our understanding of the pathophysiology of complex polygenic disorders like hypertension. In addition, it will help us understand the intricate interactions of metabolic pathways involved in these processes, as we should be able to identify specific gene-gene interactions by analyzing the behaviors of functional gene variants.

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