Regulatory Elements Required for Human Angiotensinogen Expression in HepG2 Cells Are Dispensable in Transgenic Mice

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Abstract—Previous researchers have identified two sequences present upstream (angiotensinogen gene–activating element [AGE2]) and downstream (d61–2) of the human angiotensinogen gene that act as cell-specific enhancers of transcription in transiently transfected HepG2 cells. To examine the importance of these two sequences in regulating tissue- and cell-specific expression of the gene in vivo, we generated transgenic mice containing the mutations in the context of a genomic transgene previously shown to exhibit appropriate tissue and cell specificity. The ability of these sequences to enhance transcription of a basal human angiotensinogen promoter was confirmed in transient transfection assays in HepG2 cells, and mutations within the AGE2 and d61–2 sequences abolished transactivation of the promoter. Tissue- and cell-specific expression was examined in three lines of transgenic mice carrying the d61–2 mutation, two lines of transgenic mice carrying the AGE2 mutation, and three founder transgenic mice carrying a double-mutant construct. Although the absolute levels of expression varied among lines, the pattern of tissue-specific expression was essentially unaltered by the mutations. In situ hybridization confirmed that the mutations were also dispensable for proximal tubule-specific expression within the kidney. Finally, a comparison of transgene expression with transgene copy number revealed a direct proportionality in liver (R = .77, P = .0014) and kidney (R = .76, P = .0024). These results clearly demonstrate that these sites, which strongly induce promoter activity in cells in culture, are not required for appropriate expression of the gene when present in a genomic construct in vivo. (Hypertension. 1998;31:734-740.)

Key Words: mice, transgenic ■ transfection ■ gene expression ■ mutagenesis ■ in situ hybridization

The RAS plays an important role in the regulation of blood pressure and body fluid homeostasis. AGT, the only known precursor of the RAS, is cleaved by renin and then by ACE to produce Ang II, the major effector peptide of the system. Ang II causes increases in vascular tone and sodium reabsorption through activation of signal transduction cascades initiated at specific high-affinity cell surface receptors located throughout the cardiovascular system. Linkage between HAGT and essential hypertension and preeclampsia and an association between variants of the HAGT gene with increased plasma AGT have been reported.1-3 Further support for AGT as a genetic determinant of blood pressure stems from transgenic and gene-targeted animal models that demonstrate increases in plasma AGT lead to high blood pressure4-8 and from antisense experiments demonstrating that central administration of angiotensinogen antisense oligonucleotides reduces blood pressure in the spontaneously hypertensive rat.9 Because plasma concentrations of AGT are close to the Km value of renin,10 small increases in plasma AGT concentration can potentially result in a substantial increase in plasma Ang II. Taken together, these data strongly suggest that abnormalities in the regulation of AGT expression may contribute to the pathogenesis of essential hypertension and therefore underscore the importance of gaining an understanding of the fundamental mechanisms regulating the expression of this gene in vivo.

We previously generated and characterized transgenic mice containing an HAGT genomic transgene containing 1.2 and 1.4 kb of 5′- and 3′-flanking sequence, respectively.11 Driven by its endogenous promoter, the HAGT transgene exhibits appropriate tissue- and cell-specific expression, and the mice exhibit high levels of circulating HAGT. This results in acute hypertension after intravenous administration of purified human renin11 and chronic hypertension in double transgenic mice containing the HAGT and human renin genes.2 Furthermore, the presence of the HAGT genomic transgene (in the presence of the human renin gene) rescued abnormalities present in angiotensinogen knockout mice, including postnatal lethality, hypotension, and renal abnormalities.4 In addition to providing a model system for studying the pathogenesis of hypertension initiated by the RAS, these results provided evidence to suggest that sufficient regulatory elements needed to drive appropriate expression of the HAGT gene in vivo were present within the transgene.

Numerous studies have been performed with the goal of identifying regulatory elements controlling the expression of...
**Selected Abbreviations and Acronyms**

- ACE = angiotensin-converting enzyme
- AGE2 = angiotensinogen gene–activating element
- AGT = angiotensinogen
- Ang = angiotensin
- HAGT = human angiotensinogen gene
- HAGT = human angiotensinogen protein
- PCR = polymerase chain reaction
- RAS = renin-angiotensin system

HAGT (for reviews, see Refs 12 and 13); and among these, two sequences have been identified that strongly stimulate reporter gene activity in transient transfection assays and therefore may be considered enhancers. The first sequence is an AGE2 that lies at position −76 to −65 in the 5′-flanking region of the mouse AGT gene and contains the palindromic CTCTGTACAGAG as the core element.14 The second sequence (d61–2) was found in the 3′-flanking region of the HAGT gene and contains the core sequence ACTTT at position +2194 to +2198.15 These two sequences exhibited significant enhancement of reporter gene expression in transient transfection assays performed in HepG2 cells (10- and 50-fold stimulation of the basal angiotensinogen promoter, respectively) but not in cells of extrahepatic origin, and mutations in their core elements abolished enhanced reporter gene activity. It is particularly interesting to note that Morishita et al16 recently demonstrated that injection of double-stranded decoy oligonucleotides spanning the corresponding AGE2 region of the rat AGT gene into the liver of spontaneously hypertensive rats resulted in a decrease in liver AGT mRNA and plasma AGT levels and a transient reduction in blood pressure. These results highlight the potential importance of these sequences in regulating a key gene controlling blood pressure. We therefore wanted to examine the importance of these sequences in vivo and hypothesized that they may play a role in regulating tissue- and cell-specific expression of HAGT. In the present study, we generated transgenic mice harboring HAGT with mutations in the key elements of AGE2 and d61–2 to determine their effect on regulation of HAGT expression.

**Methods**

**Generation of DNA Constructs**

The original wild-type HAGT genomic DNA (pHuAWT), including all five exons, four introns, and 1.2 kb of upstream and 1.4 kb of downstream flanking sequence that we used previously in transgenic mice,17 was used as the starting plasmid for this study. To optimize the mutagenesis, an internal deletion of a 5-kb BamHI fragment was generated containing a portion of intron 1, all of exon II, and a portion of intron 2. This plasmid (pHAGTΔII) was used as the template for mutagenesis. Mutations of the AGE2 and d61–2 sequences in pHAGTΔII were performed through double-stranded, site-directed mutagenesis using the Chameleon kit (Stratagene) with the instructions provided by the manufacturer. Two mutagenic primers were used: 5′-CTGAGGGTGCACCGCGCGGTGTGAAATACC-3′ for the AGE2 site and 5′-CCCTCCTCTCCATGAGGTTTGCAGGCAGC for the d61–2 site. One selection primer, 5′-CCCTCCTCTCCATGAGGTTTGCAGGCAGC-3′, hybridizing to the same strand as the mutagenic primers, was designed to target a unique Nhel endonuclease site and to create a unique BssHII endonuclease site in the plasmid backbone. The resulting plasmids, pHuA5HΔII for the AGE2 mutant and pHuA3TΔII for the d61–2 mutant, were sequenced using the following primers: 5′-CTGGGAACAGC-3′ for the AGE2 site and 5′-CCCTCCTCTCCATGAGGTTTGCAGGCAGC-3′ for the d61–2 site. One selection primer, 5′-CTGAGGGTGCACCGCGCGGTGTGAAATACC-3′, hybridizing to the same strand as the mutagenic primers, was designed to target a unique Nhel endonuclease site and to create a unique BssHII endonuclease site in the plasmid backbone. The resulting plasmids, pHuA5HΔII for the AGE2 mutant and pHuA3TΔII for the d61–2 mutant, were sequenced using the following primers: 5′-

![Figure 1. Map of the transgenes. The AGE2 region in the 5′-flanking DNA and d61–2 region in the 3′-flanking DNA is expanded. The mutations used in the HuA5H and HuA3T constructs are shown.](Image)

![Figure 2. Transfection constructs and transfection results. A, Constructs transfected into HepG2 cells are shown. The presence of mutations is indicated by Δ5H and Δ3T. The pHuA51 construct comprises the region −51 to +22 of HAGT containing the basal promoter. The pHuA138 construct comprises the region −138 to +22 of HAGT containing the AGE2 site and basal promoter. The pHuA138Δ5H construct has the same mutation as that used in the HuA5H transgene. The pHuA51−3E and pHuA51−3EΔ3T constructs comprise the region +2166 to +2231 containing the d61–2 enhancer fused to the −51 to +22 basal HAGT promoter. The pHuA51−3EΔ3T construct contains the same mutation as the HuA3T transgene. B, Transfection analysis of AGE2-containing constructs. Relative luciferase activity for each construct is shown after normalization to pHuA51, which is set at 1.0. †P<.05 vs pHuA51. *P<.05 vs pHuA138. C, Transfection analysis of d61–2-containing constructs. Relative luciferase activity for each construct is shown after normalization to pHuA51, which is set at 1.0. †P<.05 vs pHuA51. *P<.05 vs pHuA51−3E.)
promoter region (−51 to +22) of HAGT. This region was first amplified with PCR using the oligonucleotides 5′-CGTCAAGCT
TGCCTGATGACC-3′ (+22 downstream, HindIII site underlined) and 5′-GGGAGGCTTGCTCCATCCC-3′ (−51 upstream, SacI site underlined) and cloned into pGL2-Basic (Promega) as a SacI-to-HindIII fragment. The −138 to +22 region containing the AGE2 sequence was amplified through the use of PCR from the pHuA-WT or pHuA51 vector using the +22 downstream primer (see above) and the primer 5′-GGGGGTACCTGGCATCT GTCC-3′ (−138 upstream, SacI site underlined). The amplified product was cloned into pGL2-Basic as a SacI-to-HindIII fragment as above. The d61–2 segment was cloned through PCR amplification of pHuA-WT or pHuA3T using the primers 5′-CGGGGTACCTGGCATCT
CAAACGAAAGTGCA-3′ (+2166 upstream, KpnI site underlined) and 5′-GGGAGGCTTGCTCCATCCC-3′ (+2231 downstream, SacI site underlined). This region was cloned into pHuA51 as a KpnI-to-SacI fragment. All cloning junctions were confirmed by DNA sequence analysis.

### Transient Expression Analysis

Plasmid DNA was purified on cesium chloride density gradients before transfection into human hepatocarcinoma (HepG2) cells. Monolayer cultures (60% to 80% confluent) in 60-mm dishes were washed with HBSS before the addition of a 3-mL Lipofectin-DNA mixture in Opti-MEM media using the protocol provided by the manufacturer (GIBCO BRL). After 20 hours, 6 mL of Eagle’s minimum essential medium, with 15% FBS, was added, and incubation was continued for an additional 36 hours. Then, 10 μg of luciferase plasmid DNA and 2 μg of SV40-promoter-β-galactosidase plasmid were cotransfected. β-Galactosidase and luciferase activity assays were performed using the Galaco-light kit (TROPIX) and luciferase assay system (Promega), respectively. Luminescence was measured with a Monolight 2010 automatic luminometer, and β-galactosidase activity was used to normalize for transfection efficiency. Data was calculated as relative luciferase activity compared with pHuA51. The data represent the mean ± SEM of four independent experiments for the AGE2 mutation and five independent experiments for the d61–2 mutation. The data were analyzed by one-way ANOVA followed by Student’s modified t test with Bonferroni’s correction for multiple comparisons between mean values with the use of the SigmaStat Software package (Jandel Scientific). P<.05 was considered significant.

### Generation of Transgenic Mice

The transgene constructs in pHuA5H, pHuA3T (Fig 1), and pHuAΔ53D were separated from the vector through digestion with NheI, purified on an agarose gel, and then recovered with the use of a SpinBind kit (FMC Bioproducts). Transgenes were microinjected at a concentration of 2 mg/μL in 10 mmol/L Tris·HCl, pH 7.5, and 0.1 mmol/L EDTA made with embryo culture certified water (Sigma Chemical) into one-cell fertilized mouse embryos obtained from superovulated C57BL/6J X SJL/J (B6SJL F2) mice according to standard procedures.17,18 All mice were fed standard mouse chow and water ad libitum. Care of the mice used in the experiments met or exceeded the standards set forth by the National Institutes of Health guidelines for the care and use of experimental animals. All the procedures were approved by the University Animal Care and Use Committee at the University of Iowa.

### Characterization of Transgenic Mice

Transgenic founders and offspring were identified through PCR analysis of genomic DNA purified from tail biopsies as described previously.13 The same primer set was used to detect transgenic mice containing the wild-type, AGE2, and d61–2 mutations. Southern blot analysis was performed to confirm transgenic founders, using 10 μg of BglII and BamHI-digested tail genomic DNA. A 32P-labeled DNA fragment spanning exon 2 and part of intron 2 of HAGT was used as a probe, and the presence of a 2.9-kb band was diagnostic of the transgene as described previously.13 The same digests were used to quantify relative copy number. The endogenous mouse angiotensinogen gene was detected through digestion with SacI and hybridizing with a probe specific for the mouse angiotensinogen gene as described previously.13 To confirm that the mutations as designed were

2166 to 2231, respectively, of mouse AGT. The AGE2 site in the HAGT enhancer drives the luciferase reporter gene in corresponding transgenic lines was examined through the use of regression analysis with Sigma-STAT software (Jandel Scientific). All samples were normalized for loading using an internal standard: mouse angiotensinogen for the Southern blots and GAPDH for the Northern blots. Samples from different films were corrected using identical internal control samples present on each blot.

### Results

**HAGT Enhancer Elements Induce Reporter Gene Transcription in HepG2 Cells**

The relative position and sequences of the 5′ AGE2 site and 3′ d61–2 site are shown in Fig 1. The d61–2 site was originally identified in HAGT, whereas the AGE2 site was identified in mouse AGT. The AGE2 site in the HAGT gene was identified based on its homology with the mouse site (both are palindromes containing the central CTGT sequence) and its relative position from the transcription start site. To confirm the transcriptional inducibility of the HAGT gene in HepG2 and the AGE2 and d61–2 sequences, the corresponding sequences (−138 to −51 and +2166 to +2231, respectively, of HAGT) were PCR amplified and placed upstream of an expression vector containing the basal HAGT promoter (−51 to +22) driving the luciferase reporter gene (pHuA138 and pHuA53E, respectively, Fig 2A). Transfection constructs containing the identical mutations previously shown to disrupt AGE2 and d61–2 activity (pHuA138Δ5H and pHuA51–3Δ3T, respectively) also were gen-

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Generation of Transgenic Mice With Mutations in HAGT

The HAGT transgene used in these studies was derived from an HAGT genomic clone that we demonstrated previously to be expressed in an appropriate tissue- and cell-specific fashion in transgenic mice.5 Site-directed mutations were made in the 5’ AGE2 site (HuA5H) or the 3’ d61–2 enhancer (HuA3T) (Fig 1). Four transgenic founders (of 38 pups born) containing the HuA5H construct and 6 transgenic founders (of 36 pups born) containing the HuA3T construct were identified through PCR (data not shown) and confirmed through Southern blotting (Fig 3). This analysis revealed that each founder varied with respect to transgene copy number. The presence of the 5H and 3T mutations in the integrated transgenes was confirmed by sequence analysis after PCR amplification of genomic DNA from founders or transgenic offspring representing each transgenic line.

Expression of HAGT was measured by Northern blot hybridization in two transgenic lines of HuA5H and three transgenic lines of HuA3T. Representative Northern blots are shown in Figs 4 and 5. This analysis revealed that qualitatively, the overall expression pattern of HAGT among different lines of HuA5H and HuA3T transgenic mice was very similar and could not be easily distinguished compared with mice carrying the HuAWT construct. The relative expression of HAGT in these animals is summarized in the Table. In each case, expression was robust in liver and kidney, moderate in heart and white and brown adipose tissue, and low but detectable in brain and adrenal gland. There was essentially no HAGT mRNA detected in spleen, submandibular gland, or lung; the only exception was the presence of low levels of HAGT mRNA in the lung of female, but not male, HuA5H mice (Table). This result is unlikely to be due to a position effect because the same finding was observed in two independent lines of mice with different insertion sites. HAGT mRNA also was detected in testes and ovary (Table). No HAGT expression was detected in any nontransgenic littermates.

HAGT expression in the kidney of HuA5H and HuA3T transgenic mice was reproducibly higher in male than in female mice (Figs 4 and 5). This sexually dimorphic expression pattern is consistent with what we previously observed in mice carrying the wild-type construct and suggests that androgen responsiveness is preserved in mice that carry the HuA5H and HuA3T constructs.13 Except for the known androgen-responsive expression of HAGT in kidney and the differential expression in the lung of HuA5H mice, there essentially was no difference in the expression of the transgene between males and females in heart, brain, adrenal gland, or brown adipose tissue (data not shown). Each of the results presented above suggests that the AGE2 and d61–2 sequences are not required for the appropriate tissue-specific expression of the HAGT genomic transgenes.

In kidney, HAGT expression is restricted specifically to proximal convoluted tubule cells of the renal cortex. To determine whether this cell-type-specific expression of the HAGT gene was altered in HuA5H and HuA3T transgenic mice, in situ hybridization analysis was performed on kidney sections from representative HuAWT, HuA3T, and HuA5H mice (Fig 6). As in HuAWT mice, HAGT expression was restricted to the proximal convoluted tubule cells of the renal cortex in both HuA5H and HuA3T mice. No expression was detected in renal medulla, glomeruli, or blood vessels or in the...
kidney of nontransgenic mice. Similarly, no hybridization was detected when a control sense RNA probe was hybridized to serial kidney sections from transgenic mice (data not shown). These results clearly demonstrate that the AGE2 and d61–2 sequences are dispensable for appropriate cell-specific and androgen-regulated expression of HAGT in kidney.

Transgenes integrate in the genome at a single site but in tandem head-to-tail arrays (transgenic techniques reviewed in Ref 18). We were therefore concerned that a sequence present in the transgene would be able to influence the expression of the next transgene along the array and considered the possibility that a functional d61–2 sequence in the 3′ end of a HuA5H transgene could influence other transgenes in the array to functionally compensate for a loss of the 5′ AGE2, and vice versa. We tested this directly by generating transgenic mice with a double AGE2/d61–2 mutation. Four founder mice containing this transgene were killed without establishment of stable transgenic lines and were analyzed for tissue-specific expression (Fig 5 and Table). One founder contained a very low transgene copy number and did not express HAGT mRNA. HAGT expression in the other three founders (two males and one female) was similar to that observed in mice containing either the wild-type or single-mutant constructs, with the highest expression in liver and kidney and lower-level expression in heart, brain, adrenal gland, adipose tissue, testes, and ovary. Little or no expression was evident in submandibular gland, spleen, or lung.

Copy Number Dependency of HAGT Expression in Transgenic Mice

We previously observed a correlation between copy number and HAGT expression in transgenic mice containing the wild-type construct. To determine whether the expression of HAGT was also copy number dependent in HuA5H and HuA3T mice, we compared HAGT expression in liver and kidney from mice with different transgene copy numbers. We analyzed samples from four lines of HuAWT mice, five lines of HuA3T mice, and four lines of HuA5H mice. Relative Southern blot intensity was used as an index of copy number because the determination of absolute copy number is quite difficult to do accurately, even with internal standards. The intensity of the HAGT hybridization signal on Northern blots of liver RNA was compared directly with sister Southern blots containing genomic DNA from the same mice. Southern blots were normalized to the intensity of the endogenous mouse AGT gene and Northern blots were normalized for GAPDH expression. There was a clear trend toward higher transgene expression in both liver (Fig 7A) and kidney (Fig 7B) in mice containing higher transgene copy numbers. Regression

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*Expression in males greater than that in females.
†Expression in females only.
‡Founders (A and C are males, B is a female).

Figure 6. In situ hybridization was performed on frozen sections of kidney tissue as described in the text. A, C, and E, Bright-field photomicrographs. B, D, and F, Respective dark-field photomicrographs. A and B, HuAWT. C and D, HuA5H. E and F, HuA3T. Exposure times are 6 weeks for HuAWT and 24 days for HuASH and HuA3T.
polyadenylation site. Nibu et al. previously demonstrated that this symmetry (CTCTGTACAGAG). In the human gene, the site consists of a 12-bp palindrome that exhibits perfect dyad symmetry (CTCTGTACAGAG). We show herein that like the mouse AGE2 site, the human AGE2 site is in the same relative position; although the sequence differs somewhat (GCCTGTGCACAGGC), it shares the same dyad symmetry and the central CTGT–ACAG sequence. We show here that the AGF2 protein may interact with a ubiquitous transcription factor (AGF3) binding at position +3 to +14 (AGE3) relative to the start site of transcription because mutation of this site causes a loss of enhancer-like activity in HepG2 cells. These results do not appear to be the result of a transgene location artifact because a similar expression pattern of each HAGT gene variant was observed in multiple independent transgenic lines. In fact, the only difference observed between the constructs was a low level of HAGT gene expression in the lung of female HuASH mice. Because no HAGT gene expression in lung was detected in the original HuAWT and HuA3T mice, this suggests that the AGE2 element may impart some negative influence on expression in the lung or may interact with hormone response elements. We also tested the possibility that one site, but not both sites, is required for targeting appropriate expression in vivo. Similar to the single mutant constructs, HAGT expression in three independent transgenic founders containing the double-mutant construct could not be distinguished from its expression in mice containing the wild-type construct. Because enhancers can function over large distances in an orientation-independent manner, we thought it important to formally rule out effects on expression of an intact d61–2 site on a neighboring HuA5H transgene (and an intact AGE2 site on a neighboring HuA3T transgene) within the concatameric insertion.

In total, our findings strongly suggest that the AGE2 and d61–2 elements are not required for tissue- and cell-specific expression of HAGT in vivo. In this context, however, it is interesting to note that the expression of the mutant constructs was generally higher than that of the wild-type construct. This was initially surprising given that these sequences appear to function as enhancers of transcription in vitro. In an effort to account for the apparent contradiction, we examined whether expression of HAGT in liver and kidney was proportional to transgene copy number and found a positive correlation (Fig. 7). Except in some rare cases, there is no way to predict, a priori, the level of transgene expression based on transgene copy number. In the case of β-globin, the expression of a mimilocus transgene consisting of ~25 kb of 5'-flanking DNA and 20 kb of 3'-flanking DNA from the globin locus and containing several lineage-specific DNAses I–hypersensitive sites was shown to be position independent and proportionate to copy number. It is thought that DNAses I–hypersensitive sites may form a locus control region that may function to insulate the gene from influences from neighboring genes at the insertion site and act in concert with, or as enhancers of, transcription, probably by modulating local chromatin structure. The ability of certain cis-acting sites to manipulate that the ACTTT sequence, which does not overlap with these sites, was required for enhancer-like activity. We confirmed that this sequence was required for transcriptional induction of the basal HAGT promoter in HepG2 cells.

We previously generated transgenic mice containing HAGT that exhibited a cell-type and tissue-specific expression pattern closely resembling the expression pattern of HAGT in humans. Because both the AGE2 and d61–2 sequences appear to function with some cell-type specificity, we considered them to be candidates for targeting appropriate cell specificity of HAGT in vivo. However, our mutagenesis studies did not reveal any significant alteration in overall tissue- or cell-type-specific expression of HAGT compared with the original HuAWT transgenic mice despite the fact that the mutations caused a loss of enhancer-like activity in HepG2 cells. This analysis performed on all data revealed a correlation between copy number and expression in both liver ($R^2 = .79, P = .0014$) and kidney ($R^2 = .76, P = .0024$).

**Discussion**

Among the numerous regulatory elements identified in AGT are two that could be potentially characterized as enhancer sequences because they can strongly stimulate transcriptional activity of a reporter gene in transient transfection assays. The first is AGE2, which was shown to bind the liver- and HepG2-specific transcription factor AGF2; this site can induce a 10-fold increase in reporter gene expression in HepG2 cells. The AGE2 site in the mouse and rat AGT gene consists of a 12-bp palindrome that exhibits perfect dyad symmetry (CTCTGTACAGAG). In the human gene, the site is in the same relative position; although the sequence differs somewhat (GCCTGTGCACAGGC), it shares the same dyad symmetry and the central CTGT–ACAG sequence. We show herein that like the mouse AGE2 site, the human AGE2 site can stimulate an 11-fold induction in basal promoter activity here that the same dyad symmetry and the central CTGT–ACAG sequence. We show herein that like the mouse AGE2 site, the human AGE2 site can stimulate an 11-fold induction in basal promoter activity that is significantly attenuated when the palindrome is mutated (Fig. 2). Additional data suggesting the importance of AGE2 were reported by Morishita et al., who showed that double-stranded decoy oligonucleotides spanning the rat AGE2 site caused a transient reduction in liver AGT mRNA, plasma AGT, and plasma Ang II and a decrease in blood pressure when injected into spontaneously hypertensive rats. Presumably, these results from a competition between the gene and decoy or oligonucleotides for the AGF2 transcription factor, although effects on other unknown gene products that are also regulated by the AGF2 factor cannot be ruled out. In addition, the AGF2 protein may interact with a ubiquitous transcription factor (AGF3) binding at position +3 to +14 (AGE3) relative to the start site of transcription because mutation of this site abolishes AGE2/AGF2-mediated transcriptional induction.

The second site we investigated is the d61–2 site, which lies in the 3'-flanking region of the gene just downstream of the second polyadenylation site. Nibu et al. previously demonstrated that this 24-bp sequence could strongly stimulate the basal human angiotensinogen promoter nearly 50-fold in a position- and orientation-independent manner, consistent with its definition as an enhancer of transcription. Importantly, this sequence stimulated transcription only in HepG2 cells, not in cell lines derived from extrahepatic tissues. Although the sequence contains potential binding sites for AP-3 and C/EBP, mutagenesis studies revealed that the ACTTT sequence, which does not overlap with these sites, was required for enhancer-like activity. We confirmed that this sequence was required for transcriptional induction of the basal HAGT promoter in HepG2 cells.

We previously generated transgenic mice containing HAGT that exhibited a cell-type and tissue-specific expression pattern closely resembling the expression pattern of HAGT in humans. Because both the AGE2 and d61–2 sequences appear to function with some cell-type specificity, we considered them to be candidates for targeting appropriate cell specificity of HAGT in vivo. However, our mutagenesis studies did not reveal any significant alteration in overall tissue- or cell-type-specific expression of HAGT compared with the original HuAWT transgenic mice despite the fact that the mutations caused a loss of enhancer-like activity in HepG2 cells. These results do not appear to be the result of a transgene location artifact because a similar expression pattern of each HAGT gene variant was observed in multiple independent transgenic lines. In fact, the only difference observed between the constructs was a low level of HAGT gene expression in the lung of female HuASH mice. Because no HAGT gene expression in lung was detected in the original HuAWT and HuA3T mice, this suggests that the AGE2 element may impart some negative influence on expression in the lung or may interact with hormone response elements. We also tested the possibility that one site, but not both sites, is required for targeting appropriate expression in vivo. Similar to the single mutant constructs, HAGT expression in three independent transgenic founders containing the double-mutant construct could not be distinguished from its expression in mice containing the wild-type construct. Because enhancers can function over large distances in an orientation-independent manner, we thought it was important to formally rule out effects on expression of an intact d61–2 site on a neighboring HuA5H transgene (and an intact AGE2 site on a neighboring HuA3T transgene) within the concatameric insertion.

In total, our findings strongly suggest that the AGE2 and d61–2 elements are not required for tissue- and cell-specific expression of HAGT in vivo. In this context, however, it is interesting to note that the expression of the mutant constructs was generally higher than that of the wild-type construct. This was initially surprising given that these sequences appear to function as enhancers of transcription in vitro. In an effort to account for the apparent contradiction, we examined whether expression of HAGT in liver and kidney was proportional to transgene copy number and found a positive correlation (Fig. 7). Except in some rare cases, there is no way to predict, a priori, the level of transgene expression based on transgene copy number. In the case of β-globin, the expression of a mimilocus transgene consisting of ~25 kb of 5'-flanking DNA and 20 kb of 3'-flanking DNA from the globin locus and containing several lineage-specific DNAses I–hypersensitive sites was shown to be position independent and proportionate to copy number. It is thought that DNAses I–hypersensitive sites may form a locus control region that may function to insulate the gene from influences from neighboring genes at the insertion site and act in concert with, or as enhancers of, transcription, probably by modulating local chromatin structure. The ability of certain cis-acting sites to manipulate the ACTTT sequence, which does not overlap with these sites, was required for enhancer-like activity. We confirmed that this sequence was required for transcriptional induction of the basal HAGT promoter in HepG2 cells.

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chromatin structure, may be the key feature of position-independent, copy number–dependent expression. Clearly, it will now be important to perform experiments to address whether such nuclease-hypersensitive sites are present in AGT.

It is interesting to note that two sequences shown to enhance promoter activity in transfected cells do not appear to be necessary for expression of the gene in vivo. Although discordance between the regulation of gene expression in vitro and in vivo has been reported in many cases (for a review, see Ref 24), the mechanisms responsible remain unclear. Indeed, cells in culture lack the complex physiological environment of cells in vivo, such as the interplay between the neural and endocrine systems. Therefore, one potential explanation for the discordance is the difference in the differentiated character of immortalized cells. HepG2 cells are derived from a human hepatocellular carcinoma, and although this cell line retains many liver-specific features, such as the synthesis of liver-specific proteins, including AGT, it also loses some characteristic features of hepatocytes, such as lower expression of C/EBP and other transcription factors. Moreover, although we cannot formally rule out species-specific differences in transcription factors regulating AGT, both human and mouse AGE2 apparently function similarly in human HepG2 cells (Fig 2 and Ref of 14). An alternative explanation is that sequences necessary for promoter function in the context of promoter-reporter gene fusion constructs may differ substantially from their importance in the context of constructs, which more closely resemble their normal genomic structure. Therefore, other sequences present within the genomic construct, such as introns, may compensate for a loss of the AGE2 or d61–2 sequences in vivo. Introns have been reported to contain transcriptional regulatory elements and to assist in alignment of nucleosomes in chromatin.

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References
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