

The Kidney Androgen-regulated Protein Promoter Confers Renal Proximal Tubule Cell-specific and Highly Androgen-responsive Expression on the Human Angiotensinogen Gene in Transgenic Mice*

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Transgenic mice were generated containing a 1542-base pair fragment of the kidney androgen-regulated protein (KAP) promoter fused to the human angiotensinogen (*HAGT*) gene with the goal of specifically targeting inducible expression of renin-angiotensin system components to the kidney. High level expression of both *KAP-HAGT* and endogenous *KAP* mRNA was evident in the kidney of male mice from two independent transgenic lines. Renal expression of the transgene in female mice was undetectable under basal conditions but could be strongly induced by administration of testosterone. Testosterone treatment did not cause a transcriptional induction in any other tissues examined. However, an analysis of six androgen target tissues in males revealed that the transgene was expressed in epididymis. No other extra-renal expression of the transgene was detected. *In situ* hybridization demonstrated that expression of *HAGT* (and *KAP*) mRNA in males and testosterone-treated females was restricted to proximal tubule epithelial cells in the renal cortex. Although there was no detectable human angiotensinogen protein in plasma, it was evident in the urine, consistent with a pathway of synthesis in proximal tubule cells and release into the tubular lumen. These results demonstrate that 1542 base pairs of the *KAP* promoter is sufficient to drive expression of a heterologous reporter gene in a tissue-specific, cell-specific, and androgen-regulated fashion in transgenic mice.

The renin-angiotensin system (RAS)¹ is a classical endocrine system activated by the release of renin from the kidney and angiotensinogen (AGT) from the liver. In blood, renin proteolytically cleaves AGT to form angiotensin I (Ang-I) which is further processed by angiotensin converting enzyme to form Ang-II, a potent vasoconstrictor and antinatriuretic peptide. The RAS has been implicated in the genetic basis of hypertension and pre-eclampsia (1–4). Our understanding of the RAS in normal and pathophysiological regulation of blood pressure has been complicated by the fact that in addition to its actions as an endocrine system, certain individual tissues, such as the kidney (5–7), heart (8, 9), brain (10), and vasculature (11), contain all the components of the RAS cascade and therefore have the potential for local synthesis and action of Ang-II. In the kidney, for example, renin, *AGT* and *ACE* mRNAs, and proteins are synthesized in juxtaglomerular cells, proximal convoluted tubule (PCT) cells, and endothelial and tubular cells, respectively, and Ang-II type-1 (AT-1) and type-2 (AT-2) receptors are localized in glomeruli, collecting ducts, tubules, and vasa recta (12–18). The intrarenal RAS has been postulated to regulate various aspects of renal function including blood flow, natriuresis, and tubular-glomerular feedback, and may therefore participate in the pathogenesis of hypertension (19–21). Our current understanding of the relative importance of the intrarenal *versus* systemic RAS comes largely from pharmacological studies (22) which have been limited by the specificity of inhibitors, the ability to deliver agents to specific regions of the kidney, and their differential actions in the kidney and systemic circulation. Indeed, progress in our understanding of the role of the intrarenal RAS has been hampered by the lack of tools that separate the effects of the endocrine (blood borne) RAS from individual tissue RAS.

Normally, circulating AGT is derived from the liver where it is expressed at a high level and constitutively released. Transgenic mice containing a human *AGT* (*HAGT*) gene construct containing its own endogenous promoter express the transgene in hepatocytes of the liver, but also in PCT cells of the kidney, and in a number of other tissues (18). Double transgenic mice containing both the *HAGT* gene and also the human renin gene, encoding the species-specific processing protease for *HAGT*, are chronically hypertensive (23, 24) due to co-activation of both endocrine and tissue RAS. Clearly, the identifica-

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¹ The abbreviations used are: RAS, renin-angiotensin system; AGT, angiotensinogen; Ang-I or II, angiotensin I or II; PCT, proximal convoluted tubule; KAP, kidney androgen-regulated protein; bp, base pair(s); PCR, polymerase chain reaction; kb, kilobase pair(s); *HAGT*, human AGT.

tion and characterization of a promoter capable of specifically targeting renal PCT cells would lead to the development of a novel mechanism to specifically activate the intrarenal RAS independently of the systemic RAS.

The kidney androgen-regulated protein (KAP) was originally identified as an abundant 20,000-Da polypeptide product derived from *in vitro* translation of mouse kidney RNA (25). Although the function of the KAP protein remains unknown, it is encoded by a highly abundant 850-bp mRNA which accumulates to 4–5% of the total poly(A) mRNA in kidney of androgen-stimulated mice, suggesting the use of a highly active and efficient promoter (26). Androgen treatment causes a 3–4-fold stimulation of KAP mRNA (26) and a similar accumulation of angiotensinogen mRNA (18) in the kidney. *In situ* hybridization studies showed that KAP mRNA is localized in PCT cells of the renal cortex in normal male and testosterone-induced female mice (27); and its expression is under complex hormonal control involving androgens, estrogens, and pituitary hormones (28, 29). Based on the cellular localization of KAP mRNA in the kidney and its induction by androgen, we hypothesized that the KAP promoter and regulatory sequences would specifically target expression of the *HAGT* gene to the renal PCT cells. We show herein that under the control of 1542 bp of the KAP promoter, *HAGT* gene expression is restricted to renal PCT cells and is strongly induced in female mice in response to androgen treatment.

MATERIALS AND METHODS

Generation of Transgenic Mice—The transgene used herein consists of 1542 bp of the KAP promoter fused to the coding region of the *HAGT* gene. In the first step of the construction, a *SacI* to *XbaI* fragment encoding a portion of the 5'-flanking region of the KAP gene (–1542 to –466) was excised from a λ phage genomic clone (30) and joined with a PCR amplified fragment encoding DNA from the same *XbaI* site (–466) to coordinate –1 relative to the start site of transcription. This PCR fragment was engineered to contain a *BglII* site downstream of the PCR product. The first A in the *BglII* recognition sequence (AGATCT) became the transcription start site (+1) of the transgene. The resulting *SacI* to *BglII* fragment extending from –1542 to +6 was then cloned first into pGL2-Basic (Promega, Madison, WI) to form the plasmid 1542-KAP-Luc, and then subcloned as a *KpnI* to *HindIII* fragment into pBluescript II SK– to form the plasmid 1542-KAP-SK. The *HAGT* coding region was cloned from a previously described genomic clone (18) as a *BglII* to *NheI* fragment into the unique *BglII* and *SpeI* sites in 1542-KAP-SK to form the plasmid 1542-KAP-*HAGT*. *NheI* and *SpeI* have compatible ends. The *BglII* site in *HAGT* resides within intron 1, 70 bp upstream of exon II, and in this construct forms the 5'-untranslated region of the gene. Translation initiation of *HAGT* starts 4 bp into exon II. All cloning junctions were confirmed by sequence analysis. The transgene segment of the final plasmid was excised by digestion with *KpnI* and *NotI* and the transgene segment was purified away from the remainder of the plasmid backbone by agarose gel electrophoresis. The transgene DNA was recovered using the Qia-Quick purification kit (Qiagen) and was microinjected at a concentration of 2 ng/ μ l in 10 mM Tris-Cl, pH 7.5, 0.1 mM EDTA made with embryo culture-certified water into one-cell fertilized mouse embryos obtained from superovulated C57BL/6J X SJL/J (B6SJL F2) mice using standard procedures (31, 32). All transgenic mice were heterozygous for the KAP-*HAGT* construct.

Female mice were treated with testosterone by the administration of a 5-mg testosterone pellet designed for continuous release for 21 days (catalog number A-151, Innovative Research of America, Sarasota, FL). Mice were anesthetized with the inhalation anesthetic metofane and the pellet was implanted subcutaneously in the back and tunneled to the nape of the neck using a 10-gauge trocar. The incision was closed with a 6-0 silk suture and the mice were allowed to recover on a heating pad. The duration of the procedure was less than 5 min. The testosterone treatment was allowed to proceed for 5 days at which time experimental and sham operated control mice were killed. 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 in their guidelines for the care and use of experimental animals. All procedures were approved by the University

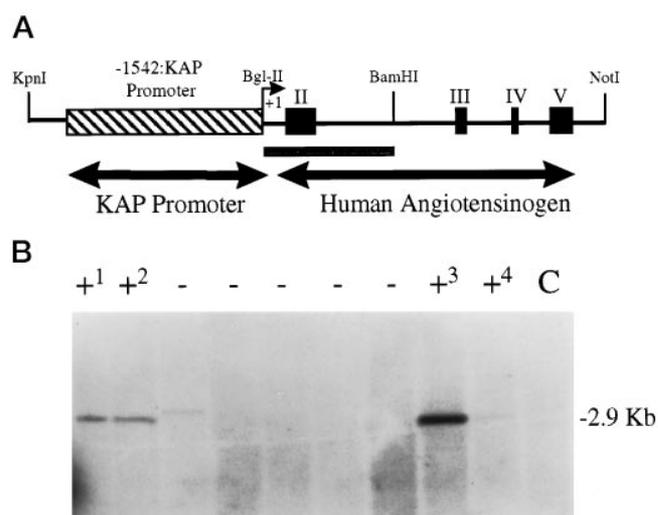


FIG. 1. Schematic map and identification of transgenic founders. Panel A, a schematic map of the transgene is shown. The KAP promoter is shown as the cross-hatched box and *HAGT* exons are shown in filled boxes. The start site of transcription is shown by an arrow and is indicated as +1. The transgene was excised as a *KpnI* to *NotI* fragment for microinjection. The thin gray bar below the map represents the probe used for the Southern blot shown in Panel B. Panel B, a Southern blot on DNA isolated from tail biopsy samples is shown. Genomic DNA was digested with *BglII* and *BamHI* and probed with the segment indicated in Panel A. A 2.9-kb band is diagnostic of the transgene. Transgenic founders are +1, 1827/1; +2, 1841/1; +3, 1848/3; +4, 1854/1. C, genomic DNA from a known nontransgenic B6SJL mouse.

Animal Care and Use Committee at the University of Iowa.

Analysis of Nucleic Acids—Genomic DNA was purified from tail biopsies and subjected to Southern blot analysis for the identification of founder animals or to PCR analysis for the identification of transgenic offspring (33). To identify transgenic founders, 10 μ g of tail genomic DNA was digested with *BglII* and *BamHI* and probed with a genomic segment encompassing exon 2 and intron 2 of *HAGT* (see Fig. 1). A 2.9-kb band was diagnostic of the presence of the transgene. There was no significant cross-reactivity of this probe with mouse genomic DNA. PCR analysis was performed on approximately 10–50 ng of tail DNA using primers specific for *HAGT* and amplified a 539-bp segment internal to exon 2 as described previously (18).

Tissue-specific expression studies were performed by Northern blot analysis using: 1) a *HAGT* cDNA probe derived from exon 2 at nucleotide coordinates 302–840 relative to the start site of transcription (18), and 2) a KAP cDNA probe derived by cloning a reverse transcriptase-PCR product encompassing coordinates 93–521. The oligonucleotides used to clone the KAP cDNA were 5'-ACTGTGGCTTTCCCCTGTC-3' and 5'-CTTCCTCGTTCTTTCTTCTTTG-3'. Both probes were cloned using an AT cloning kit (Invitrogen); and the orientation of each probe in the vector was confirmed by sequence analysis. Single-stranded antisense RNA was generated by SP6 transcription of the *HAGT* vector and by T7 of the KAP vector as described previously (33). Total tissue RNAs were isolated by homogenization in guanidine isothiocyanate followed by phenol emulsion extraction at pH 4.0 using a modification of the method previously described (34, 35). Homogenization was scaled up to 2.5 ml to increase RNA yield and quality. To ensure the specific detection of *HAGT* and KAP transcripts, Northern blots were treated with 1.0 μ g/ml RNase A in 2 \times standard saline citrate for 15 min at room temperature. We have previously demonstrated that this procedure removes nonspecific hybridization of single-stranded RNA probes (18, 33).

Primer extension was performed using a modification of the procedure previously reported (36). A 30-base oligonucleotide with the sequence 5'-AAATATCATTTTGCAAAGGGTGAAAGGTGG-3' was end labeled using [γ - 32 P]ATP and T4 polynucleotide kinase. This oligonucleotide was designed antisense to KAP-*HAGT* mRNA in the 5'-untranslated region so that a 37-base extension would result if transcription was initiated at the major KAP transcriptional start site. The labeled oligonucleotide was purified from a denaturing 8% polyacrylamide, 7 M urea gel, eluted into 0.5 M ammonium acetate and 10 mM magnesium acetate, and ethanol precipitated. 50,000 dpm of labeled oligonucleotide was hybridized with 40 μ g of total kidney RNA in a total

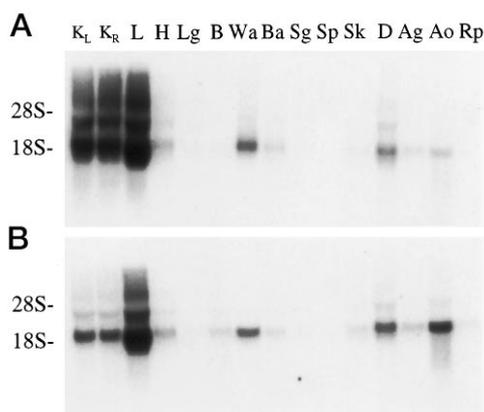


FIG. 2. Expression of *HAGT* from its endogenous promoter. The tissue-specific expression of *HAGT* in male (A) and female (B) transgenic mice containing a genomic construct consisting of all exons, introns, and 1.2 kb of *HAGT* 5'-flanking DNA and 1.4 kb of *HAGT* 3'-flanking is shown. *K_L*, left kidney; *K_R*, right kidney; *L*, liver; *H*, heart; *Lg*, lung; *B*, brain; *Wa*, white adipose tissue; *Ba*, brown adipose tissue; *Sg*, submandibular gland; *Sp*, spleen; *Sk*, skeletal muscle; *D*, diaphragm; *Ag*, adrenal gland; *Ao*, aorta; *Rp*, reproductive tissues (testes in A; ovary in B). Exposure time was 7 days.

volume of 20 μ l containing Sequenase reaction buffer (U. S. Biochemical Corp.) in a thermal cycler (MJ Research). We used a thermal profile in which the samples were first incubated at 95 $^{\circ}$ C for 2 min, followed by a gradual ramped cooling cycle to 65 $^{\circ}$ C over 6 min, incubation at 65 $^{\circ}$ C for 90 min, followed by a gradual cooling to room temperature at a rate of 1.5 $^{\circ}$ C/min. The annealing mixture was diluted into avian myeloblastosis virus reverse transcriptase buffer containing potassium chloride (provided by the manufacturer) in a volume of 40 μ l containing 0.25 mM dATP, dCTP, dGTP, TTP, and 2.5 units of avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories) and incubated at 42 $^{\circ}$ C for 90 min. The primer extension products were purified by ethanol precipitation and run on 6% polyacrylamide, 7 M urea sequencing gels. The same end-labeled oligonucleotide was also used to sequence the 1542-KAP-*HAGT* plasmid DNA using the Sequenase kit using the protocol recommended by the manufacturer (U. S. Biochemical Corp.) for using pre-labeled primers. The primer extension products and sequencing reactions were run in adjacent lanes on the same gel.

In Situ Hybridization—*In situ* hybridization was used to examine the cellular localization of KAP and KAP-*HAGT* mRNA in the kidney of transgenic mice. Tissues were removed and immediately frozen in liquid nitrogen. Frozen sections were cut 8 μ m on a Reichert-Jung cryostat and were hybridized to antisense and sense *HAGT* or KAP RNA probes labeled as described previously, except that digoxigenin-labeled uridine triphosphate (UTP) (studies performed at the Population Council) or 3 H-labeled UTP (studies performed in Iowa) was used as a label (17, 28, 37). The partial cDNA clones described above were used for *in situ* hybridization. No *HAGT* signal was detected in kidney tissue from non-transgenic mice. The 3 H-labeled sections were stained with hematoxylin and eosin. Detection of digoxigenin-labeled probes was by enzyme immunoassay and enzyme-catalyzed color reaction using the protocol provided by the manufacturer (Boehringer Mannheim). Sense and antisense probes were used on adjacent sections from the same tissue block.

Angiotensinogen Assays—Mouse and human angiotensinogen protein were determined in plasma as described previously (18). The assay differentiates mouse angiotensinogen from human angiotensinogen on the basis of the species-specificity of the biochemical reaction between renin and angiotensinogen (38). Briefly, transgenic KAP-*HAGT* and negative control littermates were sacrificed by CO₂ asphyxiation. Approximately 0.5 ml of fresh blood was collected from the aorta and placed in chilled tubes and EDTA was added to a final concentration of 2.5 mmol/liter. Plasma was obtained by centrifugation at 14,000 rpm in a microcentrifuge for 5 min and 150 μ l of plasma was immediately frozen at -80 $^{\circ}$ C. Radioimmunoassays were performed using the RIANEN Angiotensin I ¹²⁵I-labeled RIA kit (DuPont Co., Billerica, MA) using the directions and reagents supplied by the manufacturer. Plasma samples were thawed in an ice bath and 3 μ l of dimercaprol, 3 μ l of 8-hydroxyquinoline, and 300 μ l of maleate buffer were added to the plasma (all reagents were obtained with the radioimmunoassay kit). Samples were then split into 2 tubes. Tube A was incubated without any further additives and used endogenous mouse renin. Tube B was

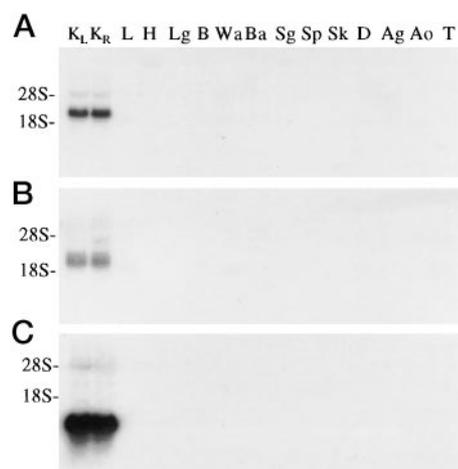


FIG. 3. Expression of the KAP-*HAGT* transgene in male transgenic mice. Northern blots of total tissue RNA from a representative male KAP-*HAGT* line 1827/1 (A and C) and KAP-*HAGT* line 1848/3 (B) probed with antisense RNA probes for *HAGT* (A and B) and endogenous KAP (Panel C). *K_L*, left kidney; *K_R*, right kidney; *L*, liver; *H*, heart; *Lg*, lung; *B*, brain; *Wa*, white adipose tissue; *Ba*, brown adipose tissue; *Sg*, submandibular gland; *Sp*, spleen; *Sk*, skeletal muscle; *D*, diaphragm; *Ag*, adrenal gland; *Ao*, aorta; *T*, testes. Exposure time was 1 day.

incubated with 17 μ g (3.33 units/mg specific activity) of purified human renin in normal saline (Scripps Laboratories, San Diego, CA). Both samples were incubated at 37 $^{\circ}$ C. Samples were removed from incubation at 0, 15, 30, 45, 60, 120, and 240 min. Samples were appropriately diluted with reagent blank so that the radioimmunoassay results were on the linear portion of a standard curve. The amount of angiotensin-I generated at each time point was then obtained by comparison to a standard curve generated each time the assay was performed. The angiotensin-I produced was then plotted *versus* time and saturation kinetics (due to conversion of all angiotensinogen to angiotensin-I) was observed by 60 min (data not shown). Plasma levels of angiotensinogen were then extrapolated using the 1:1 molar relationship between angiotensin-I and its precursor, angiotensinogen, using the 120-min time point. The amount of human angiotensinogen was calculated by subtracting the total angiotensinogen in tube A (mouse angiotensinogen) from tube B (human plus mouse angiotensinogen). To detect urinary angiotensinogen, the above assay was used except 150 μ l of urine replaced the plasma. No other changes were made in the assay protocol.

RESULTS

Transgenic mice containing a KAP-*HAGT* transgene were developed to accomplish two specific goals. The first was to determine if 1542 bp of the KAP promoter would target the expression of a heterologous gene specifically to PCT cells of the kidney. The second and long term goal of these studies is to develop a model to examine the functional importance of the intrarenal RAS by expressing *HAGT* within the kidney, but not in any other extra-renal tissues. We chose the KAP promoter for these studies by virtue of its kidney-specific expression and because, like angiotensinogen, KAP is expressed in PCT cells and its expression is responsive to androgens. Therefore, expression of *HAGT* under KAP promoter control would not change the overall cellular expression normally exhibited by *AGT* in the kidney.

Transgenic mice were generated with a construct containing 1542 bp of the KAP promoter fused to a 10.3-kb *HAGT* genomic clone encompassing exons II, III, IV, and V, the intervening introns, a 70-bp segment derived from the 3'-end of intron I, and the native 3'-end of the *HAGT* gene containing the poly(A) sites (Fig. 1A). This fusion results in the utilization of DNA normally present in intron I as a 5'-untranslated region. Of 58 live born offspring, 4 transgenic founders containing this construct were identified by both PCR (data not shown) and Southern blot (Fig. 1B). Three of these founders were successfully

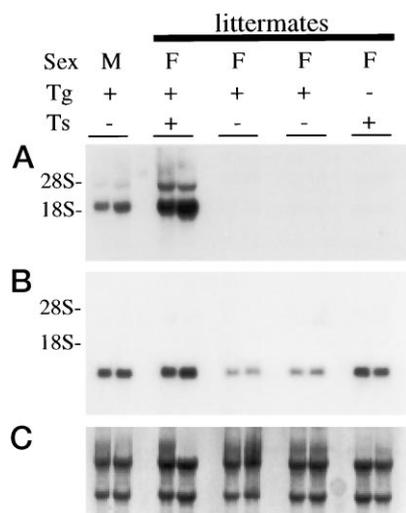


FIG. 4. Induction of renal KAP-HAGT mRNA by testosterone. Northern blots of total kidney RNA from male (M) and female (F) mice are probed for expression of *HAGT* (A) and endogenous KAP (B). Each pair of samples was derived from the left and right kidney, respectively. Tg+, transgenic; Tg-, nontransgenic; Ts+, testosterone-treated, Ts-, untreated controls. Panel C is the methylene blue stain of the filter used in panel A used to visualize 18 S and 28 S ribosomal RNA and to demonstrate equal loading of RNAs in each lane. All female mice used in this experiment were from the same litter. Exposure time was 1 day.

bred to establish transgenic lines, two of which, derived from founders 1827/1 (Fig. 1, +¹) and 1848/3 (Fig. 1B, +³), transmitted the transgene to approximately 50% of both males and females, indicating insertion in autosomes. However, the transgene in the third line, derived from founder 1841/1, was transmitted to 100% of males (of 26 tested) but 0% of females (of 15 tested), indicating an insertion into the Y chromosome. This line was discontinued as there was no evidence of transgene expression (data not shown).

Fig. 2 shows the expression of *HAGT* in male and female transgenic mice containing a genomic construct controlled by its own endogenous promoter (18). Under the control of 1.2 kb of its own promoter, *HAGT* mRNA is expressed at the highest level in liver, at a moderate level in kidney, white adipose tissue, diaphragm, and aorta, and at low levels in heart, brown adipose tissue, skeletal muscle, and reproductive tissues (Fig. 2). In contrast, under control of the KAP promoter, expression of *HAGT* mRNA is evident in kidney, but not in liver, heart, lung, brain, adipose tissue, submandibular gland, spleen, muscle, adrenal gland, or vascular tissue (Fig. 3, A and B). This kidney-specific pattern of expression was observed in two independent lines of KAP-*HAGT* mice, although the level of expression in line 1827/1 (Fig. 3A) was reproducibly higher than in line 1848/3 (Fig. 3B). As with the expression of many transgenes, there was no correlation between transgene copy number, which was higher in 1848/3 (Fig. 1), and level of transgene expression, which was higher in 1827/1 (Fig. 3). Importantly, the expression of *HAGT* in these mice paralleled the tissue-specific expression of the endogenous KAP mRNA (Fig. 3C).

The level of *HAGT* mRNA in kidney when driven by its endogenous promoter is approximately 4-fold higher in male mice than in female mice (Fig. 2, compare panel A to B), consistent with the androgen responsiveness reported previously by us (18). Similarly, expression of endogenous KAP mRNA in the kidney is about 4-fold higher in male than in untreated-female mice (Fig. 4B). It is therefore interesting to note that there was essentially no detectable expression of the KAP-*HAGT* transgene in the kidney of untreated-female mice (Fig. 4A). This suggests the possibility that the 1542-bp seg-

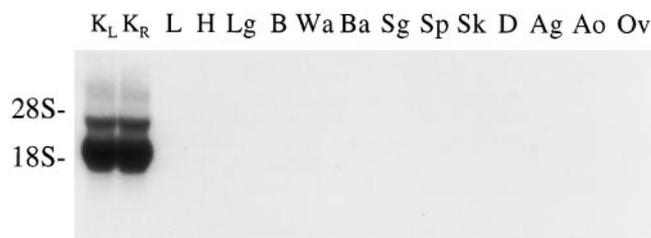


FIG. 5. Tissue-specific expression of KAP-HAGT in testosterone-treated female mice. A Northern blot showing the tissue-specific expression of KAP-HAGT mRNA in a testosterone-treated KAP-*HAGT* line 1827/1 female mouse is shown. K_L, left kidney; K_R, right kidney; L, liver; H, heart; Lg, lung; B, brain; Wa, white adipose tissue; Ba, brown adipose tissue; Sg, submandibular gland; Sp, spleen; Sk, skeletal muscle; D, diaphragm; Ag, adrenal gland; Ao, aorta; Ov, ovary. Exposure time was 1 day.

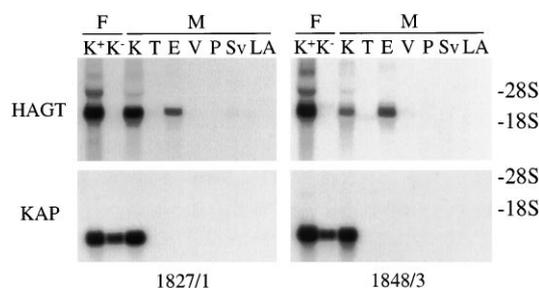


FIG. 6. HAGT expression in androgen responsive tissues. Northern blots of total tissue RNA from representative male KAP-*HAGT* line 1827/1 (left panels) and 1848/3 (right panels) mice probed with antisense RNA probes for *HAGT* (top panels) and endogenous KAP (bottom panels). Kidney RNA samples from an untreated female (K⁻), T-treated female (K⁺), and male (K) are shown as controls. T, testes; E, epididymis; V, vas deferens; P, prostate; Sv, seminal vesicle; LA, levator ani muscle. Exposure time was 1 day for the *HAGT* probe and 1 h for the KAP probe. The position of the 18 S and 28 S markers are indicated.

ment of the KAP promoter employed in this construct may have a strong dependence on androgen for expression. To test this directly, female transgenic littermates were either left untreated or were administered a testosterone (T) pellet (9.2 mg/kg/day) for a period of 5 days. The level of endogenous KAP and transgene mRNA was then examined in kidney RNA samples from control male, T-treated female, and control female mice. Representative samples from line 1827/1 are shown in Fig. 4. As expected, T-treatment caused a 3–4-fold induction in endogenous KAP mRNA (Fig. 4B), consistent with the responsiveness of the KAP promoter to androgens as previously reported (26). This induction resulted in an approximately equal level of KAP mRNA in males and T-treated females. This is in stark contrast to the marked induction (estimated to be at least 100-fold) of KAP-*HAGT* transgene mRNA in the kidney of the T-treated female mice (Fig. 4A), leading to the accumulation of significantly higher levels of transgene mRNA than in kidneys of untreated male mice. This finding was reproducible in other T-treated 1827/1 female mice ($n = 3$), and was similarly observed in females from line 1848/3 ($n = 2$, data not shown). Moreover, despite the marked increase in transgene expression in the kidney, there was no evidence of transgene induction in the other tissues examined (Fig. 5).

Given the strong androgen induction of transgene expression in this model we set out to rigorously assess whether the transgene was expressed in other androgen-responsive tissues in male mice. To this end, we assayed total RNA isolated from testes, epididymis, vas deferens, prostate, seminal vesicle, and levator ani muscle for expression of *HAGT* and endogenous KAP. This analysis revealed that *HAGT* mRNA is expressed in epididymis but not the other tissues tested (Fig. 6). Although

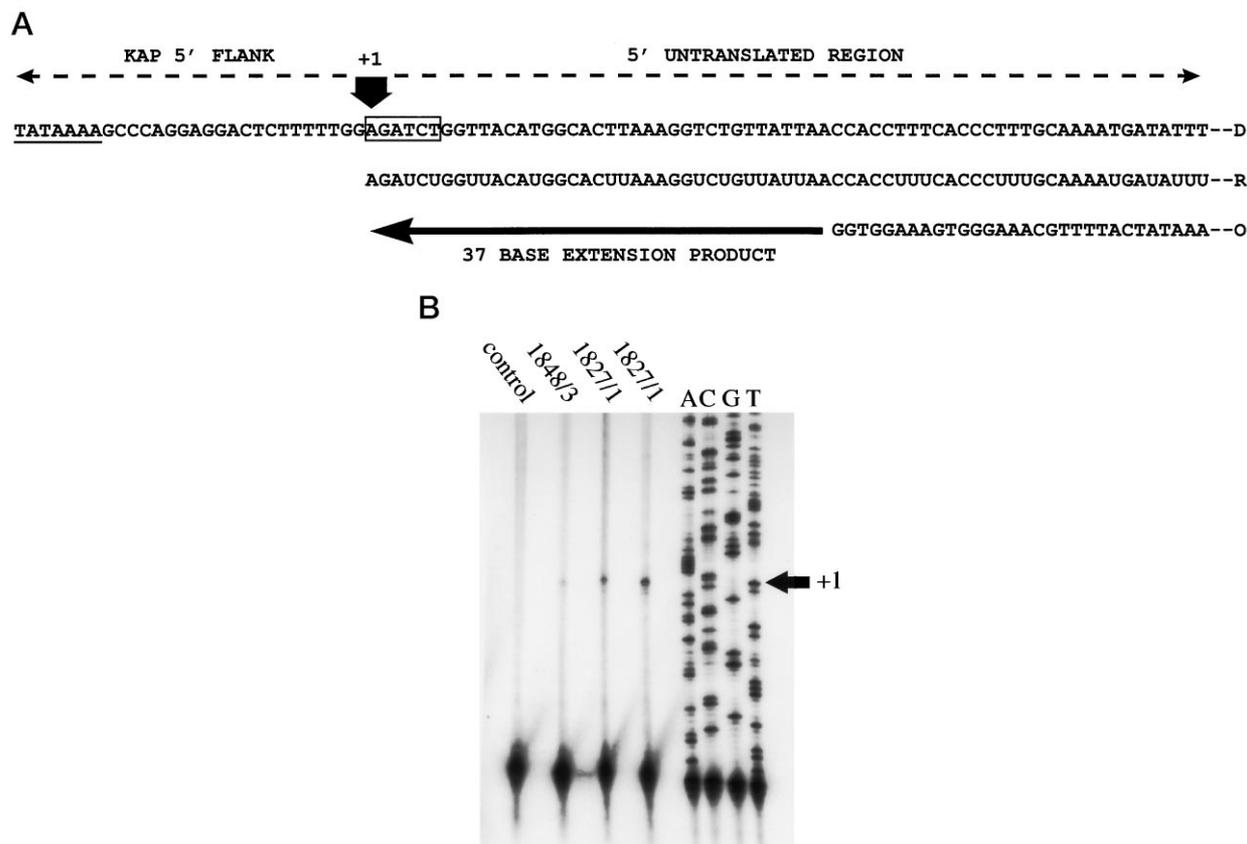


FIG. 7. **Primer extension analysis.** *Panel A*, schematic of the primer extension experiment is shown. The *top line* of sequence (labeled *D*) shows a segment of the transgene DNA from the TATA box (*underlined*) through +67 is shown. The second line of sequence (labeled *R*) shows the predicted mRNA sequence based on transcription from the KAP promoter (+1). The third line of sequence (labeled *O*) shows the sequence of the oligonucleotide primer used for primer extension. Extension of the primer results in a 37-base extension product if the major start site of the KAP promoter is used. *Panel B*, primer extension analysis on four kidney RNA samples is shown. Two samples are from line 1827/1, one sample is from line 1848/3, and one sample is from a nontransgenic control. A sequence ladder was run in adjacent lanes to accurately map the transcription start site (+1).

endogenous KAP mRNA was not detected in epididymis on parallel Northern blots (Fig. 6), it was detected when an reverse transcriptase-PCR based assay was used (data not shown).

Primer extension was performed to determine if transcription of *HAGT* occurred faithfully from the KAP promoter. A 30-base antisense oligonucleotide hybridizing in the 5'-untranslated region of the *KAP-HAGT* mRNA was used as a primer for extension toward the transcription start site. The strategy for the primer extension is shown in Fig. 7A. A 37-base extension product is indicative of the utilization of the KAP promoter. To ensure accurate sizing of the extension product a sequence ladder was run in an adjacent lane using the same primer and purified transgene DNA as a template. The data in Fig. 7B clearly demonstrates the production of a primer extension product which comigrates in the sequence ladder with position +1 from kidney RNA from line 1827/1 and line 1848/3. There was no detectable primer extension product from kidney RNA from a nontransgenic control.

In situ hybridization was performed to assess whether the KAP promoter could target *HAGT* expression to PCT cells (Fig. 8). Sense and antisense RNA probes were hybridized to frozen sections of kidney tissue from line 1848/3. Endogenous KAP expression is clearly evident in the cortical region in epithelial cells surrounding the proximal tubules (Fig. 8B). Expression of *HAGT* exhibited a qualitatively similar distribution (Fig. 8A), but at a level severalfold lower than endogenous KAP mRNA. No expression was evident when a labeled sense strand *HAGT* probe was used (Fig. 8C), or when an *HAGT* antisense probe

was hybridized to kidney sections from a nontransgenic mouse (Fig. 8D). An identical pattern of cell-specific expression was evident in transgenic line 1827/1 and in female mice treated with testosterone (data not shown).

Finally, we determined if functional HAGT protein was synthesized and released in the transgenic mice. To determine if HAGT protein was present in the systemic circulation, plasma angiotensin-I levels were measured after proteolysis of mouse angiotensinogen by mouse renin, and human angiotensinogen by human renin as described previously (18). The fidelity and specificity of the assay was confirmed by the observation of basal levels of mouse AGT, but high levels of HAGT protein in the plasma of mice containing a genomic *HAGT* transgene (18) (Table I). In *KAP-HAGT* transgenic mice, there was no significant difference in the plasma level of either mouse or human AGT when compared with nontransgenic littermates (Table I). The HAGT protein detected in the nontransgenic controls represents the background of the assay. Given the PCT cell localization of *HAGT* mRNA in this model, it was reasonable to speculate that AGT produced by the PCT cells may be released into the tubular lumen where the protein would be either cleaved by renin to form angiotensin-I or be excreted into the urine. Whereas there was no significant difference in the level of mouse AGT in the urine of nontransgenic and *KAP-HAGT* transgenic mice, HAGT was clearly observed in the urine of both male and T-treated female 1827/1 mice (Table II). The HAGT level in males was 10-fold higher than background (the HAGT level in nontransgenic mice) and in T-treated females was more than 100-fold greater than background. Although the

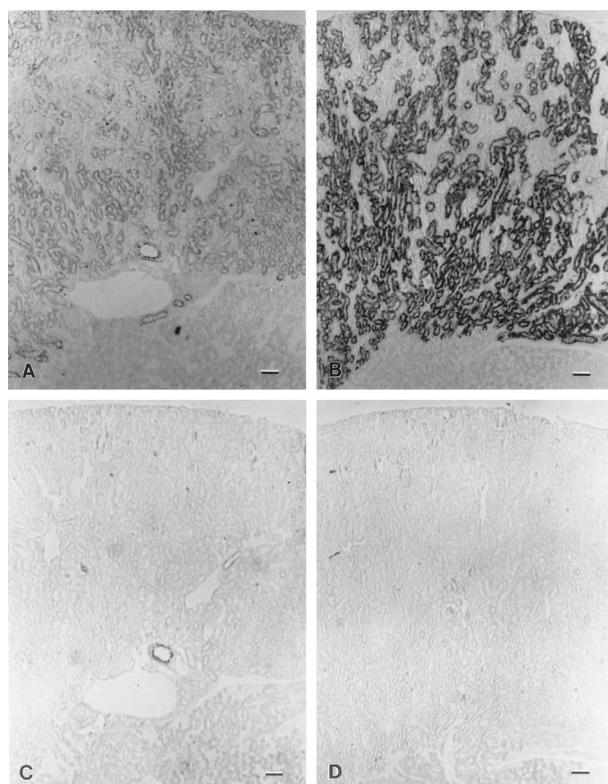


FIG. 8. Cell-specific expression of KAP-HAGT expression. *In situ* hybridization was performed on frozen kidney sections from male mice of transgenic line 1848/3. KAP and HAGT sense and antisense probes were labeled with digoxigenin-UTP and detection of transgene expressing cells was by enzyme immunoassay and enzyme-catalyzed color reaction. Panel A is a transgenic kidney hybridized with an HAGT antisense probe. Panel B is a nontransgenic kidney hybridized with an antisense KAP probe. Panel C is a transgenic kidney hybridized with a sense strand HAGT probe. Panel D is a nontransgenic kidney hybridized with an antisense HAGT probe. The bar represents 100 μ m.

TABLE I
Plasma angiotensinogen in KAP-HAGT transgenic mice

Strain	Mouse AGT	Human AGT	<i>n</i>
	<i>pmol/ml</i>		
Nontransgenic	29.2 \pm 7.2	12.2 \pm 4.3	4
HAGT 204/3	43.2 \pm 7.1	5859.5 \pm 276.0 ^a	4
KAP-HAGT 1848/3 δ	41.2 \pm 6.9	22.1 \pm 3.4	4
KAP-HAGT 1848/3 η + T	21.9 \pm 4.8	12.3 \pm 1.2	4
KAP-HAGT 1827/1 δ	24.6 \pm 3.7	10.4 \pm 2.0	4

^a *p* < 0.001 versus nontransgenic.

TABLE II
Urinary angiotensinogen in KAP-HAGT transgenic mice

Strain	Mouse AGT	Human AGT	<i>n</i>
	<i>pmol/ml</i>		
Nontransgenic δ	0.47 \pm 0.10	0.54 \pm 0.11	3
Nontransgenic η + T	0.05 \pm 0.05	0.03 \pm 0.03	2
KAP-HAGT 1827/1 δ	0.60 \pm 0.01	5.40 \pm 0.70	2
KAP-HAGT 1827/1 η + T	0.24 \pm 0.11	4.76 \pm 1.16	3
Nontransgenic (all mice)	0.30 \pm 0.12	0.34 \pm 0.14	5
KAP-HAGT 1827/1 (all mice)	0.38 \pm 0.11	5.01 \pm 0.69 ^a	5

^a *p* < 0.001 versus nontransgenic.

significance of the sexual dimorphism in urinary AGT remains unclear, the data clearly demonstrate that both HAGT protein and transgene mRNA are synthesized by PCT cells. Moreover, these results suggest that PCT-specific synthesis of HAGT in the KAP-HAGT mice does not result in its release into the systemic circulation. That the protein is functional was demonstrated by the enzymatic conversion of HAGT to angiotensin-I by human renin in the assay.

DISCUSSION

We have demonstrated that 1542 bp of the KAP promoter can faithfully target a highly spatially restricted pattern of cell-specific expression on a heterologous reporter gene in transgenic mice. While the expression of the transgene is normally undetectable in the kidney of female mice, its expression can be dramatically induced in response to testosterone treatment. Therefore, the KAP promoter should provide a novel tool with which to: 1) target the production of heterologous proteins specifically to the proximal convoluted tubule cells of the kidney, and 2) provide temporal control of expression in female mice by the administration (and potentially withdrawal) of androgens.

Regulation of the KAP Promoter—Transgenic expression of HAGT was achieved using a 1542-bp segment of the KAP gene 5'-flanking region as its promoter. Like the expression of the endogenous KAP gene in normal mice, HAGT expression in transgenic mice was localized to the PCT cells of the kidney, and was androgen responsive. Both the KAP and HAGT promoters normally target expression to an overlapping spectrum of tissues; the KAP promoter restricts expression to a small subset of HAGT positive cell types. In addition, expression of both genes respond similarly to androgen in kidney. Therefore, we considered the HAGT gene to be an ideal reporter to assay KAP promoter function. Interestingly, an alignment of 1542 bp of the KAP promoter with the known sequence of the HAGT promoter (−1222 to +1) revealed several segments of sequence homology, suggesting the potential for some conservation of promoter function. Indeed, our results demonstrate that the DNA promoter fragment used in the transgene construct contains sufficient sequence information for appropriately restricted tissue and cell specificity, as well as for androgen responsiveness in kidney. That tissue-specific expression of the transgene is appropriately controlled by the KAP promoter is also supported by its expression in the uterus during late pregnancy. The KAP gene is normally expressed in the mouse uterus during the 3 days prior to birth (39) and reverse transcriptase-PCR experiments suggest that the transgene is similarly expressed late in pregnancy (data not shown). Expression of the transgene in epididymis is interesting in light of its unusually high expression when compared with endogenous KAP mRNA. This high level expression is not due to position artifacts since high level epididymal expression of the transgene was evident in both transgenic lines which differ in both copy number and insertion site. Alternatively, this may either reflect the lack of an important negative regulatory element in the transgene that is normally present in the KAP gene or promoter region, or the presence of positive (epididymal-specific) regulatory element(s) in the HAGT portion of the transgene. Supporting the latter is our finding of HAGT mRNA expression in the epididymis of transgenic mice containing an HAGT genomic construct with its own promoter (data not shown).

Unlike normal KAP expression in the kidney of females, there was no evidence of basal expression of the HAGT transgene, and its induction by testosterone was unexpectedly high. The possibility that the transgene has an intact response to androgen but lacks sequences used for the normal KAP response to estrogen could account for these results. A subpopulation of the PCT cells, those in the juxtamedullary region (S3 cells) express KAP in the absence of androgen, as this is the site of KAP mRNA localization in females, castrated males, and Tfm/Y androgen receptor-deficient mice (28, 29). Furthermore, in Tfm/Y mice, estrogen stimulates KAP mRNA expression in S3 cells, showing that the KAP promoter responds to estrogens in a cell-specific manner. The presence of circulating estrogen may account for the higher basal expression of endogenous

KAP in females, and the absence of sequences responsive to estrogen in the transgene may be responsible for the absence of transgene expression in untreated females. It also remains possible that the unexpectedly high magnitude of transgene induction caused by testosterone (100-fold for the transgene versus 4-fold for KAP) may be due to sequences present both within the KAP promoter and within *HAGT*, which is itself androgen responsive in the kidney. Such a mechanism could potentially involve the presence of androgen-responsive elements within the coding or intronic regions of *HAGT*, or involve an androgen-mediated increase in transgene mRNA stability, as described previously in other genes (40–42).

Conclusion and Future Studies—Previous attempts to characterize specific sequences involved in the regulation of KAP gene expression were unsuccessful when either the 1542-bp promoter fragment or other promoter truncations were studied in cell transfection experiments using reporter gene assays. KAP promoter fragments were unable to direct reporter gene expression in a number of cell types in which androgen regulation of control constructs was evident. It is possible that the transcription factors required for KAP gene expression, while present in PCT cells *in vivo*, are not present in these established cell lines. This transgenic model, therefore, will be invaluable for further studies examining the complex hormonal regulation of the KAP promoter.

In addition, we feel this model will provide a novel opportunity to examine the importance of kidney-specific expression of angiotensinogen in the regulation of arterial blood pressure. As indicated above, we previously reported that transgenic mice containing genes encoding human renin and *HAGT* driven by their endogenous promoters exhibit chronic hypertension due to the high levels of circulating and tissue Ang-II (23). A major limitation in our understanding of how the RAS regulates blood pressure has been the previous inability to pharmacologically or physiologically distinguish between the effects of the RAS as an endocrine system, derived from hepatic angiotensinogen with the blood borne generation of Ang-II, from its effects within the parenchyma of organs such as the kidney, which express all components of the RAS itself. Since the cellular expression of KAP and *HAGT* are identical in the kidney, we will be able to employ this model to specifically activate the intrarenal RAS independent of its circulating effects. The biochemical data showing the absence of circulating, but presence of urinary *HAGT* protein further supports our ability to separate renal-specific effects of the RAS from its circulating effects. Therefore, to accomplish our overall goal, we are currently breeding the KAP-*HAGT* mice with transgenic mice expressing the human renin gene within the kidney. Both transgenes are required because of a strict species-specificity of the enzymatic reaction between renin and its substrate, angiotensinogen (38). Moreover, the unexpectedly robust response to androgen should provide a tool to regulate the intrarenal production of *HAGT*, and therefore Ang-II, in female mice.

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