Appropriate Tissue- and Cell-specific Expression of a Single Copy Human Angiotensinogen Transgene Specifically Targeted Upstream of the HPRT Locus by Homologous Recombination*

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Development of experimental models by genetic manipulation in mice has proven to be very useful in determining the significance of particular genes in the development of or susceptibility to hypertension. Advances in molecular genetics, transgenic mouse technology, and physiological measurements in mice provided an opportunity to go a step further and develop models to analyze the physiological significance of specific gene variants potentially causing hypertension. In this report, we describe the development of a human angiotensinogen transgenic mouse model generated by targeting the human angiotensinogen gene upstream of the mouse HPRT locus by homologous recombination. The main benefit of this transgenic mouse model is that the human angiotensinogen gene is inserted into the mouse genome as a single copy at a predefined locus and in a specific orientation—a process that can be repeated utilizing other variants of this gene. We establish the validity of this approach by showing that the hAGT<sup>hprt</sup> mice have normal tissue- and cell-specific expression of the human angiotensinogen gene and normally produce and process the hAGT protein at physiological levels.

Hypertension is a multifactorial disorder exhibiting polygenic inheritance. Since the angiotensinogen (AGT)<sup>1</sup> gene was shown to be genetically linked to hypertension, there has been a search for specific mutations in the human AGT sequence that could be causative of the disorder (1). Whereas a number of single nucleotide polymorphisms have been identified in the coding region, in introns, and within the promoter region of the gene, only one haplotype has been reported by independent research teams to associate with the hypertensive phenotype. This haplotype consists of two single base change alleles which exist in strong linkage disequilibrium, a G → A substitution at position −6 of the promoter and a T → C substitution in codon 235 changing it from methionine to threonine (2). The above-mentioned polymorphisms were derived from studies in which the frequency of the −6A/235Thr allele was analyzed with respect to blood pressure and other cardiovascular disorders like plasma angiotensinogen levels, coronary atherosclerosis, or coronary heart disease (1–8). However, among dozens of linkage, association, and case-control studies on this subject, substantial controversy remains regarding the significance of this genetic alteration in hypertension (Ref. 9–13; and reviewed in Ref. 14).

The number of reports and their conflicting conclusions illustrate the difficulties encountered in addressing and understanding the genetics of polygenic diseases. Corvol et al. (15) recently described that it is very difficult to rigorously prove the significance of a genetic polymorphism utilizing only linkage analysis when the phenotype is polygenic and multifactorial in nature and when the populations are ethnically diverse. For example, the frequency of the −6A/235Thr allele is disproportionate among different ethnic groups, being prominent in African and Japanese populations whereas the −6G/235Met haplotype is predominant in people of European descent (2, 11, 16, 17). Because of these issues, studies must be performed in large populations to ensure adequate statistical power. Evidence of linkage is generally a statistical one governed by the modest excess of one allele in hypertensive over normotensive subjects. Thus, these studies can only establish an association between a phenotype and a gene but not prove causality. It is therefore essential to establish the significance of a genetic mutation experimentally by performing biochemical and functional studies.

Understanding these limitations, we developed an experimental system using gene targeting to accurately assess the physiological significance of gene variation in the human AGT gene. The system is based on studies by us and others which established that: 1) the human renin and human AGT genes can functionally complement abnormalities in the mouse AGT gene induced by gene targeting (18) and 2) that gene targeting can effectively deliver a transgene in a single copy to a selected locus in the genome (Ref. 19; and reviewed in Ref. 20). Here we report the development and characterization of a transgenic mouse model in which gene targeting at the HPRT locus was used to selectively target a single copy of the human AGT gene to a known site in the genome. We demonstrate that insertion of the single copy transgene upstream of HPRT does not affect the overall tissue- and cell-specific expression or hormonal regulation of human AGT. This study provides an important proof-of-principal that the functional significance of allelic variation in human AGT can be assessed by examining mice carrying transgenes targeted in a single copy to an identical insertion site.
MATERIALS AND METHODS

Generation of the Model—Genomic human AGT construct spanning all five exons and accompanying introns as well as 1.5 kb of 5' and 0.3 kb of 3' region was subcloned as a Nco I fragment into a pMP8SKB HPRT vector (19), resulting in a 27-kb pMPGLM targeting vector. 107 of BK4 ES cells were electroporated in ES cell medium (Dulbecco's modified Eagle's-Ham's medium with l-glutamine and glucose (4500 mg/l)) with 10 mg/ml of Pvu I linearized pMPGLM plasmid using voltage and capacitance settings of 250V and 500 microfarads, 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 (1000 u/ml) and 20% fetal calf serum. After 24 h, the media was substituted with selective HAT media (ES cell media containing LIF, fetal calf serum, hypoxanthine (0.016 mg/ml), aminopterin (0.175 µg/ml), and thymidine (0.0048 mg/ml)), and the cells were grown for one week. HAT-resistant colonies were isolated, expanded, and a portion used for DNA analysis. Chimeric mice were generated using routine procedures.

Genomic DNA preparations from ES cells or mouse tails were used in a PCR reaction with oligonucleotides that specifically amplify 550 bp of human angiotensinogen sequence (5'-TGGTGCTAGTGCCTGCTAAACCTTGACACCG-3' and 5'-CAAGGAACGCAGCTTCTACGTCGTA-3') as described previously (21). Approximately 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 radiolabeled DNA probe which hybridizes to a 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).

Analysis of Expression—Tissues were harvested following CO2 asphyxiation and snap-frozen in liquid N2, and RNA was prepared using TRI-Reagent (Molecular Research Corp.) 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 per assay, and samples were resolved on 0.8% agarose gel. Membranes were probed overnight with a radiolabeled RNA probe spanning nucleotides 302–931 of the human AGT cDNA sequence. After washing, the signals were detected using x-ray film (Kodak) or Storm 820 phosphorimager system (Molecular Dynamics).

RPA was performed using HybSpeed RPA kit (Ambion) according to the manufacturer's specifications. Each RNA sample was incubated with the human AGT and mouse actin probes (specific activity ~20,000 cpm) which resulted in protected fragments of 518 and 250 bp, respectively. All samples were run on the same gel and bands quantified using Storm 820 PhosphorImager™ system and ImageQuant Version 4.0 software (Molecular Dynamics). Data was normalized using mouse actin and analyzed using SigmaStat graphing program.

For immunocytochemical analysis, kidney and liver segments were isolated from transgenic mice or nontransgenic littermates and fixed in 4% paraformaldehyde for 2 h, transferred to 30% sucrose overnight, and kept at 4 °C overnight. Next day, tissues were frozen in OCT and sectioned at 8–10 µm. Slides were rinsed with Superblock (Pierce) for 5 min followed by a 10 min incubation with 0.1% Triton X-100 in Superblock at room temperature. Permeabilized sections were then incubated with rabbit anti-human AGT primary antibody overnight at 4 °C. The slides were washed with phosphate-buffered saline (3× for 10 min), incubated with secondary Cy3-labeled donkey anti-rabbit antibody at 37 °C for 2 h, and washed again 3× for 40 min in phosphate-buffered saline. Confocal microscopy was performed using a Bio-Rad MRC-1024 Hercules Laser Scanning Confocal Microscope equipped with a Kr/Ar laser. Equal volume whole plasma samples (5 µl) were loaded onto 10% polyacrylamide gels and electrophoretically transferred onto nylon membranes. The membranes were either stained with Cooomassie Blue to visualize total protein or used to detect human AGT protein. For human AGT protein detection, the membrane was probed with 1:1000 dilution of primary rabbit anti-human AGT and detected with colorimetric assay utilizing horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (ECL Western blotting analysis system, Amersham Pharmacia Biotech).

Endocrinology—Mouse and human AGT can be differentiated in the plasma on the basis of the strict species-specificity of the biochemical reaction between renin and AGT (22). Plasma samples were collected from mice immediately following CO2 asphyxiation. Approximately 50 µl of whole blood was collected at each bleed and placed in chilled tubes containing 2.5 µl of 0.5 mol/l EDTA. The specimens were then immediately centrifuged at 12,000 rpm for 10 min at 4 °C, and two 250-µl plasma samples were obtained and immediately frozen at −80 °C.

FIG. 1. Schematic of gene targeting at the HPRT locus. A, schematic of the targeting vector pMPGLM with human AGT sequence represented as a thick line, mouse HPRT locus sequence as a thin line, and pBluescript SK− sequence as a crosshatched box. Boxes represent exons, and arrows indicate location of transcription start sites for the human AGT and mouse HPRT genes (P—Pvu I, N—Not I; B—BamHI). B, homologous recombination event between BK4 HPRT locus and the targeting vector results in integration of a single copy of a human AGT gene at the HPRT locus as shown in panel C. Arrows, →, human AGT-specific primer set.

Generation of the hAGT gene by PCR using hAGT-specific primer set (A) and by Southern blot using rsa probe (B). Note that clone 7 contains two insertions, one upstream of HPRT, and one at another undefined location in the genome. A cross between a male chimeric mouse with a high degree of chimerism, derived from clone 1, and three C57BL/6J females resulted in 15 offspring that were analyzed for the presence of the hAGT transgene by PCR (C) and for single copy integration at the HPRT locus by Southern blot (D). M, 100-bp ladder; −, water negative control; +, plasmid carrying human AGT gene positive control; B or BK4, BK4 cell DNA; ES, DNA from clone 3; R1, R1 cell DNA with wild-type HPRT locus.

Plasma levels of AGT were then extrapolated using the 1:1 molar immunoassay results were on the linear portion of the standard curve. Immunoassay results were on the linear portion of the standard curve. Expression of Single Copy Transgene at the HPRT Locus

FIG. 2. ES cell targeting and development of transgenic mice. HAT-resistant ES cell clones were analyzed for the presence of the hAGT gene by PCR using hAGT-specific primer set (A) and by Southern blot using rsa probe (B). Note that clone 7 contains two insertions, one upstream of HPRT, and one at another undefined location in the genome. A cross between a male chimeric mouse with a high degree of chimerism, derived from clone 1, and three C57BL/6J females resulted in 15 offspring that were analyzed for the presence of the hAGT transgene by PCR (C) and for single copy integration at the HPRT locus by Southern blot (D). M, 100-bp ladder; −, water negative control; +, plasmid carrying human AGT gene positive control; B or BK4, BK4 cell DNA; ES, DNA from clone 3; R1, R1 cell DNA with wild-type HPRT locus.

Plasma levels of AGT were then extrapolated using the 1:1 molar relationship between Ang-I and its precursor, AGT, using the formula: ng Ang-I/ml × 0.77 pmol/ng Ang-I × 0.05 µg hAGT/pm. Physioogy—Blood pressure was measured in conscious, freely moving mice surgically instrumented with a left common carotid artery catheter (0.04 in outer diameter × 0.025 in inner diameter, Microrenchathane; Brainstem Science Inc., Braintree, MA) as described (25). The blood pressure response to infusions of purified recombinant human renin were made via a catheter implanted into the right jugular vein as described (24). Recombinant human renin was the kind gift of Drs. Walter Fischli and Klaus Lindpaintner at F. Hoffman-LaRoche, Basel Switzerland. To assess specificity of the response, mice were given captropil (20 mg/kg, intravenously) or a known pressor response dose of Ang-II (10 mg/kg) (25).
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Tissue-specific analysis of human AGT expression. Tissues were isolated from hAGT<sup>hprt</sup> (A and B) and regular transgenic hAGT mice (C and D), and the expression of hAGT gene was analyzed by Northern blot analysis in both male (A and C) and female (B and D) mice. Amounts of RNA used in each blot are represented at the bottom of each figure by the segments of methylene blue-stained filters indicating 28 S rRNA. L, liver; H, heart; Sp, spleen; Lg, lung; K<sub>l</sub>, left kidney; K<sub>r</sub>, right kidney; Sg, submandibular gland; B, brain; D, diaphragm; Sk, skeletal muscle; WA, white adipose tissue; BA, brown adipose tissue; Ad, adrenal gland; Ao, aorta; T, testis; O, ovaries; U, uterus.

TABLE I

| Clones screened by Southern blot | Clones with single recombination | Percent positive |
|---------------------------------|---------------------------------|-----------------|
| HAT resistant colonies | Polymerase chain reaction positive clones | BK4 ES cells | 34 | 33 | 23 | 22 | 96 |

RESULTS

To target the human AGT in a single copy upstream of the HPRT locus, we generated a targeting vector consisting of a segment of genomic DNA containing the complete human AGT coding sequence into the pMP8SKB vector (19). The targeting vector was transfected into BK4 cells, an HPRT-deficient E14Tg2a ES cell line. This cell line lacks the promoter and first two exons of HPRT, rendering it sensitive in HAT media. The targeting vector contains two regions of homology upstream and downstream of HPRT to facilitate recombination, the promoter and first two exons of HPRT, HPRT locus, and downstream of HPRT to facilitate recombination, the promoter and first two exons of HPRT, rendering it sensitive in HAT media.

Upon establishing the hAGT<sup>hprt</sup> transgenic line, we analyzed the tissue-specific expression of the transgene and compared it with a previously established multicopy human AGT transgenic mouse line generated by standard pronuclear injection (Fig. 3) (21, 26). Abundant human AGT mRNA was detected in liver and kidney of both the multicopy and hAGT<sup>hprt</sup> transgenes, with lower levels detected in tissues such as heart, brain, white and brown adipose tissue, diaphragm, and aorta. Only minor anomalies were observed, including diminished expression in adrenal gland of both male and female hAGT<sup>hprt</sup> mice and increased expression in kidney of male hAGT<sup>hprt</sup> mice. These results strongly suggest that integration of the human AGT transgene upstream of the HPRT locus does not have a significant effect on its expression.

Cell-specific expression of the human AGT protein was tested by immunohistochemistry in two of the most abundantly expressing tissues—liver and kidney. Fluorescent signals specific to human AGT protein expression were restricted to proximal tubule cells of the renal cortex (Fig. 4) and hepatocytes of the liver (Fig. 5). These are both well established sites of AGT expression indicating correct cell-specific expression of the transgene. It is interesting to note that, whereas male and homozygous female transgenic mice had uniformly high expression of human AGT throughout the liver, the expression pattern in heterozygous female transgenic mice was variegated. This likely reflects the functional random inactivation of genes located on the X-chromosome of female mice. A similarly variegated pattern was observed in the kidney. Our quantification of the level of hAGT<sup>hprt</sup> mRNA in liver and kidney is also consistent with a pattern of random X-chromosome inactivation. The level of hepatic (Fig. 6A) and renal (Fig. 6B) hAGT<sup>hprt</sup> mRNA in homozygous female transgenic mice was twice that of heterozygous female mice.

Angiotensinogen expression is known to be regulated by androgens in kidney (27), and the level of human AGT mRNA in kidneys of male hAGT<sup>hprt</sup> mice was higher than that of female mice (Fig. 3). This was examined in more detail in hAGT<sup>hprt</sup> mice by measuring human AGT expression in kidney and liver following castration and subsequent administration of a subcutaneous pellet containing testosterone. Human AGT mRNA in kidney was reduced following castration, when compared with sham-operated control, and was restored to baseline levels by testosterone (Fig. 7). As expected, expression of the transgene in liver was unaffected by the same protocol.

We next determined if human AGT protein from hAGT<sup>hprt</sup>
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mice is correctly processed in the liver and secreted into plasma. Similar to hepatic hAG'T<sup>hprt</sup> mRNA, the levels of circulating human AGT, as detected by its conversion to Ang-I in plasma of a mouse. Therefore, to formally prove the physiologic levels of the transgene products.

Because of species-specificity in renin-angiotensin enzymatic reaction, human AGT protein is essentially inert in the plasma of a mouse. Therefore, to formally prove the physiologic activity of human AGT present in the circulation, we measured blood pressure responses to acute intravenous infusion of purified recombinant human renin. Administration of 500 ng of human renin induced an acute increase in blood pressure (ΔBP = 25.6 ± 2.0 mmHg, n = 6) in hAG'T<sup>hprt</sup> mice (Fig. 8 A). This pressor response could be repeatedly observed by additional infusion cycles (data not shown).

**Fig. 6. Relative expression of human AGT.** Relative expression of human AGT mRNA was analyzed by RNase protection assay using mouse actin as an internal control and quantified as described under "Material and Methods." Homozygous (+/+) female mice had about 2-fold higher expression in both liver (panel A, *, p = 0.011; n = 5) and kidney (panel B, *, p = 0.022; n = 5) as compared with heterozygous (+/-) transgenic female mice. C, analysis of plasma hAGT levels by measuring Ang-I (AI) conversion using radioimmunoassay similarly showed significantly decreased levels of hAGT protein in heterozygous transgenic female hAG'T<sup>hprt</sup> mice (n = 6) as compared with both transgenic male (*, p = 0.021; n = 6) and homozygous female mice (*, p = 0.021; n = 6). **Fig. 7. Androgen responsiveness of human AGT expression.** Androgen responsiveness of hAGT was analyzed in male transgenic hAG'T<sup>hprt</sup> mice. Six mice were separated into two groups. Two mice were sham-operated (S) and used as a control and four mice were castrated. After 4 days, the sham-operated mice and two of the castrated mice (C) were sacrificed, and liver and kidney tissues were isolated and frozen.

The other two castrated mice received subcutaneous testosterone pellets (CT) and were sacrificed 3 days following implantation, at which point liver and kidney tissues were isolated and frozen. RNA from all tissues were prepared at the same time, and levels of hAGT message were analyzed by Northern blot. Amounts of RNA used in the blot are represented at the bottom of the figure by a segment of a methylene blue-stained filter indicating 28 S RNA.
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In the context of expressing a single copy transgene at the HPRT locus, it is essential to consider the methodologies employed, as well as the implications for in vivo studies. The transgene expression is typically regulated in a tissue-specific and copy-number proportional manner. This ensures that the transgene expression is consistent with the endogenous HPRT expression, facilitating its proper integration and functionality.

**Fig. 8. Identification of human AGT protein in plasma.** Identification of hAGT protein in plasma was achieved by Western blot analysis of 5-μl samples of whole plasma. Two identical polyacrylamide protein gels were run simultaneously. One was stained with Coomassie Blue to indicate total protein amount used in the assay (A), and the other was used for Western blot analysis using hAGT antibody (B). M, protein size marker as indicated in daltons; B, 5 μg of bovine serum albumin; F, heterozygous hAGThprt transgenic female; Tg, regular hAGT transgenic mouse containing approximately 6–8 copies of the transgene.

**Fig. 9. Pressor response to acute human renin infusion.** The pressor response to acute human renin infusion was measured in catheterized freely moving mice. A, 500 ng of purified human renin induced a pressor response (ΔBP = 25.6 ± 2.0 mmHg; n = 6) which persisted for about 30 min. B, administration of the ACE inhibitor captopril abolished the response completely. C, purified Ang-II infusion induced an acute pressor response (ΔBP = 38.6 ± 4.4; n = 7). Arrow indicates the point of human renin or Ang-II infusion.

**DISCUSSION**

We selectively targeted the human AGT gene to the mouse HPRT locus by employing ES cells harboring a deletion in the endogenous HPRT gene and a special targeting vector capable of restoring its full functionality upon homologous recombination (19, 28). The use of gene targeting is essential in developing a model for studying effects of allelic gene variants in vivo because it nullifies the copy number and positional effects associated with transgene expression in transgenic mouse models generated by pronuclear injection. It allows reproducible insertion of the single copy of a transgene in a predetermined locus, permitting direct comparison between individually generated transgenic lines. Furthermore, independently derived transgenic mice generated by gene targeting are genetically identical, abolishing the need for multiple transgenic mouse lines harboring the same construct. The largest benefit of our strategy is the highly reproducible efficiency in selecting single homologous recombination events. This should allow for relatively easy development of a number of transgenic ES cell clones that harbor specific variants of a gene of interest, and a subsequent generation of transgenic mice.

Of course, we must recognize certain drawbacks of using a nonnative locus, such as HPRT, for gene targeting. Exogenous genes may not harbor all of the sequences necessary and sufficient for proper regulation of expression and may therefore be influenced by cis-acting regulatory elements in the vicinity of the HPRT gene. Although the HPRT gene is essentially ubiquitously expressed, three studies reported preferential expression of certain transgenes in some but not other tissues (19, 29, 30). However, two of those studies used a different methodology in their targeting by employing the neomycin resistance gene and G418 selection. Moreover, while transgene expression in the animals was studied in two reports, the third utilized teratocarcinoma cells derived from targeted ES cells. In addition, it is possible that the transgenes in those studies lacked all of the elements required for their normal expression. Our previous studies indicate that the 13.8-kb genomic human AGT transgene, identical to the one used in this study, is expressed in a tissue- and cell-specific and copy-number proportional fashion in transgenic mice, suggesting that all essential regulatory elements are present in the transgene (26). Another difference is that the transgene in our model is transcribed in the reverse orientation relative to the endogenous HPRT locus, which has been shown to have a beneficial effect on the expression of a transgene (30).

Another potential drawback of targeting the HPRT locus is that it is located on the X chromosome and the transgene expression is affected by random X inactivation, as we have demonstrated in our mice. This means that one can effectively achieve an equivalent of 1 copy (in the case of male and homozygous transgenic female mice) or 0.5 copy (in the case of heterozygous transgenic female mice) transgene expression. This should not be of a great concern in employing such mice in the analysis of transgene variants as long as it is considered in the final data analysis. For example, one could compare two or more lines of mice transgenic for different gene variants and could stratify the data according to mouse gender and/or zygosity. In fact, it is conceivable that lower levels of transgene expression might be beneficial in identifying modest molecular and physiologic changes between specific allelic variants.

Our data indicate that the human AGT gene was apparently unaffected by the HPRT locus. It exhibited correct tissue and cell specificity and was properly regulated by the changes in
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testosterone levels. Furthermore, the human AGT protein was normally processed, and its plasma concentration was proportional to the level of human AGT mRNA indicating proper regulation. The protein in plasma is readily cleaved by human renin, resulting in the acute pressor response which could be easily detected by changes in blood pressure. We also showed that we are capable of detecting relatively small differences in expression levels of both RNA and protein, comparable with the proposed differences among –6G/235Met and –6A/235Thr alleles (31). This model should, therefore, be suitable in the analysis of the most significant human AGT variants described to date.

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