Effects of intron conversion in the human *CYP11B2* gene on its transcription and blood pressure regulation in transgenic mice

Received for publication, February 13, 2020, and in revised form, June 13, 2020 Published, Papers in Press, June 15, 2020, DOI 10.1074/jbc.RA120.013047

Brahmaraju Mopidevi[1], **Indu Sivankutty**[1], **Shoujin Hao**[2], **Nicholas R. Ferreri**[2], **and Ashok Kumar**[1,*]

From the [1]*Department of Pathology, New York Medical College, Valhalla, New York, USA and* [2]*Department of Pharmacology, New York Medical College, Valhalla, New York, USA*

Edited by F. Peter Guengerich

The human cytochrome P450 family 11 subfamily B member 2 (*hCYP11B2*) gene encodes aldosterone synthase, the rate-limiting enzyme in the biosynthesis of aldosterone. In some humans, *hCYP11B2* undergoes a unique intron conversion whose function is largely unclear. The intron conversion is formed by a replacement of the segment of DNA within intron 2 of *hCYP11B2* with the corresponding region of the *hCYP11B1* gene. We show here that the intron conversion is located in an open chromatin form and binds more strongly to the transcriptional regulators histone acetyltransferase P300 (p300), NFκB, and CCAAT enhancer–binding protein α (CEBPα). Reporter constructs containing the intron conversion had increased promoter activity on transient transfection in H295R cells compared with WT intron 2. We generated humanized transgenic (TG) mice containing all the introns, exons, and 5′- and 3′-flanking regions of the *hCYP11B2* gene containing either the intron conversion or WT intron 2. We found that TG mice containing the intron conversion have (*a*) increased plasma aldosterone levels, (*b*) increased *hCYP11B2* mRNA and protein levels, and (*c*) increased blood pressure compared with TG mice containing WT intron 2. Results of a ChIP assay showed that chromatin obtained from the adrenals of TG mice containing the intron conversion binds more strongly to p300, NFκB, and CEBPα than to WT intron 2. These results uncover a functional role of intron conversion in *hCYP11B2* and suggest a new paradigm in blood pressure regulation.

Hypertension is a serious risk factor for heart failure, myocardial infarction, vascular disease, stroke, and renal failure (1). Hypertension affects about 50 million Americans at a rate of 25–30% in adult Caucasians and 1 billion people worldwide (2). Like other complex diseases, environmental factors and genetic predisposition contribute to hypertension. The renin-angiotensin-aldosterone system plays a significant role in the modulation of blood pressure (3–5). Aldosterone is a mineralocorticoid synthesized from cholesterol in zona glomerulosa of the mammalian adrenal cortex (6). About 15% of patients having essential hypertension (7–9) and 22% of patients having resistant hypertension (10) have an inappropriate excess of aldosterone. This results in an increase in age-related blood pressure and cardiovascular risk. Milliez *et al.* (11) showed that patients with aldosterone excess have markedly higher rates of stroke, atrial fibrillation, and myocardial infarction as compared with patients with essential hypertension when they were matched with blood pressure elevation.

Aldosterone synthase catalyzes final steps in aldosterone biosynthesis and converts 11-deoxycorticosterone to aldosterone through intermediate products corticosterone and 18-hydroxycorticosterone (12). It is encoded by the *CYP11B2* gene, which is one of the potential risk loci for cardiovascular diseases (13). The amino acid sequence of human CYP11B2 is ∼93% similar to 11β-hydroxylase that is encoded by the *CYP11B1* gene. *CYP11B1* and *CYP11B2* genes are localized in tandem on human chromosome 8q21–22 (14–16) and are separated by about 40-kb nucleotide sequence. In the adrenal cortex, *CYP11B2* gene expression is confined to zona glomerulosa, whereas the *CYP11B1* gene is expressed in zona reticularis and zona fasciculata.

Human *CYP11B2* gene has a −344 T/C (rs1799998) polymorphism in its promoter and variant −344T is associated with hypertension (17–24). In addition, the *hCYP11B2* gene has an intron conversion in intron2 in which part of the WT intron2 (WT intron2) is replaced by the corresponding region of intron2 of the *hCYP11B1* gene (25). In *CYP11B2* gene, the WT intron2 can occur with either −344T or −344C whereas intron conversion almost always occurs with variant −344T (25). The frequency of −344T and intron conversion is higher in hypertensive human subjects as compared with normotensives (18, 26–28). The intron conversion has also been associated with increased aldosterone level in plasma (19, 29, 30) and tetrahydroaldosterone (18, 31, 32) level in urine of human subjects. The association of polymorphisms at *CYP11B1* and *CYP11B2* loci with the risk of essential hypertension was analyzed by Alvarez-Mardazo *et al.* (33). These authors used two large white case-control samples for discovery (n = 3340) and confirmation (n = 2929) and found that *CYP11B2* intron conversion has the strongest association with hypertension in both cohorts as well as in combined analysis (odds ratio = 1.16, $p = 8.54 \times 10^{-5}$). Taken together, these studies suggest that −344T and intron conversion are associated with increased blood pressure in Caucasian, African, Japanese, and Indian populations (18, 19, 22, 34). However, molecular mechanisms involved in increased blood pressure by intron conversion of *CYP11B2* gene are not known. We show here that: (*a*) intron conversion sequence binds more strongly to transcription

This article contains supporting information.

* For correspondence: Ashok Kumar, akumar5@nymc.edu.

factors p300, NFκB, and CEBPα as compared with WT intron; (*b*) reporter constructs containing intron conversion sequence have increased promoter activity on transient transfections; (*c*) transgenic mice containing intron conversion sequence have increased plasma aldosterone levels, increased hCYP11B2 mRNA and protein level, and increased blood pressure as compared with transgenic mice containing WT-intron2; and (*d*) chromatin prepared from the adrenals of TG mice containing intron conversion sequence binds more strongly to transcription factors p300, NFκB, and CEBPα as compared with WT intron2.

Results

hCYP11B2 intron conversion (IC) region has stronger homology with the nucleotide sequence of intron2 of hCYP11B1 gene

The genomic organization of all the introns and exons of the WT and IC *hCYP11B2* gene is shown in Fig. 1*A*. This figure also shows (*a*) the position of intron conversion region marked as IC in the second intron and (*b*) location of unique restriction enzyme sites FspA1 and AccIII that were used for the generation of transgenic mice. To understand the role of IC in the regulation of *CYP11B2* gene expression and blood pressure, we first determined the nucleotide sequence of intron2 of WT and IC of *hCYP11B2* gene. A portion of the sequence of intron2 of some hypertensive subjects has homology with *CYP11B1* and not with *CYP11B2* gene (Fig. 1*B*). A comparison of the nucleotide sequence of intron2 of *WT-CYP11B2*, *IC-CYP11B2*, and *CYP11B1* is presented in Fig. S1. Nucleotide sequences of intron2 of *WT-CYP11B2* and *IC-CYP11B2* have strong homology. However, about 180 bp region located between nucleotides 2095 and 2280 does not have strong homology with hCYP11B2. On the other hand, this region has almost complete homology with intron2 sequence of the *hCYP11B1* gene (Fig. S2).

The intron conversion sequence of the hCYP11B2 gene is located in an open chromatin region in adrenal glands

To analyze whether IC region has any role in transcriptional regulation, we mapped the regulatory overlap of the intron conversion region with H3K4me1, H3K4me3, and H3K27ac histone marks and DNase hypersensitivity sites track using UCSC genome browser because these regions represent enhancer regions. These data were obtained from the ENCODE (Encyclopedia of DNA Elements) and Roadmap Epigenome projects (35, 36) and the location of the intron conversion region is marked with *yellow* (Fig. 1*C*). Results of this analysis suggested that intron conversion region of the *hCYP11B2* gene is located in an open chromatin region in the adrenal gland and is therefore capable of binding with transcription factors and modulate the expression of this gene.

Intron conversion sequence has stronger homology with consensus binding sites of p300, CEBPα, and NFκB as compared with WT intron2

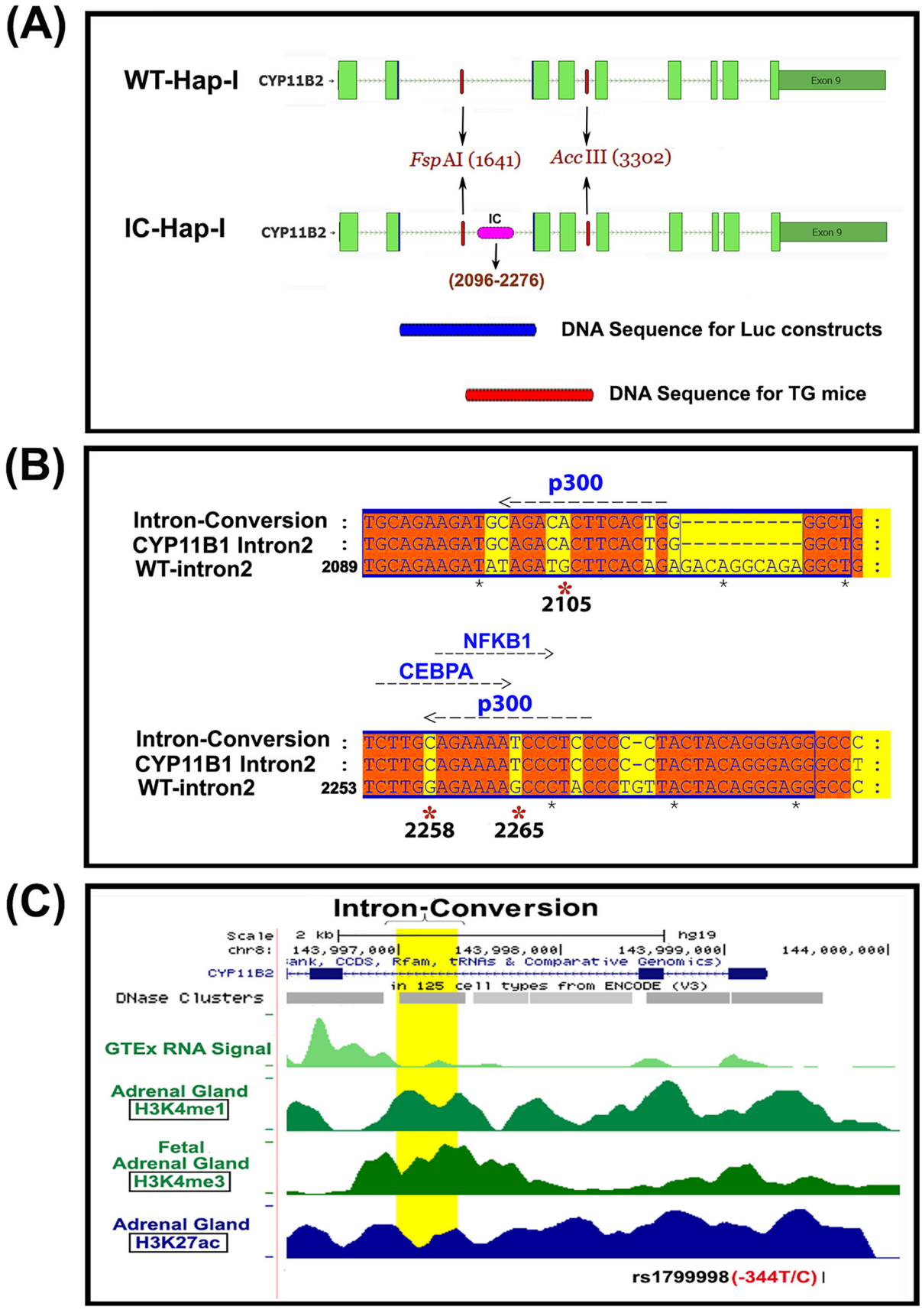
In silico analysis of transcription factor–binding site reveals that nucleotide sequence in intron conversion has homology with consensus binding sites of transcription factors p300, CEBPα, and NFκB. A comparison of consensus binding sites of these transcription factors with the nucleotide sequence present in WT intron2 and intron conversion is shown in Fig. 2, where mismatched nucleotides are marked in *red* letters. Our transcription factor–binding site prediction indicates that the intron conversion sequence has stronger homology with consensus transcription factor–binding sites as compared with WT-intron2. This suggests that transcription factors may bind more strongly to the DNA sequence present in intron conversion as compared with WT intron2 (Fig. 2). This conclusion is confirmed by the results of transient transfection and ChIP assay described later.

Reporter constructs containing intron conversion sequence of the hCYP11B2 gene have increased promoter activity as compared with WT intron2 on transient transfection in H295R cells

To understand the role of intron conversion of the *hCYP11B2* gene on transcriptional regulation, we performed transient transfection assay. We synthesized three Luc reporter constructs: one with 2.5 kb CYP11B2 promoter containing −344T in its promoter and named as Cyp-Luc (Fig. 3*Ai*). The full-length WT intron2 and intron conversion sequences were PCR amplified as shown in Fig. 1*A* and attached in front of the 2.5 kb *CYP11B2* promoter containing −344T. These reporter constructs were named WT-Hap-I-Luc and IC-Hap-I-Luc, respectively, as shown in Fig. 3, *A*, *ii* and *iii*. Transient transfection shows that reporter construct IC-Hap-I-Luc has significantly increased relative luciferase activity under basal conditions as well as after co-transfection with the HNF3β and NFκB (Fig. 3*B*) expression vectors as compared with the reporter construct containing WT-Hap-I-Luc and Cyp-Luc in adrenal carcinoma cells (H295R). These studies suggest that intron conversion increases the *hCYP11B2* gene expression as compared with WT intron2 in adrenal cells.

Generation of transgenic mice containing WT-Hap-I and IC-Hap-I of hCYP11B2 gene

To understand the role of intron conversion of *hCYP11B2* gene on transcriptional regulation in an *in vivo* situation, we generated two lines of transgenic mice containing either WT intron2 (WT-Hap-I) or intron conversion (IC-Hap-I). For this purpose, WT-Hap-I DNA was cut with FspAI and AccIII and swapped with PCR-amplified FspAI-AccIII fragment obtained from a hypertensive patient that has IC sequence (Fig. 1*A* and Fig. S1). We used the knockin strategy at the HPRT locus described by Bronson *et al.* (37) to generate transgenic animals. We selectively targeted the *hCYP11B2* gene containing intron conversion 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. This allows reproducible insertion of the single copy of a transgene in a predetermined locus, permitting direct comparison between individually generated transgenic lines. We confirmed that our transgenic animals contain WT intron2 and intron conversion by sequence determination of the promoter and intron conversion region. We have further analyzed *hCYP11B2* gene copy number in our transgenic


(A)
WT-Hap-I
CYP11B2
Exon 9
FspAI (1641)
AccIII (3302)
IC-Hap-I
CYP11B2
IC
Exon 9
(2096-2276)
DNA Sequence for Luc constructs
DNA Sequence for TG mice
(B)
p300
Intron-Conversion : TGCAGAAGATGCAGACACTTCACTGG----------GGCTG :
CYP11B1 Intron2 : TGCAGAAGATGCAGACACTTCACTGG----------GGCTG :
WT-intron2 2089 TGCAGAAGATATAGATGCTTCACAGAGACAGGCAGAGGCTG :
2105
NFKB1
CEBPA
p300
Intron-Conversion : TCTTGCAGAAAATCCCTCCCCC-CTACTACAGGGAGGGCCC :
CYP11B1 Intron2 : TCTTGCAGAAAATCCCTCCCCC-CTACTACAGGGAGGGCCT :
WT-intron2 2253 TCTTGGAGAAAAGCCCTACCCTGTTACTACAGGGAGGGCCC :
2258
2265
(C)
Intron-Conversion
Scale 2 kb hg19
chr8: 143,997,000 143,998,000 143,999,000 144,000,000
ank, CCDS, Rfam, tRNAs & Comparative Genomics)
CYP11B2
in 125 cell types from ENCODE (V3)
DNase Clusters
GTEx RNA Signal
Adrenal Gland
H3K4me1
Fetal
Adrenal Gland
H3K4me3
Adrenal Gland
H3K27ac
rs1799998 (-344T/C)

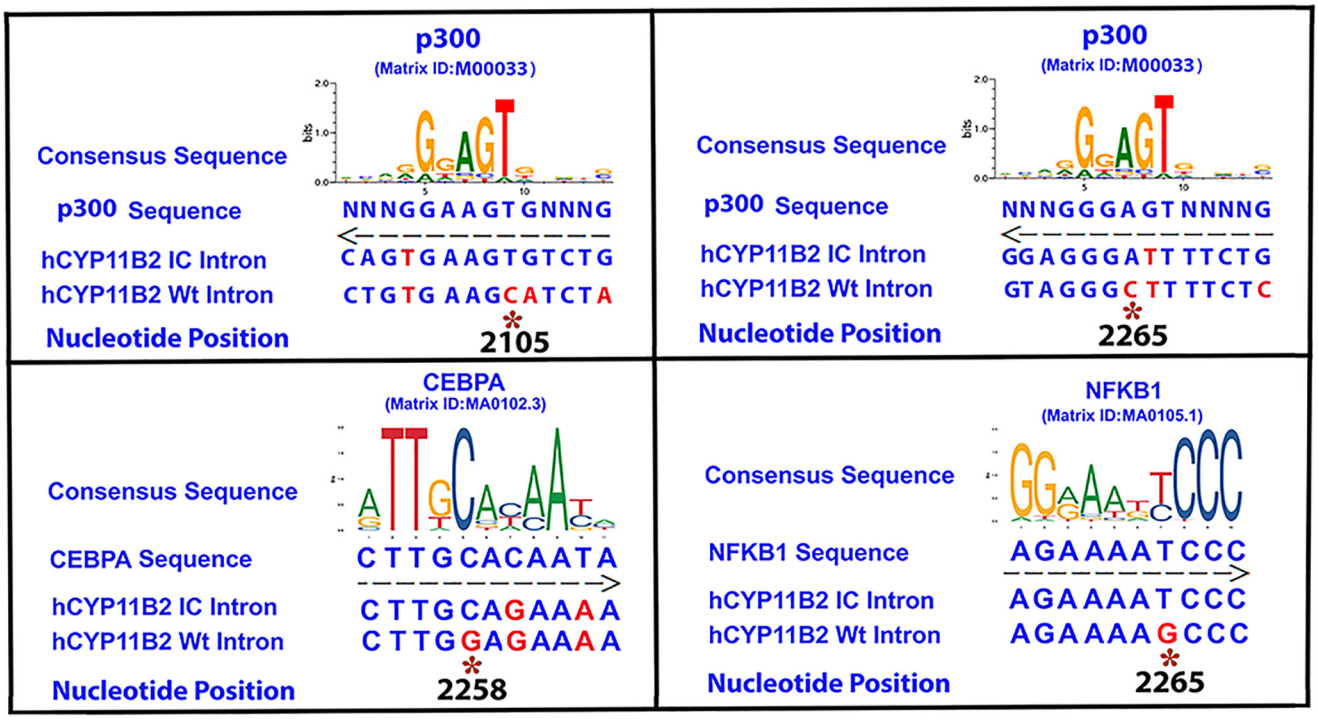


Figure 2. *In silico* transcription factor–binding prediction. Transcription factors p300, CEBPα, and NFκB1 bind more strongly to the intron conversion sequence as compared with WT intron2. Mismatched nucleotides to the consensus transcription factor–binding sites are marked in *red letters*. The most important mismatch nucleotide positions are marked with *asterisks*. Numbers correspond to nucleotide position starting from the transcription initiation site.

animals using human and mouse specific primers by qPCR method (Fig. S3) as described previously (20). Results of this experiment showed that transgenic animals contain only one copy of the *hCYP11B2* gene. We initially developed three transgenic lines from each construct but after analysis kept one line each for future studies. Transgenic animals were routinely analyzed by three sets of human *CYP11B2* gene-specific primers. The previously identified specific *hCYP11B2* intron2 variants that are present in WT-Hap-I and IC-Hap-I within the FspAI-AccIII region are shown in Table 1.

hCYP11B2 mRNA is increased in the adrenals, kidney, and brain tissues of transgenic mice containing IC-Hap-I of the hCYP11B2 gene as compared with the WT-Hap-I

Quantitative real-time PCR was performed using human CYP11B2, mouse CYP11B1, CYP11B2, and mouse GAPDH gene–specific primers to determine *CYP11B2* mRNA levels in different tissues of TG mice. The quantitation of mRNA level in TG mice with IC-Hap-I was performed using the mRNA level in WT-Hap-I as one. Results of these experiments show that *hCYP11B2* mRNA level in adrenals of transgenic mice containing intron conversion is increased by about 2.34-fold as compared with the transgenic mice containing WT-Hap-I (p = 0.0018) (Fig. 4*A*). On the other hand, the intron conversion did not have any effect on the expression of either *mCyp11B2* or *mCyp11B1* genes in adrenals of TG mice (Fig. 4, *B* and *C*). Similarly, the *hCYP11B2* mRNA levels in IC-Hap-I are increased by 1.56-fold in kidney (p = 0.0013) and 1.35-fold (p = 0.0024) in brain tissue as compared with WT-Hap-I (Fig. S4). Further, we have analyzed the *hCYP11B2* gene expression in adrenals of female transgenic mice with one copy of the knockin gene at HPRT locus. The results indicate that the *hCYP11B2* mRNA levels increased by about 1.64-fold in Hap-I as compared with the transgenic mice containing WT-Hap-I (p = 0.0016) (Fig. S5).

The hCYP11B2 protein level is increased in the adrenals of transgenic mice containing IC-Hap-I as compared with WT-Hap-I of hCYP11B2 gene

Because the above-mentioned experiments suggested that hCYP11B2 mRNA is increased in adrenals of transgenic mice containing IC-Hap-I as compared with WT-Hap-I, we were interested to determine whether hCYP11B2 protein level is also increased in these animals. Protein extracts from adrenals of male transgenic mice containing either WT-Hap-I or IC-Hap-I were resolved on 12% SDS-PAGE and electroblotted onto Immobilon-P transfer membranes. The membranes were developed using commercially available monoclonal antibodies for hCYP11B2 (Abcam, cat. no. EPR-10494) and mouse β-actin (Sigma-Aldrich,

Figure 1. *A*, pictorial representation of *hCYP11B2* gene with all exons (*green*) and introns. The number in the parentheses is the position of the nucleotide from the transcription start site. *Blue* and *red bars* show the DNA sequence used for the generation of luciferase constructs and transgenic mice. *IC* is the intron conversion region. *B*, comparison of partial DNA sequence of intron conversion (*lane 1*) with WT intron2 (*lane 3*) of *hCYP11B2* and *hCYP11B1* (*lane 2*) from the white population. *Numbers* correspond to nucleotide positions starting from transcription start site. Transcription factor–binding sites and its direction of binding are shown as *broken arrows*. *C*, regulatory overlap of intron conversion region with H3K4me1, H3K4me3, and H3K27ac histone marks and DNase hypersensitivity sites data obtained from the ENCODE and Roadmap Epigenome projects accessed through the UCSC genome browser from human adrenal gland. Location of the intron conversion region is marked with *yellow*.

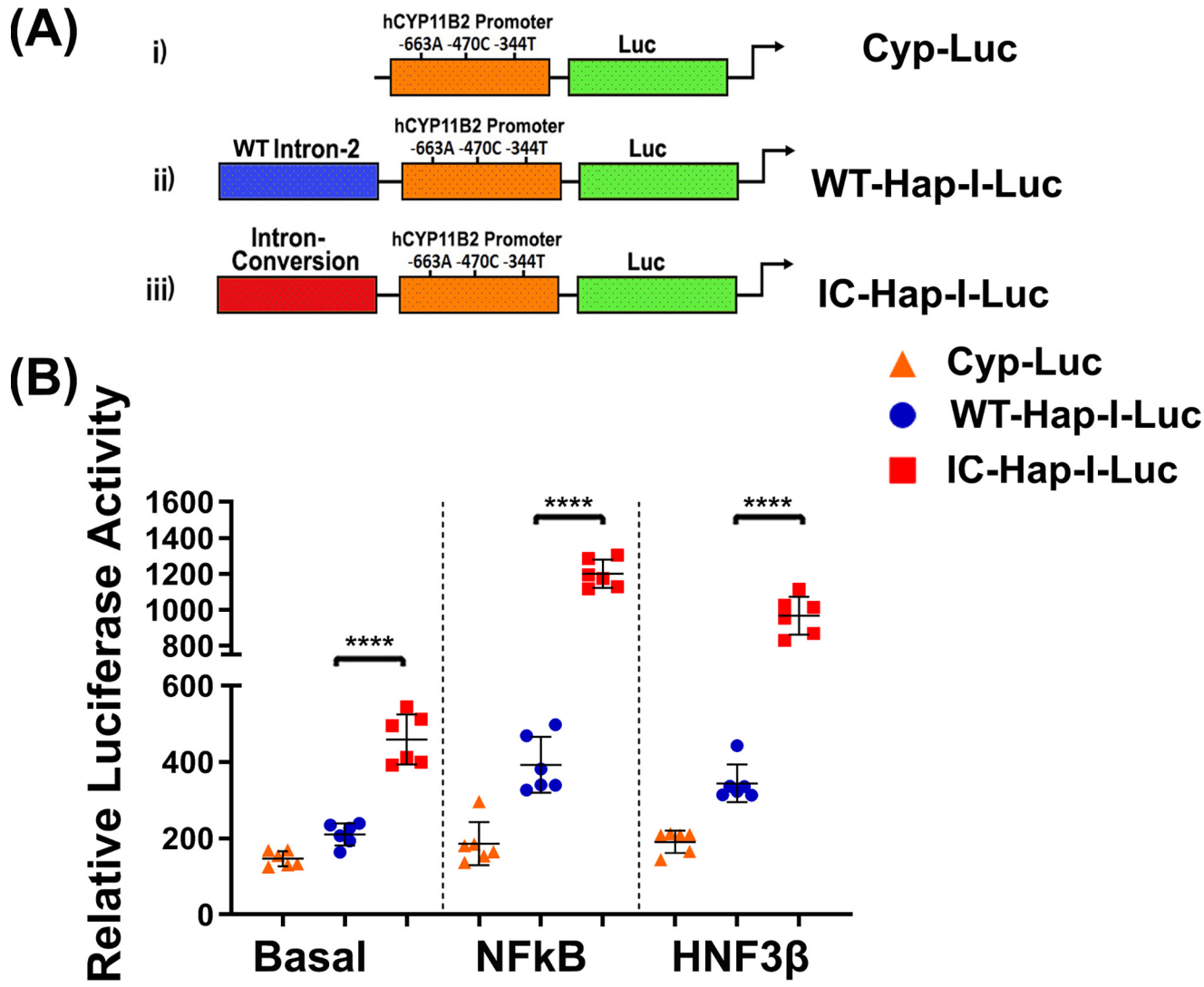


Figure 3. *A*, reporter constructs of the *hCYP11B2* gene used in transient transfections. *B*, relative luciferase activity of intron conversion (IC-Hap-I) as compared with WT-Hap-I and Cyp-Luc under basal conditions and after co-transfection with NFκB and HNF3β expression vectors in adrenal carcinoma cells (H295R). The cells were harvested 48 h post transfection followed by measurement of the luciferase activity. Results are shown as mean ± S.D. ($n = 6$). ****, $p < 0.0001$ as compared with the WT intron2 reporter construct.

cat. no. A2228, 200 μl). The immune complexes were detected by using secondary antibody conjugated with IRDye800 or IRDye700 and images were captured using an Odyssey Imaging System (LI-COR). Quantitation of protein level from three experiments showed that hCYP11B2 level is increased about 1.63-fold in adrenals of transgenic mice containing IC-Hap-I as compared with the transgenic mice containing WT-Hap-I ($p = 0.0004$). A typical Western blotting along with β-actin is shown in Fig. 5*A* and its quantification is shown in Fig. 5*B*.

In vivo ChIP assay shows increased binding of transcription factors to the chromatin of TG mice containing IC-Hap-I of the hCYP11B2 gene as compared with WT-Hap-I

To compare the binding of transcription factors with the nucleotide sequences present in IC-Hap-I and WT-Hap-I, we performed ChIP assay using chromatin extract from the adrenals of TG mice. The ChIP assay was performed using CEBPα, p300, and NFκB antibodies to identify the differential transcriptional activity of the *hCYP11B2* gene *in vivo* in our two transgenic lines. Our ChIP assay indicates that transcription factors CEBPα, p300, and NFκB have significantly increased binding (2.02-, 3.2-, and 2.35-fold) to the IC-Hap-I as compared with WT-Hap-I (Fig. 6, *A–C*).

Blood pressure is increased in transgenic mice containing IC-Hap-I of the hCYP11B2 gene as compared with WT-Hap-I

We next determined the effect of IC and WT intron2 of the *hCYP11B2* gene on blood pressure of transgenic mice using telemetry. Butz and Davisson (38) have provided details of blood pressure measurement using telemetry, and we have previously used this procedure (39, 40). After the surgical procedure, transgenic mice were moved to their home cages. Mice were housed in standard polypropylene cages placed in a temperature- and humidity-controlled facility, maintained in a 12:12 h light-dark cycle (6 AM to 6 PM lights on), and were fed standard mouse chow (Teklad LM-485) with water available *ad libitum*.

Table 1
***hCYP11B2* intron2 variants that are present in WT-Hap-I and IC-Hap-I within the FspAI-AccIII region. MAF is the minor allele frequency and NA is not available**

Variant	Chromosome	Position(GRCh38)	Minor Allele	MAF	IC-Hap-I	WT-Hap-I
rs10090037	8	142916048	C	0.106962	C	G
rs13257680	8	142915893	T	0.301693	T	C
rs879123526	8	142915742	G	0.0106	G	A
rs542092383	8	142915741	C	0.0777	C	T
rs1563881469	8	142915737	C	NA	C	T
rs1563881467	8	142915736	A	NA	A	G
rs1563881457	8	142915719–142915727	—	NA	—	AGACAGGCA
rs1156891906	8	142915717	G	NA	G	A
rs13250178	8	142915684	A	0.2165	A	G
rs13257267	8	142915595	G	NA	G	C
rs13257266	8	142915594	T	NA	T	C
rs78004363	8	142915583	C	0.0351	C	G
rs80147915	8	142915576	T	NA	T	G
rs1217407622	8	142915567	C	NA	C	T
rs1199746308	8	142915566	C	NA	C	G
rs1341948893	8	142915565	C	NA	C	T
rs13263682	8	142915519	G	0.48065	G	T

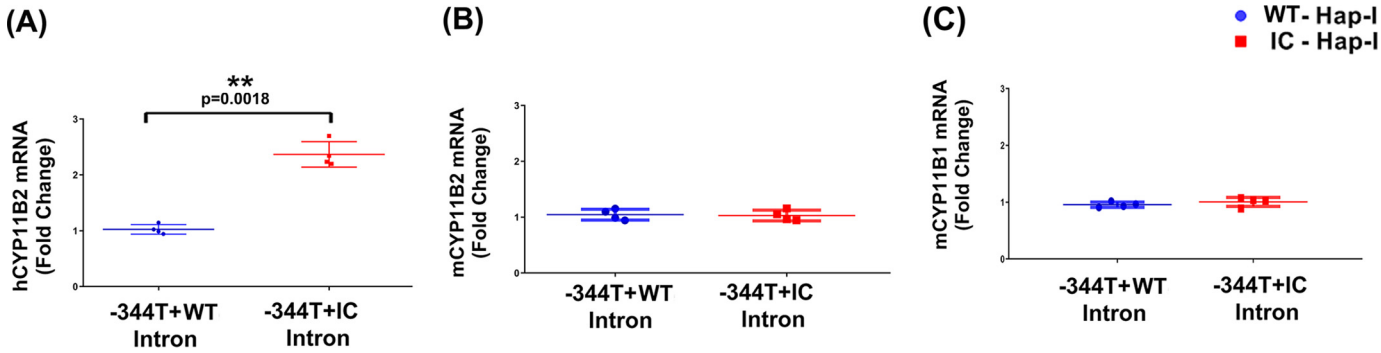


Figure 4. *A–C*, human and mouse CYP11B2 (*A* and *B*), and mouse Cyp11B1 (*C*) mRNA levels in the adrenal gland of transgenic mice containing either IC-Hap-I or WT-Hap-I, as determined by qPCR ($n = 4$); **, $p = 0.0018$ *versus* WT-Hap-I.

After 1 week of recovery from the surgical procedure, BP readings were recorded every 10 min using Data-Science instrument. Mean BP values were calculated for every 2 h from the values taken over 6 days. Systolic BP (SBP), diastolic BP (DBP), mean arterial pressure (MAP), and heart rate (HR) were recorded using DATAQUEST software. For statistical analyses, 6 days of baseline values were used. The systolic, diastolic, mean arterial pressure, and heart rate of male transgenic mice are shown in Fig. 7, *A–D*. Blood pressure was measured in 8- to 12-week-old conscious mice for 24 h over a period of 6 days (n = 5-6). Results of this experiment showed that transgenic mice containing IC-Hap-I (*red line*) had increased blood pressure as compared with transgenic mice containing WT-Hap-I (*blue line*). The BP of control male C57 animals is shown with the *black line* (Fig. 7).

High salt treatment increases systolic, diastolic, and mean arterial blood pressure and heart rate in transgenic mice containing IC-Hap-I of the hCYP11B2 gene as compared with WT-Hap-I

We next examined the effect of high-salt (4% NaCl for 6 weeks) diet on blood pressure of transgenic mice. Under high-salt conditions, the average systolic BP in TG mice containing IC-Hap-I was 139 mmHg and in WT-Hap-I was 132 mmHg whereas in control C57 the blood pressure was 116 mmHg. The average diastolic BP in TG mice containing IC-Hap-I was 120 mmHg and in WT-Hap-I was 101 mmHg whereas in control C57 the blood pressure was 95 mmHg. The average mean arterial pressure in TG mice containing IC-Hap-I was 130 mmHg and in WT-Hap-I was 119 mmHg whereas in control C57 the blood pressure was 98 mmHg. Similarly, the average heart rate in TG mice containing IC-Hap-I was 636 bpm and in WT-Hap-I was 602 bpm whereas in control C57 the heart rate was 537 bpm (Fig. 8). Taken together, the results of these experiments showed that SBP, DBP, MAP, and HR increased in in TG mice containing IC-Hap-I as compared with WT-Hap-I after high-salt treatment.

Transgenic mice containing IC-Hap-I have increased plasma aldosterone levels as compared with WT-Hap-I and C57 mice

We have also measured plasma aldosterone levels in mice containing IC-Hap-I, WT-Hap-I, and control C57BL6 animals. We have found that plasma aldosterone levels in transgenic mice with IC-Hap-I are significantly elevated as compared with transgenic mice with WT-Hap-I or control C57 mice (Fig. S6).

Effect of intron conversion of CYP11B2 gene on urinary sodium, chloride, potassium, and osmolarity levels

We have determined sodium, chloride, potassium, osmolarity, and aldosterone levels in the urine of TG animals fed normal salt diet containing either WT-Hap-I or IC-Hap-I. Results of this experiment (Fig. S7) showed that sodium and chloride

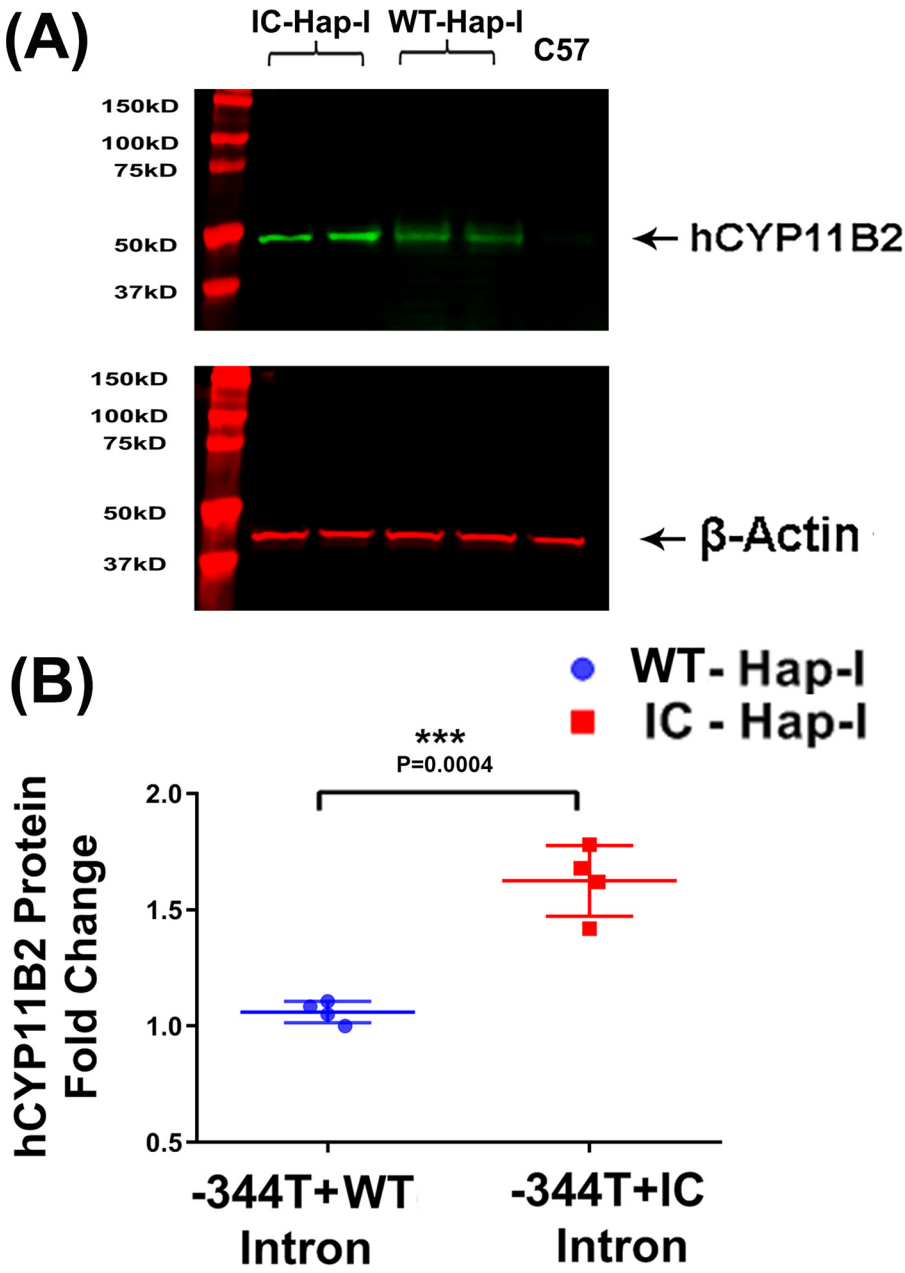


Figure 5. *A*, human CYP11B2 protein levels in the adrenal gland of transgenic mice containing either IC-Hap-I or WT-Hap-I, as determined by Western blot analysis. *B*, quantification of protein levels from the adrenal glands. Quantitation of hCYP11B2 protein was performed after normalization with β-actin. Results are shown as mean ± S.D. ($n = 4$); ***, $p = 0.0004$ *versus* WT-Hap-I.

are excreted at statistically significant reduced level in the urine of IC-Hap-I animals as compared with WT-Hap-I animals. This suggests that sodium is retained more in the kidneys of TG animals containing IC-Hap-I. However, no significant differences were observed in the urine potassium and osmolarity levels between WT-Hap-I or IC-Hap-I transgenic mice.

Discussion

To understand the functional role of intron conversion in transcriptional regulation of the *hCYP11B2* gene, we have determined the nucleotide sequence of WT intron2 and intron conversion. Our first key finding of this study is that intron conversion sequence in *hCYP11B2* gene is located in an open chromatin region and therefore may act as an enhancer. Our *in silico* studies suggest that the nucleotide sequence of the intron conversion region has stronger homology with consensus binding sites of transcription factors p300, NFκB, and CEBPα as compared with WT intron2. This suggests that increased binding of transcription factors may lead to increased transcriptional activation because of intron conversion sequence. In accordance with this observation, our transient transfection studies show that reporter constructs containing intron2 with intron conversion sequence has increased transcriptional activity as compared with the WT intron2 in human adrenal cells (H295R). Therefore, our second key finding of the present

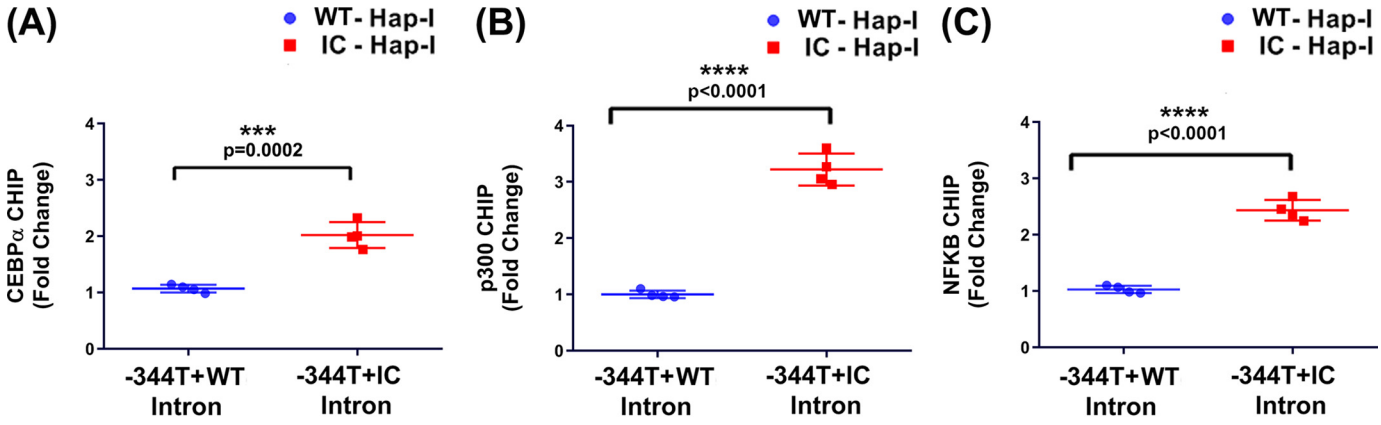


Figure 6. ChIP assay shows stronger binding of CEBPα, p300, and NFκB to the chromatin obtained from the adrenal glands of transgenic mice containing IC-Hap-I as compared with WT-Hap-I of *hCYP11B2* gene. *A–C*, show the quantification of chromatin immunoprecipitated DNA by qPCR in the presence of CEBPα, p300, and NFκB antibodies, respectively. The Ct values obtained from the immunoprecipitated DNA were normalized to the Ct values from the input DNA. Results are shown as mean ± S.D. ($n = 4$); ***, $p = 0.0002$; ****, $p < 0.0001$ *versus* WT-Hap-I.

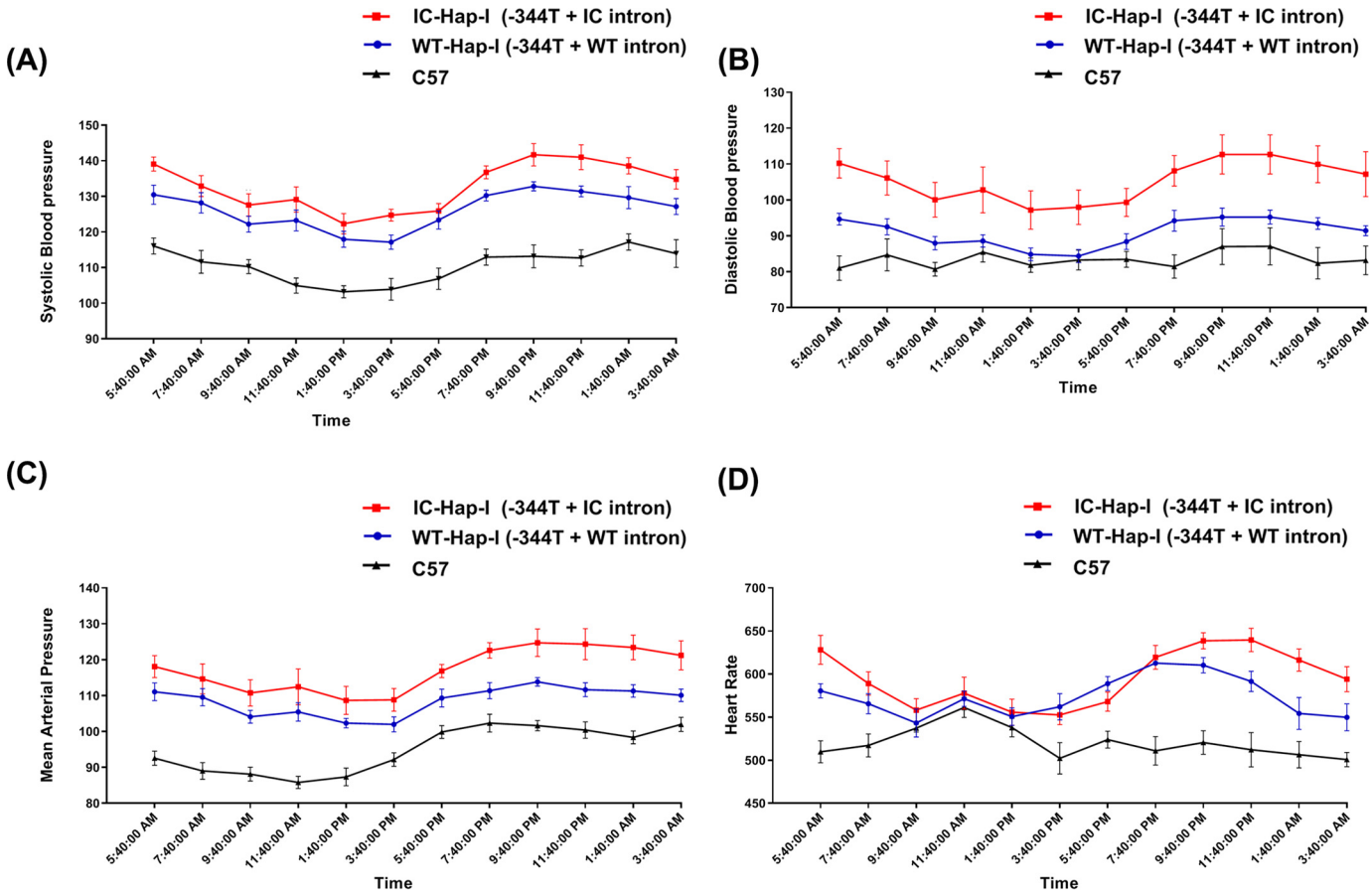


Figure 7. *A*, systolic blood pressure. *B*, diastolic blood pressure. *C*, mean arterial pressure. *D*, heart rate tracing for 24 h over a period of 6 days using telemetry probes. Tracings for transgenic mice containing IC-Hap-I and WT-Hap-I of human *CYP11B2* gene are shown in *red* and *blue*, and of control C57Bl6 is shown in *black*, respectively ($n = 5$-6). $p < 0.0001$ between IC-Hap-I *versus* WT-Hap-I for systolic, diastolic, mean arterial pressure, and heart rate.

study is that the nucleotide sequence of the intron conversion region of the *hCYP11B2* gene has increased transcriptional activity in human adrenal cells as compared with WT intron2.

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 in an *in vivo* situation. To avoid random integration of transgene in mice, Bronson *et al.* (37) have developed a method to selectively target single copy of a gene at the HPRT locus into the mouse genome. We (20, 39) and others (41, 42) have used this strategy to generate transgenic mice containing a single copy of the human angiotensinogen and human *CYP11B2* genes. We have used the same strategy to generate transgenic mice containing

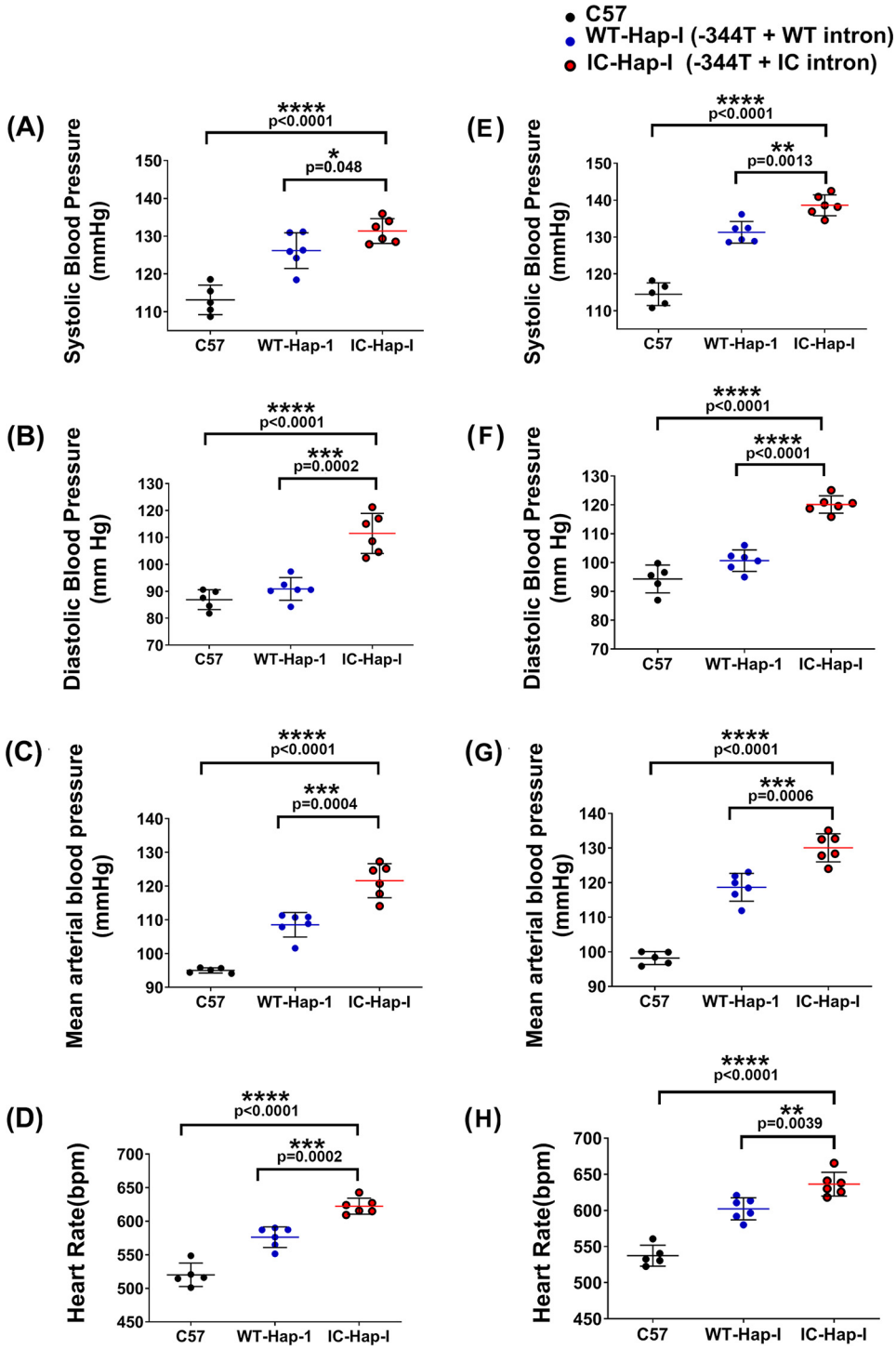


Figure 8. Effect of normal salt (*A–D*) and high salt (*E–H*) diet on systolic (*A* and *E*), diastolic (*B* and *F*), mean arterial (*C* and *G*) blood pressures and heart rate (*D* and *H*) of transgenic mice containing IC-Hap-I, WT-Hap-I of *hCYP11B2* gene and control C57 ($n = 5–6$).

either WT intron2 or intron conversion of the *hCYP11B2* gene. These novel transgenic mice contain all the exons, introns, and 3′- and 5′-flanking regions of the human *CYP11B2* gene. The third key finding of the present study is that TG mice containing intron conversion have increased *hCYP11B2* mRNA and protein levels in the adrenal tissue as compared with WT intron2 (Figs. 4 and 5). Increased *CYP11B2* expression in TG mice containing intron conversion was accompanied by elevated circulating plasma aldosterone levels as compared with WT intron2 (Fig. S6). On the other hand there was no effect on the expression of mouse *CYP11B1* and *CYP11B2* genes in our TG mice. We have measured blood pressure in transgenic mice and our fourth key finding of this study is that TG mice containing IC-Hap-I have higher systolic, diastolic, and mean arterial blood pressure as compared with TG mice containing WT-Hap-I. Moreover, high-salt treatment (4% salt diet) for 6 weeks

further increases the blood pressure in TG mice. However, increase in blood pressure is more prominent in TG mice containing IC-Hap-I as compared with WT-Hap-I and control C57. We have also shown that plasma aldosterone levels are increased in TG animals containing IC-Hap-1 as compared with WT-Hap-1. Similarly, we have found that TG animals with IC-Hap-1 have decreased sodium and chloride excretion in the urine as compared with WT-Hap-1 animals. These may be important contributing factors for increased BP in IC-Hap-1 TG mice.

In an earlier study, Makhanova *et al.* (43) have generated TG mice ($AS^{hi/hi}$) that have increased expression of the *CYP11B2* gene as a result of replacing the relatively unstable 3′ UTR of its mRNA with the more stable 3′-UTR of bovine growth hormone. (This strategy stabilizes the corresponding mRNA and increases the steady-state level of a gene product in all of the tissues where it is normally expressed but not in other tissues.) Their experiments showed that a modest increase in CYP11B2 expression did not affect blood pressure in animals fed a normal salt diet, but makes their blood pressure sensitive to high salt and to angiotensin-II infusions on an increased salt diet. They also demonstrated that their angiotensin-II/salt–dependent hypertension is accompanied by increased cardiac hypertrophy and oxidative stress. These data, together with their previous finding that a decreased level of CYP11B2 decreases blood pressure in mice on a low-salt diet (44), indicate that genetic differences in CYP11B2 levels in humans are likely to affect how BP responds to changes in dietary salt. Recently, Gu *et al.* (45) have generated transgenic mice where human *CYP11B2* cDNA was attached in front of ~2 kb of the human *CYP11B1* promoter. These authors have shown that plasma aldosterone level was increased about 18-fold in these mice after high-salt treatment (45). However, because these animals do not contain introns of the *CYP11B2* gene and are regulated by the hCYP11B1 promoter, it is possible that *hCYP11B2* gene is not regulated in a physiological manner in these mice. Although, Gu *et al.* showed that BP is increased in their TG mice, BP was measured by the tail-cuff method which is not very accurate. On the other hand, our TG mice have *hCYP11B2* gene with all the introns, exons, and 5′- and 3′-flanking regions and are therefore regulated in a physiological manner and BP was measured by telemetry.

To confirm that transcription factors indeed bind more strongly to the chromatin in the adrenals of TG animals containing IC-Hap-I of the *hCYP11B2* gene as compared with WT-Hap-I, we performed ChIP assay using chromatin from adrenals of TG mice containing either WT or intron conversion sequence (Fig. 6). Results of this experiment show that transcription factors p300, CEBPα, and NFκB bind more strongly to the chromatin obtained from the adrenal glands of TG mice containing IC-Hap-I as compared with WT-Hap-I. Previous microarray analysis has shown that transcription factors C/EBP, NFκB and p300 are expressed in zona glomerulosa of rat and human adrenals (46, 47).

P300 functions as histone acetyltransferase and regulates transcription via chromatin remodeling. It acetylates all four core histones in nucleosomes, and histone acetylation gives an epigenetic tag for transcriptional activation. P300 also functions as a transcriptional co-activator for SMAD4 in the TGFβ signaling pathway, which plays an important role in cardiac and renal fibrosis. Hypertension is accompanied with low-grade inflammation, and NFκB plays an important role in inflammation-induced gene expression. NFκB belongs to a family of master regulatory proinflammatory transcription factors canonically defined by the p50/p65 heterodimers. Following the entry into the nucleus, NFκB binds to DNA cis-regulatory elements at promoters and enhancers and activate proinflammatory transcription. NFκB interacts with transcriptional co-activators including histone acetyltransferases (p300) and histone deacetylases. Recent studies have shown that NFκB is also involved in the formation of super-enhancers and modulates the expression of multiple genes (48). CEBPα is an intron-less transcription factor that contains a basic leucine zipper domain and recognizes the CCAAT motif in the promoters and enhancers of target genes. It modulates the expression of genes involved in cell cycle regulation and body weight homeostasis. Its role in the regulation of genes in liver and adipose tissue is well known. CEBPα family of transcription factors interact with NFκB and modulate the expression of genes involved in inflammation. However, the role of these transcription factors in the regulation of adrenal-specific genes has not been studied.

Previous genome-wide association study/studies (GWAS) studies have shown that a single nucleotide polymorphism (rs62524579), located about 60 kb from the transcriptional start site of the *CYP11B2* gene in its promoter, is associated with high blood pressure (49). Recent studies using genomic DNA from over 1million people have confirmed this observation (50). However, it is not clear whether this is a functional polymorphism or it is in linkage disequilibrium (LD) with some other polymorphism which is the functional polymorphism. Surprisingly, our LD analysis shows that rs62524579 is in complete LD with −344T/C (rs1799998) and with the nucleotide sequence that is present within the intron conversion region in the European population. It will be important to understand the role of rs62524579 in the regulation of blood pressure in different ethnic groups.

In conclusion, intron conversion in intron2 almost always occurs with variant −344T in the promoter of *hCYP11B2* gene and this haplotype is associated with hypertension. We have previously shown that reporter constructs containing −344T have increased promoter activity on transient transfection in human adrenal cells, and transgenic mice containing −344T have increased hCYP11B2 mRNA in adrenals and increased blood pressure as compared with TG animals containing −344C (20). In the present study, we show that intron conversion polymorphism in intron2 of the hCYP11B2 gene is also involved in the regulation of its expression and increases BP in TG animals as compared with WT intron2. Thus, a combinatorial interaction between transcription factors binding to the promoter and intron conversion sequences is involved in transcriptional regulation of *hCYP11B2* gene and in the regulation of blood pressure.

Experimental Procedures

DNA sequence of hCYP11B2, hCYP11B2 intron conversion, and hCYP11B1 gene from the Caucasian population

To understand the role of intron conversion in transcriptional regulation of *hCYP11B2* gene, first we wanted to identify

which nucleotide sequence is replaced with the corresponding *hCYP11B1* during intron conversion. For this purpose, we used genomic DNA from Caucasian hypertensive subjects and identified DNA samples that had either WT intron or IC region. We used two separate PCRs, one to amplify the WT intron2 and another to amplify the IC as described by Davies *et al.* (18). The forward primer for amplifying the WT intron2 was 5′-TGGAGAAAAGCCCTACCCTGT-3′, whereas 5′-CAGAAAATCCCTCCCCCCTA-3′ was used for the intron conversion. The same reverse primer: 5′-AGGAACCTCTGCACGGCC-3′ was used for both the PCRs. After identifying the WT and IC DNA samples, we determined nucleotide sequence from promoter to the end of WT and IC intron2. For this purpose, we used CYP11B2_-192F (5′-CATCCTCAGACCAGCAGGACTTG-3′) as forward primer and CYP11B2_int2R (5′-CTACAGAGGCCAGGGCAGAG-3′) as antisense primer. For amplification of this region of intron2 from hCYP11B1 gene, we used CYP11B1_-180F (5′-CTTTTCCCCTGTCTACGCTCATGC-3′) as forward primer and CYP11B1_int2R (5′-CTACAGAGGCCAGGGCAGAG-3′) as reverse primer. This set of primer is specific in the sense that it amplifies only CYP11B1 and not the *CYP11B2* gene. The full-length intron2 of CYP11B1, CYP11B2, and IC is presented in Fig. S1 and these sequences were used for the generation of Luc constructs and transgenic animals.

Bioinformatics analysis

We mapped the regulatory overlap of the intron conversion region with H3K4me1, H3K4me3, and H3K27ac histone marks, and DNase hypersensitivity sites track using the UCSC genome browser. These data were obtained from the ENCODE (Encyclopedia of DNA Elements), and Roadmap Epigenome projects (35, 36). In silico analysis of transcription factor–binding site predictions were performed by using JASPAR database (RRID: SCR_003030).

In vitro cell culture and transient transfections

The *hCYP11B2* reporter construct containing 2.5-kb promoter having −344T was amplified by PCR of the genomic DNA as described previously (20). XhoI and HindIII sites were added in the forward and reverse primers, respectively, to directionally clone the amplified sequences in the pGL4-basic vector lacking eukaryotic promoter and enhancer sequences (Promega). Full-length WT intron2 was amplified from the BAC clone, and the intron conversion sequence was amplified from the patient samples that are positive for intron conversion. These full-length CYP11B2 WT and intron conversion introns were directionally cloned in front of the 2.5-kb promoter using restriction enzymes Kpn1 and Nhe1 respectively. The generated hCYP11B2-*Luc* constructs were characterized by restriction digestion and sequencing. Expression vector RSV–β-gal was obtained from Promega. CEBPα, p300, and NFκB expression vectors were obtained from Transomic Technologies. Plasmid DNAs for transient transfection were prepared by using the Qiagen Midi Plasmid Kit and transient transfections were performed in H295R cells as described previously (20). Briefly, reporter DNA (1 μg) and β-gal 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 h 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 luciferase activity in a Turners Design 20/20 luminometer using a luciferase assay system (Promega) as described by the manufacturer. Luciferase activity was normalized to β-gal activity that was determined using the β-glo assay system (Promega).

Generation of transgenic mice

We have obtained 11.2-kb DNA encompassing *hCYP11B2* gene from a BAC clone (RP11-304E16) by PCR amplification using 5′-**GCGGCCGC**ATGTCAATGGAAACTGGAAGCTGAAAGGC-3′ and 5′-**GCGGCCGC**GGCTTAGGCAAGGATTTCATGACCGAG-3′ as forward and reverse primers, respectively (20). These primers have Not1 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 DNA was initially cloned into the TOPO XL Cloning vector (Invitrogen) as per the instructions of the manufacturer and sequenced to confirm the integrity of the *hCYP11B2* gene. This clone contains −344T allele in its promoter and WT intron2 and therefore corresponds to WT intron2 of the *hCYP11B2* gene. This construct was named as hCYP11B2-WT-Hap-I. Intron 2 of the *hCYP11B2* gene contains a 1600-bp sequence encompassing intron conversion region which is recognized by unique restriction enzymes FspAI and AccIII. Thus, the hCYP11B2-WT-Hap-I plasmid was treated with restriction enzymes FspAI and AccIII to remove this 1600-bp fragment. We next amplified 4-kb fragment containing intron conversion of the hCYP11B2 gene from the genomic DNA of a patient who had intron conversion. The amplified DNA was treated with restriction enzymes FspAI and AccIII, and 1600-bp DNA fragment was gel purified. This fragment was then ligated in the original linearized plasmid hCYP11B2-WT-Hap-I to give IC-Hap-I. The resulting DNA was sequenced to confirm the authenticity of the intron conversion sequence and integrity of the −344T promoter. The generated plasmid was designated as hCYP11B2-IC-Hap-I. The TopoXL vectors containing hCYP11B2-WT-Hap-I and hCYP11B2-IC-Hap-I were treated with Not1 restriction enzyme and released fragments were subcloned in Not1 restriction site of the pMP8SKB vector (obtained from Dr. Sarah Bronson's laboratory) to produce pMP8phCYP11B2-WT-Hap-I and pMP8phCYP11B2-IC-Hap-I. These plasmids contain a unique Pvu1 site close to the Not1 cloning site so that the cloned DNA can be linearized after Pvu1 digestion for electroporation in BK4 cells. Finally, the pMP8phCYP11B2-WT-Hap-I and pMP8phCYP11B2-IC-Hap-I containing full-length *CYP11B2* genes containing either WT intron2 or intron conversion were linearized with

Pvu1 restriction enzyme and linearized fragments were used for electroporation in BK4 ES cells to generate transgenic mice. After electroporation, ES cells were grown in selective HAT medium and HAT-resistant colonies were isolated and expanded. DNA from different ES cells was amplified using *hCYP11B2* specific primers. The PCR products were analyzed by 0.8% agarose gel electrophoresis and ES cells containing *hCYP11B2* gene were identified. ES cells containing WT-Hap-I and IC-Hap-I of the *hCYP11B2* gene were used to generate transgenic mice as described previously (20). We have confirmed the presence of *hCYP11B2* gene containing intron conversion by PCR amplification of the tail DNAs of these transgenic animals using two sets of *hCYP11B2* gene-specific primers. We also confirmed the authenticity of transgenic animals containing WT-Hap-I and IC-Hap-I of the *hCYP11B2* gene by sequence determination. We have also analyzed *hCYP11B2* gene copy number in our transgenic animals using human- and mouse-specific primers by qPCR as described earlier (20). We initially developed three transgenic lines from each construct but after analysis kept one line each for future studies. Transgenic animals were routinely analyzed by three sets of human *CYP11B2* gene-specific primers. 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 Animal Care and Use Committee at the New York Medical College.

Blood pressure measurement

The mice were fed with either standard mice chow or high-salt diet (4% NaCl) and had access to water *ad libitum*. All experiments were performed following an IACUC-approved protocol in accordance with the NIH Guide for the Care and Use of Laboratory Animals. A radiotelemetric system from Data Science International (St. Paul, MN, USA) was used for this procedure. BP was measured in the conscious state by telemetry as described previously (20). 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 region. The 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 in 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 min using Data-Science instrument. Mean BP values were calculated for every 2 h from the values taken over 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 min by averaging over 10 s and stored on a hard disk. SBP, DBP, MAP, and HR were recorded using DATAQUEST software. For statistical analyses, 6 days of baseline values were used and data were analyzed using one-way analysis of variance for each time point.

Quantitative Real-Time PCR

Adrenals from 2- to 4-month-old male transgenic mice containing either CYP11B2 WT-Hap-I or IC-Hap-I were harvested after CO_2 asphyxiation and snap-frozen in liquid nitrogen, and mRNA level was quantitated by RT-qPCR using human (PPH01239F) and mouse (PPM57638A) *CYP11B2*–specific primers.

In vivo chromatin immunoprecipitation assay

The ChIP assay was performed using CEBPα, P300, and NFκB antibodies and EZ ChIP kit from Millipore as described previously (20).

Immunoblotting

Adrenal tissue from male transgenic mice containing either WT-Hap-I or IC-Hap-I was homogenized and hCYP11B2 protein levels were determined by Western blot analysis as described previously (20).

Aldosterone ELISA

ALPCO aldosterone ELISA kit was used to assay serum and urine aldosterone levels as per manufacturer's protocol.

Metabolic cage experiments

Mice were acclimatized in metabolic cages for 5 days and were given free access to food and water. Daily food and water intake, body weight, and urine volume were determined and sodium chloride and potassium were measured in urine using a SmartLyte ISE analyzer (Diamond Diagnostics). Urine osmolality was determined using a Precision Systems micro-osmometer.

Statistical analysis

The nonparametric tests were performed using the Mann–Whitney U test for two groups and Kruskal Wallis test for three groups Mixed effects regression model was used to test for differences in SBP, DBP, MAP, and HR between the groups. Statistical analyses were carried out using SAS, R, GraphPad Prism. $p < 0.05$ was considered statistically significant.

Data Availability

The data used in the current study are available in this article.

Acknowledgments—Transgenic animals were made at Dartmouth Medical Center. We acknowledge the help of the Dartmouth Mouse Modeling Shared Resource which receives support from the Norris Cotton Cancer Center shared grant P30CA023108.

Author contributions—B. M. and A. K. conceptualization; B. M., I. S., S. H., N. R. F., and A. K. data curation; B. M. and I. S. software; B. M., I. S., and A. K. formal analysis; B. M., I. S., S. H., N. R. F., and A. K. validation; B. M., I. S., S. H., N. R. F., and A. K. investigation; B. M., I. S., S. H., N. R. F., and A. K. visualization; B. M., I. S., and A. K. methodology; B. M., I. S., and A. K. writing-original draft;

B. M. and A. K. project administration; B. M., I. S., and A. K. writing-review and editing; A. K. resources; A. K. supervision; A. K. funding acquisition.

Funding and additional information—This work was supported by NHLBI, National Institutes of Health Grants RO1 HL 122742 and RO1 HL 130344 (to A. K.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: Hap, haplotype; TG, transgenic; IC, intron conversion; ES cell, embryonic stem cell; qPCR, quantitative PCR; BP, blood pressure; SBP, systolic BP; DBP, diastolic BP; MAP, mean arterial pressure; HR, heart rate; LD, linkage disequilibrium.

References

1. Galis, Z. S., Thrasher, T., Reid, D. M., Stanley, D. V., and Oh, Y. S. (2013) Investing in high blood pressure research: A national institutes of health perspective. *Hypertension* **61,** 757–761 CrossRef Medline
2. Lawes, C. M., Vander, H. S., and Rodgers, A, International Society of Hypertension. (2008) Global burden of blood-pressure-related disease, 2001. *Lancet* **371,** 1513–1518 CrossRef Medline
3. Luft, F. C. (1998) Molecular genetics of human hypertension. *J. Hypertens.* **16,** 1871–1878 CrossRef Medline
4. Luft, F. C., Mervaala, E., Müller, D. N., Gross, V., Schmidt, F., Park, J. K., Schmitz, C., Lippoldt, A., Breu, V., Dechend, R., Dragun, D., Schneider, W., Ganten, D., and Haller, H. (1999) Hypertension-induced end-organ damage: A new transgenic approach to an old problem. *Hypertension* **33,** 212–218 CrossRef Medline
5. Griendling, K. K., Murphy, T. J., and Alexander, R. W. (1993) Molecular biology of the renin-angiotensin system. *Circulation* **87,** 1816–1828 CrossRef Medline
6. Stachenko, J., and Giroud, C. J. (1959) Functional zonation of the adrenal cortex: Pathways of corticosteroid biogenesis. *Endocrinology* **64,** 730–742 CrossRef Medline
7. Young, W. F. (2007) Primary aldosteronism: Renaissance of a syndrome. *Clin. Endocrinol. (Oxf)* **66,** 607–618 CrossRef Medline
8. Rossi, G. P., Bernini, G., Caliumi, C., Desideri, G., Fabris, B., Ferri, C., Ganzaroli, C., Giacchetti, G., Letizia, C., Maccario, M., Mallamaci, F., Mannelli, M., Mattarello, M. J., Moretti, A., Palumbo, G., *et al.* (2006) A prospective study of the prevalence of primary aldosteronism in 1,125 hypertensive patients. *J. Am. Coll. Cardiol.* **48,** 2293–2300 CrossRef Medline
9. Mulatero, P., Stowasser, M., Loh, K. C., Fardella, C. E., Gordon, R. D., Mosso, L., Gomez-Sanchez, C. E., Veglio, F., and Young, W. F., Jr. (2004) Increased diagnosis of primary aldosteronism, including surgically correctable forms, in centers from five continents. *J. Clin. Endocrinol. Metab.* **89,** 1045–1050 CrossRef Medline
10. Calhoun, D. A., Nishizaka, M. K., Zaman, M. A., Thakkar, R. B., and Weissmann, P. (2002) Hyperaldosteronism among black and white subjects with resistant hypertension. *Hypertension* **40,** 892–896 CrossRef Medline
11. Milliez, P., Girerd, X., Plouin, P. F., Blacher, J., Safar, M. E., and Mourad, J. J. (2005) Evidence for an increased rate of cardiovascular events in patients with primary aldosteronism. *J. Am. Coll. Cardiol.* **45,** 1243–1248 CrossRef Medline
12. Kawamoto, T., Mitsuuchi, Y., Toda, K., Yokoyama, Y., Miyahara, K., Miura, S., Ohnishi, T., Ichikawa, Y., Nakao, K., and Imura, H. (1992) Role of steroid 11 beta-hydroxylase and steroid 18-hydroxylase in the biosynthesis of glucocorticoids and mineralocorticoids in humans. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 1458–1462 CrossRef Medline
13. Davies, E., and Kenyon, C. J. (2003) CYP11B2 polymorphisms and cardiovascular risk factors. *J. Hypertens.* **21,** 1249–1253 CrossRef Medline
14. Mornet, E., Dupont, J., Vitek, A., and White, P. C. (1989) Characterization of two genes encoding human steroid 11 β-hydroxylase (P-450$_{11\beta}$). *J. Biol. Chem.* **264,** 20961–20967 Medline
15. Chua, S. C., Szabo, P., Vitek, A., Grzeschik, K. H., John, M., and White, P. C. (1987) Cloning of cDNA encoding steroid 11 beta-hydroxylase (P450c11). *Proc. Natl. Acad. Sci. U. S. A.* **84,** 7193–7197 CrossRef Medline
16. Wagner, M. J., Ge, Y., Siciliano, M., and Wells, D. E. (1991) A hybrid cell mapping panel for regional localization of probes to human chromosome 8. *Genomics* **10,** 114–125 CrossRef Medline
17. Casiglia, E., Tikhonoff, V., Mazza, A., Rynkiewicz, A., Limon, J., Caffi, S., Guglielmi, F., Martini, B., Basso, G., Winnicki, M., Pessina, A. C., and Somers, V. K. (2005) C-344T polymorphism of the aldosterone synthase gene and blood pressure in the elderly: A population-based study. *J. Hypertens.* **23,** 1991–1996 CrossRef Medline
18. Davies, E., Holloway, C. D., Ingram, M. C., Inglis, G. C., Friel, E. C., Morrison, C., Anderson, N. H., Fraser, R., and Connell, J. M. (1999) Aldosterone excretion rate and blood pressure in essential hypertension are related to polymorphic differences in the aldosterone synthase gene CYP11B2. *Hypertension* **33,** 703–707 CrossRef Medline
19. Iwai, N., Kajimoto, K., Tomoike, H., and Takashima, N. (2007) Polymorphism of CYP11B2 determines salt sensitivity in Japanese. *Hypertension* **49,** 825–831 CrossRef Medline
20. Mopidevi, B., Kaw, M. K., Puri, N., Ponnala, M., Jain, S., Rana, A., Keetha, N. R., Khuder, S. A., Fiering, S. N., and Kumar, A. (2015) Variable transcriptional regulation of the human aldosterone synthase gene causes salt-dependent high blood pressure in transgenic mice. *Circ. Cardiovasc. Genet.* **8,** 30–39 CrossRef Medline
21. Munshi, A., Sharma, V., Kaul, S., Rajeshwar, K., Babu, M. S., Shafi, G., Anila, A. N., Balakrishna, N., Alladi, S., and Jyothy, A. (2010) Association of the −344C/T aldosterone synthase (CYP11B2) gene variant with hypertension and stroke. *J. Neurol. Sci.* **296,** 34–38 CrossRef Medline
22. Rajan, S., Ramu, P., Umamaheswaran, G., and Adithan, C. (2010) Association of aldosterone synthase (CYP11B2 C-344T) gene polymorphism & susceptibility to essential hypertension in a south Indian Tamil population. *Indian J Med. Res.* **132,** 379–385 Medline
23. Russo, P., Siani, A., Venezia, A., Iacone, R., Russo, O., Barba, G., D'Elia, L., Cappuccio, F. P., and Strazzullo, P. (2002) Interaction between the C(-344) T polymorphism of CYP11B2 and age in the regulation of blood pressure and plasma aldosterone levels: Cross-sectional and longitudinal findings of the Olivetti Prospective Heart Study. *J. Hypertens.* **20,** 1785–1792 CrossRef Medline
24. Sookoian, S., Gianotti, T. F., González, C. D., and Pirola, C. J. (2007) Association of the C-344T aldosterone synthase gene variant with essential hypertension: A meta-analysis. *J. Hypertens.* **25,** 5–13 CrossRef Medline
25. White, P. C., and Slutsker, L. (1995) Haplotype analysis of CYP11B2. *Endocr. Res.* **21,** 437–442 CrossRef Medline
26. Brand, E., Chatelain, N., Mulatero, P., Féry, I., Curnow, K., Jeunemaitre, X., Corvol, P., Pascoe, L., and Soubrier, F. (1998) Structural analysis and evaluation of the aldosterone synthase gene in hypertension. *Hypertension* **32,** 198–204 CrossRef Medline
27. Castellano, M., Glorioso, N., Cusi, D., Sarzani, R., Fabris, B., Opocher, G., Zoccali, C., Golin, R., Veglio, F., Volpe, M., Mantero, F., Fallo, F., Rossi, G. P., Barlassina, C., Tizzoni, L., *et al.* (2003) Genetic polymorphism of the renin-angiotensin-aldosterone system and arterial hypertension in the Italian population: The GENIPER Project. *J. Hypertens.* **21,** 1853–1860 CrossRef Medline
28. Henderson, S. O., Haiman, C. A., and Mack, W. (2004) Multiple polymorphisms in the renin-angiotensin-aldosterone system (ACE, CYP11B2, AGTR1) and their contribution to hypertension in African Americans and Latinos in the multiethnic cohort. *Am. J. Med. Sci.* **328,** 266–273 CrossRef Medline
29. Nicod, J., Bruhin, D., Auer, L., Vogt, B., Frey, F. J., and Ferrari, P. (2003) A biallelic gene polymorphism of CYP11B2 predicts increased aldosterone to renin ratio in selected hypertensive patients. *J. Clin. Endocrinol. Metab.* **88,** 2495–2500 CrossRef Medline

30. Paillard, F., Chansel, D., Brand, E., Benetos, A., Thomas, F., Czekalski, S., Ardaillou, R., and Soubrier, F. (1999) Genotype-phenotype relationships for the renin-angiotensin-aldosterone system in a normal population. *Hypertension* **34,** 423–429 CrossRef Medline
31. Hautanena, A., Lankinen, L., Kupari, M., Jänne, O. A., Adlercreutz, H., Nikkilä, H., and White, P. C. (1998) Associations between aldosterone synthase gene polymorphism and the adrenocortical function in males. *J. Intern. Med.* **244,** 11–18 CrossRef Medline
32. Staessen, J. A., Li, Y., and Thijs, L. (2007) Meta-analysis of blood pressure and the CYP11B2 polymorphism highlights the need for better designed studies. *J. Hypertens.* **25,** 37–39 CrossRef Medline
33. Alvarez-Madrazo, S., Mackenzie, S. M., Davies, E., Fraser, R., Lee, W. K., Brown, M., Caulfield, M. J., Dominiczak, A. F., Farrall, M., Lathrop, M., Hedner, T., Melander, O., Munroe, P. B., Samani, N., Stewart, P. M., *et al.* (2013) Common polymorphisms in the CYP11B1 and CYP11B2 genes: Evidence for a digenic influence on hypertension. *Hypertension* **61,** 232–239 CrossRef Medline
34. Tiago, A. D., Badenhorst, D., Nkeh, B., Candy, G. P., Brooksbank, R., Sareli, P., Libhaber, E., Samani, N. J., Woodiwiss, A. J., and Norton, G. R. (2003) Impact of renin-angiotensin-aldosterone system gene variants on the severity of hypertension in patients with newly diagnosed hypertension. *Am. J. Hypertens.* **16,** 1006–1010 CrossRef Medline
35. Bernstein, B. E., Stamatoyannopoulos, J. A., Costello, J. F., Ren, B., Milosavljevic, A., Meissner, A., Kellis, M., Marra, M. A., Beaudet, A. L., Ecker, J. R., Farnham, P. J., Hirst, M., Lander, E. S., Mikkelsen, T. S., and Thomson, J. A. (2010) The NIH Roadmap Epigenomics Mapping Consortium. *Nat. Biotechnol.* **28,** 1045–1048 CrossRef Medline
36. ENCODE Project Consortium. (2012) An integrated encyclopedia of DNA elements in the human genome. *Nature* **489,** 57–74 CrossRef Medline
37. Bronson, S. K., Plaehn, E. G., Kluckman, K. D., Hagaman, J. R., Maeda, N., and Smithies, O. (1996) Single-copy transgenic mice with chosen-site integration. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 9067–9072 CrossRef Medline
38. Butz, G. M., and Davisson, R. L. (2001) Long-term telemetric measurement of cardiovascular parameters in awake mice: A physiological genomics tool. *Physiol. Genomics* **5,** 89–97 CrossRef Medline
39. Jain, S., Vinukonda, G., Fiering, S. N., and Kumar, A. (2008) A haplotype of human angiotensinogen gene containing -217A increases blood pressure in transgenic mice compared with -217G. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295,** R1849–R1857 CrossRef Medline
40. Jain, S., Tillinger, A., Mopidevi, B., Pandey, V. G., Chauhan, C. K., Fiering, S. N., Warming, S., and Kumar, A. (2010) Transgenic mice with -6A haplotype of the human angiotensinogen gene have increased blood pressure compared with -6G haplotype. *J. Biol. Chem.* **285,** 41172–41186 CrossRef Medline
41. Cvetkovic, B., Yang, B., Williamson, R. A., and Sigmund, C. D. (2000) Appropriate tissue- and cell-specific expression of a single copy human angiotensinogen transgene specifically targeted upstream of the HPRT locus by homologous recombination. *J. Biol. Chem.* **275,** 1073–1078 CrossRef Medline
42. Cvetkovic, B., Keen, H. L., Zhang, X., Davis, D., Yang, B., and Sigmund, C. D. (2002) Physiological significance of two common haplotypes of human angiotensinogen using gene targeting in the mouse. *Physiol. Genomics* **11,** 253–262 CrossRef Medline
43. Makhanova, N., Hagaman, J., Kim, H. S., and Smithies, O. (2008) Salt-sensitive blood pressure in mice with increased expression of aldosterone synthase. *Hypertension* **51,** 134–140 CrossRef Medline
44. Makhanova, N., Sequeira-Lopez, M. L., Gomez, R. A., Kim, H. S., and Smithies, O. (2006) Disturbed homeostasis in sodium-restricted mice heterozygous and homozygous for aldosterone synthase gene disruption. *Hypertension* **48,** 1151–1159 CrossRef Medline
45. Gu, H., Ma, Z., Wang, J., Zhu, T., Du, N., Shatara, A., Yi, X., Kowala, M. C., and Du, Y. (2017) Salt-dependent blood pressure in human aldosterone synthase-transgenic mice. *Sci. Rep.* **7,** 492 CrossRef Medline
46. Romero, D. G., Plonczynski, M. W., Welsh, B. L., Gomez-Sanchez, C. E., Zhou, M. Y., and Gomez-Sanchez, E. P. (2007) Gene expression profile in rat adrenal zona glomerulosa cells stimulated with aldosterone secretagogues. *Physiol. Genomics* **32,** 117–127 CrossRef Medline
47. Zhou, J., Shaikh, L. H., Neogi, S. G., McFarlane, I., Zhao, W., Figg, N., Brighton, C. A., Maniero, C., Teo, A. E., Azizan, E. A., and Brown, M. J. (2015) DACH1, a zona glomerulosa selective gene in the human adrenal, activates transforming growth factor-β signaling and suppresses aldosterone secretion. *Hypertension* **65,** 1103–1110 CrossRef Medline
48. Brown, J. D., Lin, C. Y., Duan, Q., Griffin, G., Federation, A., Paranal, R. M., Bair, S., Newton, G., Lichtman, A., Kung, A., Yang, T., Wang, H., Luscinskas, F. W., Croce, K., Bradner, J. E., *et al.* (2014) NF-κB directs dynamic super enhancer formation in inflammation and atherogenesis. *Mol. Cell* **56,** 219–231 CrossRef Medline
49. Warren, H. R., Evangelou, E., Cabrera, C. P., Gao, H., Ren, M., Mifsud, B., Ntalla, I., Surendran, P., Liu, C., Cook, J. P., Kraja, A. T., Drenos, F., Loh, M., Verweij, N., Marten, J., *et al.* (2017) Genome-wide association analysis identifies novel blood pressure loci and offers biological insights into cardiovascular risk. *Nat. Genet.* **49,** 403–415 CrossRef Medline
50. Evangelou, E., Warren, H. R., Mosen-Ansorena, D., Mifsud, B., Pazoki, R., Gao, H., Ntritsos, G., Dimou, N., Cabrera, C. P., Karaman, I., Ng, F. L., Evangelou, M., Witkowska, K., Tzanis, E., Hellwege, J. N., *et al.* (2018) Genetic analysis of over 1 million people identifies 535 new loci associated with blood pressure traits. *Nat. Genet.* **50,** 1412–1425 CrossRef Medline