THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 20, Issue of May 14, pp. 14210–14217, 1999

*Printed in U.S.A.*

# Homeostasis in Mice with Genetically Decreased Angiotensinogen Is Primarily by an Increased Number of Renin-producing Cells*

(Received for publication, January 22, 1999, and in revised form, March 1, 1999)

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**Here we investigate the biochemical, molecular, and cellular changes directed toward blood pressure homeostasis that occur in the endocrine branch of the renin-angiotensin system of mice having one angiotensinogen gene inactivated. No compensatory up-regulation of the remaining normal allele occurs in the liver, the main tissue of angiotensinogen synthesis. No significant changes occur in expression of the genes coding for the angiotensin converting enzyme or the major pressor-mediating receptor for angiotensin, but plasma renin concentration in the mice having only one copy of the angiotensinogen gene is greater than twice wild-type. This increase is mediated primarily by a modest increase in the proportion of renal glomeruli producing renin in their juxtaglomerular apparatus and by four times wild-type numbers of renin-producing cells along afferent arterioles of the glomeruli rather than by up-regulating renin production in cells already committed to its synthesis.**

An essential feature of complex organisms is the ability to maintain near constancy of their internal environments. Homeostasis is maintained by the operation of sophisticated systems that permit desirable physiological changes in biological variables, but that also act homeostatically if external factors cause undesirable changes in the variables. Genetic heterogeneity, such as is inherent to all outbred species including humans, also tends to cause variation in the internal environment. Yet the extent and types of homeostatic changes induced by naturally occurring genetic differences have not received much attention. We have recently been carrying out experiments aimed at identifying genes whose quantitative expression affects an important biological variable, blood pressure. To this end, we have used gene targeting in mice to alter the number of functional copies of several candidate genes, and so to produce systematic changes in their expression of the same order of magnitude as those occurring naturally in humans. Since the resulting mice are in other respects wild-type, their homeostatic systems are intact, and the mice can be used to investigate what compensations have been induced by the genetically determined differences in expression of the "titrated" genes. Because the causative genetic changes are life long, any induced compensations are categorically comparable with the lifelong adjustments that individual humans make in adjusting to the genetic heterogeneity inherent to our species.

The renin-angiotensin system (RAS)[1] is critical for controlling blood pressure and salt balance in mammals. Angiotensinogen (AGT) is the sole source of angiotensin II (AngII), the major active peptide of the system. AGT is synthesized primarily in the liver and is secreted constitutively into the blood stream. It is the substrate for renin, a highly specific protease whose only known substrate is AGT. The majority of renin synthesis and secretion into the blood stream in normal mature animals is by modified smooth muscle cells in the juxtaglomerular apparatus (JGA) of the kidney. The action of renin on AGT produces the decapeptide angiotensin I (AngI), which has no significant cardiovascular activity. Angiotensin-converting enzyme, ACE, a dipeptidase present in the blood stream as a circulating protein and in tissues as a membrane bound protein, converts AngI to the vasoactive octapeptide AngII. Genetic heterogeneity has been demonstrated at the angiotensinogen locus in humans (1, 2), and two common alleles are associated with quantitative differences in the plasma concentration of AGT and with differences in blood pressure. In previous experiments (3, 4) quantitative differences in expression in mice of the angiotensinogen gene (*Agt*) have been shown to directly cause modest changes in blood pressure. Here we explore the long term homeostatic adjustments that occur in mice attempting to restore their blood pressures to normal in the face of inheriting below normal expression of the angiotensinogen gene (*Agt*).

## EXPERIMENTAL PROCEDURES

*Animals*—Except as indicated, all mice used were F1 hybrids between the inbred strains 129 and B6 with or without a disruptive mutation in the 129-derived copy of the *Agt* gene. The mutation in the *Agt* gene was generated in embryonic stem cells from the substrain 129/OlaHsd (5). Prior to the matings to produce the F1 hybrids, the *Agt* gene mutation had been maintained for several generations on the closely related substrain 129/J. The mice were fed regular chow and handled following the National Institutes of Health guidelines for the care and use of experimental animals.

*Protein Studies*—Blood samples were rapidly withdrawn from the descending aorta of mice after exposure to an atmosphere of $CO_2$ (less than 1 min from loss of consciousness to the end of collection). The blood was collected into ice-cold microcentrifuge tubes containing EDTA and was immediately centrifuged to isolate plasma. Plasma AGT and (active) renin concentrations were determined by radioimmunoassay as described previously (4). Plasma prorenin concentrations were determined after conversion to active renin by the trypsin-Sepharose 4B

* This work was supported by National Institutes of Health Grants GM20069 and HL49277 and a grant from the W. M. Keck Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: RAS, renin-angiotensin system; AGT, angiotensinogen; AngI, angiotensin I; AngII, angiotensin II; JGA, juxtaglomerular apparatus; ACE, Angiotensin-converting enzyme; RT-PCR, reverse transcription polymerase chain reaction; bp, base pair(s).

method (6). ACE activity was measured by the cleavage of a chromogenic tripeptide (7), using serum isolated from blood collected retroorbitally without anticoagulant.

*Plasma Peptide Measurement*—Blood handling and radioimmunoassays followed published methods (8, 9) with slight modifications. Peptides were extracted with ethanol as described in the assay procedure from the Nichols Institute (San Juan Capistrano, CA) using 600 $\mu$l of plasma pooled from three individuals matched by genotype and gender. The extracted peptide samples were divided into three equal portions and dried in a vacuum centrifuge. Single portions were used for measurement of AngI, AngII, or bradykinin. Recoveries of each peptide at completion of the extraction procedure were determined by $1^{125}$-labeled tracers to be approximately 80%. By using highly specific monoclonal antibodies for the measurements, the radioimmunoassays could be carried out without further separations. The radioimmunoassays were performed with commercially available kits for AngI (DuPont), AngII (Nichols), and bradykinin (Peninsula, Belmont, CA).

*RNA Isolation*—Tissues were rapidly dissected after withdrawing blood. Total RNA was isolated conventionally (10) using the TRI REAGENT™ procedure (Molecular Research Inc., Cincinnati, OH).

*Riboprobes and Sense RNA Preparation*—DNA fragments from the *Agt*, renin, and $\beta$-actin genes were prepared by PCR using strain 129/OlaHsd mouse genomic DNA as the template and the following probes designed from published sequences: a 390-bp exon 2 fragment from the *Agt* gene (11); a 290-bp exon 9 fragment from the mouse renin gene *Ren-1*$^{d}$ (12); and a 250-bp fragment from the mouse $\beta$-actin gene (13). A 418-bp fragment corresponding to nucleotides 2523–2931 of mouse ACE cDNA (14) was cloned after RT-PCR using total RNA from the lung as template. All of the fragments were subcloned into a Bluescript(KS) vector for *in vitro* transcription (15). $^{32}$P-Labeled antisense riboprobes were synthesized by the manufacturer's protocols using a MAXI script™ transcription kit (Ambion Inc., Austin, TX). Unlabeled sense RNAs were prepared with the same transcription system. The sense RNAs were gel-purified and stored at −70 °C.

*RNase Protection Assay*—The procedure described by Azrolan and Breslow (16) with minor modifications was used for RNase protection assay. All reactions were carried out in duplicate for each sample in all experimental groups. A standard curve was generated using sense RNAs.

*Primer Extension Analysis*—Primer preparation and primer extension analysis for the mouse renin genes were slightly modified versions of published procedures (17, 18). The primer was a 38-mer oligonucleotide complementary to *Ren-1*$^{C}$ mRNA from positions 1039 through 1076 of the cDNA sequence (19). We determined that the nucleotide sequence of this region is identical in the strain B6 *Ren-1*$^{c}$ gene and the strain 129 *Ren-1*$^{d}$ and *Ren-2* genes. Autoradiographic bands were quantitated with an NIH image computer program (version 1.55).

*RT-PCR Assay*—Quantitative RT-PCR (20) was used to assess expression of the gene coding for the type 1A receptor for AngII. Total RNA from the kidney was reversely transcribed to cDNA (21). A template plasmid for preparing the internal standard was constructed by making a 120-bp deletion (*Pvu*II/*Dra*I sites) in a *Plm*I/*Sac*I fragment from the mouse type 1A receptor gene. PCR primers specific for the type 1A gene were designed from sequences of the type 1A and 1B genes (22); they are 5′-ACGAGTCCCGGAATTACACG-3′ for the sense primer and 5′-GCGTGCTCATTTTCGTAGACAGG-3′ for the antisense primer. Competitive PCR was performed in the presence of an internal standard, yielding a 320-bp fragment. The RT product corresponding to the type 1A mRNA is 440 bp long. The amount of the full-length product relative to the internal standard was determined after hybridization to labeled full-length fragment as a probe.

*Renin Immunocytochemistry in Kidney Sections*—Immunohistochemical detection of renin was as described previously (23, 24). Briefly, after deparaffinization, 7-$\mu$m kidney sections were incubated with a polyclonal renin antibody (1:10,000, gift from Dr. Tadashi Inagami, Vanderbilt University, Nashville, TN). The high specificity and characterization of this renin antibody has been documented previously (25). Immunocytochemistry was done with kidneys from the *Agt* one-copy and wild-type mice. Two to four sections per kidney were examined by direct microscopic visualization. The total number of glomeruli, the number with renin-positive JGA, and the number with renin-positive cells upstream of the JGA were counted in each section. The percentage of renin-positive JGA was determined as (the number of renin-positive JGA in all sections) × 100/(the total number of glomeruli observed). The number of cells positive for renin, including those in the JGA itself, along the afferent arterioles of glomeruli having upstream renin-positive cells was also counted in each section. The figures thus obtained from each slide were averaged for each animal.

To determine the area of juxtaglomerular apparatuses in the *Agt* one-copy and wild-type mice, 10 random fields of each section were captured with a video camera. Every section was screened using the same magnification (× 400), and only the JGA with a classic donut-shaped outline were evaluated. All the images were studied with an image analysis software (Mocha™, version 1.02, Jandel Scientific). Using the manual measurement mode, the perimeter of each JGA was outlined, and its area was determined by summing the number of pixels contained within the outline.

To determine the number of cells in the JGA that had been evaluated for area, the number of nuclei observed within the outlined perimeter was counted.

*Microvascular Dissection*—To obtain an integrated view of the distribution of renin within the kidney, the entire renal arterial tree was dissected as described previously in rats and mice (26, 27) and stained for renin. The distribution of renin within the kidney was classified as described previously (28). In a type I distribution, renin is present along the whole length of the afferent vessel. In type II, renin extends upstream from the glomerulus but does not occupy the whole length of the vessel. In type III, renin is present as rings along the afferent vessel. In type IV, renin is restricted to the classical juxtaglomerular localization. In type V, no renin is found in the arteriole.

*Statistics*—All values are expressed as mean ± S.E. The two-tailed *t* test was used for statistical evaluations.

## RESULTS

*The Experimental Animals*—The experimental animals used for investigating long term homeostatic compensations in the RAS have a single functional copy of the *Agt* gene and one disrupted by gene targeting. We refer to them as *Agt* one-copy mice, and their blood pressures are about 8 mmHg (approximately 7%) below the pressures of wild-type mice with two copies of the gene (4). Except when indicated, the mice studied were F1 progeny-derived from the two inbred strains 129 and C57BL/6 (B6) and so were genetically identical except for having different numbers of functional *Agt* genes. Because of this genetic uniformity, even small differences between the mice can be ascribed directly to the difference in *Agt* gene copy number.

*Renin-Angiotensin System Proteins and Peptides*—To determine what components are homeostatically adjusted in the *Agt* one-copy animals, the steady state concentrations of the major RAS protein components present in plasma were compared in the *Agt* one-copy animals and their wild-type controls. The resulting data, Table I and Fig. 1 (below), show two major differences. First, the plasma AGT concentration in the *Agt* one-copy mice is markedly reduced, to 33–37% of the AGT concentration in the controls ($p < 0.01$), which is also significantly less than the 50% expected if the amount of protein were directly related to gene copy number ($p < 0.01$ *versus* 50%). A possible complication affecting this observation is that the functional *Agt* gene in the one-copy animals is derived from mouse strain B6, whereas the wild-type two-copy animals have one copy from strain B6 and one from strain 129. However, a comparison of AGT levels in wild-type inbred strain B6 and 129 mice shows that B6 mice have *higher* AGT levels (558 ± 30 AngI ng/ml/h, in six females) than do 129 mice (426 ± 11 AngI ng/ml/h, in six females), so that if strain differences in *Agt* gene expression persist in the F1 hybrids, the AGT concentration in the *Agt* one-copy animals should be even more than 50% of the wild-type F1 animals. We conclude that the plasma concentration of AGT shows no evidence of any compensatory increase in the *Agt* one-copy animals.

The second major difference is that, in marked contrast to the absence of any detectable compensation in AGT plasma concentration, the plasma renin concentration in the *Agt* one-copy animals is very significantly higher (240%) than in the wild-type controls ($p < 0.001$), indicating a marked homeostatic adjustment in this component of the RAS.

In mice and humans, renin is secreted into the circulation by

TABLE I

*Plasma proteins and peptides in F1 Agt wild-type and F1 Agt one-copy mice*

AGT, renin, and prorenin: ng of AngI/ml/h. ACE activity: units/liter. AngI, AngII, and bradykinin: pg/ml. Values are means ± S.E. Values in parentheses are percent relative to wild-type. *n*, number of animals; ND, not determined; wt, wild type.

| | | Males | | Females | |
|---|---|---|---|---|---|
| | *n* | *Agt* wt | *Agt* one-copy | *Agt* wt | *Agt* one-copy |
| AGT | 6 | 498 ± 23 | 164 ± 6 (33)[a] | 550 ± 14 | 201 ± 16 (37)[a] |
| Renin | 6 | 38 ± 1 | 85 ± 6 (222)[b] | 50 ± 4 | 131 ± 12 (262)[b] |
| Prorenin | 6 | 244 ± 3 | 572 ± 51 (234)[a] | ND | ND |
| ACE | 3 | 400 ± 29 | 360 ± 15 (90)[c] | 246 ± 15 | 251 ± 11 (102)[c] |
| AngI | 3 | 59 ± 3 | 34 ± 4 (58)[b] | 60 ± 2 | 45 ± 3 (75)[b] |
| AngII | 3 | 23 ± 5 | 12 ± 1 (50)[a] | 18 ± 1 | 11 ± 2 (62)[a] |
| Bradykinin | 3 | 94 ± 4 | 96 ± 2 (103)[d] | 70 ± 2 | 64 ± 1 (93)[d] |

[a] $p < 0.01$ *versus* wild-type.
[b] $p < 0.001$ *versus* wild-type.
[c] $p = 0.15$ *versus* wild-type.
[d] $p = 0.87$ *versus* wild-type.

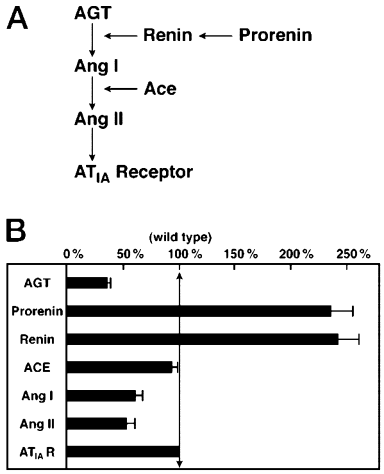


FIG. 1. **RAS proteins and peptides in *Agt* one-copy mice.** *A*, inter-relationships of the components measured in the RAS cascade. *B*, levels of the RAS components in *Agt* one-copy mice. The *bars* show means ± S.E. as percentages of those in wild-type mice (*Agt* two-copy). See Table I and Fig. 3 for details.

renin-producing cells partly as enzymatically inactive prorenin and partly as enzymatically active renin (29). The observed homeostatic increase in plasma renin could therefore be partly or wholly the consequence of a change in the proportion of the secreted protein in the active *versus* inactive form of renin. To investigate this possibility, we determined the plasma concentration of prorenin in *Agt* one-copy and in wild-type animals as well as the concentration of (active) renin. The plasma prorenin concentration in the *Agt* one-copy male animals was significantly higher (234%; $p < 0.01$) than in the wild-type controls (Table I). This increase in plasma prorenin is virtually identical to that of the plasma active renin, so that the same ratio of prorenin and active renin is observed in *Agt* one-copy and wild-type mice. We conclude that a change in the ratio of these two products is not part of the homeostatic adjustment made in the *Agt* one-copy mice.

To determine the net effect of the observed increase in plasma renin concentration combined with the observed decrease in plasma AGT concentration, we compared the steady state concentrations of AngI in *Agt* one-copy and wild-type mice. The results show that the *Agt* one-copy animals have AngI levels that are 58% and 75% of wild-type in males and females, respectively. Thus the combined effect of the two changes is a partial but not complete restoration of the AngI concentrations to the wild-type level ($p < 0.001$ for one-copy *versus* wild-type), albeit at the expense of decreasing the steady state concentration of AGT below 50% of wild-type.

An additional possible means of compensating for the less than normal AGT and AngI plasma levels in the one-copy animals would be via a homeostatically induced increase in the level of the converting enzyme ACE. Measurements of serum ACE activities (Table I), however, show no significant differences ($p = 0.15$) between the *Agt* one-copy mice and the wild-type controls. An additional indicator of possible changes in ACE function is the plasma bradykinin concentration, since this octapeptide is destroyed by the enzyme. We found that the bradykinin levels were not different between the *Agt* one-copy mice and the wild-type mice ($p = 0.87$). Thus we conclude that homeostatic compensation has not been induced at the level of the converting enzyme or of the bradykinin peptide.

The major effector peptide of the RAS is the octapeptide AngII. A measure of changes in the net status of the circulating arm of the system can therefore be obtained by comparing the steady state plasma concentrations of AngII in *Agt* one-copy and wild-type mice. The resulting data show that plasma AngII in the *Agt* one-copy males and females are, respectively, 50% and 62% of the levels in the wild-type animals. These levels are significantly less than wild-type ($p < 0.01$), indicating that homeostasis is incomplete, as is reflected by the residual differences in blood pressure between *Agt* one-copy and wild-type animals.

In summary, (Fig. 1), measurements of the expression of the protein and peptide components of the endocrine RAS show clear evidence that a major homeostatic compensation occurs in plasma renin concentrations in response to a genetic reduction in *Agt* gene expression. Other components of the system either show no changes or have changes that appear to be passive and secondary to the genetic reduction in AGT levels and the consequent homeostatic increase in renin. The final result is a steady state concentration of AngII in *Agt* one-copy animals that is still significantly less than normal.

*Renin-Angiotensin System mRNAs*—To ascertain whether the changes seen in the circulating protein components of the RAS are present at the level of transcriptional products, we used an RNase protection assay to determine the amounts of the relevant mRNAs in tissues that make the largest contribution to the plasma in *Agt* one-copy and wild-type animals. The major site of synthesis of AGT secreted into blood is the liver (30), which also contains the highest abundance of AGT mRNA. Fig. 2A presents the data for AGT mRNA in *Agt* one-copy and wild-type F1 males and females and in strain B6 and 129 inbred wild-type males. More mRNA is present in the female mice than in the males (a disparity also observed in the plasma AGT). But regardless of gender the liver AGT mRNA levels in *Agt* one-copy animals are clearly reduced compared with those in the wild-type animals (54% in males; 53% in females; $p < 0.001$). Recollecting that the single functional *Agt* gene in the one-copy animals is derived from strain B6 and noting from

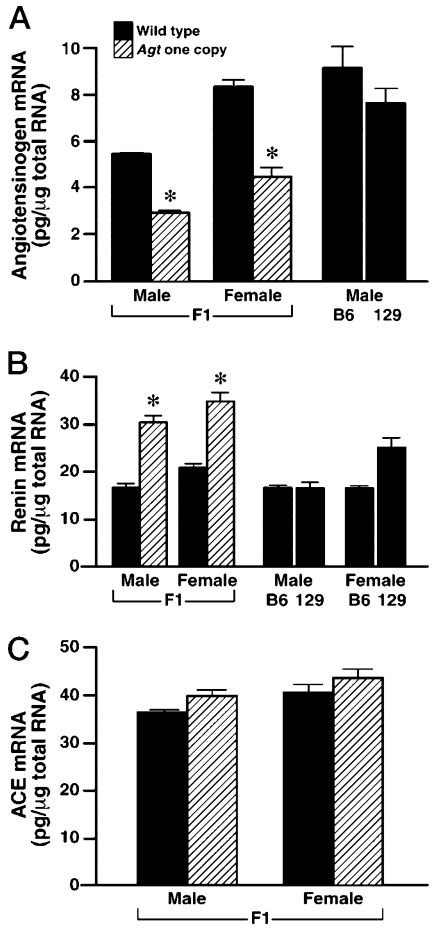


FIG. 2. **Tissue mRNA levels for primary tissues synthesizing AGT, renin, and ACE determined by RNase protection assay.** *A*, AGT mRNA in the liver. $n = 10$ for each of the four groups of F1 mice; $n = 5$ for the wild-type B6 and 129 males. *B*, renin mRNA in the kidney. $n = 10$ for each group of F1 mice; $n = 5$ for the wild-type B6 and 129 males and females. *C*, ACE mRNA in the lung. $n = 5$ for each group of F1 mice. The *bars* show means ± S.E. *Asterisks* indicate *Agt* one-copy means significantly different from wild-type means ($p < 0.001$).

Fig. 2*A* that liver AGT mRNA levels in strain B6 are 1.2 times the corresponding levels in strain 129, we conclude that the liver AGT mRNA data agree with the plasma protein data in indicating no homeostatic compensation in the *Agt* one-copy mice in the transcription of the remaining functional *Agt* gene in the primary tissue of AGT synthesis.

The high steady state plasma renin (and prorenin) concentrations observed in *Agt* one-copy animals suggest a substantially increased level of renin gene transcription. Fig. 2*B* presents the relevant data and shows that the steady state renin mRNA contents of the kidneys of the *Agt* one-copy males and females are respectively 182 and 165% of the wild-type two-copy controls ($p < 0.001$). Thus a major part of the homeostatic adjustment in renin production is a consequence of an increase in amount of renin mRNA.

Since the steady state serum ACE activities of the experimental and control mice do not differ significantly, we expected to see no differences in the ACE mRNA levels in tissues in which ACE is synthesized. The lungs are a major site of ACE synthesis in both sexes (31). In addition, a truncated form of ACE is synthesized in the testis from a testis-specific promoter (32). Fig. 2*C* presents data showing that the ACE mRNA contents of the lungs of *Agt* one-copy males and females are slightly increased (108 and 105%) relative to their two-copy controls, but the difference is not statistically significant ($p = 0.09$). The ACE mRNA level in the testis was also slightly

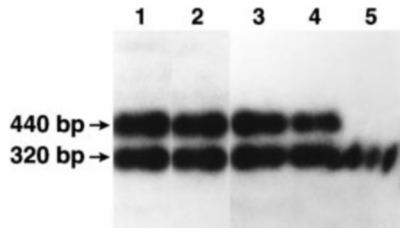


FIG. 3. **Comparisons by RT-PCR of type 1A angiotensin II receptor mRNA in *Agt* one-copy and wild type mice.** The products of 25 cycles of PCR with reverse transcripts from the kidneys of five mice are visualized. The 440-bp band is the product from the mouse type 1A receptor mRNA; the 320-bp product is from a PCR internal standard. The genotypes of the mice were: *Agt* wild-type, 1A receptor wild-type (*lane 1*); *Agt* one-copy, 1A receptor wild-type (*lane 2*); *Agt* wild-type, 1A receptor wild-type (two-copy) (*lane 3*); *Agt* wild-type, 1A receptor one-copy (*lane 4*); *Agt* wild-type, 1A receptor zero-copy (*lane 5*).

higher (data not shown), but again the difference was not significant (108% wild-type, $p = 0.65$). Thus there is no evidence for significant homeostatic compensation at the level of ACE mRNA.

In the mouse, three receptors (types 1A, 1B, and 2) control the cellular and physiologic actions of AngII (22, 33). The results of administering receptor antagonists that specifically block the actions of either the type 1 or the type 2 receptors establish that blood pressure changes are chiefly executed by the type 1 receptors (34). Genetic experiments disrupting the genes coding for the type 1A receptor gene (35, 36) or the type 1B receptor gene (37, 53) show that approximately 90% of the endocrine pressor effects of AngII are via by the type 1A receptor. Another possible means of homeostatic adjustments in the face of a chronic decrease in blood pressure would therefore be to increase expression of the type 1A receptor. However, comparison of the type 1A receptor gene expression by RT-PCR (Fig. 3) in *Agt* wild-type mice (*lane 1*) and the *Agt* one-copy mice (*lane 2*) revealed no detectable differences, although the same assay readily detected the decreased level of type 1A receptor mRNA in animals having only one copy of the 1A receptor gene (*lane 4*) in place of the normal two copies (*lane 3*), and showed no product in 1A receptor gene zero-copy mice (*lane 5*). Thus chronic homeostatic changes in expression of the type 1A angiotensin II receptor gene do not occur in the *Agt* one-copy animals.

*Other Tissues*—A great deal of work by many investigators has been directed toward assessing the possible autocrine/paracrine contributions to blood pressure homeostasis by RAS components synthesized in tissues other than those directly involved in the endocrine/circulatory aspects of the system (38, 39). Homeostatic changes in other tissues were therefore investigated in the *Agt* one-copy and wild-type animals. The data for AGT mRNA are summarized in Fig. 4*A* and show that, as in the liver, the AGT mRNA levels in the kidney, submandibular gland and testis of the *Agt* one-copy animals are close to the levels expected for animals having only a B6-derived *Agt* gene. Thus there is no evidence of significant homeostatic compensation at the level of *Agt* gene transcription in these tissues. In the brains of both males and females, the *Agt* one-copy animals likewise have lower amounts of AGT mRNA than wild-type animals, but the difference in the brain is less than in all other tissues. Further studies will be required to determine whether this is due to a compensatory change in *Agt* gene expression in the brain or to some other factors.

Fig. 4*B* presents the data for renin mRNA levels in various extra-renal tissues. With one exception, renin mRNA in extra-renal tissues does not differ between the *Agt* one-copy and wild-type animals. The exception is the adrenal gland of males, where the level of adrenal renin mRNA in *Agt* one-copy males

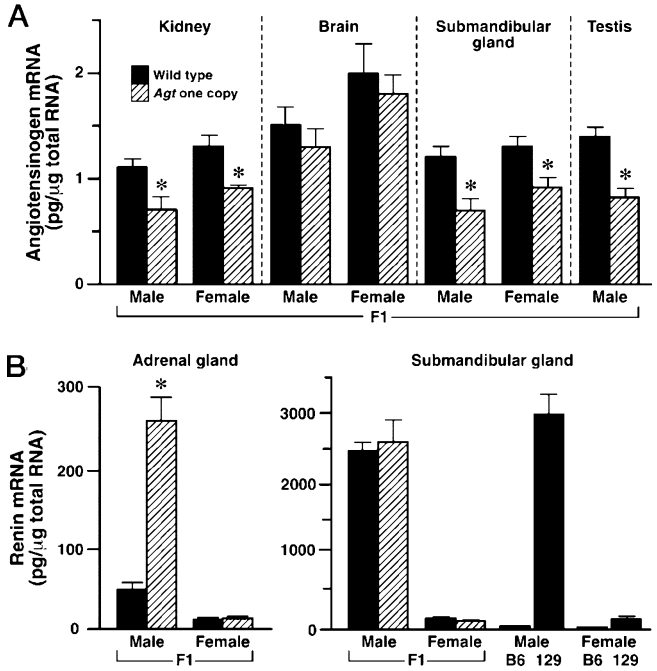


FIG. 4. **Tissue mRNA levels for secondary tissues synthesizing AGT and renin determined by RNase protection assay.** *A*, AGT mRNA levels in the kidney, brain, submandibular gland, and testis; $n = 10$ for each group. *B*, renin mRNA levels in the adrenal gland and submandibular gland; $n = 10$ for each group. The *bars* show means ± S.E. *Asterisks* indicate *Agt* one-copy means significantly different from wild-type ($p < 0.001$).

is four times that of wild-type males ($p < 0.001$). However, no difference in adrenal renin mRNA is seen between the *Agt* one-copy and wild-type females ($p = 0.48$). Note also that *Agt* wild-type males have approximately four times the adrenal renin mRNA of wild-type females, so that gender-related differences in renin expression are seen in the adrenal glands irrespective of their *Agt* genotypes.

*Gender-related Effects*—Some strains of mice including 129 have a duplicated renin locus that includes a closely linked androgen-responsive renin gene (*Ren-2*) in addition to a gender-indifferent gene (*Ren-1*); male mice carrying the *Ren-2* gene synthesize large amounts of mRNA in their submandibular glands and secrete renin into their saliva (40, 41). Other strains, including B6 (and humans), have only the *Ren-1* gene, and so have much lower salivary gland renin mRNA levels, comparable with the levels seen in females. The *Ren-2* gene has not been described in any species other than mouse. The F1 mice used in the present study have the single strain B6-derived *Ren-1*$^c$ gene and the two closely linked strain 129-derived *Ren-2* and *Ren-1*$^d$ genes. We therefore carried out experiments to determine to what extent the homeostatic changes in renin expression seen in the *Agt* one-copy animals differed by locus as well as by gender.

The relative contribution to kidney renin mRNA of the *Ren-1* and *Ren-2* genes was determined by a primer extension analysis. We found that in the *Agt* one-copy females 20% of the renin mRNA was derived from the *Ren-2* gene, which does not differ significantly from the percentage in their wild-type sisters or in a 50/50 mixture of the two female parental mRNAs. In the *Agt* one-copy males 40% of the mRNA was from the *Ren-2* gene, which is greater than that in their wild-type brothers (10%) or in a 50/50 mixture of the two male parental mRNAs (10%). Thus, in *Agt* one-copy females the homeostatic increase in renin expression is mediated by the two renin genes in the same proportion as they are expressed in their wild-type sisters, but in males a greater proportion of the increase is mediated by the androgen-sensitive *Ren-2* gene.

*Cellular Responses*—A great preponderance of evidence supports the view that the kidney is the chief source of circulating active renin and its enzymatically inactive precursor prorenin in humans and mice (42). In the normal kidney, renin is produced by modified smooth muscle cells that are associated mainly with the juxtaglomerular apparatus (JGA) and to a much lesser extent with the afferent upstream (proximal) portion of the glomerular arterioles. Several, not necessarily mutually exclusive, mechanisms could therefore mediate the chronic homeostatic elevation of kidney renin mRNA that we observe. Transcription of one or more of the renin genes could be up-regulated in the usual renin-producing cells of the kidney, or the number of these or other cells capable of synthesizing renin could be increased. To help distinguish between these various possibilities we determined by immunohistochemistry the number and distribution of renin-containing cells in the kidneys of *Agt* one-copy animals and wild-type controls.

The immunohistochemical results are illustrated and diagrammed in Fig. 5. In wild-type animals the majority of renin-producing cells are confined to the juxtaglomerular end of the arterioles in the manner typical of a classical JGA (Fig. 5, *A* and *C*). This distribution of expression corresponds to the type IV pattern (28) shown diagrammatically in the *bottom panel* of Fig. 5. A much smaller proportion of wild-type glomeruli have additional renin-staining cells extending along the afferent arterioles as well as being present in the JGA (types II and III). Some wild-type glomeruli have no renin-staining cells (type V). In the *Agt* one-copy animals (Fig. 5, *B* and *D*), there is a considerable increase in the proportion of glomeruli having renin-staining cells extending along the afferent arteriole as well as being present in the JGA (types II, III, and mixed types II/III and III/IV).

A summary of a statistical analysis of these immunochemical data is presented in the upper portion of Table II, using combined data from 12-week-old F1 *Agt* one-copy ($n = 5$) and wild-type mice ($n = 5$) and from 10-week-old F2 litter mates having the same genotypes ($n = 5$ and 3, respectively); the F1 and F2 data were essentially indistinguishable. This analysis shows: (i) that the proportion of glomeruli having renin-staining cells in the classical JGA region is somewhat (about 25%) greater in the *Agt* one-copy mice than in wild-type ($p < 0.05$);

(ii) that in the *Agt* one-copy mice the number of afferent arterioles with renin-staining cells upstream from the glomerulus is three times that of wild-type mice, and this is highly significant ($p < 0.0001$); (iii) that the number of renin-staining cells along the individual afferent arterioles of the *Agt* one-copy mice is also increased significantly (1.4 times wild-type; $p < 0.05$); (iv) that the total number of renin-staining cells along the afferent arterioles of the *Agt* one-copy mice is more than 4 times wild-type ($p < 0.0001$).

To assess the possible occurrence of a hyperplastic response in the renin-containing cells of the JGA of the *Agt* one-copy mice, the number of renin-staining cells in a sampling of glomeruli having classic donut-shaped JGA was determined by counting nuclei within the renin-staining areas in *Agt* one-copy mice, and this was compared with wild type using the 10-week-old F2 litter mates. No difference was observed (lower part of Table II, $p > 0.7$). To assess the possible occurrence of a hypertrophic response, the area of renin staining was determined for each JGA. Again no difference between the two genotypes was observed (Table II, $p > 0.3$). The sizes of the individual renin-producing cells, as judged by the area per cell, are indistinguishable in the classic JGA of the two genotypes. The intensity of renin-staining per cell was likewise not observably different in the two genotypes.

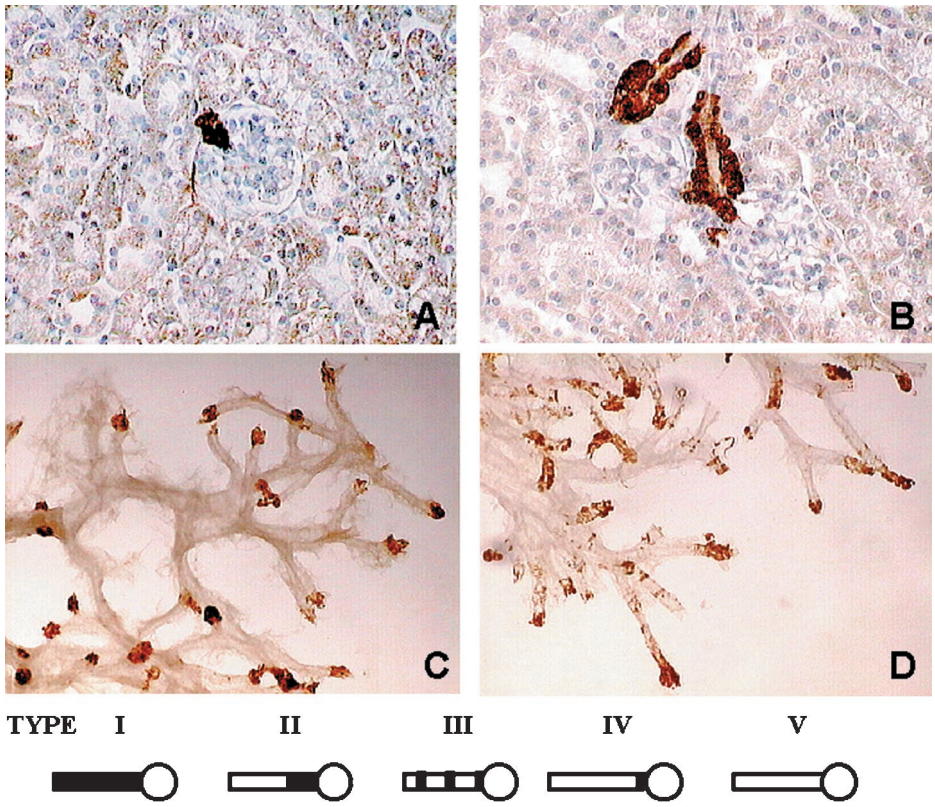


FIG. 5. **Renin immunolocalization in the kidneys.** *A*, kidney tissue section from a wild-type mouse. Renin staining (*brown*) in this type IV glomerulus is confined to the JGA area. *B*, kidney tissue section from an *Agt* one-copy mouse. Renin immunostaining in these type II glomeruli includes the JGA area but also extends along the afferent arterioles in the direction of the interlobular artery. *C*, renin immunolocalization within the renal arteriole tree microdissected from a wild-type mouse. Renin is present mainly in the JGA area (type IV). Renin is not present in interlobular arteries. *D*, renin immunolocalization in the renal arteriole tree microdissected from an *Agt* one-copy mouse. In addition to renin staining in the juxtaglomerular region (type IV), renin is present in cells along the afferent vessels in distributions corresponding to types II, III, and mixed types (types II/III and III/IV). Renin is present in interlobular arteries. The diagram below *A–D* illustrates the types of distribution I through V.

TABLE II

*Renin-staining cells in kidneys of Agt wild-type and Agt one-copy mice*

Values are means ± S.E. Areas are pixels × $10^3$. Values in parentheses are percent relative to wild-type. Counts are averages from two to four sections from each kidney. *n*, number of animals; wt, wild-type.

| | *n* | *Agt* wt | *n* | *Agt* one-copy |
|---|---|---|---|---|
| Percent glomeruli with renin-staining JGA | 8 | 45 ± 2 | 10 | 56 ± 5 (124)[a] |
| Afferent arterioles with upstream staining cells | 8 | 5.0 ± 0.6 | 10 | 15.5 ± 1.4 (310)[b] |
| Renin-staining cells per afferent arteriole | 8 | 6.4 ± 0.7 | 10 | 9.2 ± 0.9 (143)[a] |
| Staining cells along afferent arterioles | 8 | 32 ± 5 | 10 | 141 ± 17 (441)[b] |
| Renin-staining cells per classic JGA | 3 | 4.1 ± 0.3 | 5 | 4.2 ± 0.1 (103)[c] |
| Area of JGA | 3 | 1.35 ± 0.03 | 5 | 1.48 ± 0.09 (110)[c] |
| Area per JGA cell | 3 | 0.30 ± 0.04 | 5 | 0.35 ± 0.02 (119)[c] |

[a] $p < 0.05$ *versus* wild-type.
[b] $p < 0.0001$ *versus* wild-type.
[c] $p > 0.4$ *versus* wild-type.

Overall these data show that the higher plasma renin levels and the greater amount of total kidney renin mRNA in the *Agt* one-copy animals relative to wild type is mediated by their having a somewhat greater proportion of glomeruli with renin-expressing cells in the JGA region and a severalfold greater number of renin-expressing cells along the afferent arterioles of their renal glomeruli rather than by hyperplasia or hypertrophy of JGA cells already committed to renin synthesis or by up-regulation of renin gene expression in these cells.

## DISCUSSION

The main purpose of the present study was to determine the major long term adjustments directed toward homeostasis that occur in mice inheriting a precisely determined genetic variation with a blood pressure lowering tendency, namely inactivation of one copy of the *Agt* gene. The first finding is that in the liver, the prime site of AGT synthesis, no homeostatic up-regulation of the remaining functional *Agt* gene can be detected at the mRNA level. Thus the single copy of the *Agt* gene in the experimental animals yields essentially half the amount of mRNA achieved by the two copies in the wild-type controls. This absence of autoregulation of a normal functional gene to compensate for some unusual behavior in its homologous allele appears to be very widespread and probably universal in genes that do not code for products that act directly with their own regulatory machinery. There is a considerable body of data from previous studies showing that the expression of many genes is directly and precisely proportional to gene copy number. Epstein (43), for example, compiled from previous studies convincing evidence in support of this proportionality in humans and mice having trisomies, monosomies, and deletions involving over 40 different loci mainly coding for enzymes and plasma proteins. And we, in "gene titration" experiments with mice, have demonstrated a direct proportionality between gene copy number and expression with the genes coding for AGT (3, 4), for ACE (44) and for the natriuretic peptide receptor A (45). In none of these instances is the normal gene up-regulated in the absence of a functional homologue, while three copies of a gene produce very close to 1.5 times the amount of immediate gene product resulting from two copies, indicating a similar

absence of down-regulation. These observations do not exclude the existence of mechanisms to up-regulate or down-regulate genes via less direct and more complex pathways, but they do exclude the general occurrence of *auto*regulation. The implication is that most genetic variants which affect expression will not be corrected by adjusting the transcription of either the variant gene or its nonvariant homologue.

The second finding is that the major route whereby homeostasis in the endocrine side of the RAS is attempted in the *Agt* one-copy animals is through an increase in plasma renin concentration mediated by a modest increase in the proportion of glomeruli with renin-producing cells in their JGA and by the presence of considerably greater numbers of renin-producing cells along the afferent glomerular arterioles in the *Agt* one-copy animals than in the wild-type animals, rather than by hyperplasia or hypertrophy of JGA cells already committed to renin synthesis or by an increase in their renin content. No significant changes were detected in expression of the *Ace* gene or of the gene coding for the type 1A AngII receptor, which mediates most of the blood pressure-related functions of the system.

Comment is required on our observation of differences between the relative expression of the *Ren-1* and *Ren-2* in the male but not the female *Agt* one-copy and wild-type mice. In assessing the relevance of these findings to the overall problem of the homeostasis of blood pressure, it is important to recollect that the androgen-responsive *Ren-2* locus has been reported only in the mouse and then not in all strains. Past work by others (17, 18) has shown that the *Ren-1*:*Ren-2* expression ratio differs markedly between different cell types in males but not in females, ranging from around 1:1 in the male kidney to 1:100 in the male salivary glands. The relative increase in *Ren-2* expression seen in the kidneys of male but not the female *Agt* one-copy animals suggests that the additional renin-positive cells along the afferent arterioles have some features like cells in the salivary glands and so show gender differences in our F1 mice.

Comment is also required on the 4-fold greater amounts of renin mRNA observed in the adrenals of the male *Agt* one-copy animals relative to the expression in their wild-type brothers. At least two arguments suggest that this increase in renin expression does not represent a general mechanism for achieving homeostasis in the circulatory arm of the RAS. First, the effect is male-limited and is therefore again likely a consequence of the mouse strain-specific androgen-responsive *Ren-2* gene. Second, the plasma active renin concentration in the *Agt* one-copy males is actually not as much increased (2.2 times wild type) as in the one-copy females (2.6 times wild type). However, we do not exclude the possibility that the increased adrenal expression of renin in the males can act on a local arm of the RAS (46), and the phenomenon merits further investigation.

Our finding that homeostasis in the genetically modified animals is accompanied by considerably greater numbers of renin-producing cells in the kidney alters the nature of subsequent questions. In place of asking how up-regulation of the renin genes in cells already producing renin is induced and executed in the *Agt* one-copy animals, the question becomes what mechanism leads to the observed presence of more renin-producing cells. No definite answer is available. However, the changes seen in the normal rat kidney during the period from late gestation to early maturity and in adult kidneys exposed to experimentally lowered blood pressures suggest some interesting possibilities. Initially in the developing rat kidney (28) the afferent glomerular arterioles stain positively for renin along the whole of their lengths (type I, as illustrated in Fig. 5). Shortly after birth, type I-staining glomeruli are much less frequent, but glomeruli can be seen in which the renin staining extends less far along the arterioles from the glomerulus (type II). These in turn partly disappear, and glomeruli are seen that show the classic adult pattern, with renin staining confined to the JGA (type IV), together with glomeruli showing no renin staining (type V). The type of renin distribution that is severalfold more frequent in the *Agt* one-copy mice than in wild type has a staining pattern very similar to the type II pattern in the rat. This similarity suggests the possibility that in the *Agt* one-copy mice more of the renin-producing cells initially present along the afferent arterioles persist into adult life and fewer glomeruli cease to produce renin in their JGA.

A second possibility is that additional renin-producing cells are formed in the *Agt* one-copy mice from non-renin-producing smooth muscle cells. This type of metaplastic/metamorphic conversion/recruitment has been described in rat kidneys exposed to blood pressures reduced by partial arterial ligation (47, 48) and in the kidneys of rats treated with high doses of ACE inhibitor (23, 49). Cell recruitment has also been demonstrated in other systems, for example during estrogen-induced synthesis of apolipoprotein II in avian hepatocytes (50), during glucose-induced synthesis of pro-insulin in purified pancreatic beta cells (51), and during thyrotropin-induced formation of intracellular colloid droplets in thyroid follicular cells (52).

In conclusion, it is clear that much remains to be done if we are to understand how homeostasis is achieved in the face of quantitative genetic variations that are likely to be common in the genes which control our internal environment. The present results suggest that lifelong genetically determined disturbances may be corrected by homeostatic adjustments in the extent to which different cell populations are retained (or discarded) during development while still allowing disappearance (or reappearance) of the same cells in maturity if circumstances change. Attempts to determine the signals and mechanisms involved in genetic homeostasis should not only look for chronic up-regulation or down-regulation of immediately relevant genes, but should also look for possible shifts in the relative frequencies of the different types of cells that participate in the system.

*Acknowledgments*—We thank Drs. Cora-Jean Edgell and Thomas C. Coffman for helpful discussions and comments. We also thank Hui-Ying Li, Mei-Hong Lin, Jenny Lynch, and Vasantha Reddi for their technical help.

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