TA Repeat Variation, Npr1 Expression, and Blood Pressure Impact of the Ace Locus

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Abstract—The activity of the atrial natriuretic peptide receptor (Npr1) is altered in spontaneously hypertensive rats (SHR) in relation to its mRNA levels, suggesting abnormal transcriptional control in hypertension. A single-stranded conformational polymorphism caused by a repetitive dinucleotide segment of 10 TA in BN-Lx and of 40 TA in SHR was localized at position −943 relative to the transcription start site of the Npr1 gene, downstream of a putative cGMP-regulatory region, and was the only sequence difference noted between the two strains. Transient transfections of −1520 to −920 Npr1 promoter-SV40-luciferase fusion vector showed that the construct from BN-Lx stimulated the SV40 promoter, whereas that from SHR slightly inhibited it. In contrast to the BN-Lx construct, the activity of the SHR fragment was refractory to downregulation by atrial natriuretic peptide. Genotype-phenotype correlation studies in recombinant inbred strains (RIS) derived from BN-Lx and SHR crosses revealed significant correlations of the TA repeat with basal guanylyl cyclase activity and Npr1 mRNA levels. The correlations were heightened by a locus on chromosome 10 containing the Ace gene. The highest basal guanylyl cyclase activity and Npr1 mRNA values were found in RIS with both genes (Npr1/Ace) of BN genotypes, whereas the lowest were recorded in RIS, with the SHR genotypes at both loci. This was inversely correlated with diastolic blood pressure in these strains. In conclusion, the longer TA repeat unit in the promoter of Npr1 of SHR, in tandem with a putative cGMP responsive element, regulates the transcription of the Npr1 gene with consequences on diastolic blood pressure. (Hypertension. 2003;41:16-24.)

Key Words: rats, spontaneously hypertensive ■ natriuretic peptides ■ cyclic GMP ■ genes ■ angiotensin-converting enzyme

Atrial natriuretic peptide (ANP) is a cardiac hormone acting on the vasculature, the kidney, the adrenal glands, and the nervous system through specific membrane receptors possessing intrinsic cGMP-synthesizing activity.1–5 These physiological functions of ANP oppose those of the renin-angiotensin system.

The natriuretic peptide receptor (NPR) family includes 3 major receptor subtypes. NPR-A, also called GC-A because of its guanylyl cyclase activity, or Npr1 for its gene designation, is a transmembrane protein with an extracellular binding site for ANP, a single short transmembrane domain separating the protein into two halves, and an intracellular domain containing a regulatory kinase-like domain and a catalytic GC domain at its C-terminal end.6

Downregulation of Npr has been demonstrated in vivo7–9 and in vitro.10 Increased cGMP downregulates both NPR density and ANP-stimulated cGMP synthesis.8,11,12 Receptor desensitization appears to be a mechanism13,14 of apparent receptor desensitization, but transfection experiments with Npr1 cDNA have revealed that early desensitization by ANP pretreatment can be due to dephosphorylation of NPR-A/ GC-A protein.15 Other studies have shown that ANP can suppress the transcriptional activity of Npr1 in vascular smooth muscle cells and in inner medullary collecting duct cells through a putative cGMP-regulatory element,16,17 leading to a reduction in its mRNA levels. Our recent investigations have narrowed down the region and defined the consensus sequence of the cGMP-responsive element.18

Essential hypertension is a multifactorial disease controlled by multiple genes as well as environmental factors.19 Linkage studies have been performed in the F2 generation of several crosses of genetically hypertensive rat models with normotensive strains. The identification of quantitative trait loci (QTL) in these animal models has unveiled candidate genes. One of these is the Npr1 gene on rat chromosome 2.19–23 Data from congenic strains have confirmed the existence of a blood pressure QTL at the Npr1 locus,24 whereas the Na-K-ATPase α1-subunit (Atp1a1) in the vicinity of the Npr1 gene, which was initially proposed,25 has recently been excluded as a blood pressure QTL.26,27

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The involvement of Npr1 in hypertension is further supported by recent studies showing the development of hypertension in knock-out mice lacking Npr1, which is not altered by salt intake, and diuresis and natriuresis induced by volume expansion are abolished in Npr1-deficient mice, indicating that natriuretic peptides released from the heart act mainly through Npr1.

In hypertension, data on ANP binding sites are conflicting, as both increased and decreased density have been reported. For instance, increased receptor density or affinity is observed in young spontaneously hypertensive rats (SHR), whereas older animals demonstrate reduced receptor density. We have reported previously that a heightened response to ANP in the glomeruli and aortic smooth muscle cells of young SHR compared with Wistar-Kyoto (WKY) rats is accompanied by elevated receptor density. The results suggest abnormalities at this level.

We report here that a dinucleotide TA repeat in the promoter region of Npr1 specific to SHR affects the basal transcriptional activity of the Npr1 gene. Its impact on transcription as well as on diastolic blood pressure (DBP) in recombinant inbred strains (RIS) originating from reciprocal crosses of BN-Lx and SHR points to interactions of QTL on chromosomes 2 and 10.

Methods

Animals

The progenitor strains (BN-Lx/Cub and SHR/Oba) and their RIS were obtained from Prague, Czech Republic. This RIS panel, representing 31 fully inbred homozygous strains, is the only one available to study the genetics of hypertension in rats. The rats used in our experiment were housed at a constant temperature under a 12 hour:12 hour dark/light cycle and fed normal Purina rat chow with free access to tap water. All animals studied (n = 4 to 6 per strain) were 12 weeks old. They were brought to Montreal for isolation of glomeruli and adrenals, as described previously. The blood pressure data were available for all strains from previous records. Npr1 and Ac gene expression was determined on genomic DNA from the 31 original strains, and biochemical data were obtained in age-matched male rats from 18 randomly selected RIS (9 H×B and 9 B×H reciprocal crosses). Eight RIS had the Npr1 LN normotensive genotype, and 10 had the SHR hypertensive phenotype; there was an equal distribution of RIS with BN and SHR genotypes at the Ac locus (9 each). Rats were purchased from different suppliers for screening of the Npr1 genotypes. The protocols for handling and maintaining the rats were approved by the CHUM animal care committee.

DNA and Chemicals

Plasmid DNA was amplified according to Sambrook et al. All polymerase chain reaction (PCR)-amplified DNA fragments were sequenced by the dye-exchange chain termination method, with the use of the T7 Sequenase sequencing kit (Amersham Pharmacia Biotech). Before cell transfection, the plasmids were purified by cesium chloride gradient ultracentrifugation. Restriction enzymes were from Gibco BRL. Synthetic rat ANP (Promega), which carries the luciferase gene, was sequenced by the dideoxy-chain termination method, with the use of the T7 Sequenase sequencing kit (Amersham Pharmacia Biotech). Polyacrylamide gel electrophoresis and autoradiography were performed in a Bio-Rad multiphorImager (Molecular Dynamics).

Polymerase Chain Reaction and Single-Stranded Conformational Polymorphism Screening

DNA was extracted from the livers of different rat strains, as described by Ausubel et al. Oligonucleotides of 20 bases were synthesized in sense and antisense orientations based on the reported rat Npr1 gene sequence. Their sequences are listed in Table 1.

Oligonucleotides S27 and A14 were used for Npr1 genotyping of various rat strains. The lengths of the DNA products flanked by these primers were 140 bp for BN and 200 bp for SHR. PCR amplification was performed in a Perkin Elmer Cetus DNA thermal cycler according to the manufacturer’s protocol (Gibco BRL). Aliquots of the amplification reactions were run on 6% acrylamide single-stranded conformational polymorphism (SSCP) gels in TBE (0.089 mol/L Tris–boric acid, 2 mmol/L EDTA buffer, pH 8.0) containing 10% glycerol for SSCP, or run on 6% acrylamide gel in TBE for non-SSCP analysis. Radioactive bands were detected with a PhosphorImager (Molecular Dynamics).

Construction of the Npr1 Promoter and Reporter Gene

Fragments of the Npr1 gene 5’ upstream region were generated by PCR and then subcloned into the reporter vector pGL3b (basic) (Promega), which carries the luciferase gene. The −1520 pGL3b Luc fragments, which start at base −1520 with respect to the transcription start site as given in GenBank sequence J05677, directly upstream of the Npr1 gene, were constructed using primers listed in Table 1.

Fragments of the Npr1 gene not directly upstream of the transcription start site were generated by PCR and then subcloned into the reporter vector pGL3p (promoter, Promega), which carries the luciferase gene downstream of the SV40 promoter sequence. The −1520 primer described in Table 1 was used along with an antisense primer at position −920 to generate the −1520 to −920 fragment. The full −1520 to 0 DNA fragment described above was cleaved with Ndel to generate the −1520 to −1290 fragment. A −1205 primer (Table 1) with the −920 antisense primer generated the corresponding fragment. All fragments were sequenced. No single base substitution was found, indicating no Taq polymerase errors. Amplified plasmids were purified by cesium chloride–ethidium bromide gradient ultracentrifugation.

Transfection of Cultured Cells, Luciferase, and β-Galactosidase Assays

Mouse NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s high glucose medium supplemented with 10% calf serum (Life Technologies) and 2% penicillin/streptomycin at 37°C in a 5% CO2–controlled atmosphere. DNA was transiently transfected into cells according to the calcium phosphate precipitation procedure. The cells were initially plated in 6-well plates (Nunc) at a density of 2 to 3×104 cells per well in 2 mL of medium. After 24 hours, they were transfected in duplicate with 2 μg/well of purified plasmid. Transfection efficiency was monitored by cotransfecting 3 μg of a control plasmid, pCMV-β Gal (Clontech), which expresses β-galactosidase. The culture medium was changed 24 hours later, and the cells were or were not preincubated with 100 mmol/L ANP for the time indicated. They were then collected, and expression of the reporter gene was assayed. β-Galactosidase activity in cell extracts was measured by using o-nitrophenyl β-D-galactopyranoside (ONPG) as substrate, as described by Sambrook et al. Luciferase activity of the cell extracts was measured with the Promega Luciferase Assay System.

Determination of Npr1 mRNA Levels by Quantitative Reverse Transcription–PCR

Total RNA was extracted from the adrenal glands of RIS rats. Total RNA (0.5 μg) was titrated with increasing amounts of mutated Npr1 cDNA by quantitative reverse transcriptase (RT)-PCR, as described previously.

Statistical and Genetic Analyses

Statistical analyses were carried out with the unpaired Student t test or 2-way ANOVA as appropriate. Differences were considered to be significant at P<0.05. A genome-wide scan was performed with a simple and composite interval-mapping technique (MapManager QTb21 software). The markers used are those indicated in http://www.ratmap.gen.gu.se.

Composite interval mapping is a refine-
ment that absorbs effects associated with known or suspected QTL during analysis of other QTL. Thresholds for significance were obtained with permutation tests carried out according to Doerge and Churchill.48 Suggestive, significant, and highly significant values, taken from the guidelines of Lander and Kruglyak, 49 corresponded to the 37th, 95th, and 99.9th percentiles, respectively. These values represent likelihood ratio statistics for finding an association between a trait and a locus marker more than by chance.

Results

Identification of a Dinucleotide TA Repeat Polymorphism in the Promoter Region of Npr1

From the previously reported gene sequence of rat Npr1,42 we screened the 1520-bp 5′-flanking region of the Npr1 gene by PCR-SSCP technology. A unique PCR-SSCP between BN-Lx and SHRp was localized between positions −1010 and −871 from the transcription start site (Figure 1A). Sequence analysis of this polymorphic fragment revealed the presence of a dinucleotide TA repeat of 40 in SHR and of 10 in BN-Lx (Figure 1B). Sequence analysis of this polymorphic fragment revealed the presence of a dinucleotide TA repeat of 40 in SHR and of 10 in BN-Lx (Figure 1B). A TA repeat of 10 was also present in the sequence published by the Yamaguchi group,42 but the rat strain used in their studies was not specified. Sequencing of several 1520-bp clones from BN-Lx and SHR, including a putative cGMP-responsive element,16,18 confirmed that the TA repeat localized at position −943 from the transcription start site was the only difference between the two strains.

Genotyping With the Dinucleotide TA Repeat Unit of the Npr1 Promoter

We used the TA repeat in the Npr1 promoter to genotype individual RIS to analyze the genotype-phenotype relation of this TA expansion with Npr1 mRNA levels, protein activity, and blood pressure. As shown in Figure 1C, PCR amplification with primers flanking the TA repeat (S27 and A14 in Table 1) gave DNA products that migrated distinctly on acrylamide gel electrophoresis, with SHR-PCR products migrating more slowly than BN-Lx DNA, defining a long (L) and a short (S) allele (Figure 1C). The amplification of genomic DNA from 31 RIS revealed an almost equal distribution of this TA repeat, with 15 strains bearing the SHR (homozygous LL or HH for hypertensive strain) genotype, and 16, the BN-Lx (homozygous SS or BB alleles for BN-Lx strain) genotype.

We then investigated the polymorphism of the TA repeat in various rat strains. Allelic variants that differed with respect to the repeat number of TA units were regrouped into 3 major genotypes: a short (S) allele of 10 TA, a set of alleles of intermediate (I) lengths consisting of 25 to 32 TA, and a set of alleles composed of dinucleotide repeats >34 TA (L = long length). Table 2 summarizes the genotypes observed in several rat strains studied in hypertension research. BN-Lx and BN rats were homozygotes for the short allele (SS),

<table>
<thead>
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<th>Table 1: Oligonucleotides Used for PCR-SSCP and Genotyping</th>
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<tr>
<td>Name</td>
</tr>
<tr>
<td>S17</td>
</tr>
<tr>
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</table>

PCR oligonucleotides used for construction of the Npr1 promoter

Bgl II sites are underlined.

Genotyping With the Dinucleotide TA Repeat Unit of the Npr1 Promoter

We used the TA repeat in the Npr1 promoter to genotype individual RIS to analyze the genotype-phenotype relation of this TA expansion with Npr1 mRNA levels, protein activity, and blood pressure. As shown in Figure 1C, PCR amplification with primers flanking the TA repeat (S27 and A14 in Table 1) gave DNA products that migrated distinctly on acrylamide gel electrophoresis, with SHR-PCR products migrating more slowly than BN-Lx DNA, defining a long (L) and a short (S) allele (Figure 1C). The amplification of genomic DNA from 31 RIS revealed an almost equal distribution of this TA repeat, with 15 strains bearing the SHR (homozygous LL or HH for hypertensive strain) genotype, and 16, the BN-Lx (homozygous SS or BB alleles for BN-Lx strain) genotype.

We then investigated the polymorphism of the TA repeat in various rat strains. Allelic variants that differed with respect to the repeat number of TA units were regrouped into 3 major genotypes: a short (S) allele of 10 TA, a set of alleles of intermediate (I) lengths consisting of 25 to 32 TA, and a set of alleles composed of dinucleotide repeats >34 TA (L = long length). Table 2 summarizes the genotypes observed in several rat strains studied in hypertension research. BN-Lx and BN rats were homozygotes for the short allele (SS),
whereas SHR from different commercial suppliers were all homozygotes for the long allele (LL), as was the SHR progenitor strain of the RIS panel used in this study. We observed that the WKY and Wistar strains obtained from different commercial sources exhibited significant variability in the number of their TA repeat units. We even noted heterozygocity at this locus in 1 male Wistar rat (Table 2). The heterogeneity of the Wistar and WKY strains contrasted with the relative homogeneity of SHR of 3 different origins where only microheterogeneity was observed. Buffalo rats were homozygous for SS, Milan normotensive and hypertensive strains were both homozygous for II, and the Dahl salt-sensitive strain was homozygous for SS.

**TABLE 2. Npr1 Genotypes in Various Rat Strains Studied in Hypertension Research**

<table>
<thead>
<tr>
<th>Rat Strain</th>
<th>Npr1 Genotype</th>
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<td>BN.Lx (2m)</td>
<td>SS, SS (corresponding to BB genotype)</td>
</tr>
<tr>
<td>SHRp (2m)</td>
<td>LL, LL (corresponding to HH genotype)</td>
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<tr>
<td>SHRt (1m, 2f)</td>
<td>LL, LL, LL</td>
</tr>
<tr>
<td>SH Rc (1m)</td>
<td>LL</td>
</tr>
<tr>
<td>Wistarcr (2m)</td>
<td>SI, II</td>
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<tr>
<td>Wistarhsd (2m)</td>
<td>SS, II</td>
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<tr>
<td>Buffalo (1m)</td>
<td>SS</td>
</tr>
<tr>
<td>MNS (2m)</td>
<td>II, II</td>
</tr>
<tr>
<td>MHS (1m)</td>
<td>II</td>
</tr>
<tr>
<td>Dahl S (2m)</td>
<td>SS, SS</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the number of male (m) or female (f) rats genotyped for the TA repeat unit. S indicates 10 TA; I, the set of alleles containing 25–32 TA repeats; and L, the set of alleles containing 34–40 TA. SHRp, SHRt, and SH Rc indicate the source of the SHR substrate (p indicates progenitor strain of the RIS panel; t, Taconic Farms; and cr, Charles River). Wistar substrains came from Charles River (cr), Harlan Sprague Dawley (hsd), or Taconic Farms (t).

**Figure 1.** A, Schematic representation of promoter region of the GC-A gene. Open arrow at right side of box indicates transcription start site. Arrow heads over the gene represent sense and antisense primers with their identification numbers (not to scale). Lower shaded box represents the location of the polymorphic fragment. Unique restriction sites are shown underneath the promoter. B, Sequence analysis of polymorphic PCR fragments from SHR and BN-Lx DNA. Bases are identified on top of sequencing gel. Lines on both sides of the gel cover the region of TA repeats. No other difference in sequence was observed. C, Polymorphism of TA repeat in the panel of RIS. PCR amplified DNA fragments containing the TA repeat were analyzed by PAGE. Template DNA was extracted from progenitor strains BN-Lx (B allele) and SHR (H allele) (extreme two right lanes) and from RIS. Strains are identified on top of gel.

**Figure 2A.** A, Schematic representation of promoter region of the GC-A gene. Open arrow at right side of box indicates transcription start site. Arrow heads over the gene represent sense and antisense primers with their identification numbers (not to scale). Lower shaded box represents the location of the polymorphic fragment. Unique restriction sites are shown underneath the promoter. B, Sequence analysis of polymorphic PCR fragments from SHR and BN-Lx DNA. Bases are identified on top of sequencing gel. Lines on both sides of the gel cover the region of TA repeats. No other difference in sequence was observed. C, Polymorphism of TA repeat in the panel of RIS. PCR amplified DNA fragments containing the TA repeat were analyzed by PAGE. Template DNA was extracted from progenitor strains BN-Lx (B allele) and SHR (H allele) (extreme two right lanes) and from RIS. Strains are identified on top of gel.
and SHR fragments were compared (Figure 2B). The −1520 to −1290 fragment spanning the putative cGMP-response element without the TA repeats from either BN.Lx (10 TA) or SHRp (40 TA) stimulated SV40 promoter activity by about 2-fold (Figure 2B). The −1205 to −920 fragments of BN-Lx and SHR containing the TA repeat inhibited the SV40 promoter by ≈75% each, indicating the presence of cis-acting negative sequences in this region but with no significant difference between them despite differences in the length of their TA repeats. On the other hand, when the −1520 to −920 fragment, which contained both the cGMP-response element and the TA repeat, was studied, a significant difference between BN-Lx and SHR (P = 0.0048) was revealed. The overall effect remained positive in the case of BN-Lx, with a 52 ± 14% increase of SV40 promoter activity, whereas in SHR the positive component was completely neutralized by the presence of the long TA repeat unit (−8.0 ± 11%). These results suggest that the long TA repeat found in SHR could repress the activity of the cGMP-response element under basal conditions. We then tested the overall effect of the promoter fragments in downregulating Npr1 expression after ANP stimulation. Transfected cells were therefore preincubated with ANP. As shown in Figure 2C, fragments containing only the TA repeats from BN-Lx or SHR reduced luciferase activity as in the preceding experiment in cells incubated without ANP. The addition of the hormone did not significantly change the effect of this negative cis element. Increased activity by the −1520 to −920 fragment of the BN-Lx promoter was again observed, and this stimulation was inhibited significantly (2-fold) by preincubation with ANP. In contrast, the fragment originating from SHR caused a slight reduction of luciferase transcription and activity and was not changed by ANP treatment.

Effects of Npr1 and Ace Loci on DBP in RIS

Previous total genome scans did not reveal any significant QTL for blood pressure at the Ace locus in this RIS panel, whereas there was a significant QTL for DBP (LOD score of 3.2; P = 0.0001, significant threshold = 2.4, CI 41 cM) on chromosome 2 at the peak marker D2N91 (or D1Arb24), which is at an 18-cM distance from the Npr1 gene.39 As Npr1 and Ace locus interactions have been demonstrated in other crosses,20,21 we evaluated their effects on RIS blood pressure. Including Ace (Dep1 marker on rat chromosome 10) into the model led to the appearance of a major QTL for DBP on chromosome 2, with a maximum LOD score of 4.4 (P = 7.5 × 10⁻⁶), which fell within the category of highly significant LOD score, considering a highly significant
threshold of 4.3. We also tested the other markers distal to Dcp1 on chromosome 10 for possible interactions with DBP QTL on chromosome 2. Only Dcp1 increased the chromosome 2 DBP QTL. Furthermore, the region involved in this interaction did not include NOS 2 (nitric oxide synthase 2) located within 22 cM from Ace. Figure 3A shows the effect of Npr1 and Ace alleles separately on DBP in RIS. Strains of the RIS panel bearing the Npr1 hypertensive (HH) genotype had significantly higher (112.9±2.6 versus 103.4±2.2 mm Hg; P=0.009) DBP than RIS with the BN.Lx (BB) genotype of Npr1. On the other hand, there was no difference in DBP in RIS grouped by Ace alleles (109.1±2.6 versus 106.8±2.7 mm Hg, HH versus BB genotypes respectively, P=NS). Figure 3B shows the effect of the Npr1 locus on DBP for each Ace genotype. The data suggest that the Ace locus, which does not have any significant effects on blood pressure on its own (Figure 3A), in this cross increased the apparent effect of the Npr1 genotype on DBP (ANOVA, P=0.03), resulting in the combined effect of the two loci, which accounted for an up to 18.3 mm Hg difference of DBP in RIS.

**Genotype-Phenotype Correlations of the Dinucleotide TA Repeat Allele With GC Activity and mRNA Levels in RIS**

The impact of the TA repeat allele was evaluated in vivo by determining genotype-phenotype associations for BN-Lx and SHR progenitors as well as for their RIS. High particulate GC activity and cGMP elevation in response to ANP have been previously reported in glomeruli, and we have investigated this phenotype in SHR and BN-Lx progenitor strains as well as in their RIS genotyped for the TA repeat allele. Maximal ANP-stimulated GC activity was significantly lower (P<0.001) in isolated glomeruli from SHR compared with BN-Lx rats (Table 3). Basal GC activity in the glomeruli of RIS bearing the HH genotype of Npr1 was lower than in the BN-Lx progenitor strain or RIS with the Npr1 BB genotype, but the difference did not reach statistical significance. ANP-stimulated GC activity was not different between strains bearing BB or HH Npr1 genotypes. There was no difference in basal GC activity in RIS grouped by Ace alleles, whereas RIS with HH Ace alleles had significantly lower ANP-stimulated GC activity than RIS bearing Ace BB alleles (P=0.037 and P=0.004 for 10⁻⁸ mol/L and 10⁻⁷ mol/L ANP stimulation, respectively). When both genotypes were considered, the lowest basal GC activity was observed in glomeruli of RIS bearing both Npr1 and Ace genes of hypertensive origin (HH homozygotes) with the highest values of both loci of homozygous BB strains. The difference was highly significant (P<0.001). Basal GC values of RIS, which were homozygous BB in one locus and HH in the other, fell between strains bearing the same alleles at both loci. Furthermore, ANP-stimulated GC activity was also the lowest in HH homozygous strains at both loci and the highest in RIS with the BB genotype at both loci (Table 3). On the other hand, the Ace HH genotype appeared to reduce ANP-stimulated GC activity at both concentrations of ANP and for both Npr1 genotypes. These results suggest that TA repeat length predicts basal GC activity but that its effect at least partly interacts with the Ace locus to the extent that most of the variance in GC activity is explained by these two loci.

Npr1 mRNA levels were measured in the adrenals of the same RIS by quantitative RT-PCR. As shown in Figure 4, Npr1 mRNA levels were correlated with the Npr1/Ace genotypes, with mRNA levels declining from Npr1/Ace

**| Genotype | ANP-Stimulated | ANP-Stimulated | ANP-Stimulated |
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<td>10⁻⁷ mol/L</td>
<td>10⁻⁸ mol/L</td>
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<td>17.5±1.6*</td>
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</table>

*P<0.05; **P<0.004; ***P<0.001.
BB,BB>BB,HH>HH,BB>HH,HH. The pattern was concordant with basal GC activity determined in glomeruli (Table 3). Furthermore, regression analysis showed significant inverse correlations between basal GC activity and DBP (r = −0.950, P < 0.04), Npr1 mRNA levels and DBP (r = −0.975, P < 0.005), and a significant direct correlation between basal GC activity and Npr1 mRNA levels (r = 0.964, P < 0.04).

Discussion
The current study identified an expanded dinucleotide TA repeat in the Npr1 promoter of SHR, which is located downstream of a recently proposed cGMP response element. Tandem repetitive DNA sequences, such as the TA dinucleotide repeat unit identified here, are known to show extensive variation in the number of repeats.50,51 It has been demonstrated that the nature of the TA dinucleotide repeat, rather than its flanking sequences, is responsible for generating intraspecies and interspecies variability.52 Allelic variability is most likely the result of slipped-strand mispairing during DNA replication.53 We observed heterogeneity in the number of TA repeats within the Wistar and WKY strains from different commercial sources (Table 2), but only the BN allele (B) or the SHR allele (H) was detected in the panel of RIS derived from BN-Lx and SHR (Figure 1).

Tandem repeat sequences in certain genes have been implicated in the regulation of their expression. For instance, a CA repeat confers an inhibitory effect on cPLA2 promoter activity.54 The class III insulin-linked polymorphic region located in the 5′-flanking region of the insulin gene affects its transcription.55 The present study shows that the TA polymorphism upstream of a cGMP response element affects Npr1 transcription and downregulation by ANP (Figures 2B and 2C). Dinucleotide repeats such as TA repeats are known to have a high potential to form alternative DNA, such as Z-DNA, H-DNA, and cruciform DNA56,57; they may either enhance58,59 or repress promoter activity.60 Our results suggest that the long stretch of the TA repeat may alter the activity of the cGMP response element located upstream. Such gene repression may alter feedback mechanisms of the vasodilatory system in SHR and may be involved in the pathogenesis of hypertension in this strain. Our results also suggest that the Npr1 gene does not underlie hypertension or that some other regions of the Npr1 gene are altered in the Milan hypertensive or the Dahl salt-sensitive strains which bear the II and SS genotypes, respectively (Table 2).

Furthermore, the Npr1 genotype by itself did not explain the difference in ANP-stimulated GC activity between progenitor strains, indicating that other factors are involved in its regulation. One such factor could be the Ace locus that interacts with the Npr1 locus in regulating systolic blood pressure20 and heart weight in two distinct F2 populations. Our results showed that indeed RIS with both loci with alleles from BN (Npr1 BB and Ace BB) demonstrated the highest basal and ANP-stimulated GC activity, and RIS with both loci with alleles from SHR (HH,HH) had the lowest activities. In these RIS, the HH genotype in both loci elicited a 3-fold decrease in GC-A mRNA levels as well as in basal GC activity associated with a 18 mm Hg increment in DBP. In the same cross, the Ace alleles were without any effect on blood pressure, in contrast to other F2 hybrids from SHRSP×WKY and SHR×WKY crosses.72,23 In RIS, Npr1 alleles alone accounted for a 8-mm Hg difference, whereas rats homozygous for SHR alleles at both Npr1 and Ace loci gained an additional 10 mm Hg over Npr1 HH homozygotes, suggesting a more than additive effect. This type of interaction could also explain, at least in part, the contradictory data reported in the literature. For instance, no Ace and Npr1 locus interactions were noted in F2 (WKY×SHR) in the regulation of blood pressure.20 The TA repeat variations observed in the WKY substrains depicted in this study could offer an explanation. Of course, as in other genetic experiments, the possibility of yet unknown genes associated with the alleles hereby defined cannot be excluded. Furthermore, the limited number of meiosis tested in the RIS paradigm will require further ascertainment of Npr1 and Ace loci interaction in an F2 cross.

The renin-angiotensin system, with Ace as a rate-limiting step, opposes the effect of the ANP-NPR-A system on blood pressure, natriuresis, diuresis, and cellular growth. The direct effect of angiotensin II on Npr1 transcription remains to be evaluated, but in vascular smooth muscle cells, transforming growth factor-β1 (TGF-β1) could be a possible mediator. We have shown that TGF-β1 decreases Npr1 mRNA levels,76 and it is well known that TGF-β1 mediates the hypertrophic effect of angiotensin II in several cells.61 The precise mechanism by which the Npr1 and Ace loci interaction occurs awaits further investigation.62,63

Perspectives
Essential hypertension is a complex trait of polygenic and heterogeneous character, with multiple genes involved and gene-gene interactions expected. The presence of highly polymorphic alleles in the regulatory regions of genes controlling blood pressure may explain the continuum of blood pressure observed in essential hypertension. Indeed, our study demonstrates that a TA dinucleotide repeat expansion in the promoter region of the main natriuretic peptide receptor Npr1 inhibits the action of a cGMP response element, with consequences on the transcription of the receptor. This gene alteration in the regulatory region of Npr1 appears to be specific to the SHR strain, as other rat genetic models of hypertension do not bear functional dinucleotide expansion.
pointing to genetic heterogeneity of the disease. Our results also identify some of the consequences of Npr1 and Ace loci interactions at the molecular level, offering the potential to better understand the role of these major hormonal regulators of blood pressure in the pathogenesis of hypertension. Whether this type of alteration exists in human patients with essential hypertension is a possibility, as TA dinucleotide repeat segments are present in the human Npr1 promoter, and the putative cGMP response element is conserved. The locus on chromosome 2 bearing Npr1 as a candidate gene has been found in various crosses of rat strains with genetic hypertension and appears to bear several phenotypes associated with hypertension in humans. Our data in the rat suggest that locus interactions should be considered in human genetic studies of this locus.

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