Variable Transcriptional Regulation of the Human Aldosterone Synthase Gene Causes Salt-Dependent High Blood Pressure in Transgenic Mice

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Background—Aldosterone, synthesized in the adrenal cortex by the enzyme CYP11B2, induces positive sodium balance and predisposes to hypertension. Various investigators, using genomic DNA analyses, have linked -344T polymorphism in the human CYP11B2 (hCYP11B2) gene to human hypertension. hCYP11B2 gene promoter has 3 single-nucleotide polymorphisms in linkage disequilibrium: T/A at -663, T/C at -470, and C/T at -344. Variants ACT occur together and form the haplotype-I (Hap-I), whereas variants TTC constitute Hap-II. We hypothesize that these single-nucleotide polymorphisms, when present together, will lead to haplotype-dependent differences in the transcriptional regulation of the hCYP11B2 gene and affect blood pressure regulation.

Methods and Results—We evaluated differences in tissue expression in vivo and consequential effects on blood pressure stemming from the 2 haplotypes. Novel transgenic mice with the hCYP11B2 gene, targeted to the mouse HPRT locus, with either Hap-II or Hap-I variant are used in this study. Our results show increased adrenal and renal expression of hCYP11B2 in transgenic mice with Hap-I when compared with mice with Hap-II. Importantly, we observed increased baseline blood pressure in Hap-I transgenic mice, an effect accentuated by a high-salt diet. Pathophysiological effects of elevated aldosterone were corroborated by our results showing upregulation of proinflammatory markers in renal tissues from the transgenic mice with Hap-I.

Conclusions—These findings characterize the haplotype-dependent regulation of the hCYP11B2 gene where -344T serves as a reporter polymorphism and show that Hap-I leads to increased expression of hCYP11B2, with permissive effects on blood pressure and inflammatory milieu. (Circ Cardiovasc Genet. 2015;8:30-39. DOI: 10.1161/CIRCGENETICS.114.000694.)

Key Words: aldosterone ■ gene expression regulation ■ genetics ■ renin–angiotensin system

The human CYP11B2 (hCYP11B2) gene has T/C polymorphism at the -344 site in its promoter region. The -344T allele is accompanied by higher CYP11B2 gene expression in human studies when compared with the -344C allele. 2 White subjects (n=437) who were ≥65 years demonstrated higher baseline blood pressure (BP) in TT versus CC homozygotes. 3 Other investigators provide complementary evidence for the role of this polymorphism in cardiovascular disease: -344T allele is associated with increased BP with aging in Italian subjects; 4 T-allele is associated with higher excretion rate of tetrahydroaldosterone in white subjects; -344T is shown to be associated with higher BP in newly diagnosed hypertensive subjects with African ancestry; genomic DNA analysis from 406 hypertensive and 424 normotensive South-Indian subjects has found -344T-allele to be associated with hypertension (P=0.007); finally, homozygosity for -344C confers 17% lower risk for hypertension, with respect to homozygous TT subjects in meta-analyses. 5 Importantly, Iwai et al 6 have analyzed the role of 13 polymorphisms at CYP11B2 locus in 1443 Japanese subjects and concluded that only -344T allele was associated with increased aldosterone levels. Thus,
-344C/T polymorphism is associated with increased risk of cardiovascular disease that could be a consequence of differential transcriptional regulation caused by the single-nucleotide polymorphism (SNP).

The nucleotide sequence of the *CYP11B2* promoter (AAGGCC(T/C)C) has sequence homology with the transcription factor SF1 binding site (AAGGCTCA). Recombinant SF1 binds 5x stronger to an oligonucleotide containing -344C when compared with -344T. However, reporter constructs of h*CYP11B2*, with -344T or -344C, show similar baseline expression that is equally repressed when cotransfected with SF-1 expression plasmid, in human adrenocortical cells (H295R).10

We show that the promoter contains either Hap-I or Hap-II, at the mouse HPRT locus. These transgenic mice with knocked in h*CYP11B2* gene using the Hap Map data. We have further confirmed the presence of these haplotypes in human populations by analysis of the 1000 genome database.

This study was designed to test the transcriptional regulation and physiological differences between the 2 haplotypes of the h*CYP11B2* gene in vivo. To this end, we have generated transgenic mice with knocked in h*CYP11B2* gene, containing either Hap-I or Hap-II, at the mouse HPRT locus. These transgenic mice contain 11.2 kb of DNA encompassing 2.5 kb of the 5'-flanking region (containing either Hap-I or Hap-II), 2.9 kb of the 3'-flanking region, and 5.86 kb of the coding region containing all the 9 exons and 8 introns of h*CYP11B2* gene. We show here, in our novel transgenic mice with h*CYP11B2* transgene, increased CYP11B2 gene expression in renal and adrenal tissues of mice with Hap-I (-344T) when compared with mice with Hap-II (-344C). Importantly, the Hap-II transgenic mice have significantly increased baseline BP, an effect accentuated by high-salt diet, as opposed to mice with Hap-I. Thus, this is the first study characterizing the haplotype-dependent regulation of the h*CYP11B2* gene, in an in vivo setting, with examination of the consequent cardiovascular effects.

**Methods**

**Patient Selection**

We have analyzed the genomic DNA from 277 normotensive (151 men and 126 women) and 295 hypertensive subjects (179 men and 116 women). All of these subjects were recruited from the outpatient department of Westchester Medical Center (Valhalla, NY). All case and control subjects gave informed consent before participating in the research. The Institutional Review Board at the New York Medical College approved the research protocol for animal use. Individuals were excluded if they had a previous history of coronary artery disease, peripheral vascular disease, cerebrovascular disease, secondary hypertension, diabetes mellitus, or renal diseases. For a period of 30 minutes before BP measurement, no exercise, alcohol, caffeine, or smoking were allowed. BP was measured by using a conventional mercury sphygmomanometer. Measurements by 2 different observers were taken at the left arm with individuals in the seated position after 15 minutes of resting. The criteria for hypertension was defined as a systolic BP (SBP) of >140 mm Hg, a diastolic BP (DBP) of >90 mm Hg, or under antihypertensive therapy. The normotensives (with SBP/DBP ≤140/90 mm Hg) without a history of hypertension and without diabetes mellitus were recruited from the same population and matched for the sex and age. Clinical data of the patients were self-reported during a detailed prestudy examination and confirmed by medical charts provided by their physicians.

**Analysis of Genomic DNA in Patients**

The genomic DNA was amplified using 5'-TGG AGG GTG TAC CGT TGT CA-3' as a forward primer (h*CYP11B2*_192R) and 5'-GTC CGT TGA GGA TG-3' as a reverse primer (h*CYP11B2*_192R) to amplify the 270-bp 5'-flanking region of the h*CYP11B2* gene containing the T/C polymorphism at the -344 position of the promoter. The amplified fragment was analyzed by 3.5% agarose gel electrophoresis and was confirmed by direct sequencing. For a small cohort of samples, the genomic DNA was amplified using h*CYP11B2*_1350F 5'-ACA GCA ATG ATG CAA GGG AT-3' as a forward primer and h*CYP11B2*_192R 5'-GTC CGT CTG TGA GGA TG-3' as a reverse primer to amplify the 5'-flanking region of the h*CYP11B2* gene containing the polymorphic site at the -344, -470, and -663 positions of the promoter. The amplified products were resolved on 2% agarose gel and subsequently sequenced and confirmed by DNA sequencing.

**Plasmid Construction, Cell Culture, and Transient Transfection**

The h*CYP11B2* reporter construct containing 2.5 kb promoter having -344 T (Hap-I) and -344C (Hap-II) was polymerase chain reaction (PCR) amplified using 5'-CCG CTC GAG ATG TCA ATG GAA ACT GCA ATG ATG CAA GGG AT-3' as a forward primer and 5'-CCC AAG CTT TTC AAT GCT GCC TCC ACC CTG-3' as the reverse primer. The Hap-I was obtained from the bacterial artificial chromosome clone RP11-51E16 that contains T at -344 position, and Hap-II was obtained from the human genomic sample containing -344C, -470T, and -663T in the promoter. The restriction enzymes, Xhol and HindIII sites, were used in the forward and reverse primers, respectively, to directionally clone the amplified templates in the pGL4 basic vector lacking eukaryotic promoter and enhancer sequences (Promega). Expression vector RSV-β-gal was obtained from Promega. Plasmid DNAs for transient transfection were prepared by using the Qiagen midi plasmid kit under conditions described by the manufacturer. The human adrenocortical carcinoma cells (H295R) were routinely cultured as a monolayer and maintained in 100-mm tissue culture dishes with the complete media containing the basal 1:1 mixture of Dulbecco's Modified Eagle's and Ham's F12 (DMEM/F12; Gibco-Invitrogen) media, supplemented with 25% Nu-Serum IV (BD Biosciences), 1% ITS Premix (insulin, 6.25 μg/mL; transferrin, 6.25 μg/mL; selenium, 6.25 ng/mL; bovine serum albumin, 1.25 mg/mL; and linoleic acid, 5.35 μg/mL; BD Biosciences) and 0.5% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin mix; Gibco-Invitrogen) in a humidified atmosphere of 95% air and 5% CO2, at 37°C in an incubator. The growth media were changed every other day, and on 90% confluence, the cells were plated at ~70% density and grown in the complete media in 6-well tissue culture plates under the same growth conditions. For transient transfections, reporter DNA (1 μg) and β-galactosidase DNA (10 ng) were mixed with pBluescript DNA to a final weight of 1.2 μg of DNA. Transient transfections were performed with Attractene Transfection Reagent (Qiagen) following the manufacturer's protocol. The cells were harvested 48 hours post transfection, and whole-cell extracts were prepared by resuspension in 200 μL of lysis buffer (Promega). An aliquot of the cell extract was used to measure the luciferase activity using a Turners Design 20/20 luminometer using a luciferase assay system (Promega) as described by the manufacturer. Luciferase activity was normalized to β-galactosidase activity that was determined using the β-glo assay system (Promega).
Preparation and Modification of hCYP11B2 Plasmid for Generation of Transgenic Mice

DNA (11.2 kb) encompassing the hCYP11B2 gene was obtained from a bacterial artificial chromosome clone (RP11-304E16) by PCR amplification using 5'-GGGCGCGCCATGTCATAAGGAACTGCTAGCAG-3' and 5'-GGGCGCGCCGCTAAAGCGATTTCATGACCGAG-3' as forward and reverse primers, respectively. These primers have NotI restriction enzyme site for further subcloning purpose (Not1 site is shown in bold letters). The amplified DNA contains 2.5 kb of the 5'-flanking region, 2.9 kb of the 3'-flanking region, and 5.86 kb coding region (containing all the exons and introns) of hCYP11B2 gene.

The amplified PCR product was initially cloned into the TOPO XL Cloning vector (Invitrogen) as per the instructions of the manufacturer and sequenced completely, which authenticated the integrity of the hCYP11B2 gene. This clone contains -344T allele and therefore corresponds to Hap-I of hCYP11B2 gene and was designated as hCYP11B2 Hap-I. The promoter sequence of this clone has a 750-bp region encompassing polymorphic sites at -344, -470, and -663, which is recognized by unique restriction enzymes Apal and Psl. Thus, hCYP11B2 -344-Hap-I plasmid DNA was treated with restriction enzymes Apal and Psl to remove this 750-bp fragment. Then, a 1100-bp fragment was amplified from the genomic DNA of a human subject containing Hap-I (-344C, -470T, and -663T) of the hCYP11B2 gene. The amplified fragment was treated with restriction enzymes Apal and Psl, and the 750-bp DNA fragment was gel purified. This fragment was then ligated in the original linearized plasmid hCYP11B2 Hap-I. The resulting DNA was sequenced to confirm that it contains -344C allele (Hap-II) and was designated as hCYP11B2 Hap-II. The Topo XL vectors containing hCYP11B2 Hap-I and hCYP11B2 Hap-II were treated with NotI restriction enzyme, and released fragments were subcloned in the Not-I restriction site in pMP8SKB vector (obtained from Dr Sarah Bronson laboratory) to produce pMP8shCYP11B2-Hap-I and pMP8shCYP11B2-Hap-II. This DNA contains a unique Pvu-I site close to the Not-I cloning site, so that the cloned DNA can be linearized after Pvu-I digestion for electroporation in BK4 stem cells. This Pvu-I site can also be used as a diagnostic tool to analyze the cloned DNA in this vector because it will show an additional band after Pvu-I–NotI digestion. Finally, the plasmids pMP8shCYP11B2-Hap-I and pMP8shCYP11B2-Hap-II containing full-length CYP11B2 with either -344T or -344C alleles were linearized with Pvu-I restriction enzyme, columns from Qiagen purified linearized fragments.

Generation of Transgenic Mice

The linearized plasmids were used for electroproporation in BK4 ES cells in Dr Fiering laboratory to generate transgenic mice.11 After electroporation, embryonic stem (ES) cells were grown in selective hypoxanthine–aminopterin–thymidine resistant colonies were isolated and expanded. DNA from different ES cells was amplified using hCYP11B2-specific primers. The PCR product was analyzed by 0.8% agarose gel electrophoresis, and ES cells containing hCYP11B2 gene were identified. ES cells (containing either Hap-I or Hap-II of the hCYP11B2 gene) were used to generate transgenic mice, on the C57/BL6 background. Transgenic animals were routinely analyzed for the presence of hCYP11B2 gene by PCR amplification of the tail DNAs of these transgenic animals using 2 sets of hCYP11B2 gene–specific primers. Finally, we confirmed transgenic animals containing Hap-I and Hap-II of hCYP11B2 gene by sequence determination of the promoter region. We initially developed 3 transgenic lines from each construct, but after analysis, we kept 1 line each for future studies. These transgenic mice have single copy of the hCYP11B2 gene, as determined by quantitative PCR. Male transgenic animals were backcrossed with female C57 mice, and all experiments were performed using the heterozygous male mice with C57/BL6 background. Transgenic animals were routinely analyzed by 3 sets of hCYP11B2 gene–specific primers: first from the promoter region (forward primer 5'-ACA GCA ATG ATG CAA GGG AT-3' and reverse primer 5'-GTC CGT CGT GTC TGA GGA TG-3' amplified -1350 and -192 regions of the promoter to give a 1158-bp fragment), second from the coding region (forward primer 5'-TCC AGA AAA TCT ACC AGG AAC TG-3' and reverse primer 5'-ATG TCC ACT GAT GCT GCC TG-3' amplified 3435 and 4497 regions of the coding sequence to give a 1062-bp fragment), and third from the 3'-flanking region (forward primer 5'-GCT GTG CAG AAG TGG GAT AG T-3' and reverse primer 5'-CCC ATA AAC AAG GAA GCC ATC TCT G-3' amplified 5606 and 7208 regions of the 3'-flanking region to give a 1600-bp fragment).

BP Measurement

All experiments were performed following an Institutional Animal Care and Use Committee–approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All mice were fed with standard mice chow and had access to water ad libitum. BP was measured in the conscious state by telemetry. A radiotransmitter 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 a 2- to 3-cm incision was performed exposing the neck and upper thoracic regions. Left carotid artery was isolated, and the tip of the telemetric catheter (model TA11PA-C10) was inserted into the carotid artery and advanced into the aortic arch, with the telemetric device main body positioned into a subcutaneous pocket into the right side of the abdomen. The skin was sutured with self-dissolving sutures. The surgery was performed under aseptic conditions. After 1 week of recovery from the surgical procedure, BP readings were recorded every 10 minutes using the Data-Science instrument as described previously.12 Mean BP values were calculated for every hour from the values taken for 6 days. Before implantation of the BP device, the zero offset of the instrument was measured and the unit was soaked in 0.9% NaCl. The data were sampled every 10 minutes by averaging for 10 s and stored on a hard disk. SBP, DBP, MAP, and heart rate were recorded using DATAQUEST software. For statistical analyses, 6 days of baseline values were used and data are analyzed using 1-way ANOVA for each time point. To study the effect of Na load on BP, transgenic mice were treated first with low-Na diet (0.01% Na) for a period of 2 weeks, followed by a period of normalization (2 weeks), and then treated with high-salt diet (8% Na). BP was measured using telemetric probes during the last 6 days of each treatment.

Quantitative Real-Time PCR

Adrenal and kidney from 8-week-old male transgenic mice containing either CYP11B2 Hap-I or Hap-II were harvested after CO2 asphyxiation and stored in Allprotect Tissue Reagent (Qiagen). The total RNA was isolated using the RNeasy Plus mini kit (Qiagen). Quantitative real-time reverse transcription PCR was next performed using power SYBR Green Master Mix (Cat number 4367659; Life technologies) and an ABI 7500 Fast Real-Time PCR System thermocycler (Applied Biosystems). Human (PPM02946E) primers were purchased from SuperArray Bioscience Corporation (MD). After a 95°C incubation for 10 minutes, 40 cycles of PCR (95°C/15s; 60°C/1 m) were then performed on an ABI 7500 Fast Real-Time PCR System with 3 μL cDNA, 50 nmol/L PCR primers, and 12.5 μL SYBR Green Master Mix in 25 μL reactions. Threshold cycles for 3 replicate reactions were determined, and relative transcript abundance was calculated after normalization with mouse GAPDH.

In Vivo Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation (ChIP) assay was performed using the EZ ChIP kit from Millipore. The mice were perfused with normal saline. Adrenal and kidney tissues were excised and minced into smaller pieces, fixed with 1% formaldehyde for 20 minutes at room temperature, and washed with chilled PBS followed by their lysis. The DNA was fragmented by sonication, and 10 μL of the chromatin solution was saved as input. A 5-μg amount of RNA polymerase II antibody or rabbit immunoglobulin G was added to the 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 (as described in the manufacturer’s protocol). The chromatin fraction was extracted with SDS buffer and reverse cross-linked at 65°C for 4 to 6 hours. The DNA was then purified using Qiagen PCR purification columns (Cat number 28106). The immunoprecipitated DNA (1 µL) and the input DNA (1 µL) were subjected to 35 cycles of PCR amplification using −23F 5′-CAT CCT CAG ACC ACC AGG ACT TG-3′ as a forward and +29R 5′-ACC TCT GCT TCC TTT GCC CTG AGT G-3′ as a reverse primer when RNA polymerase II antibody was used for immunoprecipitation. This amplified the 242-bp fragment containing the RNA polymerase II–binding region of the hCYP11B2 gene promoter. The PCR-amplified products were analyzed on 2% agarose gel. Further using the same primer set, the samples were subjected to quantitative real-time PCR using SYBR Green PCR Master Mix. Threshold cycles for 3 replicate reactions were determined, and relative enriched DNA abundance was calculated after normalization with input DNA.

Immunoblotting
Adrenal and renal tissues from male transgenic mice containing either Hap-I or Hap-II animals were homogenized in 250 µL of homogenizing buffer (10 mmol/L potassium phosphate buffer, 250 mmol/L sucrose, 1 mmol/L EDTA, NP40, and protease inhibitor cocktail; Sigma Chemical) followed by high-speed centrifugation (13,000 rpm) at 4°C for 20 minutes. The supernatant was then collected and used for western blot analysis. Proteins (25 µg) were fractionated on SDS-PAGE (10% polyacrylamide) and transferred to Immobilon-P transfer membranes (Millipore). Membranes were blocked in Odyssey blocking buffer (Cat number 927-4000; LI-COR Biosciences) and immunoblotted with commercially available monoclonal antibodies for hCYP11B2 (Cat number EPR10494, Abcam, MA) and β-actin (Cat number A2228-200U; Sigma-Aldrich, St. Louis, MO). The immune complexes were detected by using secondary antibody conjugated with IRDye800 or IRDye700 (Cat number 926-32222 and Cat number 926-32213), and images were captured using an Odyssey Imaging System (LI-COR). Densitometric analysis of protein bands was performed by Gel-Pro Analyzer software from Media Cybernetics (Media Cybernetics, Inc, Pittsburgh, PA). The results were averaged and normalized with β-actin.

Immunohistochemistry
Adrenals from 3 male mice per group (Hap-I, Hap-II, and C57 control) were fixed in formalin immediately after dissection. Formalin-fixed tissues were embedded in paraffin blocks and sectioned into 5-µm thickness. Sections for immunohistochemistry were baked at 60°C for 1 hour before hydration in alcohol and treated with antigen retrieval solution. Sections were then quenched with H2O2 for 30 minutes. Using the VECTASTAIN Elite ABC Peroxidase kit PK-6101 (Vector Laboratories Inc, Burlingame, CA), sections were blocked for 1 hour with the blocking serum provided in the kit. Next, all the sections were incubated with the primary anti-CYP11B2 (1:100, rabbit monoclonal; Epitomics-an abcam company, Burlingame, CA) antibody overnight at 4°C. On day 2, sections after washing with TBS were incubated with secondary antibody provided in the kit, and finally, slides were incubated with ABC reagent (also provided in the kit). Staining was done with the Vector DAB Substrate Kit for Peroxidase (SK-4100), and after counterstaining with hematoxylin, slides were mounted with cytosel (XYL) from Thermo Scientific and cover slips from Fisher Scientific were used to cover the sections.

Aldosterone ELISA
ALPCO aldosterone ELISA kit was used to assay serum aldosterone levels in transgenic and C57 mice, as per manufacturer’s protocol.

Statistical Analysis
Continuous data are expressed as means±SEM. The normality of the data were checked by the D’Agostino–Pearson omnibus test. The nonparametric tests were performed using Mann–Whitney U test for 2 groups and Kruskal Wallis test for 3 groups. Mixed-effects regression model was used to test for differences in MAP between the groups. Haplotype associations were carried out by χ2 test using SNP and Variation Suite (Golden Helix). Allele frequencies and 95% confidence interval (CI) were calculated for each polymorphism and were tested using χ2 test. Statistical analyses were carried out using SAS, R, GraphPad Prism, SNP, and variation suite. P<0.05 was considered statistically significant.

Results
Hap-I of hCYP11B2 Gene Is Associated With Hypertension in Whites
The CEU population data from 1000 genomes were used to establish the LD of SNPs in the promoter of hCYP11B2. Analysis of the 1000-genome database reveals complete LD (r2=1) between the 3 polymorphic sites of the hCYP11B2 gene promoter (Figure 1A). These findings were consistent with the remaining populations from 1000 genomes, and the SNPs are in complete LD. We then collected patient samples and confirmed the haplotype association with human hypertension. All the samples in the study were from white population (by self-report). The genotype distributions were consistent with Hardy-Weinberg equilibrium (P=0.93). A total of 277 normotensive (151 men and 126 women) and 295 hypertensive subjects (179 men and 116 women) were recruited from the outpatient department of the Westchester Medical Center, Valhalla, NY (mean age, 59±10 years). All cases were diagnosed as having primary hypertension, and patients with secondary hypertension, diabetes mellitus, or ischemic heart disease were excluded. In all patient samples, the haplotype characterization is based on genotyping for the -344 polymorphic site. Complete LD of SNPs, forming Hap-II and Hap-I, is confirmed by genotyping 20 randomly selected samples for -470 and -663 polymorphic sites in addition to genotyping for -344. The frequency of the -344T allele was 0.56 (95% CI, 0.52–0.60) in hypertensive subjects and 0.495 (95% CI, 0.45– 0.53) in normotensive subjects. The frequency of the -344C allele was 0.44 (95% CI, 0.40–0.48) in hypertensive subjects and 0.505 (95% CI, 0.47–0.55) in normotensive subjects. We have tested for differences in allele frequencies using χ2 test, and the test results indicated significant difference in the allele frequencies (P=0.0176). Homozygotes for -344T alleles were 100 in 295 in hypertensive subjects and 68 in 277 in normotensive subjects. On the other hand, homozygotes for -344C allele were 66 in 295 in hypertensive subjects and 70 in 277 in normotensive subjects. We have used a haplotype association test using a χ2 test comparing the TCA Hap-I with CTT Hap-II and found that TCA haplotype is significantly associated with hypertension (odds ratio=1.49; 95% CI, 1.21–1.82). Results of this experiment suggested that -344T allele which is a part of Hap-I of the hCYP11B2 gene is associated with increased BP in our cohort.

Transient Transfection Shows Increased Transcription of Hap-I Versus Hap-II
We tested haplotype-dependent transcriptional regulation of the CYP11B2 gene in vitro using the H295R cells. RSV-β-gal vector was used to transfect the cells with a 2.5-kb promoter
of the hCYP11B2 gene, containing either Hap-I or Hap-II sequence. As shown in Figure 1B, hCYP11B2 is expressed in transfected H295R cells, and Hap-I transfected cells show significantly greater (P<0.05) expression when compared with cells transfected with Hap-II (Figure 1C).

CYP11B2 Human Transgene, Single Copy, Is Expressed in Transgenic Mice With Hap-II and Hap-I

We selectively targeted the hCYP11B2 gene to the mouse HPRT locus by using ES cells harboring a deletion in the endogenous HPRT gene. We used a special targeting vector capable of restoring its full functionality on homologous recombination. The use of gene targeting is essential in developing a model for studying the 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. As shown in Figure I in the Data Supplement, our transgenic animals contain single copy of the hCYP11B2 gene at the HPRT locus, for mice with both Hap-II and Hap-I.

Hap-I of the hCYP11B2 Gene Increases Transcriptional Activity In Vivo

We examined the differential transcriptional activity of the hCYP11B2 gene in vivo in our 2 transgenic lines. ChIP assay was performed using polymerase II–specific antibody on chromatin extracts from adrenal and renal tissues of the transgenic animals. As shown in Figure 2, polymerase II enrichment to the chromatin isolated from these tissues is significantly greater in mice with Hap-I of the hCYP11B2 gene when compared with mice with Hap-II. These results show increased polymerase II binding to the hCYP11B2 gene in transgenic mice with the Hap-I of the hCYP11B2 gene when compared with the gene in mice with Hap-II.
The 2 transgenes are identical except for the specified SNPs. Thus, the ChIP experiment demonstrates haplotype-dependent increased transcription of human transgene in the mice with Hap-I.

**CYP11B2 mRNA Is Increased in the Adrenal and Renal Tissues of Transgenic Mice Containing Hap-I of the hCYP11B2 gene When Compared With Mice With Hap-II**

Relative gene expression of the hCYP11B2 gene was quantified in adrenal and renal tissues of the transgenic mice using PCR and gel electrophoresis. Significantly increased \( P<0.05 \) hCYP11B2 gene expression is observed in adrenal (Figure II in the Data Supplement, 2.21-fold change) and renal (Figure II in the Data Supplement, 1.54 fold change) tissues of the transgenic mice with Hap-I when compared with the tissues from transgenic mice with Hap-II. No expression of the human transgene was observed in the regular C57 control mice. These experiments establish the presence of the transgene in the transgenic mice and its absence in the C57 controls.

Complementary experiments were conducted to quantify hCYP11B2 and mouse CYP11B2 expression in adrenal and renal tissues of the transgenic animals. As shown in Figure 3, renal and adrenal tissues from the transgenic mice with Hap-I have significantly \( P<0.05 \) elevated the levels of the hCYP11B2 transgene when compared with mice with Hap-II. This differential expression was not observed in the mouse CYP11B2 gene. The presence of the transgene did not affect endogenous mCYP11B2 expression because no significant difference in gene expression was observed in the 2 haplotypes, when compared with C57 mice (normalized threshold cycle values for mCYP11B2 in the adrenals: Hap-I, 1.76±0.26, Hap-II, 1.54±0.24, and C57, 1.63±0.1). These results indicate that the polymorphisms in the hCYP11B2 gene predisposed it to varied transcriptional regulation in the 2 haplotypes. This was confirmed with immunoblot analyses of the adrenal and renal tissues (Figure 4). Transgenic mice with Hap-I demonstrated significantly \( P<0.05 \) the elevated levels of the human transgene when probed with a human-specific anti-CYP11B2 antibody when compared with tissues from the transgenic mice with Hap-II. Plasma aldosterone levels were also significantly \( P=0.002 \) elevated in the transgenic mice with Hap-I when compared with both plasma aldosterone levels in transgenic mice with Hap-II and...
C57 mice (Figure 5). It is important to note that the transgene was not detected in naive C57 mice and the mere presence of the transgene does not increase baseline aldosterone levels in transgenic mice with Hap-II.

hCYP11B2 Is Localized to the Zona Glomerulosa in the Adrenal Glands

Immunohistochemistry shows localization of the hCYP11B2 in the zona glomerulosa of the adrenal cortex, ie, in its native environment (Figure III in the Data Supplement). The color intensity is appreciably higher in cross-sections from mice with Hap-I when compared with sections from mice with Hap-II. The absence of reactivity in tissue-sections from C57 mice points to the specificity of the technique in differentiating the hCYP11B2 protein.

BP Is Increased in Transgenic Mice Containing Hap-I of hCYP11B2 Gene When Compared With Transgenic Mice With Hap-II

We next determined the effect of Hap-I and Hap-II of hCYP11B2 gene on BP of transgenic mice. Butz and Davisson12 have provided details of BP measurement using telemetry. They have shown that this is a reliable procedure for providing high-fidelity MAP and heart-rate recordings for 50 to 60 days in mice weighing 22 g on average but as small as 17 g. No morbidity or mortality was observed in different strains of mice using this procedure. Importantly, different strains of mice fully recovered from anesthesia and surgery, as indicated by a return of normal circadian rhythms, 5 to 7 days post surgery. We have previously used this procedure11,13 and also used it in this study. The MAP of male transgenic animals containing Hap-I and Hap-II of hCYP11B2 gene is shown in Figure 6. BP was measured in 8- to 12-week-old conscious mice for 24 hours for a period of 6 days (n=6). Results of this experiment showed that transgenic mice with Hap-I (-344T allele; red line) had an average increase of 7 mm Hg in BP compared with transgenic mice containing Hap-II (-344C allele; blue line). It is important to mention that BP was increased by 9 mm Hg during the nighttime when animals were active.

Pathophysiological Effects of Haplotypes on Salt-Dependence of BP and on Inflammatory Mediators

Renin–angiotensin–aldosterone system overcativity is associated with salt-dependent hypertension. We examined the effects of increased renal levels of aldosterone on BP in the 2 transgenic lines in the presence of low- (0.01% NaCl) or high-salt (8% NaCl) diet (2 weeks of salt treatment). There was no difference in BP of transgenic mice containing either Hap-I or Hap-II of hCYP11B2 gene on a low-salt diet (Figure 7A). High-salt treatment increased the MAP in both haplotypes; however, this increase was significantly higher in transgenic mice with Hap-I when compared with mice with Hap-II (Figure 7A). On the other hand, salt treatment had no significant effect on the BP in nontransgenic C57 mice (97.8±2.8 versus 95.5±3.5 mm Hg at baseline). Na load suppresses the renin–angiotensin system, including plasma aldosterone levels. As expected, mice treated with high-salt diet showed significant reduction in plasma aldosterone levels. It is important to note, however, that the aldosterone suppression was insufficient in transgenic mice with Hap-I of the hCYP11B2 gene (Figure 7B). These results strongly suggest that inappropriately high aldosterone levels on Na loading could mediate increased BP in mice with Hap-I.

We confirmed the pathophysiological effects of elevated renal aldosterone levels via assessment of inflammatory

![Figure 6. Mean arterial pressure tracing for 24 hours using telemetry probes. Tracings for transgenic mice containing haplotype-I (Hap-I) and Hap-II of human CYP11B2 gene are shown in red and blue and of control C57 mice is shown in black, respectively (n=6; P<0.0005 between Hap-I vs Hap-II).](image-url)
markers, previously shown to be upregulated by aldosterone. As shown in Figure 8, renal expression of IL1β, MCP1, iCAM, and VCAM is significantly increased \((P<0.05)\) in mice with Hap-I of the *hCYP11B2* gene when compared with mice with Hap-II.

### Discussion

Hypertension is a complex disease encompassing multiple pathophysiological processes on a backdrop of genetic predisposition. Interindividual variation of BP, \(\leq 45\%\), can be accounted for by genetic differences in genes regulating the physiological processes governing BP. Inappropriate levels of aldosterone are observed in 15% of patients with essential hypertension\(^{14-16}\) that increases to 22% in patients with resistant hypertension.\(^{17}\) In this regard, association studies have linked *CYP11B2* polymorphism to human hypertension and cardiovascular diseases. The first key finding of the study identifies 2 polymorphic sites (-470 and -663) on the *hCYP11B2* promoter that almost always occur in LD with the -344 site. Together, -663A, -470C, and -344T form the Hap-I, -663T, -470T, and -344C are categorized as the Hap-II. This is the first report identifying these linked polymorphisms in the *hCYP11B2* gene. Importantly, Hap-I of the *hCYP11B2* gene is associated with human hypertension and shows increased transcriptional activity in vitro.

Physiological effects of enhanced transcriptional activity of the gene were tested in novel transgenic animals generated with either Hap-I or Hap-II transgene. Transgenic 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, a transgene can integrate at different sites in the chromosome, and depending on the site of integration, promoter activity may vary from experiment to experiment. In addition, different number of transgene copies may integrate in the genome of different mice and promoter activity may vary depending on the number of transgenes in a particular line. To overcome these limitations, Bronson et al\(^{18}\) have performed gene targeting at the HPRT locus to selectively target a single copy of the gene in a controlled fashion in the genome. We\(^{11}\) and others\(^{19,20}\) have used this strategy to generate transgenic mice containing a single copy of the human angiotensinogen gene containing all the exons, introns, and 3’- and 5’-flanking regions of the gene. Results of these experiments showed that insertion of the single copy transgene upstream of HPRT locus does not affect the overall tissue and cell-specific expression or hormonal regulation of human angiotensinogen gene.

The second key finding of the study is the generation of *hCYP11B2* transgenic mice where the transgene is targeted to the mouse HPRT locus. We show here that both haplotypes have a single copy of the transgene with observation of increased CYP11B2 expression in transgenic mice with Hap-I when compared with mice with Hap-II. It is noteworthy that increased CYP11B2 expression was observed in both the adrenal and the renal tissues. Whereas classical view of the mineralocorticoids
places aldosterone synthase primarily in the adrenal cortex, recently emerging evidence highlights CYP11B2 expression in extra adrenal tissues, especially kidneys. The increased tissue expression of aldosterone synthase, in Hap-I, could depend on the increased binding affinity for the transcription factors regulating CYP11B2 gene expression. In vivo ChIP assay supports this notion by showing increased polymerase II binding to the transgene in mice with Hap-I. RNA polymerase is essential for eukaryotic transcription, and increased chromatin binding of this enzyme strongly suggests enhanced transcriptional activity of the gene. Polymerase II binding and activity are aided by a plethora of transcription factors binding to both enhancer and suppressor regions of the gene. This chromatin binding of the transcription factors is favored by the sequence homology of the gene to the transcription factor. SNPs in the promoter and enhancer regions of the gene alter this binding, and thus, they have the potential to modulate gene transcription. In this regard, in silico analysis suggests that transcription factor GATA-1 may bind to oligonucleotides containing either -663A (in LD with -344T) or -663T (in LD with -344C). However, transcription factor AP4 binds more strongly to the oligonucleotide containing -663T. This suggests that AP4 may be interfering in transcriptional activation by GATA-1 resulting in reduced transcriptional activation of CYP11B2 gene containing -344C haplotype when compared with -344T haplotype. Similarly, transcription factors ETS, MYC, and USF have stronger homology with the promoter sequence containing -470C (in LD with -344T) when compared with -470T (in LD with -344C) of the hCYP11B2 gene. We can speculate that binding of these transcription factors will increase the expression of CYP11B2 gene containing -344T haplotype.

Aldosterone is the key regulator of Na balance via activation of the mineralocorticoid receptor in the principal cells of the cortical-collecting tubule. In addition, redox and structural effects of aldosterone are now well established. Chronically elevated aldosterone induces cardiac hypertrophy and fibrosis, causes vascular remodeling, including perivascular fibrosis and reduced arterial distensibility, increases the activity of cellular oxidases, and precipitates redox imbalance.21,22 Complementary clinical studies have demonstrated favorable outcomes in patients with cardiovascular diseases being treated with aldosterone antagonists (SAVE, CONSENSUS). Also, patients with excess aldosterone have substantially higher rates of atrial fibrillation, stroke, and myocardial infarction compared with patients with essential hypertension who were matched according to their level of BP elevation.23 In line with this historical evidence, the third key finding of the study shows that increased CYP11B2 expression in transgenic mice with Hap-I was accompanied by increased circulating levels of aldosterone with higher SBP, DBP, and MAP. Importantly, achievement of the physiological steady state during a Na-replete diet is critically reliant on the withdrawal of renin-angiotensin system, thereby, increasing the steepness of the pressure-natriuresis response and decreased renal aldosterone levels. Combined, these effects promote Na excretion, restoration of effective circulating volume and maintenance of the cardiovascular steady state. Haplotype-dependent overexpression of CYP11B2, apart from increasing baseline BP, will also prevent excretion of Na during dietary excesses. This notion is supported by our findings of greater BP increase in -344T haplotype when placed on a high-salt diet. Inappropriately elevated aldosterone levels have been suggested to underlie salt sensitivity in both humans and animal models. Our results are in line with these observations and provide translational bridge to the studies reporting hypertension in patients with the -344T polymorphism. Black and South Asian ancestries are at risk for salt-sensitive hypertension, and these are the cohorts where -344T has been observed to be associated with high BP. It seems plausible that the -344T Hap-I is associated with incomplete suppression of the CYP11B2 gene during a high-salt diet. This, in turn, will lead to increased BP in mice with this haplotype during Na+ excess, as observed here. It is important to mention that allele frequency of -344T is much higher in people with African, black, and South Asian ancestries when compared with whites.6,7

Aldosterone also induces the synthesis of proinflammatory molecules, such as monocyte chemoattractant protein-1, adhesion molecules, and cytokines, such as interleukin 6. In addition, aldosterone increases the production of reactive oxygen species, possibly through increased expression of key intracellular regulators, such as nuclear factor-κB. Many of the long-term structural and functional effects of aldosterone are attributed to the activation of these mediators.24 Contextually, higher CYP11B2 expression in transgenic mice with Hap-I is accompanied by upregulation of these inflammatory, adhesion, and redox markers. These findings further support our hypothesis that haplotype-dependent differential regulation of the CYP11B2 gene predisposes to pathophysiological effects observed with inappropriate levels of aldosterone.

In conclusion, we have identified novel polymorphisms in the hCYP11B2 gene that are in LD and divide the population into 2 haplotypes, termed I and II. These polymorphisms in the gene-promoter favor polymerase II binding and increase gene transcription, most likely via altered chromatin-transcription factor association. Consequential increase in tissue and plasma aldosterone bioavailability contributes to salt-sensitive hypertension and a pathophysiological setting of pro-oxidant and proinflammatory milieu in tissues in mice with the Hap-I transgene. Haplotype-dependent differential regulation and expression of CYP11B2 have important clinical implications. Patients with the Hap-I of this gene are at an increased risk of inappropriately high aldosterone levels, predisposing these patients to aldosterone-dependent long-term morbidity, especially in cohort with fluctuations in dietary Na.

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Disclosures
None.

References
Gene Polymorphisms Promote Hypertension

Human hypertension is a complex disease and multitudes of physiological processes contribute to its pathogenesis. Interindividual variabilities in the genes regulating blood pressure, compounded by environmental insults, are thought to account for majority of the cases of hypertension. Single-nucleotide polymorphisms are at the root of this genetic variability and modulate gene expression by affecting the transcriptional regulation of genes. Our study identifies such single-nucleotide polymorphisms in the human CYP11B2 with inappropriately elevated transcription in haplotype-I of this gene; consequentially, increased aldosterone bioavailability and salt-sensitive hypertension are observed in this haplotype. Thus, haplotype-I of the human CYP11B2 gene confers increased risk of human hypertension that, in turn, could predispose these patients to aldosterone-dependent long-term morbidity, especially in cohorts with high-dietary Na. Identifying individuals with this high-risk haplotype may provide more targeted therapeutic lifestyle change recommendations and aid in better management of aldosterone-associated cardiovascular disease.

CLINICAL PERSPECTIVE

Human hypertension is a complex disease and multitudes of physiological processes contribute to its pathogenesis. Interindividual variabilities in the genes regulating blood pressure, compounded by environmental insults, are thought to account for majority of the cases of hypertension. Single-nucleotide polymorphisms are at the root of this genetic variability and modulate gene expression by affecting the transcriptional regulation of genes. Our study identifies such single-nucleotide polymorphisms in the human CYP11B2 with inappropriately elevated transcription in haplotype-I of this gene; consequentially, increased aldosterone bioavailability and salt-sensitive hypertension are observed in this haplotype. Thus, haplotype-I of the human CYP11B2 gene confers increased risk of human hypertension that, in turn, could predispose these patients to aldosterone-dependent long-term morbidity, especially in cohorts with high-dietary Na. Identifying individuals with this high-risk haplotype may provide more targeted therapeutic lifestyle change recommendations and aid in better management of aldosterone-associated cardiovascular disease.
Variable Transcriptional Regulation of the Human Aldosterone Synthase Gene Causes Salt-Dependent High Blood Pressure in Transgenic Mice

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SUPPLEMENTAL MATERIAL
Fig.S1: (A) Genotyping of hCyp11b2 transgenic mice. Lanes 1-6 are PCR amplified fragments using DNA from tails of transgenic mice analyzed by agarose gel electrophoresis with two different primer sets. Lanes 1,4,5 and 6 are positive whereas lane 2 and 3 are negative for hCYP11B2. lane 7 and 8 are amplified fragments from C57 genomic DNA and hCyp11b2 BAC plasmid DNA used as a negative and positive control respectively. (B) hCYP11B2 Genomic DNA Copy number quantitation by QRT-PCR. 250 ng of genomic DNA was used in the real time PCR using hCYP11B2 Promoter specific -473F and -192R primers. The CT values were normalized with GAPDH.
Fig-S2: (A, B) hCYP11B2 Expression in adrenal gland of transgenic mice containing haplotype-I (shown as T) or haplotype-II (shown as C) by RT-PCR. Panel A shows gel electrophoresis and panel B shows quantitation of the bands. Results are shown as mean±SEM for n=6. *p<0.05 vs. haplotype II. (C, D) hCYP11B2 Expression in the kidney of transgenic mice containing haplotype-I (shown as T) or haplotype-II (shown as C), by RT-PCR. Panel C shows gel electrophoresis and panel D shows quantitation of bands. Results are shown as mean±SEM for n=6. *p<0.05 vs. haplotype II.
Fig. S3: Immunohistochemistry of sections of the adrenal gland from the transgenic mice containing haplotype-I and haplotype-II of hCYP11B2 gene and control C57 mice. hCYP11B2 stained areas are marked with arrows. ZG=Zona glomerulosa, ZF=Zona fasciculata zones are labeled.