2 Angiotensinogen Gene Polymorphisms and Hypertension

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SUMMARY

Hypertension is a serious risk factor for myocardial infarction, heart failure, vascular disease, stroke, and renal failure. Hypertension affects 50 million Americans with a prevalence rate of 25–30% in the adult Caucasian population. The incidence of hypertension and complications resulting from hypertension are even greater in the African-American population. The renin-angiotensin system plays an important role in the regulation of blood pressure, and previous studies have suggested that the angiotensinogen (*AGT*) gene locus is linked with human essential hypertension. Previous studies have suggested that a single nucleotide polymorphism that converts methionine to threonine at amino acid 235 is associated with hypertension in Caucasian population. This polymorphism is in linkage disequilibrium with A/G polymorphism at –6 position in the promoter of *AGT* gene. Reporter constructs containing variant A at –6 have increased promoter activity on transient transfection in human liver cells, suggesting that this variant may have increased transcriptional activity. However, this polymorphism is not associated with hypertension in the African-American and Chinese populations. We have found an A/G polymorphism at –217 of the human *AGT* gene promoter and have shown that frequency of allele A at –217 is significantly increased in the DNA of African-American hypertensive patients. We have also shown that: (a) reporter constructs containing *AG* gene promoter with nucleoside A at –217 have increased promoter activity on transient transfection; and (b) the C/EBP family of transcription factors and glucocorticoid receptor (GR) bind preferentially to this region of the promoter when nucleoside A is present at –217. In addition, variant –217A is always present with variants –532T, –793A, and –1074T in the human *AGT* gene promoter. We have also shown that liver enriched transcription factor HNF-3β binds more strongly when nucleoside T is present at –1074. Previous studies have shown that HNF-3β interacts with GR and plays an important role in liver-specific gene expression. These data suggest that *AGT* haplotype containing –217A, –532T, –793A, and –1074T may be involved in increased expression of this gene, and may play a role in human hypertension. It will be important to confirm this observation in future human studies and to understand the role of this haplotype in transcriptional regulation using transgenic animals.

Key Words: Human essential hypertension; molecular genetics; angiotensinogen gene; transcription factors; single nucleotide polymorphism (SNP); haplotype; C/EBP; HNF-3.

INTRODUCTION

Hypertension is a serious risk factor for myocardial infarction, heart failure, vascular disease, stroke, and renal failure. It is estimated that hypertension affects 50 million Americans with a prevalence rate of 25–30% in the adult Caucasian population. In the United States, the prevalence of hypertension is 50% greater in blacks than in whites. In blacks, hypertension appears earlier, is generally more severe, and results in high rates of morbidity and mortality from stroke, heart failure, left ventricular hypertrophy, and end-stage renal disease. Both genetic and environmental factors may contribute to the higher prevalence of hypertension in blacks. In addition, a genetic contribution to the increased prevalence of renal disease among hypertensive blacks has been proposed on the basis of family studies and studies of associated histocompatibility antigens. Hypertension is a polygenic disease, and it has been estimated by segregation analysis and twin

studies that approx 45% of the interindividual differences in blood pressure (BP) can be accounted by genetic differences *(1)*. In the past two decades, many genes that were implicated in simple (Mendelian) diseases have been identified using genetic linkage and positional cloning methods *(2)*. Although these methods have been remarkably successful in identifying high relative risk genes, they have not been successful in identifying genes that are involved in the complex forms of disease such as hypertension and diabetes type II. This failure is the result of three main features of complex diseases. First, such diseases typically vary in severity of symptoms and age of onset, which results in difficulty in defining an appropriate phenotype and selecting the best population to study. Second, they can vary in the etiological mechanisms, which might involve various biological pathways. Third, and perhaps most importantly, complex diseases are more likely to be caused by several, and even numerous genes, each with a small overall contribution and relative risk. In addition, hypertension is an arbitrary definition and not a quantitative trait that appears relatively late in life. Nothing is known about the number of genes involved, their mode of transmission, their quantitative effect on BP, their interaction with other genes, or their modulation by environmental factors. Parameters such as ethnicity and body weight increase the genetic heterogeneity and the difficulty of replication from one study to another.

GENOME-WIDE ANALYSIS TO IDENTIFY HYPERTENSION-RELATED GENES

Krushkal et al. *(3)* performed a genome-wide linkage analysis of systolic BP on 427 sibling pairs and identified four regions of the human genome that show statistical significant linkage. These regions are on chromosomes 2, 5, 6, and 15. These chromosomal regions include numerous potential candidates, such as phospholamban, estrogen receptor (ER), aminopeptidase *N*, α 1B-adrenergic receptor, dopamine receptor type 1A (DR-1A), calmodulin, and sodium-calcium exchanger. However, none of these genes has been confirmed to be associated with human hypertension.

Hunt et al. *(4)* performed a genome-wide analysis using 2959 individuals in 500 white families from the National Heart, Lung and Blood Institute (NHLBI) family heart study and identified five regions of the genome with logarithm of odds (LOD) scores higher than 2.0 for hypertension. These included chromosomes 1, 7, 12, and 15. On the other hand, chromosome 6 showed the best evidence for linkage with systolic BP (SBP). It is important to note that angiotensinogen and renin genes are located on chromosome 1.

Kristjansson et al. *(5)* performed a genome-wide scan with 904 microsatellite markers using 120 extended Icelandic families with 490 hypertensive patients. After adding 5 markers, they found linkage to chromosome 18q with an allele-sharing LOD score of 4.60. These results provide evidence for a novel susceptibility gene for essential hypertension on chromosome 18q, and show that it is possible to study the genetics of essential hypertension without stratifying by subphenotypes. Although melanocortin receptor 4 is located in this region of the chromosome, it is not known whether this gene is involved in hypertension.

Rice et al. *(6)* performed a genome-wide scan on 317 black individuals from 114 families and 519 white individuals from 99 families using a multipoint variance-components linkage model and a panel of 509 markers. Promising results were primarily, but not exclusively, found in the black families. Linkage evidence ($p < 0.0023$) with baseline BP replicated other studies within a 1-LOD interval on 2p, 3p.3 and 12q.33.

Levy et al. *(7)* performed a genome-wide scan in the largest families from two generations of Framingham Heart study participants. Genotyping was performed on 1702 subjects from 332 large families, and BP data were available for 1585 (93%) genotyped subjects who contributed 12,588 longitudinal BP observations. For diastolic BP, LOD scores greater than 2.0 were identified on chromosome 17 (74cM, LOD 2.1) and chromosome 18 (7cM, LOD 2.1). Using a genome-wide scan, they found strong evidence for a BP quantitative trait locus on chromosome 17. The stroke-prone, spontaneously hypertensive rat and the Dahl salt-sensitive, hypertensive rat proved to be useful tools in identifying quantitative trait loci (QTL) that contribute to BP variations. Evidence of BP QTL was found on rat chromosomes 2, 10, and X. Human chromosome 17 is syntenic with rat chromosome 10, and the rat chromosome 10QTL is located near the angiotensin-converting enzyme (ACE) locus. The ACE locus is an attractive candidate gene because of its role in the renin-angtiotensin system (RAS) and the association between several polymorphisms in the ACE locus with BP levels in some studies, although other studies failed to confirm this finding. Other potential candidate genes on this segment of chromosome 17 include the Cl-HCO exchanger, phenylethanolamine *N*-methyltransferase, nerve growth factor receptor, and pseudohypoaldosteronism type IIB genes.

Caulfield et al. *(8)* recently phenotyped 2010 affected sibling pairs drawn from 1599 severely hypertensive families in a large white European population (BRIGHT study). Linkage analysis identified a principle locus on chromosome 6q with an LOD score of 3.21, which attained genome-wide significance ($p = 0.042$). The inclusion of three further loci with LOD scores higher than 1.57 (2q, 5q, and 9q) also show genome-wide significance ($p = 0.017$) when assessed under a locus-counting analysis. There is a region of overlap with other studies on chromosome 2 (140–170 cM). This region was linked to hypertension in Chinese sibling pairs and Finnish twins, and linkage is suggested in a discordant sibling-pair screen. In addition, linkage signals are located proximally to the BRIGHT locus on chromosome 2p, documented by two genome scans in a genome scan of families of African ancestry. Furthermore, this region has also been linked to the hypertension-associated phenotype preeclampsia in some genome-wide screens. Preliminary analysis of each region with an LOD score higher than 1.57 has identified a small number of good candidates that map to the chromosome 2 and 9 regions of interest. These candidates include the genes encoding serine-threonine kinases (STK39, STK17B, chromosome 2q), a protein kinase (PKNBETA on 9q), G protein-coupled receptors (GPR21 on 9q33, GPR107 9q, and GPR1 on 2q33) and a potassium channel (KCNJ3 on 2q24.1). Several BP QTL from hypertensive rat models exhibit homology with the BRIGHT regions of interest. For instance, rat 3q11–q23 is homologous to the human 9q region, rat 2q14–q16 with the human chromosome 5p region, and rat chromosome 9q22–q34 is homologous to the human 2q locus. Examination of databases reveals many known genes and expressed sequence tags in or near the 16cM QTL interval at 17q12–21. Although no genes that have been strongly implicated in BP variation lie in this interval, potential candidate genes in the interval include the α-1 thyroid receptors, the neuronal homolog of the amiloride sensitive epithelial sodium channel, the corticotropin-releasing hormone receptor 1, insulin-like growth factor-binding protein-4, hepatocyte nuclear factor (HNF) 1-β, and the chloride/bicarbonate exchanger AE1. In addition to the chromosome 17q12–21 interval, there were two additional regions yielding LOD scores over 2.0 in multipoint analyses. One of these lies just distal on chromosome 17, and it is possible that this locus is independent of their largest peak. This second chromosome 17 peak overlaps the locus

encoding the ACE locus, a much-studied candidate gene for hypertension. The final locus that has not been previously implicated in BP variation is on chromosome 18. An interesting candidate gene in the chromosome 18 interval is the melanocortin receptor 2, which is the physiological receptor for corticotropin. Given the known effects of glucocorticoids on BP, it is of interest that receptors involved in the regulation of cortisol secretion lie in both the chromosome 17 and chromosome 18 intervals.

From this brief review it is clear that although different regions of the chromosome have been shown to be involved in the regulation of BP, these studies are often nonreproducible and genes involved in hypertension remain to be identified.

ROLE OF AGT IN HYPERTENSION

The RAS plays an important role in the regulation of BP. The octapeptide, angiotensin II (Ang II), is one of the most active vasopressor agents and is obtained by the proteolytic cleavage of a larger precursor molecule, angiotensinogen (AGT), which is primarily synthesized in the liver and to a lesser extent in the kidney, brain, heart, adrenal, fat, and vascular walls. The human AGT cDNA is 1455 nucleotides long and codes for a 485-amino acid protein *(9)*. AGT is first converted by renin to produce a decapeptide, Ang I, and is then converted to Ang II by the removal of a C-terminal dipeptide by ACE *(10)*. In experimental as well as clinical studies, chronic administration of renin-angiotensin inhibitors has proven effective in lowering BP in hypertension. Genes that encode components of the RAS are therefore potential candidate genes that may play a role in the regulation of BP.

The plasma concentration of AGT is close to the Michaelis constant of the enzymatic reaction between renin and AGT *(11)*. For this reason, a rise in plasma AGT levels can lead to a parallel increase in the formation of Ang II that may ultimately result in hypertension. In fact, recent studies have suggested a direct correlation between AGT and BP. These studies include a highly significant relationship between plasma concentration of AGT and BP in human subjects *(4)*; higher plasma AGT levels in hypertensive subjects and in offspring of hypertensive parents compared with normotensives *(12,13)*; expression of *AGT* gene in multiple tissues—such as brain, kidney, heart, adrenals, placenta, and vascular walls—that are directly involved in BP regulation *(14)*; elevation of BP in transgenic animals that overexpress *AGT* gene *(7–9)*; and reduction of BP in *AGT* gene knockout mice *(15)*. In addition, Kim et al. have introduced up to four copies of the *AGT* gene in mice, with each copy of the gene resulting in a successive increase in BP *(16)*. These results directly demonstrated that small increases in plasma AGT level can quantitatively influence the fine control of renal vascular resistance and increase BP in a gene dose-dependent manner. A similar *ACE* gene duplication in mice led to an increase in plasma ACE level but no increase in BP *(17)*, which supports the importance of the *AGT* gene in human hypertension.

M235T POLYMORPHISM OF *AGT* GENE AND HYPERTENSION

The *AGT* gene contains five exons and four introns, which span 13 kb *(18)*. An extensive study of the potential role of the *AGT* gene in human essential hypertension was performed on two large series of hypertensive siblingships yielding 379 sibling pairs. The highly polymorphic CA dinucleotide repeat marker located in the 3'-region of the *AGT* gene and the powerful affected sibling pair methodology were used to obtain evidence

of a genetic linkage between the *AGT* gene and hypertension in this study *(19)*. A 17% excess of AGT allele sharing was found in severely hypertensive sibling pairs. Whereas significant linkage was obtained in male pairs in both the Utah and Paris groups, no excess of shared AGT alleles was observed in female subjects, suggesting the influence of an epistatic hormonal phenomenon. These studies also showed that variants 235T and 174M of the *AGT* gene are associated with hypertension. From these studies, it was estimated that mutations at the AGT locus might be a predisposing factor in at least 3–6% of hypertensive individuals younger than 60 yr of age in the Caucasian population. When the same methodology was used to analyze the same hypertensive sibling pairs from Utah or Paris, it showed no linkage with other genes of the renin system: namely renin *(20)*, ACE *(21)*, or Ang II type I receptor *(22)*.

Two other linkage studies also indicate a relationship between the AGT locus and hypertension. This linkage was observed in the subgroup of patients with diastolic pressure above 100 mmHg, but there was no difference among female–female pairs. However, there was no association between hypertension and the 235T AGT variant in this population *(23)*. The same group also found linkage and association of the AGT locus with high BP in 63 affected sibling pairs of African-Caribbean origin, suggesting some similarity in the genetic basis of essential hypertension in populations of different ethnicity *(24)*. The corroboration of these linkage studies indicate that molecular variants of the *AGT* gene, such as M235T, or those in linkage disequilibrium with this variant are inherited predispositions to essential hypertension in humans.

However, results of these studies must be interpreted with caution for several reasons. First, the frequency of the 235T AGT allele varies in different ethnic groups. The 235T allele is more frequent in the Asian than in the Caucasian population and is by far the predominant allele in the African population. As a consequence, positive results may arise from population admixture, and negative results in populations in which this allele is predominant may result from the constraint of limited statistical power. Second, differences in the design of each study and the choice of the control group make any overall comparison difficult. Finally, most of the results reported to date have been obtained with relatively small numbers of patients, which can also generate false negative or false positive results.

There are divergent results on the association between variants in AGT at position 235 (235T) or 174 (174M) and hypertension in Caucasians. In their original study, Jeunemaitre et al. found that the M235T variant was more frequent in hypertensive probands from Utah and Paris, especially in the more severe index cases *(19)*. A subsequent study by the same group on 136 mild to moderate hypertensive subjects also found that the frequency of the 235T allele was increased, although the increase was significant only for patients with a family history of hypertension. Schmidt also found a higher frequency of the 235T allele in subjects with hypertension, a family history of the disease, and early onset of hypertension *(25)*.

However, other studies have not found any association between the 235T variant and hypertension. Hingorani et al. found no difference in the frequency of M235T in 223 hypertensive subjects and 187 normotensive individuals in Anglia in the United Kingdom *(26)*. A more powerful study performed in Finland by Kiema gave negative results with 508 mild hypertensives and 523 population-based controls *(27)*. On the other hand, four large studies recently showed a positive association between the M235T allele and essential hypertension. An association with severe hypertension with stronger relation-

ship for men than for women was found in a sample from the Framingham Heart study and from the Atherosclerosis Risk in Community (ARIC) study, when the effect of body mass index and triglycerides were taken into account *(28)*. The proportions of cases attributable to the 235 allele were 8% in the ARIC population and 20% in the Framingham population. In a study testing a large number of AGT alleles, the frequency of the 235T allele was 0.47 in the 477 probands of hypertensive families and 0.38 in the 364 Caucasian controls *(29)*. In a cross-section sample of 634 middle-aged subjects from the MONIC Angsburg cohort, Schunkert found that individuals carrying at least one copy of the T235 allele had high SBP and diastolic BP(DBP)s and were more likely to use antihypertensive drugs *(30)*. Finally, another case-control study involving 802 hypertensive subjects and 658 Caucasian controls has shown a significant increase in the frequency of the T235 allele in men and in women whose hypertension was diagnosed before they were 45 yr of age *(31)*. These studies emphasize the importance of sample size testing for susceptibility locus in a complex disease such as hypertension *(32)*.

The frequency of the T235 allele is invariably high in the Japanese population, ranging from 0.65 to 0.75. An association between hypertension and molecular variants of AGT in the Japanese population has been found in several independent studies *(33–35)*. All of the studies reported so far indicate that frequency of the 235T variant is higher in Japanese hypertensive subjects. A more homogeneous genetic and environmental background may explain why the results reported for Japanese populations are more uniform. However, Ishigami et al. *(36)* showed that the –20C variant of the human *AGT* gene plays an important role in hypertension in Japanese population.

Walker et al. showed a remarkably high correlation between plasma AGT concentration and elevated BP ($p < 0.0001$) in a large study involving 574 black subjects *(4)*. Bloem et al. *(37)* showed that (1) plasma AGT level is about 19% higher in black children as compared with white children; and (2) BP is normally higher and increases faster over time in black children as compared with white children. Caulfield et al. *(24)* found an association between the *AGT* gene locus and high BP in 63 affected sibling pairs of African-Carribean origin using CA dinucleotide marker. However, these workers could not find an association between variants M235T and hypertension in the African-American population. Various recent studies have suggested that although the frequency of 235T allele is increased in the African-American population (0.8–0.9), there is no association between 235T allele and hypertension in this population *(38)*.

LINKAGE DISEQUILIBRIUM BETWEEN 235T AND –6A IN *AGT* GENE

Because amino acid 235 is located far away from the renin cleavage site, this polymorphism does not explain the molecular mechanism involved in increased plasma AGT levels by the 235T variant. The human *AGT* gene has an A/G polymorphism at nucleoside –6. It has been shown that nucleoside A is present at –6 in the ancestral *AGT* gene and –6G is a neomorph. Sequence analysis of the human *AGT* gene has shown that molecular variants 235T and –6A are in complete association *(39)*. In addition, Inoue et al. synthesized reporter constructs containing 256 and 70 basepairs (bp) of the 5'-flanking region of the human *AGT* gene containing either nucleoside A or G at –6 position. Transient transfection of these reporter constructs in human liver cells (HepG2) have shown that reporter constructs containing human *AGT* gene promoter with nucleoside A at –6 have increased promoter activity compared to nucleoside G at –6. These experiments sug-

gested that increased plasma AGT levels by the 235T variant in hypertensive patients may actually be a result of increased transcriptional activity of the human *AGT* gene by nucleoside A at –6. However, the molecular mechanism involved in increased transcription of the human *AGT* gene by nucleoside A at –6 as compared with nucleoside G at –6 is not known, and transcription factors that may be involved in this process have not been identified. In addition, as discussed later, experiments using transgenic mice have shown that this polymorphism neither alters the expression of the *hAGT* gene nor increases BP in transgenic mice *(40)*.

ASSOCIATION OF THE –217A VARIANT OF *AGT* GENE WITH HYPERTENSION

Because hypertension is more common in African-American subjects, our laboratory is interested in understanding molecular mechanisms involved in hypertension in the African-American population. We have therefore analyzed 186 African-American and 127 Caucasian subjects with hypertension (mean age: 59 ± 10 yr) and 156 African-American and 135 Caucasian normotensive controls (mean age 58 ± 10 yr). All of these subjects were recruited from the outpatient department of The State University of New York Health Science Center in Brooklyn, New York and Westchester Medical Center, Valhalla, New York. All case and control subjects gave informed consent before participating in the research. All cases were diagnosed as having primary hypertension and patients with secondary hypertension, diabetes mellitus, or ischemic heart disease were excluded. The criteria for hypertension was defined as a SBP greater than 140 mmHg, a DBP greater than 90 mmHg, or under antihypertensive therapy. BP was measured twice with the subject seated and a 5-min interval between measurments. The normotensives (with SBP/DBP <140/90 mmHg) without a history of hypertension and without diabetes mellitus were recruited from the same population and matched for sex and age. All participants completed a standard questionnaire on personal medical history and family history of hypertension. All patients and control subjects were in Hardy–Weinberg equilibrium. The frequency of the –217A allele in hypertensive patients was 0.29 as compared with 0.19 in the normotensive population, which is highly significant (p = 0.0017 and OR =1.792) (Table 1). To compare the role of this polymorphic site on hypertension in the African-American and Caucasian populations, we also analyzed genomic DNA from 127 Caucasian hypertensive subjects and 135 normotensive controls. The frequency of –217A allele was 0.15 in Caucasian hypertensive subjects and 0.11 in normotensive controls which is not significant (p = 0.12) (Table 1). Statistical analysis based on the –217 A/G genotype (using A allele as a dominant model) also suggested a significant role of the –217A allele in hypertension in African-Americans (p = 0.0021 and OR = 2.015) and not in Caucasians (Table 2). Because an A/G polymorphism at –6 has previously been associated with hypertension, we also analyzed genomic DNA from the African-American and Caucasian populations for this polymorphism. The frequency of –6A allele was 0.87 in African-American hypertensive subjects and 0.85 in normotensive controls, which was not significant (p = 0.58). However, the frequency of –6A allele was marginally significant in Caucasian subjects (p = 0.06). These experiments suggested that –217A allele of the human *AGT* gene plays a significant role in essential hypertension in African-Americans *(41)*. However, if we double the number of total Caucasian subjects, then the role of –217A becomes significant. It is therefore possible that this polymorphism will be significant in Caucasian subjects if we analyze more samples.

Table 1
Statistical Analysis of –217 A/G Polymorphism of Human Angiotensinogen Based on Allele Frequency

	A allele	*G allele*	*p value*
African-American hypertensive (n = 186)	0.29	0.71	p = 0.0017 OR = 1.792
Normotensive (n = 156)	0.19	0.81	[1.247, 2.575]
Caucasian hypertensive (n = 127)	0.15	0.85	p = 0.1208 OR =1.507
Normotensive (n = 135)	0.11	0.89	[0.901, 2.522]

Table 2
Statistical Analysis of –217 A/G Polymorphism of Angiotensinogen Gene Based on the Genotype Distribution Using A Allele Dominant Model

	(AA + AG)		*GG*	*p value*
African-American hypertensive (n = 186)	12	84	90	p = 0.0021 OR = 2.015
Normotensive (n = 156)	4	50	102	[1.301, 3.121]
Caucasian hypertensive (n = 127)	4	31	92	p = 0.1433 OR =1.595
Normotensive (n = 135)	3	23	109	[0.894, 2.844]

TRANSCRIPTION FACTORS INVOLVED IN THE EXPRESSION OF *AGT* GENE

The activation of eukaryotic genes in vivo often requires the coordinated binding of multiple transcription factors to the promoter–enhancer region. It has been shown that distinct signal transduction pathways regulate activity of many of these transcription factors. In several cases, it has been shown that binding of multiple transcription factors to a specific promoter–enhancer region is cooperative and requires a unique composition and spatial arrangement of transcription factor binding sites. The assembly of these enhancer complexes is facilitated by protein–protein interactions between DNA-bound factors and protein-induced DNA bending. These features are important for transcriptional regulation because combination of multiple transcription factors generates a diverse pattern of regulation, and highly cooperative binding ensures the specificity of transcriptional control *(42–44)*. Most eukaryotic transcription factors contain one or more transactivation domains that are involved in interaction with downstream coactivators (such as CREB binding protein, CBP/p300, and steroid receptor coactivators) and this interaction plays a crucial role in transcriptional activation of a gene *(45,46)*. It is possible that polymorphisms in the promoter of a gene may affect the binding of transcription factors to this region of the promoter and alter the transcriptional regulation of the gene. For example, it has been shown that a single nucleotide polymorphism in the CD14

promoter decreases the affinity of SP1 transcription factor binding and enhances transcriptional activity *(47)*. Similarly two mutations in the promoter of human protein-C gene cause type-1 protein-c deficiency by disruption of two HNF-3 binding sites *(48)*. A polymorphism of the human matrix γ-carboxy glutamic acid protein promoter alters the binding of AP-1 complex and is associated with altered expression and serum levels of this protein *(49)*.

The nucleotide sequence of the human *AGT* gene promoter containing nucleotide sequence from –1223 to +27 and potential *cis*-acting DNA elements that may bind to different transcription factors are shown in Fig. 1. Comparatively little is known about transcriptional regulation of the human *AGT* gene expression. We have shown that the nucleotide sequence located between –99 and –91of the human *AGT* gene binds to the C/EBP family of transcription factors and this region of the promoter plays an important role in DBP and C/EBP-β-induced expression of this gene *(50)*. There is an adjacent SP1 binding site located around –87 and our recent data have suggested that SP1 and C/EBP-β cooperatively regulate the expression of human *AG* gene. It has been previously shown that human *AGT* gene has a C/A polymorphic site at –20 (located between TATA box and transcriptional initiation site). We have shown that USF binds to this sequence when nucleoside C is present at –20 and ER binds to this sequence when nucleoside A is present at –20 *(51)*. We have also shown that cotransfection of ER increases the expression of reporter constructs containing human *AGT* gene promoter with nucleoside A at –20 as compared with the same reporter constructs containing nucleoside G at this position. Orphan receptor Arp-1 also binds to this sequence and reduces ER-induced promoter activity *(52)*. Yanai et al. have also shown that the nucleotide sequence located between the TATA box and transcriptional initiation site of the human *AGT* gene binds to USF and plays a critical role in its expression *(53)*. In addition, we have shown that the liver-enriched transcription factor HNF-3 binds to the nucleotide sequence located between +10 and +20 of the human *AGT* gene promoter *(54)*. It has been shown recently that human *AGT* gene expression is increased by interleukin (IL)-6 treatment and a STAT-3 binding site is located at –274 *(55)*. Our gel shift and transient transfection assays have shown that nucleotide sequence around –240 is an HNF-1 site. HNF-1 plays an important role in liver and kidney specific expression of a gene. Nucleotide sequence of the human *AGT* gene around –217 contains a full palindromic GRE and there are three glucocorticoid receptor-binding site (GRE), half sites in other regions of the promoter (at –673, –130 and +15). We have also shown that the transcription factor CREB binds to the nucleotide sequence located between –840 and –830 of the human *AGT* gene and this sequence is involved in cAMP-induced expression of this gene *(56)*. This is a composite element and has sequence homology with a C/EBP binding site. Nucleotide sequence located between –323 and 490 contains multiple hormone receptor binding sites. There are at least three HNF-4 binding sites (DR-1) between –363 and –423. In addition, this sequence contains DR-4 sites that may be involved in binding with thyroid hormone receptor. Although it has been shown that this region of the promoter is important in the regulation of *AGT* gene expression by HNF-4 *(57)*, it is not known whether it plays any role in TR-induced expression of this gene. It has been shown previously that CREB, HNF-4, ER, USF, C/EBP and GR (which bind to different regions of the human AGT gene) interact with transcriptional coactivator CBP and coordinately regulate the expression of a gene. However, the role of CBP in transcriptional activation of human AGT gene, which is regulated by multiple transcription factors that can individually bind to CBP, is not known.

AGT gene is expressed in multiple tissues such as liver, fat, kidney, brain, and adrenals. However, molecular mechanisms involved in tissue specific expression of this gene are not known. It has been shown that the rat *AGT* gene expression is regulated by glucocorticoids, estrogens, androgens, and thyroid hormones, and by different cytokines during inflammation *(10)*. Glucocorticoids are the most important regulatory agents, and cell culture studies have shown that they increase the expression of *AGT* gene in liver and fat cells. Glucocorticoids can modulate the expression of *AGT* gene by interaction with multiple transcription factors. It has been shown that GR interacts with STAT-3 and C/EBP-β. Because IL-6 increases the expression of a gene through STAT-3 and C/EBP pathways, glucocorticoids may increase the expression of *AGT* gene through the action of IL-6. In addition, GR interacts with cAMP and increases the expression of various liver specific genes. In the case of rat *AGT* gene, a nucleotide sequence called acute phase responsive element (APRE) is located around –545 *(58)*. This nucleotide sequence is flanked by two GREs: a full palindromic sequence located around –580, and a half palindromic sequence located around –470. The nucleotide sequence of APRE has strong homology with NFκB site and partial homology with C/EBP binding site. Gel shift analysis has shown that both of these transcription factors can bind to APRE. Under basal conditions, a constitutively synthesized transcription factor (most probably C/EBP-α) binds to this sequence. However, on lipopolysaccharide (LPS) treatment, an inducible factor (most probably NFκB) binds to this sequence and displaces C/EBP-α. Transient transfection analysis has shown that presence of both of the GREs is required for maximum increase in the expression of rat *AGT* gene by IL-1 and LPS treatment. These experiments suggested that expression of the rat AG gene is increased during acute phase reaction mainly through the NFκB pathway. On the other hand, Brasier's group has shown that expression of the human *AG* gene is increased mainly by the IL-6 pathway *(55)*. However, the molecular mechanism involved in the regulation of human *AGT* gene expression by glucocorticoids is not known, and the role of GREs in this gene has not been analyzed.

The promoters of rat and human *AGT* genes are very different with respect to the location and sequence of GRE. The palindromic GRE of the rat gene has stronger homology with the consensus GRE, but is located far away from the transcriptional initiation site, at –580. On the other hand, although the GRE in the promoter of human *AGT* gene has only partial homology with consensus GRE, it is located much closer to the transcriptional initiation site, at –217. This site in the human gene is located in close proximity with the C/EBP and STAT-3 binding sites.

HUMAN *AGT* GENE PROMOTER WITH –217A VARIANT HAS INCREASED PROMOTER ACTIVITY

To understand the role of A/G polymorphism at –217 on transcription of the human *AGT* gene, we have synthesized reporter constructs where different regions of the human *AGT* gene promoter were attached to the luciferase gene in pGL3 basic vector (Promega Biotec). This vector does not contain any promoter or enhancer sequence. The reporter construct pHAGT1.3*luc* has 1223 bp of the 5'- flanking sequence and 70 bp of exon-I of the human AGT gene, and contains nucleoside A at –6 and G at –217. This construct was then mutated from –217G to –217A using a STRATAGENE site-specific mutagenesis kit. The resulting reporter construct had nucleoside A at –6 and at –217 as determined by nucleotide sequence analysis. These reporter constructs were transiently transfected in

-1223 CCAGACAAGT GATTTTTGAG GAGTCCTAT CTATAGGAAC AAAGTAATTA

-1173 AAAAAATGTA TTTCAGAATT ATACAGGCCA TGTGAGATAT GATTTTTTTA
HNF-3
-1123 AATGAAGATT TAGATAATG GGTAAAAAAG AGGTATTTGT GTGTTTGTTG
*
-1073 ATTGTTCAGT CAGTGAATGT ACAGCTTCTG CCTCATATCC AGGCACCATC

-973 TGCCATCGTG GATATGCCGT GGCTCCTTGA ACCTGCTTGT GTTGAAGCAG

-923 GATCTTCTT CCTGTCCCTT CAGTGCCCTA ATACCATGTA TTTAAGGCTG
AP-1 CREB
-873 GACACATCAC CACTCCCAAC CTGCCTCACC CACTGCGTCA CTTGTGATCA

-823 CTGGCTTCTG GCGACGTCTCA CCAAGGTCTC TGTCATGCCC TGTTATAACG
* *
-773 ACTACAAAG CAAGTCTTAC CTATAGGAAA ATAAGAATTA TAACCCTTTT

-723 ACTGGTCATG TGAAACTTAC CATTTGCAAT TTGTACAGCA TAAACACAGA

-673 ACAGCACATA TTTCAATGCC TGCATCCTGA AGGCATTTTG TTTGTGTCTT

-623 TCAATCTGGA TGTGCTATTG TTGGTGTTTA ACAGTCTCCC CAGCTACACT

-573 GGAAACTTCC AGAAGGCACT TTTCACTTGC TTGTGTGTTT TCCCCAGTGT
*
-523 CTATTAGAGG CCTTTGCACA GGGTAGGCTC TTTGGAGCAG CTGAAGGTCA

-473 CACATCCCAT GAGTGGGCAG CAGGGTCAGA AGTGGCCCCC GTGTTGCCTA
HNF-4/PPAR
-423 AGCAAGACTC TCCCCTGCCC TCTGCCCTCT GCACCTCCGG CCTGCATGTC
SP1/AP-1
-373 CCTGTGGCCT CTTGGGGGTA CATCTCCCGG GGCTGGGTCA GAAGGCCTGG
APRE
-323 GTGGTTGGCC TCAGGCTGTC ACACACCTAG GGAGATGCTC CCGTTTCTGG
HNF-1
-273 GAACCTTTGGC CCCGACTCCT GCAAACTTCG GTAAATGTGT AACTCGACCC
CEBP/GRE
-223 TGCACCGGCT CACCTCTGTTC AGCAGTGAAA CTCTGCATCG ATCACTAAGA
APRE * GRE
-173 CTTCCTGGGAA GAGGTCCCAG CGTGAGTGTC GCTTCTGGCA TCTGTCCTTC
CEBP
-123 TGGCCAGCCT GTGGT CTGGC CAAGTGATGT AACCCTCCTC TCCAGCCTGT

-73 GCACAGGCAG CCTGGGAACA GCTCCATCCC CACCCCTCAG CTATAAATAG
ERE/USF +1
-23 GGCATCGTGA CCCGGCCGGG GGAAGAAGCT GCCGTTGTTC TGGGTACTAC
* *

Fig. 1. Nucleotide sequence of the 1223 bp 5'-flanking region and 27 bp of the first exon of human angiotensinogen gene. Potential *cis*-acting DNA elements that may be involved in transcriptional regulation of this gene are underlined. The transcriptional initiation site is marked as +1.

HepG2 cells. The promoter activity was analyzed after 48 h of transfection, and normalized with the β-gal activity. Results of this experiment showed that reporter construct pHAGT1.3*luc* with nucleoside A at –217 gave a 28% increase in the promoter activity

as compared with the reporter construct pHAGT1.3*luc* with nucleoside G at –217 ($p < 0.001$) (mean value of six experiments) *(41)*. We have recently used these reporter constructs in transient transfection in Hep3B (another human liver-derived cell line) and immortalized human hepatocytes (TPH cells obtained from Dr. Ranjit Ray). TPH cells were obtained by transduction of Hepatitis C virus core protein in primary human hepatocytes, and we have shown that these cells synthesize human AGT mRNA by RT-PCR. Results of these experiments have shown that the reporter construct containing –217A had 20–30% increased basal promoter activity as compared with the reporter construct containing –217G.

NUCLEOTIDE SEQUENCE OF *AGT* GENE AROUND –217 HAS HOMOLOGY WITH GRE AND C/EBP BINDING SITE

The nucleotide sequence of human *AGT* gene promoter from –203 to –226 containing A/G polymorphic site at –217 is shown in the top line of Fig. 2. It has homology with the C/EBP binding site (the consensus C/EBP binding site TT/GNNGCAAT/G is shown in the reverse orientation in line 2). The –217 polymorphic site is the first nucleotide of the consensus C/EBP site. In addition, a consensus GRE (third line) is present in this region of the promoter. The first nucleoside of the palindromic GRE corresponds to the polymorphic site –217 of the *AGT* gene promoter. It has been shown previously that GR cooperatively interacts with other transcription factors and this combinatorial interaction is involved in glucocorticoid induced promoter activity in PEPCK and TAT genes. It has also been shown that C/EBP-β and CREB can interact with GR and cooperatively increase the GR-induced promoter activity by cAMP.

In order to examine whether polymorphism at –217 affects the binding of transcription factors to this region of the promoter, we synthesized two oligonucleotides containing either nucleoside A or G at –217 position and used them in gel shift assays. Our gel shift analysis has suggested that the oligonucleotide containing nucleoside A at –217 binds more strongly to C/EBP family of transcription factors and GR as compared to the same oligonucleotide containing nucleoside G at this position *(41)*.

GLUCOCORTICOIDS AND C/EBP INCREASE THE PROMOTER ACTIVITY OF –217A VARIANT

In order to understand the physiological significance of the GR binding site at –217 region of the *hAGT* gene promoter, we synthesized reporter constructs where six copies each of either oligonucleotide 223A or 223G were attached in front of the pGL2-luc vector (obtained from Promega). These reporter constructs were transiently transfected in HepG2 cells in the presence of an expression vector that contained rat GR coding sequence. After transfection, cells were treated with 100 n*M* dexamethasone, and promoter activity was analyzed after 24 h. Results of this experiment showed that promoter activity of reporter construct containing –217A increased by about 18-fold over the basal value. On the other hand, promoter activity of reporter construct containing –217G increased only two- to threefold. Because PGC-1 acts as a coactivator and increases GR induced promoter activity, we also cotransfected an expression vector containing hPGC-1 in this experiment.

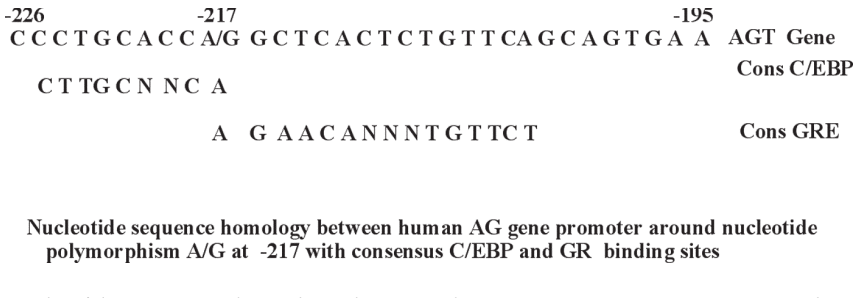

Fig. 2. Nucleotide sequence homology between human *AG* gene promoter around nucleotide polymorphism A/G at –217 with consensus C/EBP and GR-binding sites.

Results of this experiment showed that PGC-1 further increased the GR induced promoter activity. The same results were obtained by transient transfection in other cells such as human embryonic kidney cells (HEK 293) and 3T3L1. Taken together, results of these experiments suggested that reporter a construct containing six copies of an oligonucleotide containing –217A has increased GR induced promoter activity as compared with the reporter construct containing six copies of an oligonucleotide containing 217G.

Because C/EBP-β and -δ play an important role in acute phase reaction, and *AGT* gene expression is upregulated during acute phase reaction, we were interested in studying the effect of these transcription factors in the expression of human *AGT* gene containing A/G polymorphic site at –217. Expression vectors containing the coding sequence of either C/EBP-β or C/EBP-δ were cotransfected with reporter constructs containing different regions of the human AGT gene linked to the luciferase gene containing either nucleoside A or G at –217 in HepG2 cells. Transfected cells were then treated with IL-6 and promoter activity was analyzed by luciferase assay. Results of cotransfection experiments with C/EBP-β show that reporter construct pHAGT1.3*luc* containing nucleoside A at –217 had 30% increased promoter activity and reporter construct pHAGT303*luc* containing nucleoside A at –217 had 43% increased promoter activity as compared with corresponding reporter constructs containing nucleoside G at –217. Similarly, results of co-transfection experiments with C/EBP-δ show that reporter construct pHAGT1.3*luc* containing nucleoside A at –217 has 17% increased promoter activity, reporter construct pHAGT303*luc* containing nucleoside A at –217 had 32% increased promoter activity as compared with corresponding reporter constructs containing nucleoside G at –217 *(41)*. It is possible that combinatorial interaction of C/EBP, GRE, and STAT-3 is involved in the induced expression of the human *AG* gene by the IL-6 pathway, and polymorphism at –217 may play an important role in this process.

ASSOCIATION OF VARIANT –217A WITH OTHER POLYMORPHIC SITES OF *AGT* GENE

Previous studies have identified seven polymorphic sites in the 1.2 Kb promoter of human *AGT* gene *(29)*. These polymorphic sites are A/G at –6, A/C at –20, C/T at –532, C/T at –776, G/A at –793, T/A at –830, and G/T at –1074, and are shown in Fig. 3. Jeunemaitra et al. *(29)* have shown that polymorphisms at –532, –793, and –1074 always occur together. Surprisingly, these authors did not find the A/G polymorphism at –217 in the AGT gene promoter. In order to understand the linkage disequilibrium of these polymorphic sites in African-American subjects, we have performed sequence analysis of DNA samples isolated from African-American subjects and have found that variant

Fig. 3. Position of single nucleotide polymorphisms in the 1.2 kb promoter region of the human angiotensinogen gene.

-217A always occurs with –532T, –793A, and –1074G. The frequency of haplotype AA was 0.237 and that of haplotype AG was 0.086 in African-American subjects, which was statistically significant. This suggests that transcription factors binding to these sites in the AGT gene promoter may interact with each other and regulate the expression of this gene in subjects that contain allele –217A.

NUCLEOTIDE SEQUENCE AROUND –1074T OF *AGT* GENE HAS HOMOLOGY WITH HNF-3 BINDING SITE

The nucleotide sequence of *AGT* gene promoter around –1074 has partial sequence homology with the consensus HNF-3 binding site (Fig. 4). The nucleotide sequence of *AGT* gene with nucleoside G at –1074 has two mismatches with the consensus HNF-3 binding site, whereas the nucleotide sequence of *AGT* gene with nucleoside T at -1074 has one mismatch. Liver-enriched transcription factor HNF-3 belongs to the winged helix/forkhead family of transcription factors, which plays an important role in transcription of liver specific genes. Previous studies have suggested that HNF-3 acts as an accessory factor in GR-induced promoter activity of phosphoenolpyruvate carboxykinase (*PEPCK*) and *TAT* genes in the liver cells. Our unpublished data has shown that an oligonucleotide from this region of the promoter binds more strongly to HNF-3β when nucleoside T is present at –1074. It is tempting to speculate that increased binding of GR and C/EBPβ at -217A and increased binding of HNF-3β at –1074T is involved in increased expression of the *hAGT* gene in this haplotype.

HAPLOTYPE –6A: –217A: –1074 OF *AGT* GENE HAS INCREASED PROMOTER ACTIVITY

Because variant –217A always occurs in association with –532T, –793A, and –1074T, we were interested in examining whether *AGT* gene promoter containing this haplotype AA (containing nucleoside A at –6 and A at –217) has increased promoter activity as compared with the haplotype AG (containing nucleoside A at –6 and nucleoside G at –217). For this purpose, we amplified 1.3 Kb *AGT* gene promoter from a hypertensive and a normotensive subject. These amplified sequences contained –6A, –20A, –217A, –532T, –776T, –793A, and –1074T (haplotype AA) and –6A, –20A, –217G, –532C, –776T, –793G, and –1074G (haplotype AG). These amplified sequences were ligated in pGL3*luc* to produce reporter constructs containing these haplotypes. These reporter constructs were then used in transient transfections in HepG2, Hep3B, TPH, and differentiated and undifferentiated 3T3L1 cells. In all the cells, haplotype AA gave increased basal promoter activity as compared with haplotype AG. Because immortalized human liver cells are the closest representatives of the primary human liver cells, results of this experiment are

Homology between nucleotide sequence around T/G polymorphic site at -1074 of human AGT gene promoter with HNF-3 binding site

T A T T T/G A/G T/C T T/C CONS. HNF-3 SITE

T G T T T G T T T/G AGT GENE

Fig. 4. Homology between nucleotide sequence around T/G polymorphic site at –1074 of human AGT gene promoter with HNF-3 binding site

presented here. Reporter constructs (1 μg) and RSV-gal (50 ng) were cotransfected in TPH cells (400,000 cells per well) in triplicates using six-well plates in the presence of lipofectamine reagent (Qiagene). The promoter activity was analyzed after 48 h of transfection and normalized with the β-gal activity. Results of this experiment showed that the promoter activity of haplotype AA was 1.9-fold higher than that of haplotype AG.

TRANSGENIC MICE TO STUDY THE EFFECT OF POLYMORPHISMS OF *AGT* GENE IN VIVO

Transient transfection in cultured cells has provided important information about tissue-, hormonal-, and developmental-specific transcriptional regulation of a number of genes. However, such studies are often hampered by the limited selection and state of differentiation of cell lines used in transient transfection assay. For example, transcription rates of numerous liver-specific genes are greatly reduced in cultured hepatoma cells and primary hepatocytes. In many instances, cultured cells have lost functional hormone receptors (such as glucocorticoid, estrogen, and angiotensin-II receptors in HepG2 cells) and expression vectors containing coding region of these receptors have to be cotransfected to analyze the effect of a particular hormone. These experiments raise another serious problem—the number of transfected receptors may far exceed its physiological level in transfected cells, and may provide false information. Transgenic mice are at present the most rigorous system available for identifying and characterizing *cis*-acting DNA elements. In addition, transgene expression can be assessed in numerous cell types as well as during both embryonic and fetal development.

The transgenic-mice approach has been successfully used to identify *cis*-acting DNA elements involved in liver, kidney, and adipose-specific expression of the rat *PEPCK* gene *(59,60)*. These studies have also provided information about *cis*-acting DNA elements involved in developmental, glucocorticoid, and dietary-regulated expression of this gene. These transgenic experiments have complemented some of the results obtained by transient transfection assay in an in vivo setting. In some cases, mutations were made in selected *cis*-acting DNA elements and the effect of these mutations on tissue-specific expression of the mutated promoter was analyzed by the expression of transgene in mice *(60)*. In recent years, transgenic mice have been generated by the injection of fusion genes containing promoter sequences attached to the β-galactosidase gene. These transgenic mice provide a better colorimeteric assay to analyze the expression of transgene in different tissues. A fusion gene was synthesized by attaching the *lac Z* gene in front of a thymidine kinase promoter sequence attached to a NF-IL6 binding site. Transgenic mice containing this fusion gene were then used to study the effect of hypoxia in an in vivo

situation. Results of these experiments indicated that expression of the fusion gene containing the NF-IL6 sequence was increased under hypoxic conditions as measured by the β-gal assay. On the other hand, transgenic mice containing a mutated NF-IL6 site did not show an increase in β-gal activity under hypoxic conditions *(61)*. In a similar way, cell-specific expression of the mouse glycoprotein hormone β-subunit gene was analyzed by *lac Z* gene expression of expression vectors containing a different region of its promoter in transgenic mice *(62)*.

One of the main problems in these transgenic studies is that transgene can integrate at different sites in the chromosome and, depending on the site of integration, promoter activity may vary from experiment to experiment. Another problem is that a 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. However, experiments with *PEPCK* gene have shown that promoter activity did not vary significantly as a result of the number of transgenes in different animals.

In order to overcome these limitations, Sigmund's group has developed another experimental model system to assess the physiological significance of the human *AGT* gene *(63)*. In this model, gene targeting at the HPRT locus was used to selectively target a single copy of the human AGT gene to a known site in the genome. They showed that insertion of the single copy transgene upstream of HPRT does not affect the overall tissue- and cell-specific expression or hormonal regulation of human AGT. This study provides an important proof-of-principle that the functional significance of allelic variation in human AGT can be assessed by examining mice carrying transgenes targeted in a single copy to an identical insertion site. They targeted the human *AGT* gene to the mouse HPRT locus by employing embryonic stem (ES) cells harboring a deletion in the endogenous *HPRT* gene, and a special targeting vector capable of restoring its full functionality upon homologous recombination. The use of gene targeting is essential in developing a model for studying effects of allelic gene variants in vivo because it nullifies the copy number and positional effects associated with transgene expression in transgenic mouse models generated by pronuclear injection. It allows reproducible insertion of the single copy of a transgene in a predetermined locus, permitting direct comparison with individually generated transgenic lines. Furthermore, independently derived transgenic mice generated by gene targeting are genetically identical, abolishing the need for multiple transgenic mouse lines harboring the same construct. An important practical benefit of their strategy is the highly reproducible efficiency in selecting single homologous recombination events. This is because, unlike the common approach to homologous recombination in which the targeting construct carries a positive selectable marker, the HPRT system carries sequences that can repair the deletion of HPRT found in the specific ES clone that is suitable for these studies. The selection for growth in HAT medium allows only targeted clones to survive, so all clones that survive selection have been targeted. This should allow for relatively easy development of a number of transgenic ES cell clones that harbor specific variants of a gene of interest, and the subsequent generation of transgenic mice.

However, there are certain potential drawbacks of using a nonnative locus, such as HPRT, for gene targeting. Exogenous genes may not harbor all of the sequences necessary and sufficient for proper regulation of expression and may therefore be influenced by *cis*-acting regulatory elements in the vicinity of the *HPRT* gene. However, previous studies by Sigmund's group have shown that the 13.8-kb genomic human AGT transgene

is expressed in a tissue- and cell-specific and copy-number proportional fashion in transgenic mice, suggesting that all essential regulatory elements are present in the transgene. Another potential drawback of targeting the HPRT locus is that it is located on the X chromosome and the transgene expression is affected by random X inactivation. This means that one can effectively achieve an equivalent of 1°copy (in the case of male and homozygous transgenic female mice) or 0.5°copy (in the case of heterozygous transgenic female mice) transgene expression. Taken together, their data indicated that the human *AGT* gene was apparently unaffected by the HPRT locus. It exhibited correct tissue and cell specificity and was properly regulated by the changes in testosterone levels. Furthermore, the human AGT protein was normally processed, and its plasma concentration was proportional to the level of human AGT mRNA, indicating proper regulation. The protein in plasma is readily cleaved by human renin, resulting in the acute pressor response, which could be easily detected by changes in BP.

In a recent paper, this group has used this model to specifically test the physiological effects of variants at nucleoside –6 and amino acid 235 in the *AGT* gene in vivo *(40)*. For this purpose, they generated two different hAGT transgenes, representative of the two haplotypes observed in humans, by site-directed mutagenesis, and inserted both transgenes in a single copy at HPRT in the mouse genome. The identical genetic environment in both genetic background and transgene location allowed them to directly compare the transcriptional and functional activity of each haplotype and to link any significant difference in phenotype(s) to the sequence differences of the hAGT transgenes. The molecular analysis of the two hAGT haplotypes showed that variation at nucleotide –6 and amino acid 235 positions have no effect on the tissue- and cell-specific expression pattern in either males or females, and that both variants are transcribed with equal efficiency in liver, kidney, brain, and aorta. This result was unexpected, because previous reports indicated that the –6 variant affects baseline transcriptional efficiency both in vitro (in the context of a reporter construct), and in vitro, in that it may influence the steady-state level of AGT mRNA in the decidual spiral arteries of first trimester pregnant women. They have argued that there are several possibilities for this disparity. As was previously shown by the work in their laboratory, the *hAGT* gene can behave differently when studied in vitro and in vivo *(46)*. Whereas deletion mutations in the promoter and 3' enhancer of the *hAGT* gene were shown to have an obvious effect on the transcription of the gene in HepG2 cells, no effect on gene regulation was detected in vivo when these mutations were studied in transgenic mice. The reason for this remains elusive, although it is conceivable that the HepG2 cell culture model is lacking all of the factors involved in the normal regulation of the hAGT expression, because it is an immortalized cell line. It is also possible that the environment of a mouse cell does not allow for the effect of the hAGT variants to be revealed. This could result either from the absence of cellular factors that can recognize specific elements of the hAGT promoter, or the fact that those factors are significantly different from their human counterparts so that they cannot serve the same function.

It is important to recognize that Sigmund's group has specifically analyzed the effect of only one polymorphism, namely at –6 position, in the *hAGT* gene on its transcriptional regulation *(40)*. However, studies by Inoue et al. have shown the effect of only 256 bp of the 5'-flanking region of the human *AGT* gene on transient transfection in HepG2 cells. It is important to note that the effect of –6A variant was very small (only 14–19%) on transient transfection. It is possible that experiments using transgenic animals containing different haplotypes of the human *AGT* gene may provide meaningful results. It is also

important to mention that in order to examine the effect of promoter variants of the *AGT* gene on the BP in transgenic animals, one has to use double transgenic mice containing human AGT, as well as human renin genes. Very little is known about the tissue-specific regulation of human renin gene expression and therefore, although these experiments are very interesting, there are many unsolved problems. In addition, mouse physiology may be different from the human physiology and this haplotype, though important in the regulation of BP in human subjects, may not show its effect in mice.

CONCLUSIONS AND PERSPECTIVES

Hypertension is a polygenic disease, and most probably multiple genes are involved in the etiology of this disease in different ethnic groups. Although initial studies, based on microsatellite markers, had shown that renin, ACE, and angiotensin receptor type 1 genes are not involved in hypertension, recent studies have suggested that certain polymorphic variants of renin and angiotensin receptor may be involved in human essential hypertension *(64)*. It is also important to mention that other genes such as adducin *(65)*, β2-adrenergic receptor *(66)*, and G protein β3 subunit *(67)* have also been suggested to be involved in human hypertension. It will be important to examine whether interaction of these genes leads to hypertension.

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