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Haplotypes of Angiotensinogen in Essential Hypertension

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Summary

The M235T polymorphism of the angiotensinogen gene (AGT) has been associated with essential and pregnancy-induced hypertension. Generation of haplotypes can help to resolve whether the T235 allele itself predisposes to the development of hypertension or acts as a marker of an unknown causal molecular variant. We identified 10 diallelic polymorphisms at the AGT locus and genotyped both a series of 477 probands of hypertensive families and 364 controls, all French Caucasians, as well as a series of 92 hypertensives and 122 controls from Japan. Despite a large ethnic difference in gene frequency, a significant association of T235 with hypertension was observed both in Caucasians (.46 vs. .38, $P = .004$) and in Japanese (.91 vs. .76, $P = .002$). In both groups, the G→A substitution located at position −6 upstream of the initial transcription site occurred at the same frequency and in complete linkage disequilibrium with the T235 allele. No other polymorphism was found to be consistently associated with hypertension. Five informative haplotypes subdividing the T235 allele were generated. Whereas two of them were associated with hypertension in Caucasians, none of these two haplotypes (H3 and H4) reached statistical significance in Japanese. The analysis of the AGT-GT repeat revealed marked linkage disequilibriums between each of the diallelic polymorphisms and some $(GT)_n$ alleles, with similar patterns in the two populations. The strong disequilibrium between M235 and $(GT)_{16}$ explained the increased frequency of that particular allele in French controls compared with hypertensives (.42 vs. .36, $P < .01$). The haplotype combining the M235T and G-6A polymorphisms appears as the ancestral allele of the human AGT gene and as the one associated with hypertension.

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Introduction

The angiotensinogen gene (AGT) has been implicated in essential hypertension through both genetic linkage and allelic association. Positive linkage was established initially in both Utah and French Caucasians (Jeunemaitre et al. 1992) and was confirmed later in two others sets of Caucasian (Caulfield et al. 1994) and African-Carribean (Caulfield et al. 1995) hypertensive families. Apart from rare mutations that potentially affect the kinetics of angiotensinogen II generation (Inoue et al. 1995) or the secondary structure and secretion of the protein (Gimenez-Roqueplo et al. 1996), the M235T polymorphism encoding a threonine instead of a methionine at residue 235 of the mature angiotensinogen protein was significantly more common in hypertensive subjects than in normotensive controls, in both Utah and French Caucasians (Jeunemaitre et al. 1992). This association was confirmed in unselected French hypertensives participating in a clinical trial (Jeunemaitre et al. 1993) and in hypertensives with age at onset at <50 years who were selected in Germany (Schmidt et al. 1995). Two other studies found an association with the T174M polymorphism, which is in complete linkage disequilibrium with M235T. They indicated that this association could be restricted either to males (Hegele et al. 1994) or to non-overweight hypertensives (Tiret et al. 1995). Despite a strong ethnic difference in allele frequency, the association with M235T also held in the Japanese population (Hata et al. 1994; Kamitani et al. 1994; Iwai et al. 1995; Nishiuma et al. 1995). In addition, M235T was also significantly associated with preeclampsia in Caucasians as well as in Japanese (Ward et al. 1993), a finding substantiated by a later report of significant linkage between AGT and preeclampsia in pedigrees from Scotland and Iceland (Arngrimsson et al. 1993). Finally, new reports suggest that M235T could also represent a genetic risk factor for coronary-artery disease in Caucasians (Katsuya et al. 1995) and Japanese (Ishigami et al. 1995; Kamitani et al. 1995) individuals.

A variety of observations support the hypothesis that the causative mutation should affect the synthesis, function, secretion, or metabolism of angiotensinogen itself.

First, presence of the T235 allele has been associated with a 10%–20% increase in plasma angiotensinogen (Jeunemaitre et al. 1992, 1993; Bloem et al. 1995). A positive relation between plasma angiotensinogen and blood pressure (BP) is supported by physiological (Gould and Green 1971) and pharmacological (Gardes et al. 1982; Ménard et al. 1991) considerations and by the reported differences in plasma angiotensinogen concentration that are observed between hypertensives and normotensives (Fasola et al. 1968; Walker et al. 1978). More-recent results, obtained by the inactivation or duplication of the AGT gene in transgenic mice, showed a relationship between AGT gene expression, plasma angiotensinogen, and BP (Kim et al. 1995).

Whether the T235 allele directly accounts for a physiological effect or acts as a marker for a causative mutation is as yet unresolved. Three possible interpretations can account for the observed associations: (1) T235 could represent the main functional mutation within the angiotensinogen gene, which predisposes to the development of essential hypertension; (2) it could serve as a marker for yet unknown molecular variant(s), directly mediating predisposition; or (3) both T235 and the other variants could be causally implicated. Genetic methods have the potential to provide a powerful test for the presence or absence of other causal variants. If T235 acts as a marker, it is logical to assume that only a subset of the genes carrying T235 also harbor the functional mutation(s). In this case, other polymorphisms at the AGT locus can be used to subdivide T235 alleles into a series of distinct haplotypes. These haplotypes should exhibit a variable degree of association with the causal mutation(s) and, thus, with hypertension. This situation has been observed in other genetic situations, such as hemoglobinopathies (Weatherall 1995). For the AGT gene, this hypothesis was suggested by two studies (Caulfield et al. 1994, 1995) that found that different alleles of the AGT microsatellite actually could bear the susceptibility to hypertension.

To address this question, we have identified a series of 10 diallelic AGT polymorphisms and have characterized their haplotype distribution in a large series of hypertensive cases and normotensive controls of Caucasian and Japanese origin. We also genotyped the microsatellite of the AGT locus (Kotelevtsev et al. 1991), thus enabling an analysis of the disequilibrium patterns between the two types of polymorphisms. Partition of the T235 allele with diallelic markers did not unambiguously pinpoint a subset of T235 haplotypes responsible for the association with hypertension. Likewise, the only significant differences observed, in the distribution of GT alleles, between hypertensive and normotensive subjects of Caucasians or origin were attributable to the strong linkage disequilibrium existing between M235 and the $(GT)_{16}$ repeat allele. The main outcome of this search was the finding that a polymorphism occurring six residues upstream from the initiation site of transcription, G-6A, was in complete linkage disequilibrium with M235T and that the haplotype combining both polymorphisms was the one associated with hypertension.

Subjects and Methods

A. Study Subjects

The grandparents of the Utah families included in the CEPH database were used as controls for the frequency of the AGT diallelic polymorphisms and to define the corresponding haplotypes. French hypertensive patients were selected from the HYPERGENE data set of hypertensive families recruited in the Broussais Hypertension Clinic in Paris (Charru et al. 1994). Only the 477 probands (age 49.4 ± 8.4 years; 48% men) who satisfied the following main criteria were considered: Caucasian origin, hypertension established on the basis of diastolic BP ≥95 mmHg and/or the presence of an antihypertensive treatment (mean BP 158.9 ± 22.6/98.8 ± 13.3 mmHg; 73% on treatment), onset of hypertension at <55 years of age (40.3 ± 10.8 years), and body-mass index <27 kg/m^2 (25.4 ± 3.8). The absence of secondary hypertension was established by an extensive inpatient workup when the latter was clinically indicated. The absence of diabetes mellitus (mean blood glucose 5.4 ± 0.7 mmol/liter) was assessed by the presence of the following three criteria: (1) the absence of personal diagnosis of diabetes mellitus, (2) the absence of antidiabetic drug treatment, and (3) a fasting blood-glucose level <6.5 mmol/liter. Also, patients were not eligible when other factors that could affect BP—for example, renal insufficiency (mean plasma creatinine 85.8 ± 22.4 μmol/liter), excessive alcohol consumption, and estrogen use—were present.

Among these 477 probands of hypertensive families, 75 probands already had been studied in our previous analysis (Jeunemaitre et al. 1992). The clinical and biological characteristics of these 75 probands were not statistically different from those of the other 402 probands. They were not excluded from this analysis, in order to keep the maximal power of the haplotype analysis.

French normotensive patients were selected by the Institut Régional pour la Santé (IRSA) during an annual medical visit for preventive medicine. All of the 364 individuals analyzed were Caucasians and were recruited in two main centers of the regions Centre and Picardie, which are located ~200 km south and north of Paris, respectively. This control group was different from that analyzed in our previous study (Jeunemaitre et al. 1992). Normotensive individuals were selected to match the distributions of age (46.1 ± 7.6 years), gender (44% men), and body-mass index (23.6 ± 2.2 kg/m^2)

observed in the hypertensive subjects. All had sitting systolic and diastolic BP <140 and <90 mmHg (mean BP 113.8 ± 9.4/71.4 ± 6.5 mmHg), respectively, without any history of hypertension or antihypertensive treatment, diabetes mellitus (mean blood glucose 5.02 ± 0.5 mmol/liter), renal insufficiency (mean plasma creatinine 71.6 ± 12.2 μmol/liter), or cardiovascular disease and without family history (in parents and siblings) of hypertension.

Japanese hypertensives and controls have been described elsewhere (Hata et al. 1994). Patients had established hypertension (systolic BP >160 mmHg and/or diastolic BP >90 mmHg), in the absence of any secondary cause, diabetes, or renal disease. All patients and controls were ascertained at Yamanishi University Hospital.

B. Identification of Polymorphisms

1. Search for new diallelic polymorphisms.—Our initial search for polymorphisms (Jeunemaitre et al. 1992), relying on conformational variation in electrophoresis under nondenaturing conditions (Orita et al. 1989), spanned all coding segments of exons and splice junctions. In addition to the M235T and T174M missense mutations, four other polymorphisms, at positions −532, −20, −18, and −6 upstream of the transcription start site, and a polymorphism in intron 3, at position −13 relative to the beginning of exon 4, had been detected. With the same methodology, this search was extended to nucleotide −1221 upstream of the gene, in a panel of 96 French hypertensive subjects.

Each sample was electrophoresed under at least two conditions: (1) a 0.5 × Hydrolink ™ MDE™ (AT Biochem) prepared in 0.6 × TBE (1 × TBE = 90 mM Tris-borate [pH 7.8], 2 mM EDTA), run at room temperature at 400 V for 14–20 h; and (2) a 5% polyacrylamide gel (49:1 polyacrylamide:methylene-bis acrylamide) prepared in 0.5 × TBE, run at +4°C at 15 W constant power for 3–4 h. Direct sequencing of electrophoretic variants was performed as described elsewhere (Jeunemaitre et al. 1992).

Several segments of the 3′ region of the human AGT gene have been shown to contain sequences involved in the cell type-dependent activation of the gene (Nibu et al. 1994*a*, 1994*a*). A systematic search was performed, by SSCP analysis, on the region containing an enhancer core element from +2170 to +2230, using the primers indicated in table 1 and three different nondenaturing conditions. This systematic search was done in 20 M235M homozygous and 20 T235T homozygous French individuals. No electrophoretic variant was observed. In addition, direct sequencing also was performed in 16 individuals, all homozygous for the T235 allele, for the 5′ region of the gene (from nucleotide −800 to nucleotide −1), the five exons, and the entire untranslated region of the gene.

2. Genotyping.—The frequency of each variant was established in cases and controls by allele-specific oligonucleotide hybridization using the methodology described elsewhere (Jeunemaitre et al. 1992) and the primers indicated in table 2. The G-6A variant could not be resolved by this technique without ambiguity, probably because of the high GC content of that sequence. We used the mutagenically separated PCR technique, in which both normal and mutant alleles are amplified in the same tube, using different length allele-specific primers (Rust et al. 1993). The following primers were designed with one forward primer and two reverse primers in which additional deliberate differences (underlined) were introduced to correspond to the molecular variant and to reduce cross-reactions between the two alleles: FP-6, 5′-GTGTCGCTTCTGGCATCTG TCCTTCTGG-3′; RP-6A, 5′-TACCCAGAACAA-CGGCAGCTTCTTCCACT-3′; and RP-6G, 5′CCG-GTTACCTTCTGCTGTACAGCCCAGAACAACG-GCAGCTTCTTCCATC-3′.

PCR reactions were conducted in a 25-μl reaction volume containing 2.5 μl of 10 × PCR buffer (500 mM KCl, 100 mM Tris HCl pH 8.3, and 0.01% gelatine), 1.5 mM $MgCl_2$, 10 μM each of the four dNTPs, 10 pmol each of the three primers, and 0.5 units *Taq* polymerase. The first denaturation step (94°C for 5 min) was followed by 35 cycles, each of 94°C for 45 s, 62°C for 45 s, and 72°C for 45 s, and by a final extension at 72°C for 7 min. The amplification reaction yields a 187-bp and a 207-bp product for the A-6 and G-6 alleles, respectively, which were resolved on a 2% agarose gel.

3. Analysis of the GT alleles at the AGT locus.—The AGT genotypes of the highly informative dinucleotide repeat located in the 3′-flanking region of the AGT gene were established as described elsewhere (Jeunemaitre et al. 1992). To avoid any ambiguity in comparisons with allelic frequencies obtained in other studies, we classified the alleles according to their number of GT repeats and to provide in the figures a correspondence with the numbering scheme used initially by Kotelevtsev et al. (1991).

C. Statistical Analysis

Comparison of genotypic frequencies of single polymorphisms were performed by use of contingency χ^2 tests. Pairwise linkage-disequilibrium coefficients were estimated by the maximum-likelihood method, and the extent of disequilibrium was expressed as the $D' = D/D_{max}$ or D/D_{min}, according to Thompson et al. (1988). Allelic or haplotypic frequencies for one or more loci were estimated by the maximum-likelihood method, by use of a simplified version of the computer program GENEF (J.-M. Lalouel, unpublished data). In analyses involving single codominant loci, this coincides with di-

Table 1

Primers Used for Detection of Mutations in 5′ Region of AGT Gene by SSCP Analysis

Location	Forward Primer	Reverse Primer
−1221 to −1004	AGACAAGTGATTTTTGAGGAGTC	AACAACAAAGAGCAGGAAGAGATGG
−1049 to −797	CTTCTGCCTCATATCCAGGC	ACCTTGGTGAGAGTCGCCAG
−867 to − 656	ATCACCACTCCCAACCTGCC	ATGCCTTCAGGATGCAGGCA
−706 to −456	ACATTTGCAATTTGTACAGC	GCCCGCTCATGGGATGTG
−421 to −171	AAGACTCTCTCCCCTGCCCTC	GAAGTCTTAGTGATCGATGCAG
−164 to +73	AGAGGTCCCAGCGTGAGTGT	AGACCAGAAGGAGCTGAGGG
+2141 to +2258	ACAGATGTATACAATTCAGCAG	CACCTAAAACTTCAAAGGACTG

rect gene counting. Rather than estimating frequencies of a large number of unobserved haplotypes, however, we used a sequential inclusion procedure operating as follows: (1) after inclusion of the M235T polymorphism, additional polymorphisms were sequentially added one at a time; joint estimation of haplotypes yielded the minimum sets of haplotypes required to account for the observations, with all haplotypes below a frequency of 1/4N, where N is the sample size, being automatically eliminated; (2) in five instances, a rare haplotype was required to account for only one possible observed genotype, and a parsimonious solution was obtained by deleting that rare genotype. The final set of haplotypes generated remained the same, whatever the order in which markers sequentially were added, and only five genotypes required unique, rare haplotypes and consequently were excluded.

Since most of the polymorphisms were in complete linkage disequilibrium with one another, this strategy led to nearly unambiguous haplotypes. Thus, instead of a global maximum-likelihood method that would have raised more df, statistical comparisons between cases and controls were performed by use of simple χ^2 tests of homogeneity, with continuity correction.

Results

Diallelic Polymorphisms

Since our previous report, four additional variants have been detected, at positions −776, −793, −830, and −1074 (fig. 1). None occurs in a region known, so far, to affect the expression of the AGT gene. No electrophoretic variant was found either in the 80 bp of exon 5 (+1399 to +1478) by SSCP analysis or in the entire untranslated 3′ region by direct sequencing of 16 subjects homozygous for TT235.

The frequencies of seven polymorphisms located within 1 kb of the 5′ region of the gene, the M235T and T174M variants in exon 2, and one polymorphism in intron 3 were analyzed in hypertensive and normotensive subjects (table 3). The significantly higher frequency, in Japan, of T235 in hypertensive subjects compared with normotensive controls has been reported elsewhere (Hata et al. 1994). The study of French subjects confirmed the previously reported association (Jeunemaitre et al. 1992) in another set of cases and controls (.47 vs. .38, respectively; P = .004). However, the frequency of the T174M variant was not significantly different between hypertensives and controls, whether from France or from Japan. A significant increase of the G-6A variant was observed in both groups, paralleling the difference observed for M235T. One other polymorphism, C-776T, displayed a significant difference between French hypertensives and controls (.08 vs. .03, P = .001), but that association was not replicated in the Japanese groups.

Pairwise Linkage Disequilibrium

The polymorphisms were typed in the entire set of Utah CEPH grandparents, in addition to the groups

Table 2

Primers Used for Detection of ATG Polymorphisms by Specific Oligonucleotide Hybridization

Location	Primer 1	Primer 2
−20	ATAGGGCATCGTGAC	ATAGGGCCTCGTGAC
−532	GTGTGTTTTCCCCAGT	TGTGTGTTTTTCCCAGT
−776	TGTTATAACGACTACAA	TGTAGTCATTATAACAG
−793	AGGGCATGACAGAGAC	GTCTCTATCATGCCCT
−830	GTCACTTGTGATCACTG	GTCACTTGAGATCACTG
−1074	TGTTTGTTGATTGTTCA	TGAACAATAAACAAACA
Int3	ATCTCCCCAGGACCATC	GATGGTCCTTGGGAGAT

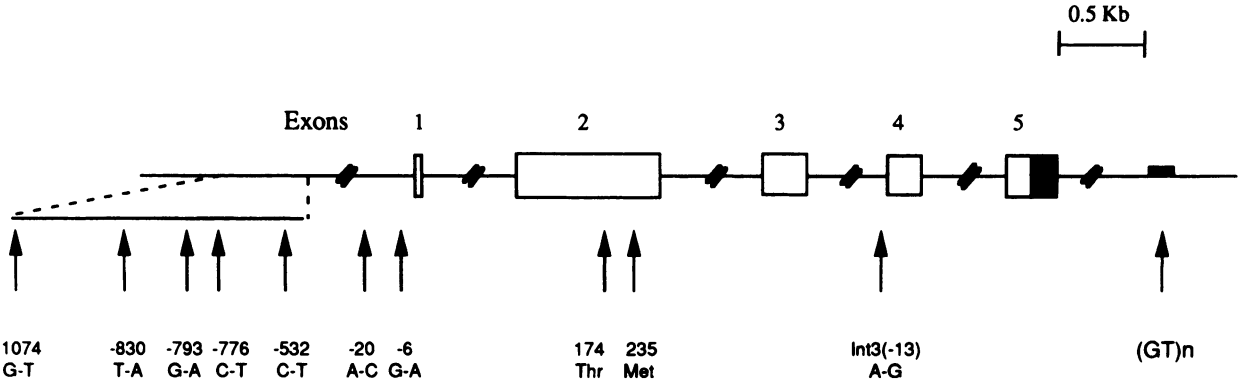

Figure 1 Schematic diagram of the human angiotensinogen gene and location of identified variants. The positions of the variants in the 5′ region are numbered by reference to the transcription-initiation site as defined by Gaillard et al. (1989). The two polymorphisms of exon 2 are at amino acid residues 174 and 235. The intron 3 polymorphism is located at position −13 upstream of the beginning of exon 4.

described above, and their patterns of linkage disequilibrium were examined. Most of the polymorphisms were in complete linkage disequilibrium with one another (table 4). The patterns of linkage disequilibrium analyzed in the CEPH group were in close agreement with those observed in French Caucasians. Despite strong ethnic differences in the frequency of the T235 allele, similar patterns of disequilibrium also were observed in the 122 normotensive Japanese (data not shown).

In order to subdivide the T235 allele into frequent haplotypes, the linkage-disequilibrium patterns were summarized in terms of the allelic associations with either T235 or M235 (table 5). The variants A-830 occurred only in genes carrying 235M. All other polymorphisms exhibited a quasi-complete linkage disequilibrium with M235T, each variant being only a subset of the T235 alleles. It is important to note that, in both populations, the G-6A polymorphism was in complete linkage disequilibrium with M235T and occurred with the same frequency. Thus, the two polymorphisms almost always were seen together. As a result, although our subsequent analyses will refer primarily to M235T, all associations pertaining to the T235 allele extend directly to the A-6 polymorphism.

Multisite Haplotypes

The distribution of the T235-associated polymorphisms into multisite haplotypes was examined first in the CEPH sample. For the purpose of the present

Table 3

Frequency of Angiotensinogen Polymorphisms in Hypertensives and Controls

	Caucasians			Japanese		
	Frequency			Frequency		
Polymorphism	Hypertensives (n = 477)	Controls (n = 364)	χ^2_2[a]	Hypertensives (n = 92)	Controls (n = 122)	χ^2_2[a]
M235T	.465	.379	12.1 (P = .004)	.913	.762	17.4 (P = .002)
G-1074T	.124	.091	4.8 (Not significant)	Not tested	.147	
T-830A	.067	.060	.8 (Not significant)	Not tested	Not tested	
G-793A	.127	.092	8.6 (P = .01)	.179	.153	3.8 (Not significant)
C-776T	.080	.034	18.8 (P = .001)	.092	.108	2.3 (Not significant)
C-532T	.122	.094	4.7 (Not significant)	Not tested	Not tested	
A-20C	.160	.175	1.0 (Not significant)	.225	.195	.7 (Not significant)
G-6A	.466	.384	11.0 (P < .01)	.900	.742	19.1 (P < .001)
T174M	.114	.117	1.0 (Not significant)	.117	.076	2.3 (Not significant)
A-13G(int3)	.082	.069	1.8 (Not significant)	.376	.287	5.0 (Not significant)

[a] Test on genotype frequencies.

Table 4

Pairwise Linkage-Disequilibrium Coefficients, between AGT Gene Polymorphisms, Estimated in Caucasian Controls

	$\pm D'$[a]								
POLYMORPHISM	M235T	G-1074T	T-830A	G-793A	C-776T	C-532T	A-20C	G-6A	T174M
G-1074T	1.00	...	...	...	...	...	...	...	...
T-830A	−1.00	−.75	...	...	...	...	...	...	...
G-793A	1.00	1.00	−.51	...	...	...	...	...	...
C-776T	1.00	.77	1.00	.77	...	...	...	...	...
C-532T	1.00	.96	−.63	.97	1.00	...	...	...	...
A-20C	1.00	−1.00	−1.00	−1.00	.57	−1.00	...	...	...
G-6A	.97	1.00	−.76	1.00	−1.00	1.00	1.00	...	...
T174M	1.00	−1.00	−1.00	1.00	.40	−1.00	.94	1.00	...
A-13G(int3)	.97	−1.00	.00	1.00	.98	−1.00	−1.00	.92	−1.00

[a] All coefficients with absolute values >.70 significantly differ from 0 ($P < 10^{-4}$).

study—that is, testing of the homogeneity of T235 haplotypes with respect to risk of hypertension—we sought to conserve df by generating a parsimonious set of haplotypes involving as many site polymorphisms as could be achieved. Although there is no formal, optimal solution to this problem, the observed patterns of multiway linkage disequilibrium yielded a rather straightforward solution. Variants at sites −1074, −793, and −532, with a few exceptions, always occurred together; only site −793 was considered in further analyses.

Sequential addition of site polymorphisms, independently of the sequence used for their incorporation, led invariably to a parsimonious set of five common haplotypes of T235, defined by five site polymorphisms (table 6). In the CEPH sample, these five sites account for 233 of 238 haplotypes tested at all sites. Only five genotypes required unique, rare haplotypes and, consequently, were excluded. Inclusion of the A-13G(Int3) polymorphism would have required retention of multiple rare haplotypes or deletion of multiple T235 genes. To conserve power and df, this polymorphism was not included in subsequent analyses. The final set of haplotypes generated remained the same, whatever the order in which markers were added sequentially. Because of the very similar linkage disequilibrium, in both populations, between M235T and the other diallelic polymorphisms, the same haplotypes were generated in both the Caucasian group and the Japanese group.

Testing Haplotypes in Hypertensives and Normotensives

The estimated frequencies of the six haplotypes identified above were compared, between hypertensive and controls, in both the French Caucasian population and the Japanese population (table 7). The strong and significant increase of H1 in normotensives that was observed in both populations simply reflects the symmetrical decrease of the T235 allele, a decrease already observed in the single-locus analysis. In Caucasians, the difference in H1 frequency (.093, $P < .001$) was explained mainly by two haplotypes: H3 (T235/T-776) and H4 (T235/A-793). This pattern was not observed in Japanese, where most of the haplotypes subdividing T235 displayed a small difference between hypertensives and controls. In this group, the only marginal significant difference was observed for the T235 haplotype not bearing any other polymorphism (i.e., H2; see table 7).

Distribution of the GT Alleles

In order to investigate the possibility of an association between a multiallelic series of alleles at this locus and hypertension, alleles of the microsatellite dinucleotide (GT) repeat at the angiotensinogen locus (Kotelevstev et al. 1991) were examined in all samples. The frequencies observed in Utah CEPH subjects were very similar to those exhibited by French controls (data not shown). To reduce df, alleles at either end of the distribution were pooled. A significant difference was observed between normotensive and hypertensive Caucasian subjects (χ^2 [7 df] = 21.56, $P < .01$). The decrease in $(GT)_{16}$ in hypertensives compared with normotensives (.358 vs. .416) accounted for most of this statistical difference (fig. 2). As a consequence, a slight increase was detected in the other alleles, but no particular shift from one to another could be observed.

A different pattern was observed in the Japanese. Compared with that observed in Caucasians, the distribution was shifted toward shorter and longer alleles, the $(GT)_{16}$ allele accounting only for 11.4% and 8.9% of the alleles in controls and hypertensives, respectively (fig. 2). When all the alleles were analyzed, a marginally significant difference was observed between normotensive and hypertensive Japanese (χ^2 [7 df] = 14.1, $P < .05$). A tendency toward longer alleles was observed in hyper-

Table 5

Association between Each Diallelic Angiotensinogen Polymorphism and T235 Allele

Polymorphism and Sample (*n*)	P_i[a]	P_{ij}[b]	$P_{j/i}$[c]
M235T:			
CEPH grandparents (152)	.388	...	...
French controls (364)	.380	...	...
Japanese controls (122)	.762		
G-1074T:			
CEPH grandparents (148)	.098	.098	1.00
French controls (360)	.091	.090	.99
Japanese controls (122)	.153	.153	1.00
T-830A:			
CEPH grandparents (139)	.090	.000	.00
French controls (344)	.060	.000	.00
Japanese controls (...)	...	...	...
G-793A:			
CEPH grandparents (138)	.098	.081	.83
French controls (343)	.092	.092	1.00
Japanese controls (122)	.147	.143	.97
C-776T:			
CEPH grandparents (137)	.048	.037	.76
French controls (338)	.034	.034	1.00
Japanese controls (114)	.110	.110	1.00
C-532T:			
CEPH grandparents (142)	.106	.106	1.00
French controls (277)	.094	.091	.97
Japanese controls (...)	...	...	...
A-20C:			
CEPH grandparents (144)	.135	.135	1.00
French controls (339)	.177	.177	1.00
Japanese controls (108)	.185	.185	1.00
G-6A:			
CEPH grandparents (145)	.369	.362	.98
French controls (364)	.386	.376	.97
Japanese controls (113)	.743	.739	.99
T174M:			
CEPH grandparents (138)	.101	.101	1.00
French controls (355)	.117	.117	1.00
Japanese controls (122)	.078	.078	1.00
A-13G(int3):			
CEPH grandparents (146)	.079	.079	1.00
French controls (362)	.069	.067	.97
Japanese controls (120)	.296	.296	1.00

[a] Frequency of variant *i*.
[b] Joint frequency of variant *i* and T235.
[c] Conditional probability that a gene carries T235, given that it carries *i*.

tensives, significant when the overall distribution was partitioned into two groups, one group more than and the other group equal to or less than the $(GT)_{17}$ (χ^2[1 df] = 8.76, P = .004).

Conditional Distributions of the GT Alleles, According to Diallelic Polymorphisms

Conditional distributions of the GT alleles, given other diallelic polymorphisms, were analyzed in the French and Japanese groups (figs. 3 and 4). Each individual variant showed a strong degree of association with GT alleles that was very similar in both populations and in hypertensive and normotensive subjects (data not shown). The highest associations were observed for M174, exhibiting 91% and 95% of $(GT)_{14}$ in French and Japanese, respectively, and for G(Int3), exhibiting 88% and 84% of $(GT)_{>20}$ in French and Japanese, respectively. Only the T(−776) variant showed a different pattern of linkage disequilibrium, mainly associated with $(GT)_{17}$ in French and with $(GT)_{\leq 13}$ in Japanese.

Since there was a significant association between T235 and hypertension, it was important to examine the conditional distributions of GT alleles within haplotypes carrying either M235 or T235. Whereas the distribution of the GT alleles for T235 was almost uniform, 65% of M235 genes exhibited allele $(GT)_{16}$ in CEPH individuals and French controls, as a consequence of the strong linkage disequilibrium between these two polymorphisms. In Japanese individuals, 38%, 21%, and 19% of M235 genes exhibited the $(GT)_{16}$, $(GT)_{17}$, and $(GT)_{13-14}$ alleles, respectively. These patterns of linkage disequilibrium also were observed in hypertensive individuals (data not shown).

The 235T/A-6 Allele—Ancestral Form of the AGT Genes

The associations between the different polymorphisms of the human AGT gene are summarized in figure 5. The broad, uniform distribution of GT alleles, as well as the presence of multiple site polymorphisms on haplotypes carrying both T235 and A-6, suggest that T235 and A-6 may mark the original form of the gene. This interpretation is supported further by the presence of this form of the gene in all primate species examined (Inoue et al. 1997).

The patterns of association exhibited by a number of diallelic polymorphisms suggest that molecular variants

Table 6

Angiotensinogen Haplotypes

	Status of Allele[b]				
Haplotype[a]	T235	M174	T-776	A-793	C-20
H1	−	−	−	−	−
H2	+	−	−	−	−
H3	+	−	+	−	−
H4	+	−	−	+	−
H5	+	−	−	−	+
H6	+	+	−	−	+

[a] Only informative haplotypes have been mentioned.
[b] A plus sign (+) denotes presence of allele; and a minus sign (−) denotes absence of allele.

Table 7

Haplotype Frequencies in Hypertensives and Controls

	Caucasians			Japanese		
	Frequency			Frequency		
Haplotype	Hypertensives (n = 914)	Controls (n = 644)	χ_1^2	Hypertensives (n = 176)	Controls (n = 198)	χ_1^2
H1: M235	.540	.633	13.88 ($P < .001$)	.091	.242	15.08 ($P < .001$)
H2: T235	.101	.079	2.32	.426	.328	3.81 ($P < .05$)
H3: T235+T-776	.073	.031	12.78 ($P < .001$)	.074	.101	.85
H4: T235+A-793	.124	.085	6.03 ($P < .01$)	.182	.146	.85
H5: T235+C-20	.048	.053	.16	.131	.101	.81
H6: T235+M174+C-20	.109	.118	.77	.097	.081	.29

have arisen by mutation on ancestral genes carrying T235 and a particular GT allele. This pattern of genetic diversity suggests a development by spatial divergence in subdivided populations, rather than by temporal divergence in a homogeneous founder population. It is intriguing that the M235 variant, which occurs mainly on $(GT)_{16}$, is strongly associated also with the G-6 substitution. The persistence of this association may have arisen by chance, or it may represent a coordinated response to natural selection.

Discussion

The AGT gene constitutes, so far, the only locus that consistently has been linked to essential hypertension in humans (Jeunemaitre et al. 1992; Caulfield et al. 1994, 1995). The fact that this linkage was established in hypertensive sibling pairs and families not selected for any intermediate phenotype other than BP itself indicates that the AGT locus must be implicated in the pathogenesis of the disease in a substantial proportion of these families. Given the high prevalence of hypertension, predisposing mutations in the AGT gene should be present at a rather high frequency in the population. Association with the common marker M235T suggests that one predisposing mutation accounts for a large proportion of the risk mediated by AGT. This hypothesis is reinforced by the association of M235T with increased plasma angiotensinogen levels (Jeunemaitre et al. 1992, 1993; Bloem et al. 1995) and by the importance of renin-substrate concentration to both plasma renin activity (Gould and Green 1971) and BP homeostasis (Gardes et al. 1982; Kim et al. 1995).

This study provides further statistical evidence for the association between the T235 allele and essential hypertension in Caucasians. This result was obtained on both a large number (n = 477) of hypertensive probands of hypertensive families and a large number (n = 364) of carefully selected normotensive subjects without family history of hypertension. The increased frequency of T235 in hypertensives (.47 vs. .38, $P < .001$) was comparable to that previously observed in the series of 217 cases and 232 controls coming from Utah and Paris (Jeunemaitre et al. 1992). Since some cases-control studies provided no support for the association between T235 and hypertension, it probably is crucial here to emphazise the importance of sample size when one wants to test such association in a complex disease such as hypertension. Indeed, a common feature of these negative studies is their small sample size and/or the uncertain or unusual ascertainment of either cases or controls (Bennett et al. 1993; Barley et al. 1994; Caulfield et al. 1994; Fornage et al. 1995). To replicate the initial difference (Jeunemaitre et al. 1992), of allele frequencies, between hypertensive and normotensive Caucasians (.47 vs. .36) with an 80% power, $\geqslant$400 cases and $\geqslant$400 controls would be required, if a codominant effect of the T235 allele is assumed, thereby confirming that small samples lack the statistical power required to detect small statistical differences. In the Japanese, the reduced heterogeneity of the pathophysiology of hypertension could be due to a more homogeneous genetic and environmental background of the population (Hata et al. 1994; Kamitani et al. 1994; Iwai et al. 1995; Nishiuma et al. 1995).

Generation of haplotypes is a powerful method for testing the hypothesis that a specific allele mediates predisposition, and it explains the observed association of the T235 allele with hypertension. Two types of genetic polymorphism can be applied to generate haplotypes: (1) diallelic polymorphisms resulting from point mutations and (2) multiallelic variation associated with a variable number of simple-sequence tandem repeats. Several arguments can be proposed in support of the use of a battery of diallelic series to partition T235 haplotypes. First, there is ample evidence that, on an evolu-

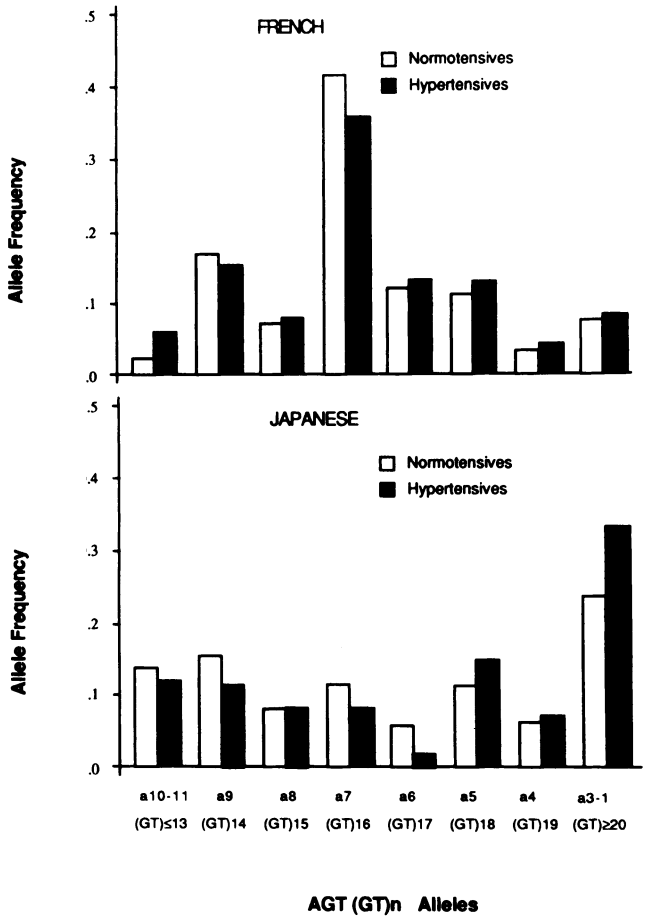

Figure 2 Distribution of AGT microsatellite alleles in French (*top*) and Japanese (*bottom*) hypertensives and normotensives. Alleles are numbered a1–a11, according to the classification adopted by Kotelevtsev et al. (1991) as well as by direct reference to the number of GT repeats in each allele. A significant difference was observed between French normotensive and hypertensive subjects (χ^2 [7 df] = 21.56, $P < .01$), mainly because of the decrease in $(GT)_{16}$ in hypertensives compared with controls (.358 vs. .416). A marginally significant difference was observed between normotensive and hypertensive Japanese (χ^2 [7 df] = 14.1, $P < .05$), explained mainly by a tendency toward longer alleles, $(GT)_{\geq 17}$, in hypertensives.

tionary time scale, single nucleotide substitutions are much more stable than are simple-tandem-repeat sequences (Stallings et al. 1991). For a common risk factor, a multiallelic series may dissipate statistical power, through both the generation of a large number of df and the functional mutation into a number of marker alleles (Hyer et al. 1991). Second, several arguments support the hypothesis that the T235 allele marks the original form of the gene and that M235 is the neomorph: (1) the strong linkage disequilibrium between M235 and one of the alleles of the AGT microsatellite marker, compared with the quasi-uniform distribution of the GT-repeat alleles among T235 genes; (2) the higher frequency of the T235 allele in three ethnic groups (Africans, Japanese, and Indian Americans), compared with only one group (northern Europeans) in which the M235 allele is more frequent (Jeunemaitre et al. 1992; Hata et al. 1994; Rotimi et al. 1994; Iwai et al. 1995; Schmidt et al. 1995); and (3) the homozygosity, for T235, in 20 chimpanzees (personal results) and all primate monkeys (gorilla, orangutan, gibbon, macaque, and baboon) analyzed by Inoue et al. (1997). Thus, an accumulation of nucleotide substitutions would be more likely to happen in the T235 allele, with its longer evolutionary time span. Consequently, the primary emphasis of our inquiry was haplotypes of T235 that are derived from diallelic series.

We extensively tried to address this question by identifying new polymorphisms at the AGT locus. A total of 10 diallelic polymorphisms were genotyped in a large series of hypertensives and controls originating from two separate ethnic origins. The linkage disequilibrium observed between these polymorphisms was very similar in both Caucasians and Japanese and led invariably to a parsimonious set of five common haplotypes of T235. No special haplotype consistently accounted for this association in both groups. However, the frequencies of the two haplotypes bearing the −776 and −793 polymorphisms were found to be statistically different between hypertensive and normotensive Caucasians. These two particular haplotypes could be in linkage disequilibrium with some mutations, present mainly in Caucasians, that would affect either the expression or the function of angiotensinogen.

Different arguments make this hypothesis unlikely. First, although not all mutations led to detectable conformation polymorphisms, only three rare mutations in the coding sequence—one at the cleavage site for renin (Inoue et al. 1995) and two others, at the 209 and 211 residues and each detected in one African Carribean family (Hixson and Powers 1995)—have been reported, so far, in the literature, in comparison with our initial study (Jeunemaitre et al. 1992). Second, we could not identify any additional mutation when all exons, splice junctions, and the 3′ UTR of the gene were sequenced in 16 individuals homozygous for the T235 allele. Third, substitutions at the −776 and −793 sites are not part of a theoretical consensus sequence that could play a role in angiotensinogen expression, although the case of this latter variant is more complex, since it is combined with two other substitutions (−1074 and −532). Little is known, however, about the transcriptional factors that bind to the AGT promoter, and the possibility that AGT expression may vary either because of these variants or because of other mutation(s) located farther upstream of the AGT gene cannot be ruled out. Finally, in the case of a true association, one would have to postulate that the haplotypes H3 (−776 and 235T) and H4 (−793C and 235T) do constitute different alleles predis-

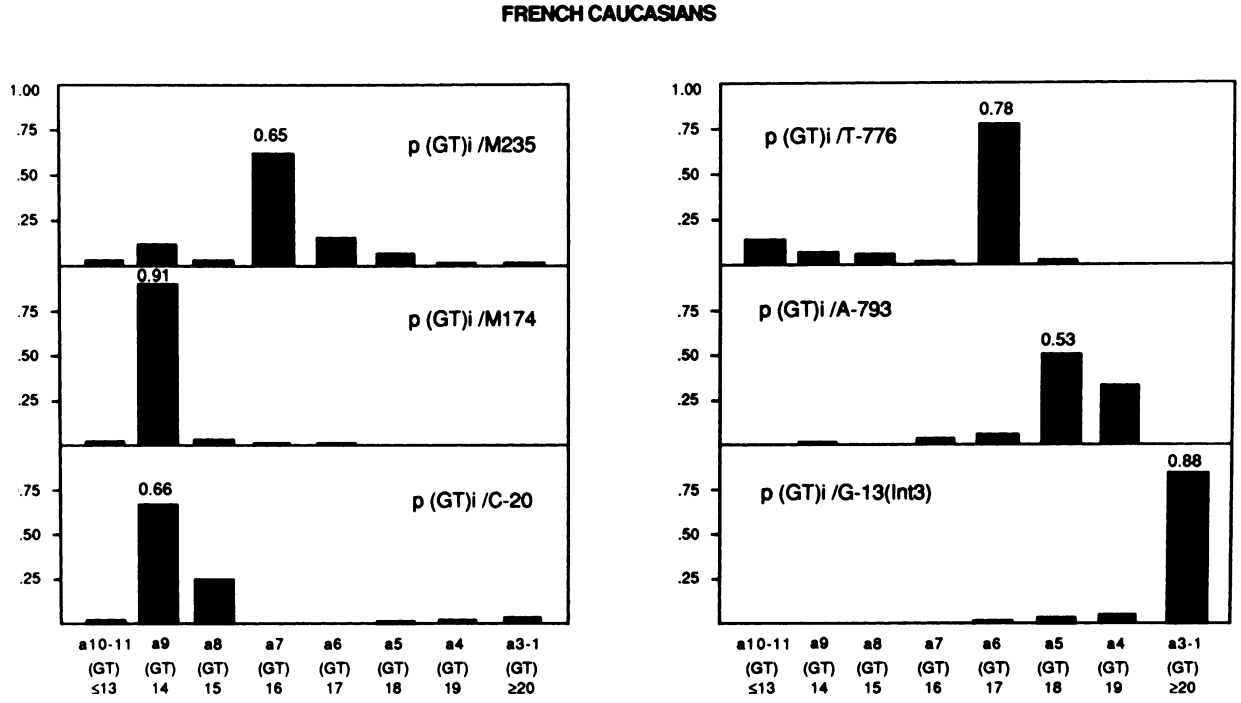

Figure 3 Conditional distributions of the AGT microsatellite alleles, according to each diallelic polymorphism in French Caucasians. For each of the diallelic polymorphisms, the conditional probability of each of the alleles of the AGT microsatellite is represented. For example, a strong linkage disequilibrium was observed, in both French and Japanese populations, between M174 and $(GT)_{14}$.

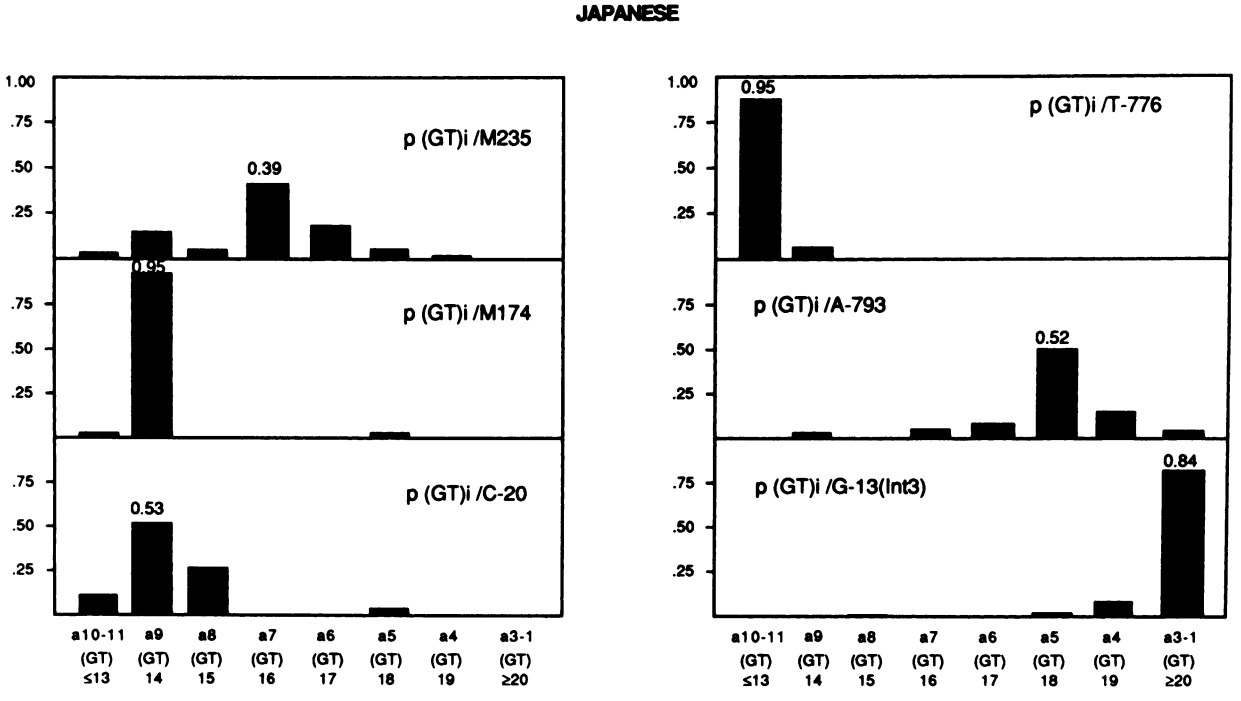

Figure 4 Conditional distributions of the AGT microsatellite alleles, according to each diallelic polymorphism in Japanese

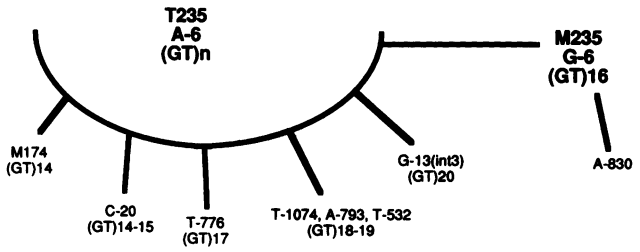

Figure 5 Diversity of the different alleles originating from a common ancestral haplotype (T235/A-6). Each of the diallelic polymorphisms originates on a common ancestral haplotype (T235/A-6), accompanied by a specific (GT) repeat allele, suggesting an evolution in space rather than in time. The M235 is one of those polymorphisms, specifically associated with the G-6 substitution in the promoter region.

posing to human hypertension in Caucasians but not in Japanese. Since the T235 allele was increased to the same extent in both populations, it would imply an unlikely genetic or environmental interaction specific to these haplotypes and to Caucasians.

Two reports have suggested the existence of specific alleles of the AGT gene that predispose to high BP. In their first study, involving 63 Caucasian families recruited in London, Caulfield et al. (1994) reported a surprisingly strong association between alleles of the AGT-GT repeat and essential hypertension. In particular, the most common allele in the reference population, a7 or $(GT)_{16}$, occurred at much lower frequency in the 63 hypertensive probands than in the 80 controls (.07 vs. .31), whereas allele a6 or $(GT)_{17}$, exhibited the opposite trend (.36 vs. .17). These results would lead to the hypothesis that two factors, one protective and the other predisposing, occur in disequilibrium with two successive alleles of the GT repeat. There are several arguments against such a complex genetic hypothesis: (1) it is not supported either by our initial study (Jeunemaitre et al. 1992) or by the present study, each of which was performed on a much larger (greater than fivefold) number of control and hypertensive subjects; (2) the only difference in frequency of the GT alleles, observed in both Utah and Paris subjects, was a decrease in the most common allele, a7 or $(GT)_{16}$, in hypertensives compared with normotensives (.34 vs. .41); (3) this difference is predicted by the linkage disequilibrium between M235 and $(GT)_{16}$ ($P[M235/(GT)_{16}] = .92$) in those populations; and (4) the linkage disequilibrium between M235 and $(GT)_{16}$ also occurs in Japanese subjects ($P[M235/(GT)_{16}] = .81$). The results obtained from Caulfield et al. (1994) therefore are inconsistent with the absence of a difference, in M235 frequency, between cases and controls in that particular study. The second study (Caulfield et al. 1995), which found marked differences in allele a8 and a9 frequency of the AGT-GT repeat in a limited number of African Carribean normotensive and hypertensive subjects, would lead to the same complex genetic hypothesis.

One significant outcome of our search for a causative mutation at the AGT locus was the finding that a polymorphism occurring six residues upstream from the initiation site of transcription was in very strong linkage disequilibrium with the polymorphism at residue 235. The frequencies of the A-6 and the T235 alleles are almost identical, with >97% concordance between the two substitutions, in both the French and the Japanese groups. As a consequence, all statistical associations observed—and interpretations proposed—for T235 directly extend to A-6. Although the Met→Thr substitution at position 235 alters the immunological recognition of the protein (Cohen et al. 1996), expression studies do not demonstrate any difference, in glycosylation, secretion, or enzymatic properties, between the two recombinant angiotensinogens (Inoue et al. 1997). By contrast, the region encompassed by positions −25 and −1 of the human AGT gene represents a transcriptionnally important cis-acting sequence (Yanai et al. 1996). However, the role of this region in AGT transcription could be complex, since different nuclear factors seem to bind at the 5′ versus 3′ parts of this cis-acting element (Yanai et al. 1996; Inoue et al. 1997). Interestingly, in vitro experiments demonstrate that the G→A substitution at position −6 affects the basal transcription rate of the AGT gene (Inoue et al. 1997). Thus, this substitution actually could represent the causative mutation explaining the association between T235 and both increased plasma angiotensinogen and, consequently, hypertension.

Until the development of genetically selected strains of animals, humans appeared to be a unique species in their apparent predisposition to hypertension, suggesting a specific interaction between genetic factors and the variation of human environment (McCarron et al. 1983). On the basis of our data, it is tempting to speculate about the role of different angiotensinogen alleles in this interaction. Through the phylogenetic history of the vertebrates, it has become clear that the renin angiotensin system has played a key role in the adaptation of living creatures to their salt environment, from marine to freshwater milieu, from aquatic to terrestrial life, maintaining BP and sodium balance through vasoconstriction and salt retention (Henderson and Deacon 1993). It is very likely that, several million years ago, the diet of hominoids was almost exclusively vegetarian (Eaton and Conner 1985), thus containing a very low level of sodium and a high potassium intake, which can constitute a strong selective environment. That selection pressure could have favored salt-retention mechanisms already developed at earlier stages of phylogeny, resulting in the selection of alleles resulting in an optimal

salt reabsorption (Denton 1984). This environmental stressor could have been even stronger for the first ancestors of human species, because of the change to the environment (from forest to savana) and to the required adaptation to upright posture. The angiotensinogen allele would be one of these genes leading to increased sodium reabsorption and increased BP. In this respect, it is interesting to note that the T235T/A-6 allele is found in chimpanzees, in which an increased salt intake causes a large rise in BP (Denton et al. 1995). More recently, increased availability and use of salt may have allowed genetic variants with a lesser effect on sodium retention and on BP to accumulate, resulting in the development of salt resistance. Nowadays, the analysis of different living human groups shows not only different frequencies of the T235 allele, according to their diversity and history, but also the residual effect of this gene on BP in a modified environment.

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