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Genetics

Genetic control of blood pressure and the angiotensinogen locus

(essential hypertension/quantitative genetic trait/gene targeting/gene disruption/gene duplication)

HYUNG-SUK KIM*†, JOHN H. KREGE*†‡, KIMBERLY D. KLUCKMAN*, JOHN R. HAGAMAN*, JEFFREY B. HODGIN*, CHRISTOPHER F. BEST§, J. CHARLES JENNETTE*, THOMAS M. COFFMAN§, NOBUYO MAEDA*, AND OLIVER SMITHIES*¶

*Department of Pathology, University of North Carolina, Chapel Hill, NC 27599-7525; ‡Department of Internal Medicine, University of North Carolina, Chapel Hill, NC 27599; and §Department of Internal Medicine, Durham Veterans Affairs Medical Center, Durham, NC 27710

Contributed by Oliver Smithies, November 30, 1994

ABSTRACT **Variants of the human angiotensinogen gene have been linked in some studies to increased circulating angiotensinogen levels and essential hypertension. To test for direct causality between genotypes at the angiotensinogen locus and blood pressures, we have studied mice carrying zero, one, two, three, or four functional copies of the murine wild-type angiotensinogen gene (*Agt*) at its normal chromosomal location. Plasma angiotensinogen levels increase progressively, although not linearly, from zero in the zero-copy animals to 145% of normal in the four-copy animals. Mice of all genotypes are normal at birth, but most zero-copy animals die before weaning. The kidneys of the zero-copy animals show pathological changes as adults, but the kidneys are normal in the other genotypes. One adult zero-copy male tested was fertile. The blood pressures of the one-copy through four-copy animals show significant and almost linear increases of approximately 8 mmHg per gene copy despite their normal compensatory mechanisms being intact. These results establish a direct causal relationship between *Agt* genotypes and blood pressures.**

Essential hypertension affects about 20% of adults in the United States. Genetic and environmental factors are important in its etiology (1). The renin–angiotensin system is one of the major systems regulating blood pressure and sodium balance, and many elements in it are potential determinants of hypertension. Plasma levels of angiotensinogen (AGT) have received particular attention for several reasons (2). First, plasma AGT levels and diastolic blood pressures are correlated in some patients (3), and associations of AGT levels and hypertension have been demonstrated in families (4, 5). Second, infusion of AGT into sodium-depleted but not sodium-repleted rats raises their blood pressures (6), while administration of antibodies against AGT lowers pressures (7). Third, transgenic mice expressing high levels of rat AGT have elevated blood pressures (8, 9). Fourth, human plasma AGT levels are near the K_m of renin (10), so that variations in AGT levels will affect the rate at which renin converts AGT to angiotensin I.

At the genetic level, cosegregation of variants at the human angiotensinogen locus (*AGT*) and hypertension has recently been described. In human hypertensive siblings, Jeunemaitre *et al.* (2) showed that a specific variant, M235T, was significantly linked to hypertension and was also associated with a modestly elevated plasma AGT concentration (about 120% of normal); they accordingly proposed that some variants of the *AGT* gene lead to an increase in AGT levels and thereby eventually to increased blood pressure. Caulfield *et al.* (11) have confirmed significant linkage between hypertension and chromosomal regions including and close to the *AGT* gene, but they could not confirm association with the M235T marker. The *AGT* gene may be important in other hypertensive diseases. Thus, Ward *et al.* (12) have demonstrated association of pre-eclampsia with the M235T variant; and Arngrimsson *et al.* (13) have found cosegregation of a marker in the 3′ flanking region of *AGT* with pre-eclampsia. These various studies show that some DNA sequences at or near the *AGT* locus affect blood pressure, but direct proof that variants of the *AGT* gene cause changes in blood pressure is lacking.

Smithies and Kim have recently described a way of using gene targeting to analyze quantitative genetic traits in living animals (14). After identification of a candidate gene, mice carrying an increased or decreased number of copies of the candidate gene are generated without altering the chromosomal location or regulatory elements of the gene; levels of gene expression above or below normal can be achieved in this way. The effects of these quantitative changes on the phenotype of interest can then be evaluated in a manner that eliminates the influences of any other genes (linked or unlinked). We here apply this approach to establish a direct causal relationship between *Agt* genotypes and blood pressures.

MATERIALS AND METHODS

Gene Targeting. Cloning of the *Agt* gene from strain 129 mouse DNA and the use of gene targeting to generate mice with a tandem duplication of the wild-type gene at its normal chromosomal location have been described previously (14). To disrupt the *Agt* gene, the targeting plasmid illustrated in Fig. 1*A* was made, using an 8-kb fragment of strain 129 genomic DNA containing exons 2 and 3 of the gene. The 5′ end of the fragment is a *Hin*dIII site in intron 1; the 3′ end of the fragment is an *Mbo* I site about 1 kb downstream of exon 3. Within exon 2 of the fragment, a *Stu* I/*Bam*HI region that codes for 114 amino acids (including all angiotensin I-coding sequences) was replaced with a neomycin-resistance gene driven by the phosphoglycerate kinase promoter (16). A copy of the *Herpes simplex* thymidine kinase gene was positioned downstream of the longer arm of homology to allow positive–negative selection (17). The targeting plasmid was linearized with *Hin*dIII and was electroporated into a subline, BK4, of the embryonic stem cell line E14TG2a (18), which is derived from mouse strain 129. The cells were cultured as described (19). Potential recombinants were identified by a PCR recombinant fragment assay (15) using the primers a and b illustrated in Fig. 1. Primer a is 5′-TGCACGGGTTCTGAGGATCCA-3′; b is 5′-TAAAGCGCATGCTCCAGACT-3′. Southern blot analysis of cell DNA digested with *Xba* I was used to confirm the correct modification. Targeted cells containing the disrupted *Agt* gene were injected into blastocysts to generate male chimeras, which were then mated with inbred females of strain C57BL/6J (B6).

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Abbreviations: AGT, angiotensinogen; B6, C57BL/6J (mouse strain).

†H.-S.K. and J.H.K. contributed equally to this work.

¶To whom reprint requests should be addressed.

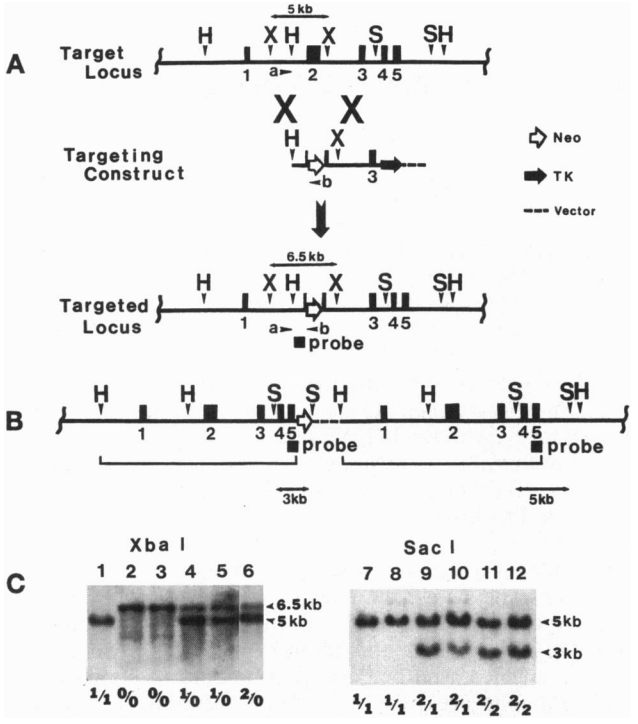

FIG. 1. (*A*) Targeted disruption of the mouse *Agt* gene. Exons 1 to 5 are shown as black boxes. Relevant restriction sites are *Hin*dIII (H), *Sac* I (S), and *Xba* I (X). A selectable marker gene conferring neomycin resistance was inserted into exon 2 at the same time as deleting the DNA coding for 114 amino acids of the mature AGT protein, including all angiotensin I-coding sequences and 17 amino acids of the leader. Arrowheads a and b indicate primers that, after PCR, yield a 1.2-kb fragment diagnostic of targeting (15). (*B*) Targeted duplication of the mouse *Agt* gene (14). Horizontal brackets indicate the duplicated region. (*C*) Southern blots of tail DNA from animals of the six *Agt* genotypes. Samples 1–6 were digested with *Xba* I; samples 7–12, with *Sac* I. The black squares in *A* and *B* indicate the two probes used.

F_1 progeny heterozygous for the disrupted *Agt* gene (1/0) and for the duplicated *Agt* gene (2/1) were mated to generate F_2 animals with all possible *Agt* genotypes.

Genotypes of Mice. Genotypes were determined by Southern blots of *Xba* I- or *Sac* I-digested tail DNA or by PCR with primers a and b (Fig. 1), except that 2/1 and 2/2 were distinguished by scoring for a simple sequence polymorphism, D8MIT56 (20), linked to the *Agt* locus (see *Results and Discussion*). To do this, PCR of tail DNA was performed with the published primers (20); PCR was followed by acrylamide gel electrophoresis to determine the presence of a 182-bp fragment characteristic of strain 129 and/or a 162-bp fragment characteristic of strain B6. The presence of only the 182-bp fragment or of both fragments was respectively scored as 2/2 or 2/1.

Plasma AGT and Renin Levels. Steady-state plasma AGT levels were determined by a radioimmunoassay for angiotensin I as described (14). Renin levels were measured identically, except that 2 μl of plasma from a nephrectomized rat was added as a source of AGT in place of 4 μl of kidney extract as a source of renin.

Blood Pressure Measurements. Blood pressures were measured in young adult mice (mean age 100 days; range 73–134 days) by a noninvasive tail-cuff method and by intra-arterial catheterization. Krege *et al.* (21) have described and compared the methods and have shown that their results are well correlated. The tail-cuff method uses a four-channel, computerized, automated system designed and built by John E. Rogers and James P. Rogers (Visitech Systems, Raleigh, NC). After training sessions on at least 7 days, the tail-cuff systolic blood pressure for each animal (without the investigator knowing its genotype) was taken as the mean of blood pressures determined during at least five subsequent daily sessions. For intra-arterial blood pressures, carotid catheterization was performed as described (21) while the animals were under general anesthesia (ketamine 2 mg and xylazine 0.2 mg per 25–30 g of body weight). At least 4 hr after surgery, blood pressure waveforms from quietly resting mice were obtained for about 10 min. Mean arterial pressure was calculated as the sum of two-thirds the mean of all waveform troughs and one-third the mean of all waveform peaks. Requirements for inclusion of intra-arterial data were a pulsatile waveform, minimal heart rate of 500 beats per minute, and survival of the mouse until the following day. The investigators knew the genotypes of 9 animals used for the intra-arterial measurements but did not know the genotypes of the other 13 animals; the data obtained in the "blinded" fashion were indistinguishable from the combined data, which we present here.

All experiments were conducted in accordance with institutional guidelines for the University of North Carolina at Chapel Hill and Duke University Medical Center. Mice were fed R-M-H 3500 autoclavable chow (Agway, Syracuse, NY).

Kidney Function Studies. Renal plasma flows and glomerular filtration rates were respectively determined from *p*-aminohippuric acid (PAH) and inulin clearances as described (22).

Histological Analysis. Tissues were collected and fixed in 10% neutral-buffered formalin immediately after the animals were sacrificed. Staining was with hematoxylin and eosin.

RESULTS AND DISCUSSION

Gene Disruption and Gene Duplication by Targeting. We have used conventional gene targeting in embryonic stem cells derived from the mouse inbred strain 129 to *disrupt* the *Agt* gene and to delete the nucleotides that encode angiotensin I. The target gene (23), targeting construct, and resulting chromosome are illustrated in Fig. 1*A*. A male chimera generated from one of the targeted embryonic stem cell colonies transmitted the disrupted *Agt* gene to its offspring.

A method for *duplicating* genes by gap-repair gene targeting and its application to the mouse *Agt* gene have been described elsewhere (14). Fig. 1*B* illustrates the resulting chromosome. The whole of the gene, including all known regulatory sequences, is tandemly duplicated at its natural chromosomal locus together with about 3 kb of DNA 5′ to the start of transcription of the gene and about 200 bp 3′ to the poly(A) addition site. The extent of the duplication is illustrated by the heavy horizontal brackets in Fig. 1*B*.

Generation of Animals Having Various Numbers of Functional *Agt* Genes. Chimeric males carrying the disrupted or the duplicated *Agt* gene were mated with inbred strain B6 females. The resulting (129 × B6)F_1 mice are genetically identical except for their *Agt* genotypes and sexes. Three *Agt* genotypes are possible in these F_1 animals: 1/0 (one normal and one disrupted copy), 1/1 (normal), and 2/1 (one duplicated and one normal copy), corresponding to one, two, and three functional copies of the *Agt* gene.

When suitable F_1 pairs are bred, three additional *Agt* genotypes arise in F_2 mice: 0/0 (both copies disrupted), 2/0 (having one chromosome with the *Agt* gene duplicated and one with the gene disrupted), and 2/2 (both copies duplicated). All six *Agt* genotypes are distinguishable at the DNA level by using Southern blots (Fig. 1*C*) or PCR with the probes and primers illustrated in Fig. 1. However, Southern blots of DNA from 2/1 and 2/2 animals differ only in the relative intensities of autoradiographic bands, unless the diagnostic restriction enzyme sites lie outside the (18-kb) duplicated *Agt* gene region. We consequently prefer to distinguish these two genotypes by

scoring for a simple sequence fragment, D8MIT56 (20), which is closely linked to the *Agt* locus and which differs in length in strain B6 (160 bp) and strain 129 (182 bp). In our F_2 mice, the singleton *Agt* gene is always derived from strain B6, and the duplicated gene is always from strain 129. In the absence of crossovers, the 2/1 and 2/2 genotypes are therefore distinguishable by PCR for the simple sequence; 2/1 animals give both the 160-bp and the 182-bp fragments; 2/2 animals give only the 182-bp fragment. Crossovers between the *Agt* gene and D8MIT56 will cause errors in this typing procedure. However, the frequency of such crossovers must be small, since we have observed none in approximately 90 meioses in which they would have been identified by observing absence of the 129-derived fragment from animals having the duplicated gene.

To study the relative survival of pups with different numbers of functional copies of *Agt*, we collected data from 39 F_1 matings yielding over 240 weanlings. The results showed no significant deviations from Mendelian expectation in the survival to weaning of pups with one, two, three, or four functional copies of the *Agt* gene, but there was a highly significant ($P < 0.001$ by χ^2) deficiency of 0/0 pups (23 expected, 3 observed). We also found a high proportion of 0/0 pups among the pups that died before weaning. We therefore sacrificed a litter from a 1/0 × 1/0 cross immediately after birth and found that the three possible genotypes were represented in Mendelian proportions (3 were 0/0, 5 were 1/0, and 2 were 1/1). Thus, absence of a functional *Agt* gene is compatible with survival to birth, but postnatal survival is severely compromised.

Plasma AGT Concentrations Versus *Agt* Genotype. We have reported (14) the steady-state plasma AGT levels in (129 × B6)F_1 animals with one, two, and three functional copies of the *Agt* gene; they were, respectively, 35%, 100% (by definition), and 124% that of the normal two-copy (1/1) animals. [The absolute level of AGT (±SEM) in normal F_1 animals is 524 ± 30 nmol/ml.] The F_2 generation of animals allows analysis of zero-copy (0/0) and four-copy (2/2) animals. As expected, the zero-copy animals ($n = 2$) have no detectable AGT in their plasma. Four-copy animals (3 males and 3 females) have an average AGT level of 144.5% ± 4.7% of the normal two-copy (1/1, F_1) levels.

The F_2 generation also includes animals of the 2/0 genotype. These animals have a duplicated *Agt* locus on one chromosome 8 and a disrupted *Agt* gene on the other. In contrast, normal (1/1) animals have a singleton *Agt* gene on each parental chromosome 8. Comparisons between these two types of animal are therefore informative with respect to the function of the duplicated *Agt* gene. We found the steady-state plasma AGT levels in 2/0 F_2 animals (3 males and 3 females) to be 55.0% ± 1.5% of the normal 1/1 F_1 level. The AGT levels of the 1/0 F_1 animals are 35% of normal. Thus, the duplicated *Agt* locus leads to the synthesis of more AGT than does *one* normal singleton *Agt* gene, in confirmation of our earlier F_1 studies (14), but to less than can be achieved with *two* normal singleton genes.

Comment is required on the plasma AGT levels of the one-copy F_1 animals, which are 35% of normal, rather than the 50% level expected from a linear relationship between AGT level and gene copy number. We determined plasma renin levels in these animals and found them to be 250% ± 5% of normal ($n = 9$). This suggests that in these animals a higher than normal proportion of plasma AGT is being converted to angiotensin I.

Blood Pressures Versus *Agt* Genotype. We measured blood pressures in two ways (21): indirectly by a noninvasive computerized tail-cuff system and directly by catheterization of a carotid artery. Fig. 2 *A* and *B* presents the tail-cuff pressures (23 males and 20 females) and the mean arterial pressures (22 *different* males) of F_2 animals having various numbers of

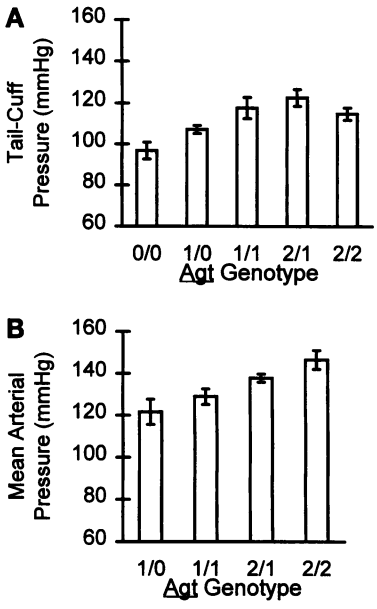

FIG. 2. Blood pressures versus *Agt* genotype (1 mmHg = 133 Pa). (*A*) Tail-cuff pressures of F_2 mice having zero ($n = 3$), one ($n = 11$), two ($n = 10$), three ($n = 8$), or four ($n = 11$) copies of the *Agt* gene. (*B*) Mean arterial pressures of F_2 mice having one ($n = 7$), two ($n = 9$), three ($n = 3$), or four ($n = 3$) functional copies of the *Agt* gene.

functional *Agt* genes. The overall picture is clear. Blood pressures increase with increasing numbers of functional *Agt* genes. Several tests establish the statistical significance of these results. Regression analysis with mean arterial pressure as the dependent variable shows a highly significant effect of *Agt* copy number (correlation coefficient 0.60; slope 8.3 ± 2.3 mmHg per copy; $P < 0.01$). Regression analysis with tail-cuff blood pressure as the dependent variable also indicates a significant effect of *Agt* copy number (correlation coefficient 0.38; slope 3.7 ± 1.4 mmHg per copy; $P < 0.02$). Because the two groups of animals used in these experiments were independent, the probability of the combined results occurring by chance can be calculated from the individual probabilities as described by Fisher (24); the combined probability is <0.0005.

Inclusion in the tail-cuff group of animals of both sexes allows the use of two-factor analysis of variance to assess any effects of gender. This analysis shows that neither gender ($P = 0.57$) nor the interaction of gender and *Agt* copy number ($P = 0.11$) is significantly correlated with tail-cuff blood pressures.

The 2/0 F_2 animals had intra-arterial blood pressures (132 ± 3 mmHg, $n = 4$) that did not differ significantly ($P > 0.6$) from the pressures of 1/1 F_2 animals (129 ± 4 mmHg, $n = 9$).

Exclusion of the Effects of Linked Genes. Some, although not all, of our F_2 mice differ systematically *only* at the *Agt* locus. The one-copy (1/0) and three-copy (2/1) F_2 animals are of this category. Thus, the wild-type *Agt* allele plus neighboring linked genes in all the one-copy and three-copy F_2 animals are derived from strain B6; their other *Agt* allele (which differs in being disrupted or duplicated) plus neighboring linked genes are derived from strain 129. Analysis of the data from these one-copy and three-copy F_2 animals can therefore be used to exclude any possible effects of linked genes. (Unlinked genes segregate randomly in the F_2 generation and so do not differ systematically between these groups of animals.) We find, in agreement with the results from the complete set of F_2 animals, that the one-copy and three-copy F_2 animals show the same effect of *Agt* gene copy numbers on blood pressures. Thus, regression analysis of the intra-arterial data from the one-copy and three-copy F_2 animals showed a slope of 8.1 ± 4.0 mmHg per copy, $n = 10$, $P < 0.05$; similar analysis of the tail-cuff data from different one-copy and three-copy F_2

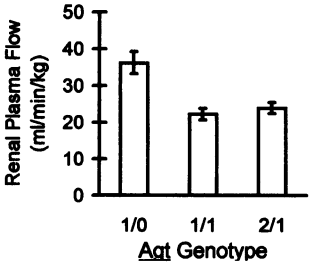

FIG. 3. Renal plasma flow versus *Agt* genotype. Renal plasma flows were measured by clearance of *p*-aminohippuric acid in F_1 mice having one ($n = 6$), two ($n = 9$), or three ($n = 6$) functional copies of the *Agt* gene (genotypes 1/0, 1/1, and 2/1).

animals showed a slope of 7.7 ± 2.1 mmHg per copy, $n = 19$, $P < 0.001$. The combined probability is <0.0005. Therefore, the demonstrated effects of *Agt* gene copy number on blood pressure are *independent of any other genetic differences* linked or unlinked to the locus.

Kidney Function. Because renal function has a strong impact on blood pressure, we performed renal studies on 1/0 (3 male, 3 female), 1/1 (5 male, 4 female), and 2/1 (3 male, 3 female) F_1 mice 208–250 days of age. Renal plasma flows were measured as clearances of *p*-aminohippuric acid. Fig. 3 presents the resulting data. Clearly, renal plasma flows in the 1/0 animals are substantially and significantly increased ($P < 0.005$) relative to the flows in two-copy (1/1) and three-copy (2/1) animals, which do not differ significantly. Since the blood pressures of the 1/0 animals are less than normal, these findings suggest that their renal vascular resistance is decreased relative to normal. As judged by inulin clearances (data not shown), the one-copy and three-copy mice have the same glomerular filtration rates as normal two-copy mice.

Zero-Copy Animals. The birth and occasional survival to adulthood of 0/0 animals completely lacking endogenous AGT is remarkable in view of the many effects known to be mediated by angiotensin II. Also, one 0/0 male tested produced three litters (average 8.3 pups per litter) of normal size, demonstrating that angiotensin is not essential for male fertility in mice.

The newborn 0/0 animals ($n = 2$) do not have any obvious pathological changes, either at the gross level or microscopically. At present, we cannot determine whether this is because

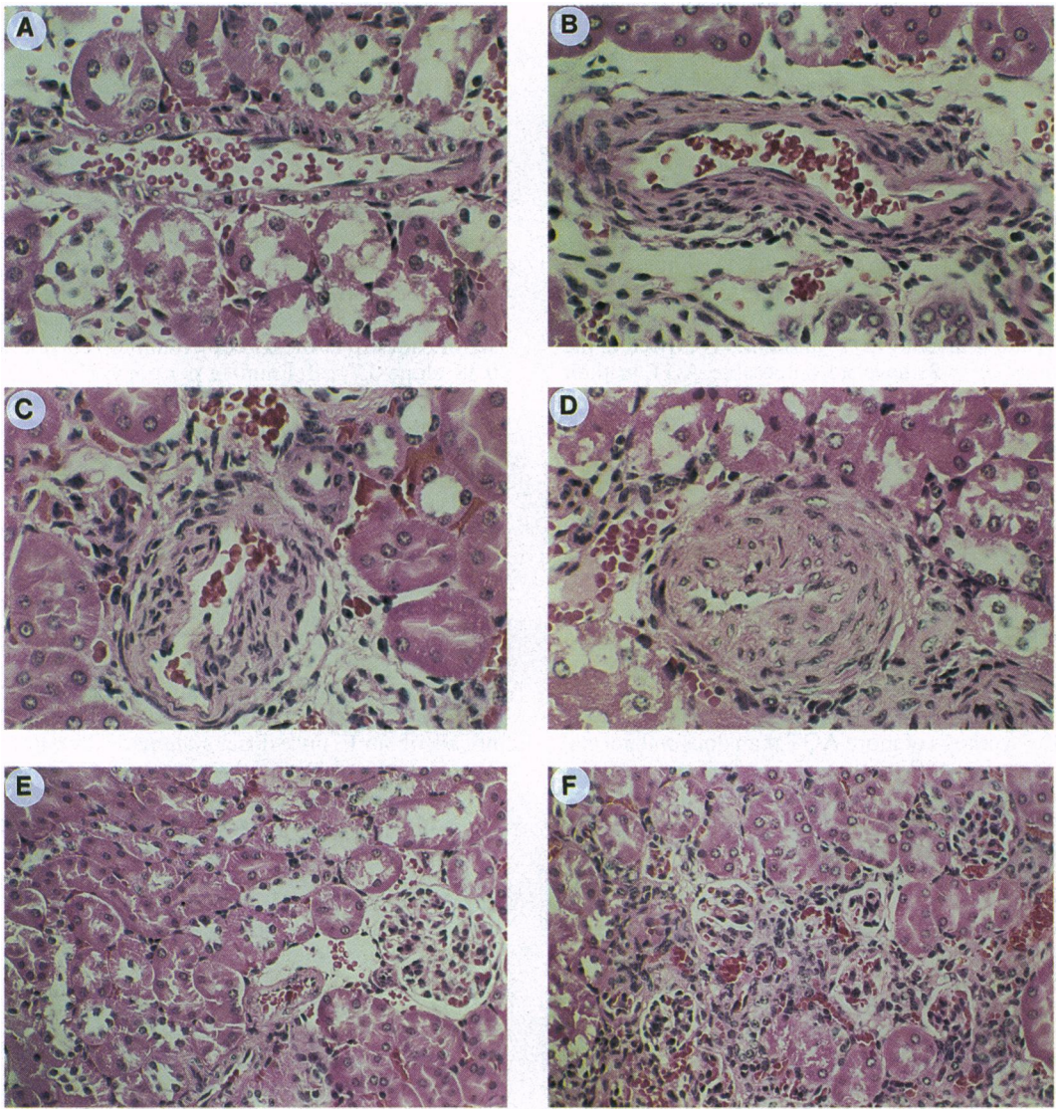

FIG. 4. Histology of kidneys from normal and zero-copy animals. (×350 in *A–D* and ×175 in *E* and *F*.) (*A*) Normal artery in wild-type kidney. (*B–D*) Arteries in zero-copy adult kidneys, showing different degrees of abnormality. (*E*) Normal cortex in wild-type kidney. (*F*) Representative area of pathological cortex from a zero-copy adult kidney.

endogenous AGT or its products are not required for normal development or because of transfer of maternally derived factors across the placenta. The kidneys of adult 0/0 animals (n = 3, 100–120 days of age) show pathological changes. Fig. 4*A* shows a normal kidney artery in oblique section. Fig. 4 *B–D* presents representative vessels from a 0/0 animal showing thickened walls caused by an increased number of cells, which appear disorganized; these changes are most pronounced in interlobular arteries. This arterial wall thickening in the 0/0 animals could be the result of an as-yet-unknown response to the absence of AGT and its products and/or to their low blood pressures. The kidneys of the 0/0 animals also have generalized cortical thinning with focal areas of severe atrophy (Fig. 4*F*), compared with normal animals (Fig. 4*E*). The atrophied areas show shrinkage and loss of tubules, interstitial fibrosis, and interstitial infiltration of chronic inflammatory cells, perhaps resulting from inadequate blood flow through arteries such as in Fig. 4*D*. The kidneys of the one-, two-, three-, and four-copy young adult animals (n = 24) appeared normal, except for one wild-type 90-day-old male that developed an isolated case of severe glomerulosclerosis.

Conclusions. Our observations on the effects of *Agt* gene expression on blood pressures can be summarized as follows. Plasma steady-state AGT levels increase progressively but not linearly with *Agt* gene copy number, ranging from zero in the zero-copy animals to approximately 145% normal in the four-copy animals. Tail-cuff systolic pressures and intracarotid mean blood pressures measured on unanesthetized F_2 animals increase progressively and significantly with increases in the number of functional *Agt* gene copies, with a change in mean arterial pressure of approximately 8 mmHg per copy. The same progressive change is seen in mice that differ systematically only at the *Agt* locus.

These observations lead to the conclusion that different levels of function of the native *Agt* gene, comparable in relative degree to those seen in human subjects, directly *cause* significant differences in the blood pressures of mice, even though they have all their homeostatic compensatory systems intact. The relevance of our findings to hypertension requires comment. As discussed in the introduction, a case could already be made that sequences at or near the human *AGT* locus affect blood pressure and are correlated with AGT levels. Missing was a direct proof of causality. Our animal data supply this proof for mice; variants of the mouse *Agt* gene that alter plasma AGT levels directly affect blood pressure.

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