CA-Repeat Polymorphism in Intron 1 of HSD11B2
Effects on Gene Expression and Salt Sensitivity

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Abstract—Mutations in the HSD11B2 gene encoding the kidney (11-HSD2) isozyme of 11β-hydroxysteroid dehydrogenase cause apparent mineralocorticoid excess, a form of familial hypertension. Because the hypertension associated with AME is of the salt-sensitive type, it seemed possible that decreases in 11-HSD2 activity might be associated with salt sensitivity. To examine this, Italians with mild hypertension underwent a protocol consisting of a rapid intravenous saline infusion and subsequent furosemide diuresis. To determine whether there were genetic associations between HSD11B2 and salt sensitivity, 198 Italians were genotyped for a CA repeat polymorphism (11 alleles) in the first intron. Increased differences in mean arterial pressure between the sodium loaded and depleted states were correlated with shorter CA repeat length (R = 0.214, P = 0.0025). The effect behaved as a recessive trait. This suggested that decreased HSD11B2 expression was associated with shorter CA repeat length. Furthermore, activity of renal 11-HSD2 as measured by an increase in the ratio of urinary-free cortisol/urinary-free cortisone was lower in 33 salt-sensitive subjects (urinary-free cortisol/urinary-free cortisone 0.89 ± 0.04 [mean ± SE]) compared with 34 salt-resistant subjects (0.71 ± 0.04, P < 0.001). However, when minigenes containing either 14 or 23 CA repeats were transfected into rabbit or human kidney cortical collecting duct cells, the construct with 14 repeats was instead expressed at levels 50% higher than those of the construct with 23 repeats, as determined by reverse transcription–polymerase chain reaction. We conclude that polymorphisms in HSD11B2 and decreased 11-HSD2 activity are associated with sensitivity to sodium loading, but a functional explanation for these associations remains to be elucidated. (Hypertension. 2000;36:187-194.)

Key Words: polymorphism ■ gene expression ■ hypertension, genetic ■ dehydrogenases ■ dinucleotide repeat

The syndrome of apparent mineralocorticoid excess (AME) is an autosomal recessive form of salt-sensitive hypertension caused by deficiency of the kidney isozyme of 11β-hydroxysteroid dehydrogenase (11-HSD2). In this disorder, cortisol is not inactivated to cortisone. As a result, cortisol, which is usually a weak mineralocorticoid in vivo, occupies the mineralocorticoid receptor in target tissues such as the distal nephron and causes excessive sodium retention and potassium excretion. The disease is caused by mutations in the HSD11B2 gene encoding the enzyme, most of which severely affect its activity.1

AME is a rare disorder, but mutations or polymorphisms with milder effects on activity might occur more frequently and could be a significant cause of hypertension in the general population. Although mutations that produce very mild effects on enzymatic activity have been identified in relatively mildly affected patients with AME,2,3 such mutations seem to be rare in the general population. Other polymorphisms have been sought but have not been associated with variations in blood pressure.4

Because the hypertension associated with AME is sensitive to sodium intake, putative frequent and milder variations in 11-HSD2 expression might also be expected to influence salt sensitivity. We have attempted to answer that question by means of both biochemical and genetic approaches. We studied individuals who had been subjected to acute salt-loading tests and found that 11-HSD2 activity (measured by urinary precursor/product ratios) was correlated with salt sensitivity. We genotyped the study participants and other subjects for highly polymorphic CA repeat polymorphism in the first intron of HSD11B2 and found that particular alleles at that locus were associated with increased sensitivity of blood pressure to salt intake. We also found that this polymorphism had significant effects on gene expression in cultured cells.
Three 37.5-mg doses of furosemide were given by mouth at 8:00 AM, and the blood pressure was measured every 15 minutes until the last 15 minutes of the infusion, when it was measured every 5 minutes. The average of these 3 blood pressure measurements was calculated. During a 2-hour recovery period, blood pressure was measured every 5 minutes. The subjects lay in a supine position, and their blood pressure was measured 3 times at 5-minute intervals, and those values were averaged. If the difference between the mean arterial pressures at the end of the salt-loading and salt-depletion period, the blood pressure of the supine subjects was measured 3 times at 5-minute intervals, and those values were averaged. If the difference between the mean arterial pressures at the end of the salt-loading and salt-depletion period was greater than the median (10 mm Hg), the patient was classified as “salt sensitive”; otherwise he or she was considered “salt resistant.”

In addition, we genotyped 153 young (24.8 ± 2.3 year) white male Germans who were fed a low-salt diet (20 mmol NaCl/day) for 2 weeks with a supplement of either 200 mmol NaCl/day (sodium) or placebo for 1 week each in a randomized double-blind crossover design as previously described. A similar independent analysis of the identical population has been published elsewhere. For purposes of this study, the patients were not divided into salt-sensitive and salt-resistant groups.

### Methods

#### Subjects

The main study population consisted of 198 white Italians (164 men) with mild hypertension (Table 1). They were taken off antihypertensive medication for 14 days and were subjected to an acute salt-loading and salt-depletion protocol after they had been given a normal sodium diet (150 mmol/day) for 3 days. In 72 patients, the normal saline (2 L) was infused over 4 hours, and the infusion was completed in 2 hours in the remainder. There was no significant difference in blood pressure response as a result of that modification, and the data from all patients were pooled for subsequent analysis. The subjects lay in a supine position, and their blood pressure was measured every 15 minutes until the last 15 minutes of the infusion, when it was measured every 5 minutes. The average of these 3 blood pressure measurements was calculated. During a 2-hour recovery period, blood pressure was checked every 15 minutes. The next day, each patient ate a low-sodium (20 mmol NaCl/day) isocaloric diet. Three 37.5-mg doses of furosemide were given by mouth at 9:00 AM, 4:00 PM, and 12:00 PM. At 9:00 AM the next morning (the end of the salt-depletion period), the blood pressure of the supine subjects was measured 3 times at 5-minute intervals, and those values were averaged. If the difference between the mean arterial pressures at the end of the salt-loading and salt-depletion period was greater than the median (10 mm Hg), the patient was classified as “salt sensitive”; otherwise he or she was considered “salt resistant.”

### Steroid Analyses

In 67 of the 198 Italian subjects (34 salt resistant; 33 salt sensitive), a 24-hour urine collection was obtained and was analyzed for urinary-free cortisol and urinary-free cortisone levels by gas chromatography and mass spectrometry via previously reported methods. The precursor/product ratio, urinary-free cortisone/urinary-free cortisone, is inversely related to 11-HSD2 activity in vivo and provides a more robust measure of the activity of this enzyme than does the urinary THF+/allo-THF/THF ratio.

### Oligonucleotides

All oligonucleotides were prepared by Biosynthesis or Gibco-BRL; their sequences are listed in Table 2.

### Identification of a CA-Repeat Microsatellite Polymorphism

Rsa I fragments of a P1 clone containing the HSD11B2 gene were analyzed by Southern blot with a 32P-labeled (CA)4 repeat oligomer. A band of 0.25 kb hybridized with the probe, and additional DNA of that size was isolated from an Rsa I digest by gel electrophoresis followed by Gelase (Epicentre) digestion. This material was cloned into pBluescriptSKII (Stratagene), and colonies were screened by hybridization with the same probe. Additional mapping of this fragment was accomplished by Southern blot analysis of various restriction digests of the P1 clone and hybridization at high stringency with the cloned insert. DNA sequence analysis was performed with Thermo-sequenase (Amersham) via the manufacturer’s protocols.

### Microsatellite Typing

Leukocyte DNA was prepared from each subject. Polymerase chain reaction (PCR) primers 1 and 2 flanked the CA microsatellite repeat. The reactions were performed with 20 to 100 ng of genomic DNA in a 25 μL reaction volume containing standard PCR buffer and Taq polymerase (Perkin-Elmer) with 4% dimethyl sulfoxide.

To confirm the genetic location of HSD11B2, 65 DNA samples from 4 large kindreds (CEPH) were typed for this polymorphism by means of a radioactive (32P) end-labeled primer. The cycling conditions were as follows: 94°C for 3 minutes, 1 cycle with a 30-second cool-down from 60°C to 50°C followed by 30 seconds at 72°C, 23 cycles of 94°C for 15 seconds, 50°C for 30 seconds, 72°C for 30 seconds, and a final 10-minute extension at 72°C. The samples were subjected to electrophoresis in 6% polyacrylamide gels with appropriate size standards, and the segregation pattern of the alleles was compared with previously mapped markers.

The Italian study population was analyzed by means of an ABI Prism 373 DNA sequencer (Applied Biosystems). The CA microsatellite primer 1 was end-labeled with a HEX fluorescent dye (Oswell). The cycling conditions were as follows: 95°C for 2 minutes, 55°C for 1 minute, 72°C for 1 minute each with a 30-second cool-down from 60°C to 50°C followed by 30 seconds at 72°C, 23 cycles of 94°C for 15 seconds, 50°C for 30 seconds, 72°C for 30 seconds, and a final 10-minute extension at 72°C. The samples were subjected to electrophoresis in 6% polyacrylamide gels with appropriate size standards, and the segregation pattern of the alleles was compared with previously mapped markers.

### 11-HSD2 Minigene Constructs

HSD11B2 minigenes containing various numbers of CA repeats and modified restriction sites to distinguish construct transcripts from those of the endogenous gene were constructed (Figure 1). First, the EcoRI, KpnI, and Spel restriction sites in the multiple cloning site of pBluescriptKSII+ (Stratagene) were destroyed by digesting the plasmid with each enzyme, rendering the DNA blunt-ended with the klenow fragment of DNA polymerase 1 and religating. Next, a 12 kb XbaI fragment from the P1 clone containing the HSD11B2 gene was cloned in the corresponding site of the vector. The resulting plasmid had unique EcoRI, KpnI, and Spel sites in the insert (pKS.EKS.P1.XbaI).

To introduce a restriction enzyme marker in the coding region of HSD11B2, the pKS.EKS.P1.XbaI construct was digested with EcoRI and KpnI to release a fragment of 3.75 kb, which was subcloned in the pNEB193 vector (New England Biolabs). An AscI site was

### TABLE 1. Demographic Characteristics of Italian Hypertensive Study Population (164 Men/34 Women)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
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</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>44.5</td>
<td>9.5</td>
<td>21.0–68.0</td>
</tr>
<tr>
<td>Basal SBP, mm Hg</td>
<td>149.7</td>
<td>14.2</td>
<td>120.0–193.0</td>
</tr>
<tr>
<td>Basal DBP, mm Hg</td>
<td>99.1</td>
<td>7.7</td>
<td>74.0–121.0</td>
</tr>
<tr>
<td>BMI</td>
<td>26.0</td>
<td>2.7</td>
<td>17.8–35.0</td>
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</tbody>
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SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index.

### TABLE 2. Oligonucleotides

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>1</td>
<td>TCA GGT CAG AAC TGG GAG GTC</td>
</tr>
<tr>
<td>2</td>
<td>TGG AGA GGG AGG CAA GAA TAT</td>
</tr>
<tr>
<td>3</td>
<td>GAA TTT CTT TGG CCG GCC GCA GCT GAC CAA G</td>
</tr>
<tr>
<td>4</td>
<td>GCA GCC ATG CAT CCT TGG CTT ATG C</td>
</tr>
<tr>
<td>5</td>
<td>GCA TGT GTG TGT CTA (CA)14 TAT GCT TGC CTC</td>
</tr>
<tr>
<td>6</td>
<td>GAG GCA AGC ATA (GT)14 TAG ACA CAC ACA TGC</td>
</tr>
<tr>
<td>7</td>
<td>GCA TGT GTG TGT CTA (CA)23 TAT GCT TGC CTC</td>
</tr>
<tr>
<td>8</td>
<td>GAG GCA AGC ATA (GT)23 TAG ACA CAC ACA TGC</td>
</tr>
<tr>
<td>9</td>
<td>CTA GAC TAG TGG CCT AGC TGG G</td>
</tr>
<tr>
<td>10</td>
<td>CCG GAA TTC CAA TCA GTG GC</td>
</tr>
<tr>
<td>11</td>
<td>GAC CAA ACC AGG AGA CAT TAG C</td>
</tr>
<tr>
<td>12</td>
<td>ATG TAG TTC TTT CCG TAG GC</td>
</tr>
</tbody>
</table>
introduced into exon 3 and an adjacent Xho I site was simultaneously destroyed via the Transformer site-directed mutagenesis kit (Clontech) with mutagenic primer 3 and selection primer 4, which destroys the Hin dIII site in the vector, thus permitting selection of mutant plasmids by digestion with Hin dIII. The mutated 3.75 kb fragment was re-inserted into the original plasmid pKS.EKS.P1.Xbal to create plasmid pKS.EKS.P1.Xbal.muAscI.

Finally, the CA repeat length variation (14 or 23 CA repeats corresponding to alleles 139 and 157 respectively; the original plasmid contains 20 repeats and corresponds to allele 151) was created by a 2-step PCR. Initially, a 2.7-kb fragment was isolated from plasmid pKS.EKS.P1.Xbal by digesting with AscI and EcoR I and subcloning in the pNEB193 vector. To generate a fragment containing 14 CA repeats, primer pairs 5/10 and 6/9 were used in PCRs as follows: 5 cycles at 95°C for 1 minute, 40°C for 1 minute, and 72°C for 1 minute; followed by 25 cycles at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. The 2 PCRs were then mixed and re-amplified; only primers 9 to 10 for 5 cycles at 95°C for 1 minute, 40°C for 1 minute, and 72°C for 1.5 minutes were used. This was followed for another 30 cycles as described above, except that the annealing temperature was 55°C. To generate a fragment with 23 CA repeats, the first PCRs were performed with primer pairs 7/10 and 8/9. In either case, fragments were digested with SpeI and EcoR I, were gel purified, and were substituted for the corresponding fragment of pKS.EKS.P1.Xbal.muAscI. The resulting plasmids will be referred to as the CA14 or CA23 constructs.

Qiagen columns were used to prepare each plasmid from 5 independent colonies. Each plasmid was subsequently dialyzed overnight with Slide-A-Lyser (Pierce Chemical Co) against Tris-EDTA (10:1) to remove excess salts. DNA concentrations were determined spectrophotometrically.

Cell Culture and Transfection
Human and rabbit kidney cortical collecting duct cells (gifts from Pierre M. Ronco) were grown in a 1:1 mixture of Ham's F12 medium and DMEM containing 5 μg/mL transferrin; 50 nmol/L sodium selenate; 50 nmol/L dexamethasone; 5 μg/mL insulin; 20 nmol/L Hepes, pH 7.4; and 2% newborn calf serum. Three independent transfections were performed in duplicate with each of the 5 plasmid preps of each construct. Eugene 6 transfection reagent was used as suggested by the manufacture (Roche). In each well of 6-well plates, 0.5 μg each of the CA 14 or 23 minigene constructs were transfected. Cells were lysed directly on the plates 24 hours after transfection via RNA-Stat-60 (Tel-Test Inc), and RNA was extracted as suggested in the manufacturer’s protocol.

Reverse Transcription–PCR
All RNA samples were treated with DNase I to remove residual DNA, and the enzyme was heat inactivated at 65°C for 10 minutes. Reverse transcription (RT) reactions were performed by means of the Thermoscript RT-PCR System (Life Technologies). Reactions contained 4 μg of RNA, 50 ng of random hexamers, 0.1 mol/L DTT, 10.0 mmol/L dNTPs, 40 U RNaseOut (an RNase inhibitor), and 15 U of ThermoScript in a volume of 20 μL. Reactions were incubated for 10 minutes at room temperature, which was followed by incubation for 50 minutes at 50°C. Reaction were heated at 85°C for 5 minutes, were cooled to room temperature, and were treated with 2 U of RnaseH. Five microliters of each RT reaction was used for PCR.

PCRs were performed by means of primers 11 and 12 located in exons 2 and 5, respectively, to produce a product of 482 bp. Transcripts originating from the transfected constructs are digested by Ascl to produce 315- and 167-bp fragments. A preliminary optimization of PCR condition was performed via a PCR optimizer kit (Invitrogen). All PCRs were performed with optimized buffer containing 7.5 mmol/L MgCl₂, pH 9.0. PCRs were assembled in a total volume of 50 μL containing 5 μL of RT reaction. 0.25 μg each of the sense and antisense primers, and 1 U Taq Polymerase (Roche) for a total of 40 cycles at 96°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute by means of a Perkin Elmer GeneAmp PCR System 9600. An RT-negative reaction was always included with each sample as a control.

PCR products were column-purified by means of a QIAquick PCR purification system kit (Qiagen), and 5 μL of each was digested with 7.5 U of Ascl overnight at 37°C in a total volume of 10 μL. Digests were resolved on 1.5% agarose gels, were stained with ethidium bromide, and were imaged with an Eagle Eye System (Stratagene). Fragments were quantified with EagleSight 3.2 analysis software (Stratagene).

Statistical analysis was performed via the Statview program (Abacus). For analyzing allele frequencies in the various genotype groups by the χ² test, only alleles carried by >5 subjects were included. In vitro expression data were analyzed by ANOVA that used construct (14 or 23 CA repeats), experiment (1-3), and plasmid preparation (1-5) as covariates.

Results
Relationship of Salt Sensitivity to 11-HSD2 Activity
Functionally, we could demonstrate differences in renal 11-HSD2 activity between individuals classified as salt sensitive or salt resistant. The urinary steroid precursor/product (urinary-free cortisol/urinary-free cortisone) ratio was significantly higher in the salt-sensitive group (0.89±0.04) compared with that in the salt-resistant group (0.71±0.04, P<0.001 by ANOVA), which implies reduced renal 11-HSD2 activity in the salt-sensitive group.

Identification of a Highly Polymorphic Microsatellite Marker in HSD11B2
A single fragment carrying a CA repeat segment was identified on a bacteriophage P1 clone carrying HSD11B2. Southern blot results of several different restriction digests of the P1 clone and various plasmid subclones suggested that this CA repeat was located within intron 1 of HSD11B2 (Figure 1). This was confirmed by complete sequencing of the intron via successive sets of custom-synthesized primers (this sequence has been deposited in GenBank accession number AF277158). While these studies were being performed, a polymorphism of similar sequence was reported to be located flanking the gene. It was unclear whether this represented a duplicated polymorphic segment or a discrepant localization of the same polymorphic locus, but we were unable to detect hybridization to any other such segments in the P1 clone (not shown).

Allele frequencies are shown in Table 3. There were marked racial differences in allele frequencies: Eighty-four percent heterozygosity in American blacks and 52% heterozygosity in whites was due to significantly increased

![Figure 1. Map of the human HSD11B2 gene showing relevant restriction sites. Exons are represented by numbered boxes, and the position of the CA repeat segment is marked.](http://hyper.ahajournals.org/doi/abs/10.1161/01.RES.0000238946.30934.69?journalCode=res)
numbers of shorter alleles in blacks ($\chi^2$ test, 51.9; 10 degrees of freedom, $P<0.0001$). Genetic mapping in 4 large kindreds confirmed the location of HSD11B2 on chromosome 16q22.11,14 and placed it between markers D16S260 and D16S398; the most likely location was between D16S260 and D16S398.

**Associations Between CA-Repeat Alleles and Salt Sensitivity**

Distributions of allele lengths for our CA-repeat HSD11B2 marker differed significantly in the Italian study population between individuals classified as salt sensitive and salt resistant ($\chi^2$ test 15.5; $P=0.0037$; 4 DF when the 5 most frequent alleles were included). Specifically, there were more short alleles among individuals in the salt-sensitive category.

Because we had no a priori hypothesis regarding a biological effect of this polymorphism, we further analyzed the data to see if this apparent effect had a particular mode of inheritance. We reasoned that if the correlation with the salt-sensitivity classification were mainly with the longer of the 2 alleles carried by an individual, this would imply a recessive effect of short alleles in increasing salt sensitivity, because the length of the shorter allele carried by a particular individual could obviously only be less than or equal to the length of his longer allele. A dominant effect of short alleles could be inferred if the correlation were more robust with the length of the shorter allele in each subject. In fact, a robust correlation was observed only with length of the longer allele ($\chi^2$ test, 11.8; $P=0.008$; 3 DF) and not with the shorter allele ($\chi^2$ test, 5.5; $P=0.14$; 3 DF) carried by each individual (Table 4).

Because these analyses required an arbitrary dichotomization of blood pressure response to salt loading and salt depletion (>10 mm Hg change, the median response), data were reanalyzed: Blood pressure change (ie, salt sensitivity) was treated as a continuous variable, and allele length was dichotomized as “short” (all alleles <153 bp, which is the median length) or “long” (≥153 bp). Differences in blood pressure change were then sought between subjects carrying 0, 1, or 2 short alleles (Figure 2; ie, subjects homozygous for long alleles, or heterozygous or homozygous for short alleles, respectively). There was no significant difference ($P=0.50$) between subjects carrying 0 or 1 short alleles, but there was a significant difference between subjects carrying 1 and 2 short alleles ($P=0.028$), and there was a trend ($P=0.10$) toward a similar difference between subjects with 0 and 2 short alleles. These results again seemed consistent with a recessive effect of short alleles on salt sensitivity. When subjects with 0 or 1 short alleles were pooled, there was again a significant difference ($P=0.024$ by ANOVA) in the blood pressure changes seen with salt loading between subjects who did (11.5 ± 1.1 mm Hg) or did not (8.0 ± 1.1 mm Hg) carry 2 short alleles.

The differences in allele frequencies between the salt-sensitive and salt-resistant groups seemed to occur over the

<table>
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<th>TABLE 4. Allele Distributions in Italian Hypertensives</th>
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<td>Allele Length</td>
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</tr>
<tr>
<td>139</td>
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<td>141</td>
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<td>157</td>
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<td>159</td>
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NaR, salt resistant (mean arterial pressure change ≥10 mm Hg with salt load); NaS, salt sensitive. $\chi^2$ test and $P$ values for each comparison are listed. For each comparison, only alleles in boldface were used for the analysis because of the small numbers of other alleles. Frequencies are in parentheses.
entire range of allele sizes, which suggests that the putative effect of allele length on blood pressure change was not subject to a threshold but instead varied continuously. Therefore, the blood pressure change in response to salt loading in each subject was plotted as a continuous variable against length of the longer allele carried by that subject (likewise as a continuous variable) and was subjected to regression analysis (Figure 3). These variables were indeed correlated (R=0.214, P=0.0025). Similarly, there was an inverse correlation between the urinary-free cortisol/urinary-free cortisone ratio and the length of the longer allele (R=0.20), but this did not reach statistical significance (P=0.09). Despite the observed correlations with blood pressure change in response to salt loading, no genotype effect on basal blood pressure was indicated by any of these analytic approaches (not shown).

The correlations observed in the study population had been made without any a priori hypothesis regarding a biologic effect of HSD11B2 allele length, and an acute salt-loading protocol in hypertensive individuals was used in the study. To extend these observations, we genotyped a population of German young men with normal blood pressure who had been studied during successive 1-week periods of ingesting a low- or high-salt diet. After this study was completed and had been initially submitted for publication, a similar study of this population was published. As was discussed above, the microsatellite polymorphism used in that study, Genbank AF071493, may be identical to that used in this study. However, the statistical analysis described in the other publication compared only the frequency of (and precursor/product ratios associated with) the 2 most common genotypes among salt-sensitive versus salt-resistant individuals and apparently did not correct for multiple comparisons; it is thus of questionable validity. When we analyzed our data on this population by means of the same approach we had used with the Italian hypertensive study population, we observed a trend (R=0.138, P=0.09) toward a greater difference in mean arterial pressure between high- and low-salt conditions when the size of the longer allele carried by each subject decreased (Figure 3). This reached statistical significance (R=0.177, P=0.03) for the difference in systolic blood pressure under high- and low-salt conditions. Moreover, there was a trend (R=0.19, P=0.11) toward higher precursor/product ratios ([THF+αTHF]/THE, corresponding to decreased 11-HSD2 activity) with the decreasing length of the longer allele. This trend was similar to that observed for urinary-free cortisol/urinary-free cortisone ratios in the Italian hypertensive population.

**Effects of CA Repeat Length on Gene Expression**

To determine whether the CA repeat had any effects on gene expression, we constructed minigenes that contained all introns and exons as well as 2.3 kb of 5′ flanking sequences. Such constructs allowed identification of effects of the repeat on expression from the native promoter as well as that of possible effects on other processes, such as the splicing of introns. Two restriction sites were modified to allow transcripts from the transfected construct to be distinguished from transcripts of the intrinsic gene. Five independent preparations of each plasmid were transfected 3 times in duplicate into each of 2 cortical collecting duct cell lines, 1 each of rabbit and human origin. RNA from each well was subjected to RT-PCR and was digested with Ascl. Ascl digests molecules corresponding to transfected HSD11B2 construct transcripts but not molecules corresponding to intrinsic transcripts, so that a fragment of 482 bp represents intrinsic transcripts and fragments of 315 and 167 bp represent construct transcripts (another Ascl site is located in exon 1 but is not included in the amplified segment). Because all HSD11B2 transcripts are amplified simultaneously with the same primers, the intrinsic transcripts serve as a competitive
hypothesis that impaired renal 11-HSD2 activity may be responsible for cortisol-induced salt retention in salt-sensitive patients.9 Similarly, increased (THF+aTHF)/THE ratios are highly correlated with sensitivity to a chronic dietary salt load in young normotensive adults.9 Because this is the case despite major differences in ages of subjects, the degree of hypertension, details of the salt-loading protocol, and the definition of salt sensitivity, we conclude that these data reflect a consistent and reproducible physiologic mechanism. The salt-loading protocols in these studies involved changes in sodium intake over hours to days. It is not yet known whether this locus might have even greater effects on blood pressure responses to more chronic changes in sodium intake.

Evidence for Influence of 11-HSD2 on Salt Sensitivity

We have recently reported the use of measuring the urinary free cortisol/urinary free cortisone ratio (urinary-free cortisol/urinary-free cortisone), which appears to be a sensitive marker of the renal 11-HSD2 enzyme.19 In this study, higher urinary-free cortisol/urinary-free cortisone ratios were observed in the salt-sensitive group when compared with those in the salt-resistant group, which is in keeping with the hypothesis that impaired renal 11-HSD2 activity may be responsible for cortisol-induced salt retention in salt-sensitive patients.9

CA Repeats and Gene Expression

These data show that decreased length of a CA repeat in the first intron of HSD11B2 increases gene expression. A related phenomenon has been observed in Long-Evans Cinnamon rats, which are susceptible to ethanol intoxication and hepatitis. These rats have decreased hepatic expression of alcohol

Discussion

Relationship of Salt Sensitivity and Essential Hypertension

Although essential hypertension is known to have a heritable component, few genetic loci (eg, those encoding angiotensinogen15,16 and α-adducin7) have thus far been identified as influencing blood pressure in normal humans. Several forms of hypertension are inherited in a simple Mendelian manner.17 In addition to AME, these include Liddle’s syndrome caused by dysregulation of the aldosterone synthase enzyme, and hypertensive forms of congenital adrenal hyperplasia. These rats have decreased hepatic expression of alcohol

Figure 4. RT-PCR of cells transfected with minigene constructs. Top: ethidium-bromide-stained agarose gel of a representative experiment in which rabbit cortical collecting duct cells were used. The 482 bp band corresponds to transcripts of the intrinsic gene, and the 315 and 167 bp bands represent transcripts from transfected constructs. Duplicate lanes are from cells transfected with constructs with 14 (CA14) or 23 (CA23) CA repeats or from sham-transfected (CTRL) cells. Middle and bottom: Mean ratios of intensity of the 315 bp vs 482 bp bands in transfected human or rabbit cells. Error bars represent 95% confidence interval.
dehydrogenase, which has been traced to increased length of a CA repeat in the first intron of the gene. CA repeats are alternating purine-pyrimidine tracts, and such segments may form Z-DNA (left-handed helices) or cruciform regions that could influence gene expression. Increased length of a similar CG repeat increases expression of corticotropin-releasing hormone reporter constructs. Variations in the length of other microsatellites also may influence gene expression. Such a polymorphism in the 5' flanking region of the insulin gene is a risk factor for diabetes. These effects are distinct from the triplet repeat flanking region of the insulin gene is a risk factor for diabetes.32,33 These effects are distinct from the triplet repeat expansions associated with many neurological diseases, most of which are in coding sequences of genes.34

The location of the CA repeat in the first intron is consistent with an effect on either gene transcription or on pre-mRNA processing. A 200-bp region containing the CA repeat does not act as an enhancer or repressor when placed in luciferase reporter constructs driven by the HSD11B2 promoter (A.K.A. and P.C.W., unpublished observations, 1999), which suggests that the effect might be posttranscriptional, but further studies will be required to answer this question.

Because microsatellites are highly polymorphic and evolve quickly, it has been suggested that such polymorphisms occurring in or near genes may serve the evolutionary purpose of fine-tuning levels of gene expression in response to environmental pressures. However, the in vivo effects of changes in gene expression may be difficult to predict from in vitro data, as is the case in this study.

Possible Functional Effects of the Intron 1 CA Repeat Polymorphism

The CA repeat polymorphism was originally identified for use in linkage studies, and it was surprising when a significant association between repeat length itself and salt sensitivity was detected in the Italian study population. Nevertheless, the similar trend seen in German young adults suggests that the associations between HSD11B2 genotype and salt sensitivity are not due to chance. The most obvious explanation for these observations would be if decreased length of the repeat somehow decreased 11-HSD2 expression or enzymatic activity. The inverse relationship between the urinary-free cortisol/urinary-free cortisone ratio and length of the longer allele at this locus in the Italian hypertensives and between the (THF+αTHF)/THE ratio and allele length in young German normotensives (although individually short of statistical significance) supports this idea. The correlation coefficients and probability value for the 2 populations (R = 0.20, P = 0.09 for Italian hypertensives; R = 0.19, P = 0.11 for German normotensives) are very similar, despite the major differences in study design. It is unlikely, therefore, that these 2 observations occurred together by chance. However, decreased length of the CA repeat is associated with higher rather than lower gene expression in cultured cells. Thus, a direct effect of CA repeat length on gene expression cannot account for the observed correlations between CA repeat length and salt sensitivity.

How can these seemingly paradoxical results be explained? We discount the trivial explanations that either the in vitro or in vivo findings represent chance associations, because the probability value for the in vitro data are highly significant, and the in vivo findings were similar to each other in 2 independent populations. In principle, it is possible that the CA repeat is part of a locus control region that affects expression in the distal nephron of 1 or more additional linked genes. However, the genes adjacent to HSD11B2 have not yet been identified, and so there is no direct evidence supporting this proposition. It is also quite possible that the CA repeat polymorphism described here is in genetic linkage disequilibrium with another unknown polymorphism in or near HSD11B2 that has an effect on gene expression or enzymatic activity that overwhelms any effect of the CA repeat itself. Finally, the CA repeat might, in principle, have indirect effects on enzymatic activity in vivo through a mechanism that cannot be discerned by our in vitro studies. To decide whether any of these explanations is plausible, it will be necessary to know much more about mechanisms regulating expression of this important enzyme.

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