Do not hallucinate.
Materials and Methods

Animals

Kidney and liver tissue samples collected from 45 days old SS/Jr and SR/Jr rats were immediately frozen on dry ice and kindly provided by Dr Garret and Dr Joe, University of Toledo College of Medicine, Toledo, Ohio (former Medical College of Ohio). Samples from 10 rats per strain (5 males and 5 females per strain, each male/female pair from different litters) were used for the analysis. Genomic DNA was isolated from both livers and kidneys of SS/Jr and SR/Jr rats using a protK approach as described elsewhere.11

Total RNA Isolation and Reverse Transcription

Total RNA was isolated from livers and kidneys of SS/Jr and SR/Jr rats using a standard TRIzol-based protocol. Oligo(dT)₁₈ (Promega Corporation)-primed cDNA was synthesized using PowerScript Reverse Transcriptase (Clontech), according to the manufacturer’s instructions, from ~2 μg of total RNA as a template, using Veriti 96-Well Thermal Cycler (Applied Biosystems).

PCR and Sequencing

Preselected amplicons (Figure 1) from genomic DNA were amplified using the following PCR setup. The PCR1 reaction contained 1 μL of stock solution DNA, 0.2 μmol/L of appropriate forward primer, 0.2 μmol/L of appropriate reverse primer (supplemental Table S1, please see http://hyper.ahajournals.org), 400 μmol/L of each dNTP, 25 mmol/L tricine, 7.0% glycerol (wt/vol), 1.6% dimethyl sulfoxide (DMSO) (wt/vol), 2 mmol/L MgCl₂, 85 mmol/L ammonium acetate pH 8.7, and 0.8 U Taq Polymerase in a total volume of 20 μL. The PCR was carried out using a touchdown thermocycling program (94°C for 60 seconds; 15 cycles of 92°C for 30 seconds, 65°C for 30 seconds [4°C gradient], and 72°C for 60 seconds; 72°C for 7 minutes, Veriti 96-Well Thermal Cycler, Applied Biosystems).

The gradient PCR reactions contained 1 μL of stock solution DNA, 0.2 μmol/L of 5’-GTCCCCGAGTTCAAAAGAAGA-3’ primer, 0.2 μmol/L of 5’-ACCCCTGATGAGGTGGATGAA-3’ primer, 7 μmol/L of each dNTP, 25 mmol/L tricine, 7.0% glycerol (wt/vol), 1.6% DMSO (wt/vol), 2 mmol/L MgCl₂, 85 mmol/L ammonium acetate pH 8.7, and 0.8 U Taq Polymerase in a total volume of 20 μL. The PCR was carried out using gradient annealing temperature program (94°C for 60 seconds; 35 cycles of 94°C for 30 seconds, 50 to 70°C for 30 seconds [4°C gradient], and 72°C for 60 seconds; 72°C for 60 seconds; 72°C for 180 seconds; Veriti 96-Well Thermal Cycler, Applied Biosystems).

All PCR samples were evaluated on a 1% agarose gel containing ethidium bromide for the presence of the proper-sized fragment. PCR fragments were sequenced using BigDye chemistry (v3.1, Applied Biosystems), according to the manufacturer’s instructions. Sequences were analyzed for polymorphisms using automated PolyPhred12 and in-house developed software, as well as by manual inspection.

KASPar Genotyping

Competitive allele-specific PCR followed by fluorescence detection (KASPar) (KBiosciences) is based on the sensitivity of KTaq polymerase to 3’end mismatches. KTaq is only able to amplify templates primed with allele-specific primer without 3’end mismatch. As a result, synthesized amplicons contain specific tails from the allele-specific primers. The reaction mix also contains labeled oligonucleotides that are complementary to these tails, and allele-specific fluorescence of these labels is activated on release by KTaq during specific amplification.

Seven sets of primers (supplemental Table S1) were designed for KASPar genotyping of known and candidate SNPs in the Atp1a1 gene. Two independent sets were designed for detection of the A1079T transversion, separately for the positive and negative strands of the DNA, respectively, and 5 sets were designed for detection of different single nucleotide polymorphisms (SNPs) localized in intronic as well as exonic parts of the Atp1a1 gene. All genomic assays are performed on nonamplified total DNA. In addition, nonamplified...
single stranded cDNA was used for detection of the putative A1079T transversion and for 2 additional SNPs localized in exonic part. Primers were tested using a DNA panel that consists of 8 wild rats and 33 inbred rat strains as well as negative controls. KASPar genotyping was performed in duplicate according to the manufacturer’s instructions (KBiosciences) with 10 ng of genomic DNA or 0.2 μL cDNA directly from the reverse transcription mix as input.

Inversion Probe Ligation Assay
The inversion probe ligation assay (IPLA), derived from the molecular inversion probe assay, is based on the specificity of T4 ligase to ligate only the probes without 3’ end mismatches and its results are independent of DNA polymerase errors. The following steps can be discerned: (1) Hybridization: Each reaction contained 2.5 μg of genomic DNA and 24 fmol of probe (supplemental Table S1) in a total volume of 18 μL. The hybridization step was performed by incubation for 5 minutes at 95°C followed by 10 minutes at 60°C, and the mixture was kept at 37°C until the ligation step was performed. (2) Ligation: A mixture containing 2U of T4 DNA Ligase (Promega Corporation) and 4 μL of Ligase 10× Buffer in total volume of 22 μL was preheated to 37°C and added to hybridized DNA. Samples were incubated for 45 minutes at 37°C. After ligation, DNA was immediately purified by precipitation. (3) Digestion of noncircularized probes: Precipitated DNA from the ligation step was treated with 20 U of Exonuclease I (New England Biolabs), 4 μL of 10× Exonuclease I reaction buffer in total volume of 40 μL. Samples were incubated for 15 minutes at 37°C. After digestion, DNA was purified by precipitation. (4) Uracil depurination and cleavage: 0.8 U of Afu Uracil-DNA Glycosylase (New England Biolabs) and 4 μL Afu UDP Reaction Buffer were added to the precipitated DNA from the previous step in a total volume of 40 μL. The reaction mixture was incubated for 10 minutes at 65°C and 20 minutes at 95°C. The concentrations of ligated allele-specific probes in the different samples are indicative of the genotype and were determined using quantitative PCR (Q-PCR). The Q-PCR reaction mixture contained 0.1 μL or 0.05 μL of DNA from the previous step, 0.5 μmol/L of each appropriate primer (supplemental Table S1), and 12.5 μL of IQ SYBR Green 2× Supermix (Biorad) in a total volume of 25 μL. PCR was performed using iCycler (Biorad) with standard conditions.
cycling conditions. The concentration of circularized probe is shown in arbitrary units (AU). One AU is defined as the average concentration of circularized probe with adenine at 3' end (A-probe) in the assay performed with genomic DNA from SR/Jr rats.

**Results**

**Gradient Annealing Temperature PCR**

Amplicons spanning the putative A1079T transversion could be amplified from genomic DNA of SR/Jr and SS/Jr strains using the whole range from 50 to 70°C as the annealing temperature. Weak nonspecific bands were observed only at 50°C and 54°C in both strains. No differences in amplification efficiency or in the presence/absence of nonspecific bands were found between SS/Jr and SR/Jr rats.

**Sequencing**

Bidirectional sequencing of the PCR products from genomic DNA (10 SS/Jr and 10 SR/Jr rats, 2 tissues [liver and kidney] per rat [6 different annealing temperatures in case of amplicon 2]), as well as cDNA (10 SS and 10 SR rats, 2 tissues per rat) did not reveal the presence of the A1079T transversion in any sample derived from SS/Jr rats (supplemental Table S1; Figure 2). Moreover, the existence of a silent C2922T (SNP1; Table) transversion, described by Herrera and Ruiz-Opazo3 in SS/Jr rats was also not confirmed by sequencing analysis. On the other hand, 1 silent exonic SNP (SNP2) and 3 intronic SNPs (SNP3–5) were confirmed to be different between SS/Jr and SR/Jr strains (Table). No sex-, litter-, or tissue-related variances within SS/Jr or SR/Jr rats were observed by sequencing of the amplicons derived from genomic DNA, as well as cDNA.

**KASPar Genotyping**

Two sets of primers, designed separately for each strand of DNA, were used for genotyping of the A1079T transversion. In line with the sequencing results, KASPar genotyping confirmed the presence of the A allele at position 1079 and the C allele at position 2922 in nonamplified genomic DNA and cDNA obtained from SS/Jr and SR/Jr rats (Table; Figure 3). As well as in the genomic DNA of 33 different rat strains (including 1 SR/JrHsd and 1 SS/JrHsd rat) and 8 wild rats. On the other hand, the presence of 4 SNPs (SNP2–5), differentiating the SS/Jr and SR/Jr strains, was confirmed by KASPar analysis using genomic DNA and for silent exonic SNP2 also using cDNA (Figure 3).

**Inversion Probe Ligation Assay**

Efficiency in circularization of the A-probe was similar in both strains indicating equal presence of the A1079 allele in the ATP1a1 gene. Furthermore, the concentration of the ligated T-probe was only detected at background levels (the same as the concentration in control samples processed without ligase) in all samples examined, again indicating that the A1079T transversion is not present (Figure 4). The source of amplifiable template for qPCR in the samples with the T-probe is probably attributable to a small amount of noncircular probes that survived the exonuclease treatment and can serve as a primer in the first few Q-PCR cycles, thereby creating the appropriate template for primers used in the qPCR reaction. Such a mechanism is supported by the finding

![Figure 3](http://hyper.ahajournals.org/)

**Figure 3.** KASPar genotyping results. A, SNP2; B, Transversion; C, SNP1. Blue dots represent samples derived from SR/Jr rats; red from SS/Jr rats and black dots represents samples processed without DNA. Values of normalized FAM fluorescence are plotted on the x axis and normalized VIC fluorescence is on the y axis. Samples located near the x axis are homozygous for FAM allele (A, T1680; B, A1079; C, C2922), and samples located near the y axis are homozygous for VIC allele (A, C1680; B, T1079; C, T2922).

![Figure 4](http://hyper.ahajournals.org/)

**Figure 4.** Inversion ligation probe assay principle and results. Principle of inversion ligation probe assay: Total genomic DNA (A) is hybridized together with a136-bp long probe containing sequence homologous to targeted genomic DNA (B) Ends of probes with perfect matching on the 3'-prime end are ligated together, resulting in circular product. Noncircularized probes with mismatch at 3'-prime end are subsequently digested with Exonuclease I. C, Circularized probe is treated with Uracll-DNA Glycosylase to depurinate the uracil residues in the probe. The mixture is subsequently heated resulting in cleavage of probe at the abasic site. D, Concentrations of ligated probes are determined using qPCR approach. Results: E, Concentration of ligated A-probe is higher compared to T-probe and is similar in both SS/Jr and SR/Jr strains, suggesting that the A1079T transversion is not present.
that in samples processed without ligase, where no circular probes can exist, similar signals are obtained (Figure 4).

Discussion

We have used different experimental approaches to assess the existence of the A1079T polymorphism in the Atp1a1 gene, including techniques that are not sensitive to potential reproducible DNA polymerase errors. Such errors were hypothesized to be responsible for misdetection of the transversion, although in all cases mentioned, the genotyping problem could be solved by a change in experimental conditions and was never caused by a robust repetitive DNA polymerase error. We failed to detect the presence of the A1079T transversion in genomic DNA and cDNA of the SS/Jr strain, using bidirectional sequencing or by KASPar genotyping—a method that is nothing more than a sophisticated variant of PASA, which was previously used to confirm the existence of A1079T.

To further exclude the possibility of putative DNA polymerase errors occurring while amplifying genomic DNA, we have used 2 different methods that do not depend on the use of a polymerase for the discrimination of the alleles—KASPar genotyping and IPLA—both failing to detect the A1079T transversion at either the genomic or mRNA/cDNA level. Although KASPar genotyping involves a specific Taq polymerase, this polymerase is not involved in the incorporation of the complementary base at the transversion position itself as this position is covered by the last base of the allele-specific oligos. IPLA is based on the sensitivity of T4 DNA ligase to mismatches with the genomic template at the 3'-end of the allele-specific ligation probes. Subsequent DNA amplification uses the successfully circularized ligation probe and not the genomic DNA as a template. Therefore, we can fully exclude a possible bias caused by DNA polymerase.

Our results clearly show that the failure to detect the A1079T transversion is caused by the absence of the transversion itself rather than technical limitations or complications of the methods used. Moreover, we also failed to detect another transversion in SS/Jr rats—C2922T—which was found by Herrera and Ruiz-Opazo in the same study and the same clone in which the A1079T transversion was found. Therefore, we conclude that the A1079T transversion, as well as the C2922T polymorphism, are most likely artifacts introduced by RT-PCR errors. However, we cannot exclude that Herrera and Ruiz-Opazo have been using a different substrain of SS/Jr, which we currently used SS strain does not contain the transversion, but we cannot exclude the presence of the A1079T transversion in the strain used by Herrera and Ruiz-Opazo, which we would be happy to test in our assays. Although the involvement of the Atp1a1 gene in hypertension attributable to another polymorphism/mechanism cannot be excluded at present, the SS and SR strains are expected to be instrumental for the discovery of novel polymorphisms and genes involved in hypertension.

Perspectives

Over the last decades, the rat has proven a versatile model for the studying complex genetics that is underlying the development of hypertension. The salt-sensitive and resistant rat strains SS and SR are important and widely used models in this research area. The A1079T transversion in the Atp1a1 gene was the first genetic factor associated with hypertension in the SS strain, however it has been a matter of debate ever since its discovery in 1990. In our independent study, we used different state-of-the-art technologies and show that the currently used SS strain does not contain the transversion, but we cannot exclude the presence of the A1079T transversion in the strain used by Herrera and Ruiz-Opazo, which would be happy to test in our assays. Although the involvement of the Atp1a1 gene in hypertension attributable to another polymorphism/mechanism cannot be excluded at present, the SS and SR strains are expected to be instrumental for the discovery of novel polymorphisms and genes involved in hypertension.

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Disclosures

None.

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The *Atp1a1* Gene From Inbred Dahl Salt Sensitive Rats Does Not Contain the A1079T Missense Transversion
Michal Mokry and Edwin Cuppen

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