The angiotensin II receptor (Agtr1a): functional regulatory polymorphisms in a locus genetically linked to blood pressure variation in the mouse

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Wong, Clifford, Nitish R. Mahapatra, Surin Chitbangonsyn, Payam Mahboubi, Manjula Mahata, Sushil K. Mahata, and Daniel T. O’Connor. The angiotensin II receptor (Agtr1a): functional regulatory polymorphisms in a locus genetically linked to blood pressure variation in the mouse. Physiol Genomics 14: 83–93, 2003. First published April 15, 2003; 10.1152/physiolgenomics.00162.2002.—Hypertension is a complex trait with multiple genetic determinants. A previous genome-wide linkage study of systolic blood pressure in a mouse genetic backcross implicated a region of chromosome 13 (LOD = 3.3 at 16.0 cM) as a determinant of blood pressure differences between a heterozygous low blood pressure strain of Mus musculus (BPL/1) and Mus spretus (SPRET); at this locus, the unexpected effect of the BPL/1 allele was to increase blood pressure. A plausible candidate locus encoding angiotensin II receptor isoform 1a (Agtr1a) is also located at 16.0 cM on chromosome 13. We therefore investigated structural and functional differences at Agtr1a between BPL/1 and SPRET, as well as the BPH/2 strain. Resequencing Agtr1a in the three strains established the exons/intron and proximal promoter structure of the mouse gene. Coding exon 3 spanned 1,960 bp (with 26 SNPs), including the 1,077-bp/359-amino acid ORF (with 5 cSNPs, all of which were synonymous). Promoter sequences revealed a consensus TATA box, conserved G/C-rich regions, and a striking, lengthy simple sequence repeat region, composed of di-, tri-, tetra-, and penta-nucleotide repeats, whose overall length varied markedly among the strains. Twenty-five other SNPs and three single nucleotide deletions differentiated the strains’ promoters, six of which were in likely functional promoter motifs. Agtr1a mRNA abundance in the adrenal gland in vivo was greater (P < 0.05) in BPL/1 than SPRET, consistent with the predicted effect of the BPL/1 allele to confer higher blood pressure when Agtr1a promoters were subcloned into luciferase reporter plasmids and transfected into PC12 chromaffin cells, basal promoter expression was higher (P < 0.001) in BPL/1 than in SPRET, consistent with the endogenous mRNA results. In summary, Agtr1a on chromosome 13 is highly polymorphic between mouse strains, although the amino acid sequence specified by the ORF is invariant, even across mouse species. We conclude that polymorphisms in the Agtr1a promoter account for differences in gene expression in vivo between BPL/1 and SPRET, in a way consistent with the effects of alleles at this locus on chromosome 13 to affect blood pressure in the mouse genome-wide linkage study.

hypertension

ESSENTIAL HYPERTENSION is a complex trait in humans, likely involving the interplay of multiple genes with the environment (6, 15). Likely candidate genes have been investigated in humans, including several encoding components of the renin-angiotensin system. The renin-angiotensin system, with angiotensin II as its primary effector molecule, plays a fundamental role in the maintenance of blood pressure and regulation of salt and water balance in the body, as well as modulating cardiac and vascular hypertrophy. Such candidate genes include renin (REN), angiotensinogen (AGT), angiotensin converting enzyme (ACE), kalirein (KLK1), and the human angiotensin II (type 1) receptor (AGTR1), with several genetic polymorphisms already linked to cardiovascular phenotypes, including the following: the ACE insertion/deletion polymorphism, which has been associated with plasma ACE activity, as well as myocardial infarction, left ventricular hypertrophy, and cardiomyopathy (37); the AGT Met235Thr amino acid substitution and G-6A promoter polymorphisms, linked to blood pressure (10, 14); and the AGTR1 receptor A1166C base substitution in the 3’-untranslated portion of the mRNA, which has been associated with arterial vasoconstriction, cardiac hypertrophy, and myocardial infarction (24, 33, 34).

In humans, two subtypes of receptors serve as binding sites for angiotensin II: type 1 (AGTR1) and type 2 (AGTR2). AGTR1 receptors are widely distributed in the body and mediate almost all of the known systemic effects of angiotensin II (32), whereas AGTR2 receptors are expressed mostly during fetal development, being much less abundant in adult tissues.

Rodent models serve as useful tools in elucidating the genetic mechanisms determining blood pressure (9, 13). It has been shown that the amino acid sequence of human AGTR1 receptor has a high degree of identity to that of the rat Agtr1 receptor (4). In both rats and mice,
two isoforms of Agtr1 receptor are expressed: Agtr1a and Agtr1b. These “a” and “b” isoforms share ~95% amino acid sequence homology (12, 25) but are encoded by different genetic loci.

The structure of rat Agtr1a has been investigated previously (22). Rat Agtr1a consists of at least three exons, with exon 3 containing the entire coding region along with 5′/H11032 exons, with exon 3 containing the entire coding region previously (22). Rat Agtr1a consists of at least three ORF but differing 5′- and 3′-untranslated sequences (22). In addition, a region of 3′-untranslated sequence further downstream may represent an additional one or more exons, as identified in one of the cDNA clones (31).

However, the molecular and exon/intron structure of the mouse Agtr1a gene has not been described in the literature. Rat and mouse Agtr1a genes are likely to share the same respective gene origins and to have co-evolved (36). Therefore, it is likely that the structure of mouse Agtr1a would be highly analogous to that of rat Agtr1a.

A genome-wide linkage scan for blood pressure loci in mice has been reported by us (35). In that study, we performed intraspecies intercrosses of an inbred hypertensive mouse strain, BPL/2 (Mus musculus genetically high blood pressure strain (BPH/2@J)), with a hypotensive strain, BPL/1 (Mus musculus genetically low blood pressure strain (BPL/1@J)), as well as two interspecies backcrosses involving a wild strain of relatively hypertensive mice, Mus spretus (SPRET). An interval linkage map for systolic blood pressure in a genome scan was created, and significant linkage was observed on chromosome 13 in a SPRET backcross to BPL/1 [(SPRET×BPL/1)×BPL/1; abbreviated “BCBPL,” backcross to BPL/1], with a LOD score of 3.3 (Fig. 1). Of note, the support interval for this linkage corresponded to the Agtr1a locus at 16.0 cM on mouse chromosome 13. Unexpectedly, the phenotypic effect of the BPL/1 allele at this locus in the backcross was an increase in systolic blood pressure (35).

Because of this significant linkage (peak LOD = 3.3 at 16.0 cM on mouse chromosome 13), suggesting the involvement of the Agtr1a locus in systolic blood pressure differences between BPL/1 and SPRET mice (35), we decided to evaluate in detail the exons and promoter region of this gene in these two strains, along with BPH/2 for additional comparison.

In this report, we describe the structure of the mouse Agtr1a locus based on the known rat Agtr1a structure, newly obtained mouse genomic sequence, and RT-PCR of the adrenal mRNA from the three mouse strains. We report the sequences of exons 1–3 and ~1 kbp of the upstream promoter region in SPRET, BPL/1, and BPH/2, and we identify polymorphic differences. We performed studies on endogenous Agtr1a gene expression via real-time RT-PCR quantification of mRNA in adrenal glands of all three strains. We also studied gene expression using Agtr1a promoter/reporter constructs transfected into chromaffin cells. Our results suggest substantial structural and functional differences between the Agtr1a promoters of BPL/1 and SPRET, which may account for quantitative differences in gene expression in vivo and hence the blood pressure linkage (35).

**METHODS**

*Mouse strains.* Twelve-week-old adult male mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The strains were BPH/2 (high blood pressure strain BPH/2@J, at inbred generation F62), BPL/1 (low blood pressure strain BPL/1@J, at inbred generation F59), and SPRET (SPRET/Ei, at inbred generation F59).

*Informatics.* The putative gene structure of the Agtr1a gene in the mouse was determined by analyzing known sequences of rat Agtr1a (rat Agtr1a cDNA, GenBank accession no. X62929; rat Agtr1a genomic DNA, GenBank accession no. S66402) deposited in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov) and aligning rat exons to mouse genome chromosome 13 contigs surrounding mouse genomic Agtr1a (mCG48688) from the Celera mouse genome database.
(http://www.celera.com) to detect conserved regions. Multiple sequence alignments were conducted with ClustalW (MacVector; Accelrys, San Diego, CA).

Interspecies (mouse/human) sequence homology plots were generated on the VISTA (“visualization tools for alignment”) (20) server at http://www-gsd.lbl.gov/vista, using 100-bp sliding windows. The genomic sequences aligned were mouse Agtr1a (Celera mCG48688, and surrounding genomic DNA contig on chromosome 13; 60- to 500-kbp segments used for alignment) and human AGTR1 (GenBank NT_006516; 883-kbp contig on chromosome 3).

Sequencing PCR primer design and optimization. Primer pairs were designed for mouse Agtr1a exons and the promoter region using mouse genome nucleotide information from the Celera mouse genome database. Targets for PCR amplification were identified and converted into FASTA format. MacVector (Accelrys) was used to design primer pairs, typically 18- to 24-mers spanning 200- to 1,000-bp target regions (amplicons), and avoiding primer dimer formation. Primer pairs with the lowest practical percent G/C content (typically <60% G/C) were chosen for annealing temperatures between 45–65°C.

Optimization of primer pairs was performed on plates containing duplicate wells of template genomic DNA (5 ng in 10 µl). The DNA templates for optimization were genomic DNA from SPRET, BPL/1, BPH/2, and a negative control (no DNA). Optimization and amplification of exon 3 was performed using AmpliTaq Gold thermostable DNA polymerase (Applied Biosystems, Foster City, CA). The mixture for an individual optimization PCR reaction (10 µl) consisted of the following final concentrations: 400 nM forward/reverse primers (Genset Oligos, La Jolla, CA), 0.2 mM each dNTP (Invitrogen, Carlsbad, CA), 0.5 U AmpliTaq Gold DNA polymerase, 1 mM MgCl2, TM buffer [1 × concentration = 34 mM Tris, pH 8, 25 mM KCl, 2.5 mM MgCl2, 8.3 mM (NH4)2SO4, 0.85 mg/ml bovine serum albumin], supplemented with 18 M2 deionized/distilled water (Sigma, St. Louis, MO), to achieve the final desired total volume (10 µl/optimization, 50 µl/amplification). PCR was performed by PTC-200 DNA Engine thermal cyclers (MJ Research, Watertown, MA). The Touchdown Profile program (MJ Research) was used, which began at annealing temperatures of 66°C and ran down to 50°C at 1°C/cycle for the first 16 PCR cycles, followed by a uniform three-step amplification profile (94°C denaturing step for 30 s, 50°C annealing step for 30 s, 72°C extension step for 30 s) for another 24 cycles, then finally holding at 10°C.

Optimization and amplification of exon 1, exon 2, and the promoter region were performed with the HotStar Taq Master Mix kit (Qiagen, Valencia, CA). The final mixture for amplification contained the following final concentrations: 1.5 mM MgCl2, 0.2 mM each dNTP, and 100 mM forward/reverse primer (Genset Oligos). The PCR protocol began with a 95°C enzyme-activating step, followed by a Touchdown program (94°C denaturing step for 30 s followed by initial annealing temperature of 70°C, subsequently run down to 55°C at 1°C/cycle, 72°C extension step for 1 min 30 s), followed by a uniform three-step amplification profile (94°C denaturing step for 30 s, 54°C annealing step for 30 s, 72°C extension step for 1 min 30 s) for another 24 cycles, then 72°C for 10 min, and finally held at 4°C.

After PCR, the products were visualized using agarose gels and ethidium bromide stain. PCR products were then purified with the QIAquick PCR purification kit (Qiagen).

Resequencing Agtr1a genomic DNA for polymorphism discovery. Genomic DNAs were prepared from mouse tails as previously described (35). Purified PCR products served as templates for resequencing, with the exception that purified PCR products inserted into the Promega (Madison, WI) pG3-Base Vector (GenBank/EMBL accession no. U47295) were used for resequencing the promoters (including the promoter microsatellites). Resequencing was performed by the UCSD Cancer Center DNA Sequencing Service with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit version 3 (Applied Biosystems). Areas resequenced included each exon (both coding exon 3 and noncoding exons 1 and 2), exon/intron borders, and the proximal promoter (1,100–1,200 bp upstream of the cap site).

Gene expression: RT-PCR for endogenous Agtr1a mRNA abundance. Total RNA was prepared from freshly frozen adrenal glands of the three strains (typical yield: 6–7 µg/pair of adrenal glands). RNA was extracted by the RNase A (Tel-Test, Friendswood, TX), followed by RNase-free DNase I (Qiagen) treatment (to eliminate residual genomic DNA). Integrity of the RNA was confirmed by the appearance of the 28S and 18S rRNA bands on ethidium bromide-stained gels. First-strand cDNA was prepared from 1 µg of total RNA template by reverse transcription with the SuperScript first-strand cDNA synthesis system for RT-PCR, using SuperScript II reverse transcriptase (Invitrogen), and random hexamer primers. mRNA/cDNA for Agtr1a was quantified by real-time RT-PCR on a ABI-7700 (Applied Biosystems) thermal cycler and fluorescent plate reader, using the Amplifluor universal detection system (Se rologicals, Norcross, GA). The mouse Agtr1a PCR primer pairs were selected from coding exon 3 of mouse Agtr1a: upstream (10 nM; beginning at the 347 bp in the ORF) 5′-GCGGAGGC-TGTTCCTGCTCACGTGTCTCA-3′, and downstream (100 nM; beginning at 417 bp in the ORF) 5′-GCGGAGGCGAGACTTCATC-3′, designed to amplify a 70-bp region. The 18-bp “Z” sequence 5′-ACTGACACCTAGCGTCACA-Z′ was incorporated into the upstream primer (just upstream of the mRNA recognition sequence), to allow incorporation of the fluorophore (5′-FAM-labeled) and quencher (3′-DABSYL-tagged) within a hairpin UniPrimer (100 nM), which only primes after the first PCR cycle. The threshold cycle was defined as that PCR cycle at which fluorescence was 10× the standard deviation of the baseline (initial) fluorescence signal.

Normalization was performed by quantitating the endogenous 18S rRNA (ng equivalents), using the following primer pairs: upstream 5′-CGCCGCTAGAGGTTAACTC-3′ (10 nM; also containing the ‘Z’ sequence, as above), and downstream 5′-TTGGAATATTCGGTGTC-3′ (100 nM), and the UniPrimer (100 nM; as above).

As a negative control, when RNA was pretreated with RNase A (Qiagen), the Agtr1a signal in the RT-PCR assay was undetectable. As a second negative control, no PCR product was obtained when cDNA was not first prepared from the mRNA by reverse transcriptase treatment.

To detect expression of endogenous Agtr1a mRNA in rat PC12 cells, RT-PCR was performed with the mouse exon 3 primers (see above), as well as the following rat primers: upstream 5′-CCTTGGCTGACTATGCTTTTGTC-3′ (bp 471–494 in rat Agtr1a cDNA clone M74054.1), and downstream 5′-ACTTCATGGGTGAGTGTCG-3′ (bp 665–645 in the same clone), to amplify a 195-bp region.

Resequencing mouse Agtr1a cDNAs to establish gene structure by RT-PCR. To establish the exon/intron borders, as well as the number of exons in the mouse Agtr1a gene, we em-
ployed RT-PCR on adrenal gland mRNA, with PCR primer pairs chosen to span exon/intron borders (exon 1, intron A, exon 2, intron B, exon 3), as well as putative far-downstream (3') exons 4.1 or 4.2 (22, 31).

Total adrenal gland RNA was isolated from the three strains, and cDNAs were obtained using SuperScript reverse transcriptase, as detailed above. The PCR primer pairs for RT-PCR and sequencing are given in Fig. 2A (see also Table 1).

**Construction of mouse Agtr1a promoter/luciferase reporter plasmids.** Promoter positions are numbered upstream (−) or downstream (+) of the cap (transcription initiation) site. Promoter fragments were PCR-amplified from genomic DNA.
DNAs of the strains. For BPL/1 and BPH/2, the primers were as follows: forward, 5’-GGTTGACAGGAGATGTTTATAG-3’; reverse, 5’-CTGTCCCCAAACAATGCCAG-3’. For SPRET, the primers were as follows: forward, 5’-GGTGACAGGCAGATGATGTTTA-3’; reverse, 5’-CTGAAGATGTGGTGACAAGGAACCTAC-3’.

The amplified regions were, therefore, -1149/+212 bp for BPH/2, -1159/+212 bp for BPL/1, and -1226/+48 bp for SPRET.

The amplified Agtr1a promoter fragments were digested on the 5’ end with EcoRI, and the 5’-overhang was made blunt with mung bean nuclease, followed by restriction digestion on the 3’ end with XhoI. The promoter fragments were then inserted 5’-3’ between the MluI (made blunt with mung bean nuclease) and XhoI sites in the polylinker of the firefly luciferase reporter vector pGL3-Basic (Promega), which lacks eukaryotic promoter and enhancer sequences and contains the cDNA for firefly luciferase (luc+). The correct insertion/orientation of the DNA fragments was verified by DNA sequencing.

The inserted promoter regions of the Agtr1a gene were, therefore, SPRET (-1184/+39 bp), BPL/1 (-1112/+39 bp), and BPH/2 (-1107/+39 bp).

The plasmids were purified on columns using a plasmid purification kit (Qiagen), prior to transfection.

Mouse Agtr1a promoter-reporter transfection and luciferase reporter activity assay. PC12 pheochromocytoma cells [grown in DMEM high glucose (Invitrogen) with 5% heat-inactivated fetal bovine serum (Gemini Bioproducts, Woodland, CA), 10% heat-inactivated horse serum (Gemini), penicillin G (100 U/ml), and streptomycin (100 μg/ml)] were transfected (at 50–60% confluence, 1 day after 1:4 splitting) with the Agtr1a promoter-firefly luciferase reporter plasmids from the three strains, by the liposome method (Superfect; Qiagen), with 1 μg of supercoiled DNA per well (12-well polystyrene plates; 2.1-cm diameter wells, Falcon Multidish; Becton-Dickinson Labware, Franklin Lakes, NJ). As an internal control in each well for transfection efficiency, the same cells were cotransfected with the Renilla luciferase expression plasmid pRL-CMV, encoding Renilla luciferase, under the control of the strong cytomegalovirus (CMV) promoter (Promega). The firefly and Renilla luciferase activities in the cell lysates were measured 16–21 h after transfection, and the results were expressed as the ratio of firefly/Renilla luciferase activity (Stop & Glo, Promega).

**RESULTS**

**Exon/intron structure.** Rat comparison clones were the Agtr1a mRNA/cDNA (GenBank accession no. 87AGTR1A POLYMORPHISMS IN THE MOUSE

| Exon 1 (1F) | Ex-1-4.1 bp 1-22 | Exon 3 (1R) | Ex-1-4.1 bp 1994-1971 | Span exons 1–3 |
| Exon 3 (2F) | Ex-1-4.1 bp 1776–1797 | Putative “exon 4.1” (2R) Exon 4.2 (6R) | Ex-1-4.1 bp 2986-2971 | Span exons 3–4.1 |
| Exon 3 (3F) | Ex-1-4.1 bp 1961–1975 | Putative “exon 4.1” (3R) Ex-1-4.1 bp 1961-1975 | Ex-1-4.1 bp 2472-2451 | Span exons 3–4.1 |
| Putative “exon 4.1” (4F) Ex-1.1-4 bp 2785–2762 | Putative “exon 4.1” (4R) Ex-1-4.1 bp 2785–2762 | Putative “exon 4.1” (5R) Ex-1-4.1 bp 2986-2971 | Ex-1-4.1 bp 2472-2451 | Span exons 3–4.1 |
| Exon 3 (5F) | Ex-1-4.1 bp 1899–1918 | Putative “exon 4.1” (5R) Ex-1-4.1 bp 2472-2451 | Ex-1-4.1 bp 2472-2451 | Span exons 3–4.1 |
| Exon 3 (6F) | Ex-1-4.2 bp 1899–1918 | Putative “exon 4.2” (6R) Ex-1-4.2 bp 2211–2188 | Ex-1-4.2 bp 2211–2188 | Span exons 3–4.2 |

**Table 1. PCR primer pairs for RT-PCR and sequencing**

![Fig. 2. Structure of the mouse Agtr1a gene. A: diagram of mouse Agtr1a gene structure. Exon/intron structure assembled based on sequencing of the mouse Agtr1a locus (Celera mCG486888), and new resequencing of mouse Agtr1a cDNA (RT-PCR products from adrenal mRNA of all three strains, with particular attention to putative exon/intron borders), with comparison to sequences in the rat Agtr1a cDNA (GenBank X62295; Ref. 22) and the known exon/intron structure of the rat Agtr1a (GenBank S66402; Ref. 31). The exon lengths are not drawn to scale. ORF, 1077 bp/359 amino acid open reading frame contained entirely within exon 3. The exon 3 lengths are 1,960 bp in BPL/1 and BPH/2 while 1,966 bp in SPRET. To establish the exon/intron borders, as well as the number of exons in the mouse Agtr1a gene, we employed RT-PCR on adrenal gland mRNA, with PCR primer pairs chosen to span exon/intron borders (exon 1, intron A, exon 2, intron B, exon 3), as well as putative far-downstream (3') exon 4.1 or "exon 4.2" (22, 31). Total adrenal gland RNA was isolated from the three strains, and cDNAs were obtained by reverse transcriptase, as detailed in the METHODS. The PCR primer pairs for RT-PCR and sequencing are shown in Table 1. B: comparative sequence homology between the mouse Agtr1a locus (current report) on mouse chromosome 13, and human AGTR1 locus on chromosome 3q22 (OMIM 106165; GenBank NT_005616) using VISTA. The genomic DNA sequence for mouse Agtr1a was established by Celera Genomics and deposited in the Celera Discovery System as mCG486888, at [http://www.celera.com](http://www.celera.com). A 60-kbp span of the local genomic region of mouse Agtr1a is shown; within the 60-kbp region shown, exon 1 spans 6749–6883 bp, exon 2 spans 15237–15326 bp, and exon 3 spans 41272–43231 bp. Within exon 3, the 1,077 bp/359 amino acid ORF spans 41325–42404 bp. Regions of >75% homology (over a 100-bp window) are shaded. Noncoding regions of >75% homology are also shaded. The major, extended region of homology (>80% over >1 kbp) is in the exon 3 ORF. UTR, untranslated regions of the exons. The genomic organization of human AGTR1 includes 5 exons spanning at least 37 kbp, with the entire ORF encoded by exon 5. Thus the major ORF alignment (B) is between mouse Agtr1a exon 3 and human AGTR1 exon 5. Mouse strains used for this sequencing included 129X1/SvJ, DBA/2J, A/J, C57BL/6J, and 129Sv/SvJm.](http://www.physiolgenomics.org)
X62295; Ref. 22) and the Agtr1a genomic DNA (GenBank accession no. S66402; Ref. 31) for determination of exon/intron borders. Alignment of the rat Agtr1a cDNA to the mouse Agtr1a 500-kbp genomic contig on chromosome 13 [Celera mCG48688 (21 kbp) and flanking contig DNA (2,000,000–2,500,000 bp)] revealed three regions of substantial sequence identity: exon 1 (~81% over ~135 bp), exon 2 (~91% over ~84 bp), and exon 3 (~91% over ~1,960 bp). In this 500-kbp mouse genomic contig, exon 1 (see panel B of the Supplemental Figure, available at the Physiological Genomics web site) spanned bp 346749–346883, exon 2 spanned bp 355237–355326 (panel C of the Supplemental Figure), and exon 3 (panel D of the Supplemental Figure) spanned bp 381272 to 383231.

Exon/intron borders were further established by RT-PCR of adrenal gland mRNA, with PCR primer pairs spanning each exon/intron border (Fig. 2A).

The deduced mouse Agtr1a gene structure includes noncoding exon 1 (135 bp), followed by intron A (~8.4 kbp), noncoding exon 2 (84 bp), intron B (~25.9 kbp), and coding exon 3 (1,960 bp). Exon 3 contains the entire 1,077 bp/359 amino acid ORF and possesses a putative downstream translational stop signal (TGA). The exon/intron borders and splice donor/acceptor sites did not differ among the three mouse strains (BPH/2, BPL/1, and SPRET), as judged by cDNA sequencing.

Our assignment of exon/intron borders was consistent with the sequence of mouse expressed sequence tag (EST) sequences deposited in public databases (e.g., BG969022). Our cDNA sequence information for mouse Agtr1a extends 416 bp beyond (3’ of) the most 3’ exon 3 sequence information previously available in the EST databases (e.g., BF383260).

One clonal version of the rat Agtr1a mRNA/cDNA also suggests a putative “fourth exon” at the 3’-untranslated portion of the mRNA (22, 31); however, the putative “exon 4” region of the rat Agtr1a cDNA is not consistent from clone to clone, and it may represent repetitive B2 elements (31). When we aligned the putative downstream “exon 4” region of the rat to the 500-kbp mouse genomic chromosome 13 contig spanning Agtr1a, we found only two relatively poor sequence identity matches: ~39% identity over ~1,057 bp (rat Agtr1a cDNA bp 1921–2937; Ref. 22) on the mouse genomic contig at 384537–385592 bp (~1.3 kbp downstream of exon 3; putative “exon 4.1”); and ~48% identity over ~129 bp (rat Agtr1a cDNA bp 2760–2888; Ref. 22) on the mouse genomic contig at 392948–393084 bp (~9.7 kbp downstream of exon 3; putative “exon 4.2”). We investigated whether putative exons 4.1 or 4.2 existed, by RT-PCR with primers designed to amplify cDNAs spanning exons 3–4.1 or 3–4.2, or 4.1 alone; no evidence of such downstream exons were detected. Neither did any of the approximately eight Agtr1a ESTs in public databases possess “exon 4” sequences. These results, coupled with the consensus polyadenylation signal in exon 3 (Fig. 2A) argue strongly against the existence of a putative “exon 4” in the mouse Agtr1a gene.

**Interspecies sequence conservation.** Comparison of mouse Agtr1a with human AGTR1 revealed a region of high (>80%) sequence homology in exon 3, corresponding to the ORF, whereas neither exons 1 nor 2 displayed substantial identity (<75% in each case). The proximal promoter (upstream of exon 1) also lacked substantial interspecies homology.

The genomic organization of human AGTR1 includes five exons spanning at least 37 kbp, with the entire ORF encoded by exon 5 (3, 5). Thus the major ORF alignment (Fig. 2B) is between mouse Agtr1a exon 3 and human AGTR1 exon 5.

**Resequencing Agtr1a for polymorphism discovery.** Noncoding exon 1 (135 bp; panel B of the Supplemental Figure) contained three single-nucleotide polymorphisms (SNPs) in SPRET compared with BPL/1 and BPH/2, although BPH/2 and BPL/1 were identical. Two SNPs in part of 3’-flanking intron A also distinguished SPRET from either BPH or BPL.

Noncoding exon 2 (84 bp; Supplemental Figure, panel C) contained three SNPs in SPRET compared with BPL/1 and BPH/2. The 189 bp in 5’-flanking intron A had four SNPs plus a 10-bp insertion/deletion differentiating the species, while the 3’-flanking 60 bp in intron B contained one interspecies SNP.

Coding exon 3 (Supplemental Figure, panel D; 1,960 bp in BPL/1 and BPH/2; 1,966 bp in SPRET) contained 26 interspecific SNPs: 5/26 in the 1,077 bp ORF (all synonymous cSNPs), and 21/26 in the 883-bp flanking regions of exon 3. In addition, there was a 10-bp insertion and 4-bp deletion in SPRET exon 3, downstream of the ORF. In 246 bp of 5’-flanking intron B, interspecific changes were one SNP and two 1-bp insertion/deletions. In 133 bp of 3’-flanking intron C, interspecific changes were three SNPs and one 1-bp insertion/deletion.

Translation of the ORF sequences showed the five SNPs located in the ORF to be synonymous (no changes in the 359 amino acids). See SwissProt/NCBI protein entry P29754.

**Resequencing the promoter region (Fig. 3 and Supplemental Figure, panel A) revealed a TATA box (TATAAATAT) at ~36/–44 bp in all three strains. There was a long simple sequence repeat region, composed of di- (CT), tri- (CTT), tetra- (CTTC or CTTT), and penta-nucleotide (CCTTT or CCTCT) repeats, from ~575/–912 bp upstream of the cap site (SPRET promoter numbering). The length of this simple sequence repeat region varied from strain to strain: the microsatellite was 338 bp long in SPRET, 264 bp in BPL/1, and 260 bp in BPH/2.

Other promoter polymorphisms (Tables 2–4 and Supplemental Figure, panel A) distinguished the strains, and created new motifs in SPRET not seen in the other two strains. C-1005T in SPRET created a

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1The Supplementary Material for this article (panels A–D for the Supplemental Figure) is available online at http://physiolgenomics.physiology.org/cgi/content/full/00162.2002/DC1.


Fig. 3. Agtr1a interstrain proximal promoter polymorphisms. The locations and lengths of large (SPRET, −575/−912 bp; BPL/1, −577/−840 bp; BPH/2, −576/−835 bp) simple sequence repeat (microsatellite) polymorphisms are shown. See panel A of the Supplemental Figure (available at the Physiological Genomics web site) for sequence alignment. The microsatellite region encompasses variable numbers of di- (CT), tri- (CTT), tetra- (CTTT) or penta- (CCTTT or CCTCT) nucleotide repeats. Promoter domains are not drawn to scale. Two G/C-rich proximal regions are also shown; these, too, vary in length among the three mouse strains.

Table 2. Agtr1 promoter polymorphisms in the three mouse strains: proximal promoter SNPs

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<thead>
<tr>
<th>Polymorphism at Nucleotide</th>
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<td>BPL/1</td>
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<td>G</td>
</tr>
<tr>
<td>−247</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>−311</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>−326</td>
<td>T</td>
<td>ΔT</td>
</tr>
<tr>
<td>−347</td>
<td>A</td>
<td>A</td>
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<tr>
<td>−525</td>
<td>T</td>
<td>T</td>
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Position numbers given according to the BPL/1 promoter, in bp upstream from the cap (transcription initiation, exon 1) site.

*Agtr1a* mRNA abundance: RT-PCR. *Agtr1a* mRNA expression in the adrenal gland differed significantly among the three strains (ANOVA, *F* = 5.63, *P* = 0.015). *Agtr1a* mRNA from BPL/1 (175 ± 2.44 ng equivalents) greatly exceeded that from SPRET (105 ± 16 ng equivalents; Bonferroni *P* < 0.05), whereas the BPH/2 (128 ± 20 ng equivalents) did not significantly (*P* > 0.05) differ from the other strains (Fig. 4).

The primers used for the real-time RT-PCR of mouse adrenal mRNA detected the endogenous *Agtr1a* mRNA in PC12 cells (89.2 ± 9.8 ng equivalents). Rat *Agtr1a*-sequence-specific PCR primers also amplified DNA fragments of appropriate size after RT-PCR of PC12 mRNA (data not shown).

Expression of the *Agtr1a* promoter in PC12 chromaffin cells. After subcloning of corresponding *Agtr1a* promoter fragments into the promoterless luciferase reporter plasmid pGL3-Basic, the promoter/reporter plasmids were transfected into PC12 pheochromocytoma cells; rodent chromaffin cells were chosen because the angiotensin II (type 1) receptor is highly expressed in such cells (8, 17, 21).

Basal (unstimulated) expression (Fig. 5) of the transfected *Agtr1a* promoter differed significantly (ANOVA, *F* = 58.9, *P* = 0.0001) among the three strains [SPRET, 6.73 ± 0.26 RLU; BPL/1, 13.16 ± 0.46 RLU; BPH/2, 10.5 ± 0.59 RLU; “RLU” is relative light units (luciferase reporter activity, firefly/renilla)] Individual strain comparisons (with Bonferroni post hoc corrections) revealed that the *Agtr1a* promoter was ~48% less efficient in SPRET than BPL/1 (*P* < 0.001; Fig. 5) and also less efficient in SPRET than BPH/2 (*P* < 0.01).

DISCUSSION

Background to the experiments: blood pressure linkage and the mouse *Agtr1a* region on chromosome 13. Common variations in blood pressure in the mouse display substantial heritability (26, 27, 35). Indeed, genetic crosses of inbred rat (9, 13) and mouse (28, 29, 35) strains differing in blood pressure reveal multiple
chromosomal regions linked to control of blood pressure, but to date few rodent blood pressure QTLs have been definitively identified by positional cloning (1). A genetic cross of a low blood pressure strain of *Mus musculus* (BPL/1) and the relatively high blood pressure (35) Mediterranean mouse species *M. spretus* (or SPRET) revealed a significant linkage (LOD = 3.3) of systolic blood pressure to a region on mouse chromosome 13, at marker D13Mit198, 16.0 cM from the p-terminus of the chromosome (Fig. 1). Since a mouse locus encoding the angiotensin II (type 1) receptor (Agtr1a) lies directly beneath the LOD peak at 16.0 cM, we decided to investigate the Agtr1a locus as a "positional candidate" for this blood pressure linkage.

Unexpectedly, the BPL/1 allele at 16.0 cM on chromosome 13 was associated with higher blood pressure in the N2 of backcross (BCBPL) offspring than the corresponding allele in SPRET (35). Thus we sought compatible changes in Agtr1a differentiating SPRET and BPL/1: that is, an alteration at Agtr1a causing greater expression in BPL/1 than in SPRET.

**Structure of the mouse Agtr1a locus.** A gene structure of Agtr1a has been tentatively described in the rat (31) composed of at least three exons, although putative rat Agtr1a exon 4 is problematic in that the exon 4 sequences may represent repetitive B2 elements (31). Thus only three exons are clear-cut in rat Agtr1a (31).

The exon/intron structure of mouse Agtr1a has not previously been clearly described. Using conserved sequence homologies between rat vascular Agtr1a cDNA clones (GenBank X62295; Ref. 22) and the emerging mouse genome contig assembly, as well as extensive resequencing of RT-PCR products from adrenal gland mRNA, we deduced the exon/intron structure for mouse Agtr1a, similar to that of the rat (Fig. 2A). The structure includes noncoding (5' untranslated) exons 1 and 2, and then exon 3, which encodes the entire ORF, as well as a consensus polyadenylation signal (AAUAAA).

In neither our own cDNA resequencing nor in the available mouse Agtr1a ESTs was there any evidence for a fourth (noncoding, 3' untranslated) exon in the mouse Agtr1a gene.

The human AGTR1 locus also displayed sequence homology with mouse Agtr1a, but substantial homology was limited to the ORF in exon 3 (Fig. 2B).

*Mice Agtr1a polymorphisms discovery by resequencing in BPL/1, BPH/2, and SPRET.* Resequencing of the exonic and promoter regions in Agtr1a identified multiple single nucleotide variations among the strains (Supplemental Figure). Several of these polymorphisms were located within the coding region of exon 3 (Supplemental Figure, panel D); however, each of these proved to be synonymous, indicating that qualitative interstrain differences in the Agtr1a protein cannot account for the systolic blood pressure linkage on mouse chromosome 13 (Fig. 1) in the genetic cross of BPL and SPRET (35).

Variations in angiotensin II (type 1) receptor exon/intron splicing occur in both the rat (22, 31) and the human (3). In the human, alternative splicing can give rise to alterations in translational efficiency and thus overall gene expression (3). However, we did not find interstrain variations in consensus (18) splice donor (GU) or acceptor (AG) dinucleotides in intron A (Supplemental Figure, panels B and C) or intron B (panels C and D). In intron A just upstream (5') of exon 2, (panel C), SPRET is deleted for a 10-bp A/T-rich region; although such pyrimidine-rich regions upstream of splice acceptor sites are common in higher organisms (18), the functional significance of this deletion in SPRET intron A is not clear.

Quantitative differences in Agtr1a expression might therefore be responsible for the systolic blood pressure

<table>
<thead>
<tr>
<th>Table 3. Repeat region (middle promoter)</th>
<th>Strain</th>
<th>BPL/1</th>
<th>BPH/2</th>
<th>SPRET</th>
</tr>
</thead>
<tbody>
<tr>
<td>repeat region (middle promoter)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Di-, tri-, tetra-, and penta-nucleotide repeat region</td>
<td>−577 to −840 bp</td>
<td>−576 to −835 bp</td>
<td>−575 to −912 bp</td>
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**Table 4. Distal promoter SNPs**

<table>
<thead>
<tr>
<th>Strain and Orthologous SNP Position/bp</th>
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<th>BPL/2</th>
<th>SPRET</th>
<th>Putative Implications</th>
</tr>
</thead>
<tbody>
<tr>
<td>G(−860)</td>
<td>G(−855)</td>
<td>A(−932)</td>
<td></td>
<td>A creates a IgH-1 site (7/7 match at −935/−929) in SPRET</td>
</tr>
<tr>
<td>T(−909)</td>
<td>T(−904)</td>
<td>G(−981)</td>
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<td>C(−933)</td>
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<td>T(−1005)</td>
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</tr>
<tr>
<td>A(−941)</td>
<td>A(−936)</td>
<td>T(−1013)</td>
<td></td>
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</tr>
<tr>
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<td>T(−940)</td>
<td>T(−1017)</td>
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</tr>
<tr>
<td>C(−965)</td>
<td>C(−960)</td>
<td>T(−1037)</td>
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<tr>
<td>C(−990)</td>
<td>C(−985)</td>
<td>G(−1062)</td>
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<tr>
<td>A(−1003)</td>
<td>A(−998)</td>
<td>G(−1075)</td>
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<tr>
<td>G(−1025)</td>
<td>A(−1120)</td>
<td>G(−1097)</td>
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<tr>
<td>C(−1028)</td>
<td>C(−1023)</td>
<td>G(−1100)</td>
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<td>G(−1052)</td>
<td>G(−1047)</td>
<td>A(−1124)</td>
<td></td>
<td></td>
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Position numbering differs among the 3 strains.
linkage on chromosome 13 between BPL/1 and SPRET (Fig. 1; see also, Ref. 35). We therefore examined the promoter sequence from the cap (transcription initiation) site out to approximately −1100 bp (Supplemental Figure, panel A) in each strain, in which we discovered a complex microsatellite (consisting of variable numbers of di-, tri-, tetra-, and penta-nucleotide repeats), whose length varied among the three strains and was longest in SPRET (Fig. 3; 338 bp long in SPRET, 264 bp in BPL/1, and 260 bp in BPH/2).

The promoter also contained 25 single nucleotide variants and 3 single nucleotide deletions differentiating the 3 strains, 23 of which were unique to SPRET variants and 3 single nucleotide deletions differentiating SPRET, 264 bp in BPL/1, and 260 bp in BPH/2).

Of note, some of the single nucleotide variants unique to SPRET altered domains that contained putative transcriptional regulatory sites (see Tables 2–4): T-55C (creating a longer G/C-rich stretch), A-102G (lengthening a G/C-rich stretch), G-128A (shortening a G/C-rich region), deletion of A at −347 in SPRET (creating a G/HF.1 site), G-932A (creating a G/H-1 site), C-1005T (creating a Stat semi-palindrome), and C-1100G (creating a C/EBP site). Murasawa et al. (21) identified an A/T-rich region of the rat Agtr1a promoter (−456/−442 bp) that functions as a negative regulatory element in chromaffin cell expression of Agtr1a.

Agtr1a endogenous gene expression studies: transcript abundance. Since the angiotensin II (type 1) receptor is expressed in the rodent adrenal gland (8), including chromaffin cells (8), where it may regulate catecholamine release (19), we studied the abundance of Agtr1a transcripts in the adrenal glands of the three strains, using RT-PCR (Fig. 4). The three strains differed significantly in Agtr1a mRNA abundance (ANOVA F = 5.77, P = 0.014). BPL/1 (at 175 ± 2.44 ng equivalents) expressed ~67% more (Bonferroni P < 0.05) Agtr1a mRNA than SPRET (at 105 ± 16 ng equivalents). Relative overexpression of Agtr1a in BPL/1 (compared to SPRET) is consistent with the action of the BPL/1 Agtr1a allele to increase systolic blood pressure in the N2 generation of the linkage study of BCBP ([SPRET×BPL/1]×BPL/1; Ref. 35).

Agtr1a is expressed in not only the adrenal medulla, but also the zona glomerulosa cells of the adrenal cortex, where it regulates mineralocorticoid biosynthesis and release (9, 13). Our RT-PCR results on total adrenal gland mRNA cannot distinguish whether differences in expression between the three strains arose from the medulla vs. the cortex.

Agtr1a promoter variants: promoter/reporter transfections. Consistent with the mRNA abundance studies (Fig. 4), the three strains’ transfected promoters differed significantly in activity (ANOVA F = 58.9, P = 0.0001). The transfected Agtr1a promoter from BPL/1 was ~95% more active (Bonferroni P < 0.001) than the orthologous region from SPRET (Fig. 5).

In conclusion, Agtr1a endogenous gene expression in vivo was diminished in SPRET compared with BPL/1

![Fig. 4. Quantitation of the endogenous Agtr1a mRNA among SPRET, BPL/1, and BPH/2. Comparisons of basal endogenous Agtr1a mRNA abundance in the adrenal gland were made by RT-PCR, using primers, probes, and conditions detailed in METHODS. The control (‘housekeeping’) RNA was 18S rRNA, enabling expression of experimental results as nanogram equivalents. The three strains differed significantly in Agtr1a mRNA abundance (ANOVA F = 5.77, P = 0.014). BPL/1 expressed significantly (Bonferroni P < 0.05) more Agtr1a mRNA than SPRET.](http://physiolgenomics.physiology.org/)

![Fig. 5. Comparison of Agtr1a promoter strengths among mouse strains: results of mouse Agtr1a promoter/luciferase reporter transfections. Basal expression in PC12 cells. Promoter fragments (based on SPRET numbering upstream from the cap/mRNA initiation site) from −1184 bp to +39 bp, were subcloned into the polylinker of the promoterless reporter plasmid pGL3-Basic, with firefly luciferase as reporter. Promoter/reporter plasmids were transfected into PC12 pheochromocytoma cells, along with the cotransfected control plasmid pRL-CMV, encoding Renilla luciferase, under the control of the strong CMV promoter. Cells were harvested 16–21 h after transfection, for luciferase assay on cell extracts. Agtr1a promoter strength values are normalized (ratioed) to the Renilla luciferase values, to control for transfection efficiency. Each experiment was conducted in triplicate. RLU, relative light units (luciferase reporter activity, firefly/Renilla). The three strains’ transfected promoters differed significantly in activity (ANOVA F = 58.9, P = 0.0001). The transfected Agtr1a promoter from BPL/1 was ~95% more active (Bonferroni P < 0.001) than the orthologous region from SPRET.](http://physiolgenomics.physiology.org/)
are associated with changes in promoter activity in vitro, as well as the circulating concentration of angiotensinogen in vivo (10). At the human ACE locus, an insertion/deletion polymorphism (as well as nearby SNPs) is associated with variations in plasma ACE activity in vivo (37). At the human AGTR1 locus, SNPs in both the promoter region (30) and the 3′-untranslated region of the mRNA (24, 33, 34) associate with clinical phenotypes, despite an invariant amino acid sequence (2). At the aldosterone synthase locus, a promoter SNP also associates with clinical phenotypes (23).

Thus our report on mouse Agtr1a functional promoter polymorphisms adds to the growing body of evidence that quantitative (rather than qualitative, amino acid changing) variations in gene expression at loci encoding components of the renin-angiotensin-aldosterone system may be genetically linked to or associated with physiological alterations in blood pressure in vivo.

We appreciate helpful discussions with T. J. Murphy (Emory University).

The mouse genomic DNA sequence for Agtr1a was established by Celera Genomics and was deposited in the Celera Discovery System at http://www.celera.com. Mouse strains used for this sequencing included 129X1/SvJ, DBA/2J, A/J, C57BL/6J, and 129S/SvlmJ.

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essential hypertension and affects basal transcription in vitro. 


