Functional Polymorphism of the Anpep Gene Increases Promoter Activity in the Dahl Salt-Resistant Rat


Abstract—We have reported that aminopeptidase N/CD13, which metabolizes angiotensin III to angiotensin IV, exhibits greater renal tubular expression in the Dahl salt-resistant (SR/Jr) rat than its salt-sensitive (SS/Jr) counterpart. In this work, aminopeptidase N (Anpep) genes from SS/Jr and SR/Jr strains were compared. The coding regions contained only silent single nucleotide polymorphisms between strains. The 5' flanking regions also contained multiple single nucleotide polymorphisms, which were analyzed by electrophoretic mobility-shift assay using renal epithelial cell (HK-2) nuclear extracts and oligonucleotides corresponding with single nucleotide polymorphism–containing regions. A unique single nucleotide polymorphism 4 nucleotides upstream of a putative CCAAT/enhancer binding protein motif (nucleotides −2256 to −2267) in the 5' flanking region of the SR/Jr Anpep gene was associated with DNA-protein complex formation, whereas the corresponding sequences in SS rats were not. A chimeric reporter gene containing ≈4.4 Kb of Anpep 5' flank from the Dahl SR/Jr rat exhibited 2.5- to 3-fold greater expression in HK-2 cells than the corresponding construct derived from the SS strain (P<0.05). Replacing the CCAAT/enhancer binding protein cis-acting element from the SS rat with that from the SR strain increased reporter gene expression by 2.5-fold (P<0.05) and abolished this difference. CCAAT/enhancer binding protein association was confirmed by chromatin immunoprecipitation and correlated with expression, suggesting selection for a functional CCAAT/enhancer binding protein polymorphism in the 5' flank of Anpep in the Dahl SR/Jr rat. These results highlight a possible association of the Anpep gene with hypertension in Dahl rat and raise the prospect that increased Anpep may play a mechanistic role in adaptation to high salt.

Key Words: CD13 ■ renal salt-handling ■ Dahl rat ■ salt-sensitive hypertension ■ polymorphism

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size differences between myeloid-specific (≈3.7 kb) and epithelial-specific (≈3.4 kb) transcript sizes.12 The immediate 5′ flank of the human *Anpep* gene contains a TATA-like element (ATATAA) 22 bases upstream of the transcriptional start site (+1) that is conserved with the orthologous porcine gene. The proximal epithelial-specific promoter is also characterized by conserved binding sites for several *trans*-acting factors, including hepatocyte nuclear factor 1 and Sp1, which are thought to play important roles in *Anpep* proximal promoter activity.13,14 An enhancer region controlling proximal promoter function has also been identified within ≈2.7 kb of the start site, which contains canonical binding sites for members of the winged helix Ets family, including CCAAT/enhancer binding protein (C/EBP).7 From the rat sequencing data, it can be deduced that the *Anpep* gene lies within quantitative trait loci (QTLs) for blood pressure identified in both Dahl salt-sensitive (SS/Jr)-Lewis and Dahl SS/Jsalt-resistant (SR/Jr) crosses (15,16 available online at http://hyper.ahajournals.org). We have reported that *Anpep* transcript abundance, protein abundance, and activity are greater in the kidneys of Dahl SR/Jr than in SS/Jr rats, raising the possibility that increased Anpep-mediated signaling, perhaps by angiotensin IV, reduces renal tubular Na uptake in adaptation to high salt.15 Consistent with this is the finding that the nonclipped kidney in the Goldblatt 2-kidney 1-clip model has a highly significant increase in membrane-bound *Anpep* activity.17,18 Based on these results, we have proposed that the *Anpep* gene may be linked to salt-sensitive hypertension in the Dahl rat. The present study was performed to further examine this possibility by comparing the *Anpep* gene between Dahl SS/Jr and SR/Jr rat strains.

**Methods**

**Rat Strains and Reagents**

Male Dahl SS (SS/Jr), Dahl SR (SR/Jr), and Lewis rats were purchased from Harlan Sprague–Dawley (Indianapolis). Animal studies were approved by the University of Illinois at Chicago animal care committee. Culture medium (DMEM F12) and T4 polynucleotide kinase were purchased from Gibco BRL. Luciferase reporter and control normal rabbit IgG or polyclonal C/EBP antibody. The resulting DNA fragments were subjected to analysis for chromatin immunoprecipitation assays and quantitation of *Anpep* expression vector was described previously.19 C/EBP dominant-negative mutant (C’30) was a HindIII and Ascl deletion of wild-type C/EBPα provided by Dr Daniel G. Tenen (Harvard Medical School, Boston, MA).20

**Electrophoretic Mobility Shift Assays**

Nuclear extracts from HK-2 cells were prepared as described previously.21 Probes for gel-shift analysis were generated by PCR using specific primers flanking each single nucleotide polymorphism (SNP; details in an online supplement available at http://hyper.ahajournals.org) and were end-labeled with [32P]ATP using polynucleotide kinase (Promega). Oligonucleotides corresponding with the binding site, wild type; WT) and a mutant form GAA TGA 3′) and were used as probes to examine fragment 9. Electrophoretic mobility-shift assay (EMSA) was performed by incubating (30 minutes, room temperature) nuclear extract (25 μg) from HK-2 cells in a total volume of 30 μL of EMSA buffer with [32P]labeled double-stranded oligonucleotides (8 fmol). For competition assays, a 100-fold excess of unlabeled oligonucleotide was added for 15 minutes before the addition of the labeled probe. Samples were run on a 6% non-denaturing polyacrylamide gel. DNA–protein complexes were visualized by autoradiography. Supershift assay was performed by adding 2 μg of C/EBPα antibody (Abcam) 10 minutes before adding the labeled probe.

**Chromatin Immunoprecipitation Assay**

Chromatin immunoprecipitation assay was performed using a kit assay (Upstate). Chromatin fragments were isolated from nuclei by sonication in SDS lysis buffer (1% SDS, 10 mmol/L of EDTA, and 50 mmol/L of Tris; pH 8.1). Fragments were preclorinated with protein aagarose beads and salmon sperm DNA followed by overnight incubation with continuous shaking at 4°C either in the presence of control normal rabbit IgG or polyclonal C/EBPα antibody. The beads were washed once each with a low-salt immune complex, high-salt immune complex, and LiCl wash buffer followed by 2 washes with 10 mM Tris/1 mM EDTA (pH 8.0) buffer and incubated at 65°C overnight in a 50-μL buffer consisting of 20 mMol/L of Tris/HCl (pH 8.0), 100 mmol/L of NaCl, and 10 mg/mL of protease K. The resulting DNA fragments were subjected to real-time PCR using primers flanking the *C/EBPα putative cis-binding sequence (palindromic) and −2272 T/C SNP in the *Anpep* promoter region (forward: 5′ GATTGGGA AAGGAAAGACA 3′ and reverse: 5′ GGAGCCATCAGAAGCC 3′) and control primers flanking the PDE4B promoter that do not contain any C/EBP or C/EBP-related transcription factor binding sites (forward: 5′ AGTGGTTATTAAACCTTAGGTTCCTCT 3′ and reverse: 5′ CCAATAA AACCGTGCATTCAAGATC 3′). Quantitative real-time PCR analysis for chromatin immunoprecipitation assays and quantitation of *C/EBPα* protein binding to the 5′ flanking region was calculated as described previously.22 ΔCt is derived by subtracting the thresh-
old cycles ($C_i$) of SS/Jr and SR/Jr rat chromatin immunoprecipitates amplified using control primers from corresponding samples ($C_T$) using primers flanking the C/EBP site and the 2272 T to C polymorphism. $\Delta C_T$ of 1 is equivalent to a 2-fold change in sensitivity.

**Statistical Analysis**

Comparisons were made by ANOVA. Data are expressed as mean ± SD. Experimental values were significantly different at $P<0.05$.

**Results**

**Identification of Anpep Gene Polymorphisms**

**Silent SNPs in Coding Region of Anpep Gene Between SS/Jr and SR/Jr Rats**

Anpep cDNA was sequenced in 7 SR/Jr and 8 SS/Jr rats. The sequences corresponded with that in Genbank (Accession No. 205108 M25073) with the exception of silent SNPs (see online Appendix). Two silent SNPs were identified at positions 2306 and 2292, that is, T/C and G/A, respectively.

**Anpep Gene Polymorphisms Within the 5' Promoter Region Between SS/Jr and SR/Jr Rats**

The 4.4-kb 5' flanking region of the Anpep gene was sequenced from the Dahl SR/Jr and SS/Jr strains. A total of 11 SNPs were identified between SR/Jr and SS/Jr strains (Figure 1).

**CEBPα Associates With 5' Flanking Region of Anpep Gene in SR/Jr but Not SS/Jr Rats**

EMSA was performed using 11 PCR-generated fragments, each containing 1 of the 11 SNPs detected between SR/Jr and SS/Jr strains (Figure 1). Inclusion of these PCR-generated DNA fragments with nuclear extract from HK-2 cells revealed complex formation only in fragment 9 from SR/Jr but not from the SS/Jr strain (Figure 2, C2272 + protein and

### Table: Polymorphisms in Anpep 5' flanking region of Anpep gene

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Position from Coding Region</th>
<th>Rat Strain</th>
<th>Sequence Change</th>
<th>Transcription Factor</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>-412 to -304</td>
<td>SS</td>
<td>GTAGAGATACATTCGCCCAATGAAAACAAAC</td>
<td>NF-Y</td>
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<tr>
<td></td>
<td></td>
<td>SR</td>
<td>GTAGAGATACATTCGCCCAATGAAAACAAAC</td>
<td>Gfi</td>
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</tr>
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<td></td>
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</tr>
<tr>
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</tbody>
</table>
FREE PROBE

Figure 2. EMSA analysis reveals interaction of a protein with fragment 9 in SR/Jr (C-2272) but not SS/Jr (T-2272) rat Anpep. Each SNP was incorporated into a separate PCR product (fragments 1 to 11). No differential binding of the probes between rat strains was detected except for fragment 9, which shows an additional DNA–protein complex in SR/Jr but not SS/Jr strains (arrow). (Fragment 9 differs between strains only in the T/C2272 SNP). The other bands present both in SS/Jr and SR/Jr strains could be nonspecific or because of binding of other transcription factors. See text for details.

Figure 3. Chromatin immunoprecipitation assay–C/EBPα transcription factor binds to the endogenous kidney Anpep promoter region of SR/Jr rats. Chromatin fragments derived from SS/Jr and SR/Jr rat kidneys were cross-linked and immunoprecipitated with polyclonal antibody against C/EBPα. Immunoprecipitates from each sample were analyzed by real-time PCR using primers spanning fragment 9 and control primers as detailed in the Methods section. The number on the y axis indicates the ΔCt (See Methods for details). Numbers on the top of bars of graph indicate the relative expression derived by 2ΔΔCt. n=5; **P<0.01 vs SS/Jr.

Discussion

The present results identify a functional polymorphism of a C/EBPα cis-element in the promoter of Anpep gene in the Dahl SR/Jr rat. The C/EBP transcription factors are a highly conserved group within the basic leucine zipper family that control genes involved in the control of cellular growth and

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differentiation, immune and inflammatory responses, and neural function/memory.23 Six C/EBP members (α to δ) have been cloned. Our data show that both C/EBPα and C/EBPβ forms of C/EBP are expressed in HK-2 cells, consistent with previous reports in the kidney.23

The essential C/EBP binding site has been reported to contain a dyad symmetrical repeat 5'-RTTGCGYAA-3', where R is A or G and Y is C or T,24 and its palindrome.24 The present results show that C/EBPα association with fragment 9 requires both TTCAGAA, a segment of the defining dyad repeat of C/EBP cis-elements, and a T SNP 4 bp upstream from this sequence. This is the first report, to our knowledge, of an SNP not within the dyad repeat regulating C/EBPα association. However, SNPs at a wide range of distances, ranging from a few to hundreds of base pairs,25,26 from the consensus binding sequences have been reported to be capable of influencing the DNA–protein association. The mechanisms of these regulations are not often clear, but may involve another protein, for example, enhancer or cofactor, binding to either DNA or C/EBPα.

Promoter activity for the 4.4-kb 5' flanking region of the Anpep gene from SR/Jr rats is greater than that from SS/Jr strain in HK-2 cells, consistent with greater Anpep transcript and protein abundance in the kidneys.4 The difference in promoter activity was present even in the presence of a dominant-negative mutant of C/EBPα, suggesting other important cis-acting elements and/or a role of C/EBPβ. Eleven SNPs are identified in the 4.4-kb 5' flanking region of the Anpep gene. EMSA was used to screen for effects of these SNPs on the DNA–protein interaction. C/EBPα was the only trans-acting factor identified in this manner as physically
associating with the functionally significant SNP-containing region. This does not, however, exclude binding by other factors with different binding affinities or in a combinatorial context in vivo.

The results raise the possibility that the Anpep gene is a salt-sensitive hypertension susceptibility gene in the Dahl rat model. The 2272 C SNP is present in the SR/Jr but not SS/Jr rat and can be inferred to map to a previously mapped QTL identified in the Dahl SS/Jr × SR/Jr cross. The 2272 C SNP is also present in the Lewis rat, a less salt-sensitive strain. We have reported previously that Anpep maps to a QTL defined in a Dahl SS/Jr–Lewis cross, suggesting that this SNP is important in differentiating the Lewis from Dahl SS/Jr rat strains. Additional strategic congenic and transgenic rat experiments are necessary to confirm a link between adaptation to high salt and the Anpep gene.

**Perspectives**

The present results identify Anpep as a new candidate gene in the Dahl rat. Although many genes have been tested, the specific genes underlying salt-sensitive hypertension in the Dahl rat remain elusive. Whether Anpep is truly important will require complementary strategies. Transgenic, congenic, and consomic strains are likely to be especially useful in the further judgment of Anpep.

A critical component to evaluating Anpep as a candidate gene is demonstrating a functional or physiological mechanism that links Anpep to the regulation of renal salt handling. In this regard, Anpep is especially intriguing, because it has been linked to RAS by metabolizing angiotensin III to angiotensin IV, a “diuretic” peptide. This raises the spectra of another level of complexity in the regulation of RAS. Significantly, it may lead to the discovery of a physiological role for angiotensin IV, which has been reported to be, as opposed to angiotensin II, a diuretic peptide, which reduces Na+ uptake in tubule cells. Time will tell whether this Anpep “pans out” as a cause of salt-sensitive hypertension in the Dahl rat and/or plays a role in human forms of hypertension.

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**Disclosures**

None.

**References**

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