A polymorphism in intron I of the human angiotensinogen gene (hAGT) affects binding by HNF3 and hAGT expression and increases blood pressure in mice

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Angiotensinogen (AGT) is the precursor of one of the most potent vasoconstrictors, peptide angiotensin II. Genome-wide association studies have shown that two A/G polymorphisms (rs2493134 and rs2004776), located at +507 and +1164 in intron I of the human AGT (hAGT) gene, are associated with hypertension. Polymorphisms of the AGT gene result in two main haplotypes. Hap-I contains the variants −217A, −6A, +507G, and +1164A and is pro-hypertensive, whereas Hap-II contains the variants −217G, −6G, +507A, and +1164G and does not affect blood pressure. The nucleotide sequence of intron I of the hAGT gene containing the +1164A variant has a stronger homology with the hepatocyte nuclear factor 3 (HNF3)–binding site than +1164G. Here we found that an oligonucleotide containing +1164A binds HNF3β more strongly than +1164G and that Hap-I–containing reporter gene constructs have increased basal and HNF3– and glucocorticoid–induced promoter activity in transiently transfected liver and kidney cells. Using a knock-in approach at the hypoxanthine-guanine phosphoribosyltransferase locus, we generated a transgenic mouse model containing the human renin (hREN) gene and either Hap-I or Hap-II. We show that transgenic animals containing Hap-I have increased blood pressure compared with those containing Hap-II. Moreover, the transcription factors glucocorticoid receptor, CCAAT enhancer–binding protein β, and HNF3β bound more strongly to chromatin obtained from the liver of transgenic animals containing Hap-I than to liver chromatin from Hap-II–containing animals. These findings suggest that, unlike Hap-II variants, Hap-I variants of the hAGT gene have increased transcription rates, resulting in elevated blood pressure.

Hypertension is a serious risk factor for myocardial infarction, heart failure, vascular disease, stroke, and renal failure (1). The renin–angiotensin system plays an important role in the regulation of blood pressure. The octapeptide angiotensin II is one of the most active vasopressor agents and is obtained by proteolytic cleavage of a larger precursor molecule, angiotensinogen (AGT),2 which is primarily synthesized in the liver, and, to a lesser extent, in fat, kidney, brain, and heart and adrenal and vascular walls (2). The AGT gene is associated with essential hypertension (3–5), cardiac hypertrophy (6), coronary atherosclerosis (7), and microangiopathy-related cerebral damage (8, 9). Initially, it was shown that polymorphism M235T (rs699, located at the +4072 position in exon II) of the hAGT gene is associated with essential hypertension, and homozygotes with variant T235 contain 15%–40% higher plasma AGT levels (3). However, the kinetics of the enzymatic reaction between human renin and recombinant AGT containing either M235 or T235 were almost the same, suggesting that M235T is not the functional polymorphism (10). A recent GWAS using Cardio-Metaobochip in 342,415 subjects found that an A/G SNP (rs2493134) located at the +507 position (in intron I) of the hAGT gene is associated with hypertension (11). Two other GWAS have shown that an A/G SNP (rs2004776) located at the +1164 position in intron I of the hAGT gene is associated with blood pressure (12, 13). The association of rs2004776 has recently been confirmed by analysis of 1 million human subjects (14). This SNP has also been associated with blood pressure in East Africans (15) and in age-related hypertension in a large population study (16). However, the molecular mechanism involved in the role of these polymorphisms in the regulation of AGT gene expression and blood pressure regulation remains to be analyzed.

The AGT locus, spanning 2 kb of its promoter and all exons and introns, has 18 polymorphisms that have an allele frequency of more than 5% in the general population. A pairwise linkage disequilibrium map between the AGT SNPs in Caucasian subjects based on the 1000 Genomes Project database and their location is shown in Fig. 1. The AGT gene has two blocks of SNPs that are in almost complete LD. The first block has SNPs located at −217, −532, −1074, −1178, −1320, and −1561. The second block consists of SNPs located at +507 (intron I), +4072 (M235T in exon II), −6, −1562, and −1670. Watkins et al. (17) performed high-density genotyping and cre-

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2 The abbreviations used are: AGT, angiotensinogen; GWAS, genome-wide association study; LD, linkage disequilibrium; C/EBP, CCAAT/enhancer-binding protein; GR, glucocorticoid receptor; TG, transgenic; BP, blood pressure; HPRT, hypoxanthine-guanine phosphoribosyltransferase; TBE, Tris borate-EDTA; cDNA, complementary DNA; Hap, haplotype; HNF, hepatocyte nuclear factor; Luc, luciferase reporter construct.
ated haplotypes across the AGT promoter in 256 cases and 126 controls from the PATH-SCOR study group. They concluded that the hAGT haplotype that contains variants −217A, −6A, and +1164A of intron I (Hap-II) predisposes to hypertension and that the haplotype containing −217G, −6G, and +1164G of intron I (Hap-II) does not have an appreciable effect on blood pressure (Fig. 2A). They also found that Hap-I is associated with increased plasma AGT levels. However, these authors did not perform a functional study of the role of these polymorphisms in expression of the hAGT gene. We have shown previously that the nucleotide sequence of the hAGT gene containing variant −217A has stronger homology with C/EBP and GR binding sites compared with −217G and that reporter constructs containing −217A have increased promoter activity on transient transfection in human liver and kidney cells compared with −217G. We have also shown that the nucleotide sequence containing variant −1670A has stronger homology with the HNF1 binding site and that an oligonucleotide containing −1670A binds more strongly to HNF3β compared with the same oligonucleotide containing +1164G; double TG animals containing hREN and Hap-I (containing −6A, −217A, +507G, and +1164A) of the hAGT gene have increased blood pressure compared with double TG animals containing hREN and Hap-II (containing −6G, −217G, +507A, and +1164G) of the hAGT gene; and the transcription factors GR, C/EBPβ, and HNF3β bind more strongly to chromatin obtained from livers of TG animals containing Hap-I compared with Hap-II.

**Results**

**SNP rs2004776 of the hAGT gene is located in an open chromatin region in HepG2 and adult liver cells**

Because functional polymorphisms are normally located in an open chromatin region, we analyzed the regulatory overlap...
of rs2004776 with histone H3 lysine 4 monomethylation (H3K4me1), histone H3 lysine 4 trimethylation (H3K4me3), and histone H3 lysine 27 acetylation (H3K27ac) histone marks and DNase hypersensitivity site data obtained from the Ency-
clopedia of DNA Elements and Roadmap Epigenome projects accessed through the University of California, Santa Cruz (UCSC) genome browser (19, 20). The tracks corresponding to
rs2004776 of hAGT gene DNase I hypersensitivity clusters from 125 cell types, DNase I uniform peaks and density signal from human hepatocellular carcinoma (HepG2) cells, formalde-
hyde-assisted isolation of regulatory elements density signal from HepG2 cells, and histone marks from adult liver and HepG2 cells are shown in Fig. 3. The location of rs2004776,
rs2493134, rs5051 and rs2493136 are also shown in Fig. 3 and are marked with blue dotted lines. The results of this analysis suggest that polymorphism rs2004776 of the hAGT
gene is located in an open chromatin region in HepG2 and human adult liver cells.

**Variant +1164A in intron I of the hAGT gene increases the promoter activity of reporter constructs**

Because the nucleotide sequence of intron I of the hAGT
gene contains an A/G SNP at +1164 (rs2004776) and putative C/EBP and GR binding sites (Fig. 2, B and C), we next examined the role of this intron in transcriptional regulation. We have
shown previously that a reporter construct containing haplo-
type –6A of the hAGT gene has increased promoter activity compared with haplotype –6G on transient transfection in human liver and kidney cells (18). We confirmed these results
(Fig. 4, B and C). To understand the role of SNP rs2004776 and intron I of the hAGT gene in its transcriptional regulation, we synthesized three additional reporter constructs (Fig. 4A)
and used them for transient transfection. The results of this exper-
iment showed that reporter construct HAP-I Luc (containing the –6A haplotype and intron I with +1164A) had increased basal as well as HNF3α- and glucocorticoid-induced promoter
activity compared with HAP-II Luc (containing the –6G haplotype and intron I with +1164G). Moreover, site-specific mutagenesis of +1164A to +1164G (HAP-III Luc) reduced the
basal as well as glucocorticoid- and HNF3α- induced promoter activity in COS-7 and HepG2 cells (Fig. 4, B and C). The results of this experiment suggest that the nucleotide sequence in
intron I of the hAGT gene acts as an enhancer and that transcription factors binding to the +1164A allele (associated with increased blood pressure) co-operatively interact with transcription factors binding to promoter polymorphisms and increase the transcription of this gene. In addition, the nucleotide sequence present in intron I increased the glucocorticoid-induced promoter activity of the reporter construct HAP-I compared with HAP-II.
An oligonucleotide containing +1164A binds more strongly to HNF3β compared with the same oligonucleotide containing +1164G

Because in silico analysis showed that the nucleotide sequence of intron I around +1164A has stronger homology with the HNF3 binding site compared with +1164G, we performed an EMSA using a nuclear extract from Hep3B cells. The results of this experiment showed that phospho-labeled oligonucleotide 1164A formed a complex with Hep3B nuclear extract (Fig. 5, lane 1, arrow), which was outcompeted in the presence of a cold oligonucleotide containing either the consensus HNF3 binding site (Fig. 5, lane 2) or cold oligonucleotide 1164A (Fig. 5, lane 3) but not in the presence of cold oligonucleotide 1164G (Fig. 5, lane 4). Moreover, the complex was reduced in the presence of HNF3β antibody (Fig. 5, lane 5).

Transgenic mice containing Hap-I of the hAGT gene have increased hAGT mRNA in the liver, increased plasma hAGT levels, and increased BP compared with TG mice containing Hap-II

Transient transfection in cultured cells provides important information about tissue-, hormone-, and development-specific expression of a number of genes. However, such studies are often hampered by the limited selection and state of differentiation of cell lines used in the transient transfection assay. TG mice are at present the most rigorous system available for identifying and characterizing cis-acting DNA elements and to understand the role of these elements in transcriptional regulation of a gene in different cell types. However, one of the main concerns in TG studies is that the transgene may integrate at different sites in the chromosome, and this may affect promoter activity from experiment to experiment. Another problem is that multiple copies of the transgene may be integrated into the genomes of different TG lines, which may result in varied promoter activity. To overcome these limitations, Bronson et al. (21) have developed a gene-targeting system to understand the in vivo regulation of gene expression. In this model, gene targeting at the HPRT locus was used to selectively target a single copy of the gene that prevents random integration. To understand the role of different haplotypes in the regulation of blood pressure in an in vivo situation, we generated double-transgenic mice containing human renin and the hAGT gene using a knock-in approach at the HPRT locus.

We generated TG animals by inserting a 14.4-kb hAGT transgene containing a 2-kb promoter with either Hap-I or Hap-II along with all exons, introns, and the 3′-flanking region of the hAGT gene at the HPRT locus essentially as described previously (22). The locations of different SNPs in Hap-I and Hap-II are shown in Fig. 2A. The resulting animals were then cross-bred with TG animals containing the hREN gene, and double-TG animals containing both the hAGT and hREN genes were selected for further experimentation. The blood pressure of 8- to 12-week-old male double-TG animals was measured by telemetry. The results of this experiment showed that TG mice containing Hap-I have increased mean arterial blood pressure compared with TG animals containing Hap-II (Fig. 6A). It has been shown previously that the renin–angiotensin reaction is species-specific in the sense that mouse renin does not cleave human AGT, and human renin does not cleave mouse Agt (23). In accordance with this observation, we found that single-transgenic mice containing the human renin gene did not have...
increased blood pressure (Fig. 6A). The BP of these TG animals was almost similar to control C57 mice.

We also measured plasma angiotensin II levels in transgenic and control C57 mice. We found that plasma angiotensin II levels were significantly up-regulated in transgenic animals containing Hap-I compared with Hap-II (Fig. 6B). In addition, plasma hAGT levels were increased in the livers of Hap-I compared with Hap-II TG animals (Fig. 7A).

The transcription factors HNF3β, C/EBPβ, and GR bind more strongly to chromatin obtained from the livers of TG animals containing Hap-I of the hAGT gene compared with Hap-II

Gel shift experiments are normally used to examine binding of a transcription factor to DNA. However, such experiments are indirect measures of regulation and do not account for conditions like competition with other binding proteins in vivo, the chromatin structure of the endogenous gene, and cascades of regulatory interactions. In contrast, the ChIP assay is a powerful tool for direct analysis of transcription factor site occupancy in vivo. Importantly, one can determine the physical association of a specific DNA-binding factor with potential control sequences in intact cells with this assay. To compare binding of transcription factors to the nucleotide sequences containing polymorphic sites in the hAGT gene present in Hap-I and II, we performed a ChIP assay using chromatin from the livers of TG animals. The results of this assay showed that the transcription factors HNF3β, C/EBPβ, and GR bind more strongly to chromatin obtained from the livers of TG animals containing Hap-I of the hAGT gene compared with Hap-II (Fig. 8).

Discussion

Like other complex diseases, hypertension is caused by an interplay of multiple genetic and environmental factors. GWAS...
have identified more than 100 loci associated with hypertension. However, GWAS normally identify a SNP that has the biggest odds ratio with the disease but does not inform whether this SNP is the functional polymorphism. More than 80% of the SNPs identified in GWAS are in the noncoding region, and this SNP is the functional polymorphism. More than 80% of the GWAS have identified more than 100 loci associated with hypertension: rs2004776 (+1164A/G) is located in an open chromatin region, especially in a DNase-hypersensitive cluster in HepG2 and human liver cells. This implies that rs2004776 is capable of binding to transcription factors and modulating the expression of this gene. Our in silico analysis suggests that variant A of rs2004776 (+1164A) has stronger homology with the HNF3 binding site compared with variant G. We therefore performed an electrophoretic mobility shift assay in the presence of HEP3B extract and phospho-labeled oligonucleotides. Our third important finding is that an oligonucleotide containing variant +1164A binds more strongly with HNF3β compared with the same oligonucleotide containing variant +1164G (Fig. 5). It is important to note that a T/G SNP at −1074 in the promoter of the hAGT gene also alters binding of HNF3β (27). Surprisingly, both variants, +1164A and −1074T, belong to the risk haplotype of the hAGT gene and show increased binding with HNF3β. The physiological reason for multiple HNF3 binding sites is not clear at this time. HNF3 belongs to the Foxa family of winged helix/forkhead box transcription factors. The Foxa family consists of “pioneer” transcription factors whose binding to promoters and enhancers enables chromatin access for other tissue-specific transcription factors (28, 29). The Foxa family of transcription factors also facilitates binding of glucocorticoid, androgen, and estrogen receptors to chromatin, which results in transcriptional activation of the gene by these hormones (30–33).

In post-natal life, members of the Foxa family control glucose metabolism in the liver (34).

Watkins et al. (17) have shown that the hAGT gene can be arranged in two major haplotypes: one that contains risk alleles (containing −6A, −217A, +507G, and +1164A) and is prehypertensive and another that does not contain the risk alleles (containing −6G, −217G, +507A, and +1164G) and is protective (called Hap-I and Hap-II in this paper). To examine the role of these haplotypes in the regulation of hAGT gene expression and blood pressure in an in vivo situation, we generated transgenic mice by knocking in the hAGT gene containing either Hap-I or Hap-II at the HPRT locus. Our next important finding is that the hAGT level is increased in the plasma of TG mice containing Hap-I of the hAGT gene compared with Hap-II (Fig. 7A). In addition, hAGT mRNA levels are increased in the livers of Hap-I TG mice compared with Hap-II (Fig. 7B). Because hAGT is not cleaved by mouse renin, we also made double-transgenic mice containing the hREN and hAGT genes. We then measured BP in 12-week-old male TG mice by telemetry and found that, on average, the systolic BP of Hap-I TG mice was about 12–13 mmHg higher compared with Hap-II TG mice (Fig. 6).

In silico analysis shows that the nucleotide sequence present in intron I of the hAGT gene has sequence homology with GRE.
and C/EBP binding sites. C/EBP plays an important role in expression of a gene in hepatocytes (35–37) and adipocytes (38), two cell types in which the AGT gene is primarily expressed. In addition, C/EBP-β and -δ are involved in IL-6– and tumor necrosis factor α–induced expression of various genes (39). Previous work has shown that binding of C/EBPβ acts like a pioneer transcription factor and opens up the chromatin to allow binding of GR to the opened chromatin (40). As a result, we found that the nucleotide sequence present in intron I increases HNF3β- and glucocorticoid-induced promoter activity on transient transfection in human liver and kidney cells (Fig. 4, C and B). Our next key finding is that GR, C/EBP, and HNF3β bind more strongly to risk variants in the promoter of the hAGT gene and co-operatively interact with transcription factors that bind to variant +1164A in intron I (Fig. 8). This increases hAGT gene expression (Fig. 7) and ultimately leads to an increase in blood pressure (Fig. 6). Our experiments also showed that, although rs2493134 (located at +507 in intron I) is not the functional polymorphism, it is in LD with other polymorphisms that alter the binding of GR, C/EBPβ, and HNF-1α and increase transcription of this gene.

The availability of transgenic mice provided us an opportunity to analyze whether transcription factors indeed bind more strongly to chromatin obtained from tissues of TG mice containing Hap-I compared with Hap-II. Our next important finding is that GR, C/EBPβ, and HNF3β bind more strongly to chromatin obtained from the livers of TG animals containing Hap-I of the hAGT gene compared with Hap-II. Because environmental factors such as stress, gender, age, high-fat diet, and high-salt diet play an important role in the regulation of blood pressure, it will be interesting to use TG animals and analyze the role of these environmental factors in the regulation of hAGT gene expression and on blood pressure regulation.

In conclusion, SNPs in the hAGT gene can be arranged in two haplotypes. Hap-I is the risk haplotype, whereas Hap-II does not have any significant effect on blood pressure. A reporter construct containing Hap-I has increased promoter activity on transient transfection. Our data suggest that increased binding of transcription factors present in the kidneys and livers of Hap-I TG mice increases AGT expression, which leads to increased angiotensin II levels and an increase in blood pressure compared with Hap-II.

Experimental procedures

Transgenic mice

Adult mice (2–4 months of age) were fed a standard chow ad libitum, were maintained in a 22 °C room with a 12-h light/dark
cycles, and received drinking water *ad libitum*. All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the institutional ethical animal care and use committee at New York Medical College.

**Cell culture**

HepG2 and COS-7 cells were routinely cultured as a monolayer and maintained in 100-mm tissue culture dishes in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO$_2$ at 37 °C. Cells were grown to 80% confluence in 12-well plates for transient transfection.

**Reporter constructs**

To understand the role of SNPs present in the *hAGT* gene, we earlier synthesized two reporter constructs containing the 2.7-kb 5′-flanking region attached to the luciferase gene. Reporter construct −6A has variants −6A, −20A, −217A, −532T, −793A, −1074T, −1178G, −1561T, −1562C, and −1670A; reporter construct −6G has variants −6G, −20A, −217G, −532C, −793G, −1074G, −1178A, −1561G, −1562G, and −1670G. To understand the role of intron I of the *hAGT* gene in transcriptional regulation, we synthesized three more constructs: HAPI, HAPII, and HAPIII. In HAPI, full-length *hAGT* intron I containing +1164A was attached in front of reporter construct −6A. In HAPII, full-length *hAGT* intron I containing +1164G was attached in front of reporter construct −6G. HAPIII is similar to HAPI, but +1164A was mutated to +1164G by site-specific mutagenesis. The expression vector RSV-β-gal was purchased from Promega.

**Transient transfection**

Transfection in HepG2 and COS-7 cell cultures in 12-well plates was carried out using Polyfect transfection reagent (Qiagen) according to the manufacturer’s protocol. Reporter DNA (500 ng) and β-gal DNA (10 ng) were used in each experiment. RSV-GR and RSV-HNF3B were used for co-transfection studies. For RSV-GR co-transfection experiments, after 2 h of transfection, the medium was changed to serum-free medium, and after 24 h of transfection, cells were treated for an additional 24 h with dexamethasone (100 nM). Cell extract preparation and luciferase or β-gal activity measurements were carried out as described previously (18).

**EMSA protocol**

Hep3B cells were grown to 90% confluence, and the nuclear extracts were prepared using the Nuclear Extract Kit following the recommended protocol (Active Motif, catalog no. 40010). The forward and reverse oligos (1 μM) were labeled separately using the Biotin 3′ End DNA Labeling Kit (Thermo Scientific). The labeled oligos were then annealed using annealing buffer at a final concentration of 250 nM. The sequences of oligonucleotides used in the EMSA were as follows: HNF3B consensus forward, TGA AGA TGT TTG CTC ATG C; HNF3B consensus reverse, GCA TGA GCA AAC ATC TTC A; 1164A forward, TGA AGA TGA TGG CTC ATG C; 1164A reverse, GCA TGA GCC ATC ATC TTC A; 1164G forward, TGA AGA TGA CGG CTC ATG C; 1164G reverse, GCA TGA GCC GTC ATC TTC A. 20 μl of binding reaction mixture containing ~2 μg of Hep3B nuclear extract and labeled oligos were prepared using the LightShift Optimization and Control Kit (Thermo Scientific) following the company protocol. The reaction mixture was then incubated at room temperature for 20–30 min. The samples were subjected to gel electrophoresis in pre-run 8% TBE gels at 0.5× TBE at 85 V for 1.5 h. The gel was then transferred to a Hybond N+ membrane soaked in 0.5× TBE at 100 V for 1 h in a cold room. The membrane was then cross-linked in a UV cross-linker at an energy of 125 ml/cm$^2$ for 1 min, followed by washes with buffers in the Chemiluminescent Detection Module Kit (Thermo Fisher). Finally, the membrane was developed using chemiluminescence.

**Preparation and modification of the hAGT plasmid for generation of transgenic mice**

The DNA (14 kb) encompassing the *hAGT* gene was PCR amplified from the haplotypes −6A and −6G of the *hAGT* gene from human bacterial artificial chromosome as described previously (18). The amplified DNA from the *hAGT* −6G haplotype contains variant +1164G in the first intron and variants

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**Figure 8. ChIP assay showing stronger binding of GR, C/EBP-β, and HNF3β to chromatin obtained from the kidneys of TG animals containing Hap-I of the hAGT gene compared with Hap-II.** A–C, quantification of chromatin-immunoprecipitated DNA by qPCR in the presence of GR, C/EBP-β, and HNF3β antibodies, respectively. The cycle threshold values obtained from the immunoprecipitated DNA were normalized to the cycle threshold values from the input DNA. Results are shown as mean ± S.D. (n = 4). *, p < 0.05 versus Hap-II.
−6G, −20A, −217G, −532C, −793G, −1074G, −1178A, −1561G, −1562G, and −1670G in the 2-kb promoter. This construct was cloned into the TOPO XL vector. The amplified DNA from the 6A haplotype contains variants +1164G in the first intron and −6A, −20A, −217A, −532T, −793A, −1074T, −1178G, −1561T, −1562C, and −1670A in the 2-kb promoter of the hAGT gene. The DNA in haplotype −6A was mutated to convert +1164G to +1164A using the QuikChange Lightning Mutagenesis Kit. The resulting DNA was sequenced to confirm that it contained +1164 A, −6A, and all promoter variants. The TOPO XL vectors containing hAGT variants were then treated with the Not1 restriction enzyme, and the resulting DNA fragments were subcloned into the pMP8SKB vector to produce pMPhAGT-Hap-I (containing variants +1164A, +507G, −6A, −20A, −217A, −532T, −793A, −1074T, −1178G, −1561T, −1562C, and −1670A) and pMPhAGT-Hap-II (containing variants +1164G, +507A, −6G, −20A, −217G, −532C, −793G, −1074G, −1178A, −1561G, −1562G, and −1670G). These plasmids were linearized with the PvuI restriction enzyme and used for generation of TG mice.

**Generation of transgenic mice**

The linearized plasmids were electroporated in BK4 ES cells and used to generate transgenic mice on the C57/BL6 background at Dartmouth Medical Center as described previously (18, 41). We confirmed the presence of the hAGT gene by PCR amplification of the tail DNAs of these transgenic animals using three sets of hAGT gene-specific primers. We also analyzed the DNA sequence of the promoter and intron I region of the hAGT gene in these TG mice. These transgenic mice had a single copy of the hAGT gene, as determined by quantitative PCR (42).

**Blood pressure measurement**

All mice were fed a standard mouse chow and had access to water *ad libitum*. Blood pressure (BP) was measured in the conscious state by telemetry. A radiotelemetry system from Data Science International (St. Paul, MN) was used for this procedure. Briefly, mice were anesthetized with ketamine and xylazine (90 and 10 mg/kg, respectively), and the left carotid artery was isolated. The tip of the telemetric catheter (model TA11PA-C10) was then inserted into the carotid artery and advanced into the aortic arch, with the telemetric device main body positioned in a subcutaneous pocket in the right flank. After 1 week of recovery from the surgical procedure, BP readings were recorded every 10 min using a Data Science International instrument as described previously (43). Mean arterial BP values were calculated for every hour from values taken over 6 days.

**Western blotting**

Blood samples from Hap-I and Hap-II hAGT transgenic mice were collected in EDTA-coated blood collection tubes, and the plasma was recovered from each sample by centrifugation at 12,000 rpm for 15 min at 4 °C. Equal volumes of plasma samples from transgenic mice were fractionated by SDS-PAGE and transferred to Immobilon-P transfer membranes to detect hAGT protein. The membranes were developed as described previously (44). Briefly, the membranes were blocked for 1 h in Odyssey blocking buffer, followed by overnight incubation at 4 °C with a 1:2000 dilution of hAGT mAb raised in rabbits (Epitomics) and then with a 1:10,000 dilution of a secondary antibody conjugated with IRDye800 or IRDye700 at room temperature for 60 min, protected from light and with gentle shaking. The blots were visualized with an Odyssey imaging system (LI-COR) and subjected to quantitative analyses using ImageQuant software (version 1.5e). The results were normalized with albumin.

**Plasma angiotensin II levels**

Plasma angiotensin II levels in transgenic and control C57 mice were determined using an enzyme immunoassay kit from Bertin Pharma. The assay was performed according to the manufacturer’s protocol.

**Quantitative Real-Time PCR of hAGT mRNA**

Livers from male transgenic mice containing either Hap-I or Hap-II of the hAGT gene were harvested following CO2 asphyxiation and stored in All Protect tissue reagent (Qiagen). RNA was isolated using the RNeasy Plus Mini Kit (Qiagen). One microgram of RNA was reverse-transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis Kit. qPCR was performed using PowerUp SYBR Green Master Mix and the CFX Connect™ real-time PCR detection system (Bio-Rad). Primers for the mouse and human AGT and mouse Gapdh genes were purchased from Integrated DNA Technologies. Following a 95 °C incubation for 10 min, 40 cycles of PCR (95 °C for 30 s, 60 °C for 30 s) were then performed, using 1 μl of cDNA, 50 nM PCR primers, and 12.5 μl of SYBR Green PCR Master Mix in 25-μl reactions. Threshold cycles for three replicate reactions were determined, and relative transcript abundance was calculated after normalization with mouse GAPDH.

**In vivo chromatin immunoprecipitation assay**

The ChIP assay was performed using the EZ ChIP kit from Millipore according to the manufacturer’s protocol as described previously (18, 41). Briefly, the mice were perfused with normal saline. Kidney tissue was excised and washed in PBS, minced into smaller pieces, fixed with 1% formaldehyde for 20 min at room temperature, and washed with chilled PBS followed by lysis. The DNA was fragmented by sonication, and 10 μl of the chromatin solution was saved as input. 5 μg amount of GR, C/EBP-β, and HNF3β antibody or rabbit IgG was added to tubes containing 900 μl of sonicated chromatin solution, and the mixture was incubated overnight at 4 °C. The antibody complexes were captured with protein A–agarose beads and subjected to serial washes. The reverse cross-linked chromatin material was purified using Qiagen PCR purification columns. The immunoprecipitated DNA (1 μl) and the input DNA (1 μl) were subjected to quantitative real-time PCR using SYBR Green PCR Master Mix. Threshold cycles for three replicate reactions were determined, and relative enriched DNA abundance was calculated after normalization with input DNA.

**Statistical analysis**

Unpaired *t* test was used to compare relative luciferase activities of reporter constructs in transient transfection experi-
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terms. Data are expressed as means ± S.D. The differences between group means from each group containing four mice were determined by a two-factor analysis of variance, followed by a Newman-Keuls post hoc analysis (NCSS LLC, Kaysville, UT). Statistically significant results are marked (*, p < 0.05).


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