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Research Article

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Mapping of a Quantitative Trait Locus for Blood Pressure on Rat Chromosome 2

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Abstract

A genetic map for rat chromosome 2 that includes five candidate genes for blood pressure regulation was constructed in a region containing a quantitative trait locus (QTL) for blood pressure. Two F₂ populations of male rats raised on high salt (8% NaCl) diet from weaning were studied: F₂(WKY × S), derived from a cross of Dahl salt-sensitive rats (S) and Wistar-Kyoto rats (WKY); and F₂(MNS × S), derived from a cross of S rats and Milan normotensive strain (MNS). In both populations a blood pressure QTL was localized between Na⁺,K⁺-ATPase α1 isoform and calmodulin-dependent protein kinase II-delta loci. The LOD score for existence of this blood pressure QTL based on the combined populations (*n* = 330) was 5.66 and accounted for 9.2% of the total variance and 26% of the genetic variance. (*J. Clin. Invest.* 1994. 94:431–436.) Key words: Dahl salt-sensitive rat • Na⁺,K⁺-ATPase α1 • interval mapping • quantitative trait locus • hypertension

Introduction

The identification of the individual loci influencing blood pressure in the rat is greatly facilitated by the advent of molecular genetics, although the rat genetic map is poorly developed compared with the mouse and human maps. Several individual loci cosegregating with blood pressure have been found in various rat models of hypertension using either the candidate gene approach or random genome scanning (1–12). Neither approach definitively identifies a locus causing blood pressure differences but only identifies a chromosomal region in which a quantitative trait locus (QTL)¹ for blood pressure is statistically likely to occur. Further genetic verification of the existence of a QTL

and “positional cloning” (13, 14) of the definitive QTL will ultimately depend on the development of congenic strains carrying the QTL-containing region in progressively smaller and smaller chromosomal fragments. This can only be accomplished if a dense genetic map is available in the chromosomal region of interest.

Our attention was initially directed to a region of rat chromosome 2 by our observation (6) that the candidate gene guanylyl cyclase A/atrial natriuretic peptide receptor A (GCA) on chromosome 2 cosegregates with blood pressure. As it turns out, GCA is at the edge of the region likely to contain the QTL. The present work builds on existing linkage groups and genetic maps for rat chromosome 2 (15–17). We localize a QTL for blood pressure by genotyping 10 informative markers on chromosome 2 in large segregating F₂ populations derived by crossing Dahl salt-sensitive rats with various control strains.

Methods

Animal procedures. Inbred Dahl salt-sensitive (SS/Jr) rats (18) were used and will be referred to hereafter as S rats. Milan normotensive strain (MNS) (19) rats were obtained from Vincent Gattone (University of Kansas) who had imported them from their founder, Giuseppe Bianchi (University of Milan, Italy). (The strain is currently available from Dr. Carl Hansen, Veterinary Resources Division, National Institutes of Health, Bethesda, MD) Wistar-Kyoto rats (WKY/NHsd), referred to hereafter as WKY, were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN).

Two F₂ populations were produced. The F₂ (WKY × S) population of 159 rats was produced by crossing WKY females with S males to produce F₁ rats, and then intercrossing F₁ × F₁ to produce F₂. The F₂ (MNS × S) population of 171 rats was produced by crossing MNS males with S females to produce F₁, which were intercrossed to produce F₂. These are the same populations used previously (6).

Only male F₂ rats were studied. F₂ rats were weaned at 30 d of age and placed on high salt (8% NaCl) diet (diet TD 82050; Teklad Premier Laboratory Diets, Madison, WI) at 37 d of age. The populations were maintained on high salt diet until the rats with the highest blood pressure had reached > 200 mmHg. This took 12 wk on high salt diet for F₂ (WKY × S) and 10 wk for F₂(MNS × S), at which point the blood pressures of the rats were studied intensively during a 7–10-d period. Blood pressure was taken by the tail cuff method (20, 21) under light ether anesthesia with the rats warmed to 28°C. At least three consecutive consistent readings were taken during three separate sessions separated

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1. Abbreviations used in this paper: AT1B, angiotensin II receptor AT1b; CAMK, calmodulin-dependent protein kinase II-delta; CPB, carboxy peptidase B; D2N35, an anonymous locus; FGG, fibrinogen gamma; GCA, guanylyl cyclase A/atrial natriuretic peptide receptor A; HSD,

3β-hydroxysteroid dehydrogenase/delta 5 isomerase; MNS, Milan normotensive strain; NAKα1, Na⁺,K⁺-ATPase α1; NEP, neutral endopeptidase/enkephalinase (EC 3.4.24.11); PRLR, prolactin receptor; QTL, quantitative trait locus; R, inbred Dahl salt-resistant rats (SR/Jr); S, inbred Dahl salt-sensitive rats (SS/Jr); SSR, simple sequence repeats; WKY, Wistar-Kyoto rats.

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Table I. Primer Sets for PCR around SSR for Loci on Rat Chromosome 2

Locus	GenBank accession number	Primers (5'→3')	Expected size (bp)	Relative size of PCR products
<i>D2N35</i>	M98802	CTGTCTAGTCAAGTGTCTGTGC CCCTAACCATAGTGGAAATCC	213	MNS > S > WKY
<i>CAMK</i>	J05072	CTCCGACAAGAAAACCTCCGCAA CAGGATCACACCAGGAACCTGAA	255	MNS = WKY > S
<i>NAKα1</i>	x53234	AACCCAGTGGTAGCTTCCATCC TACTTGTCTCGTCCAACCTACAG	187	MNS > WKY > S
<i>HSD</i>	M67465	ACTAGAGTCAGGAGGCAAAAACC ACCCTATGTGAAATGGTGATGC	217	WKY > MNS > S
<i>GCA</i>	J05677	CCCTGCGTGTTCATTCATGTGG ATCAGATTCTGAAGGCATCCG	286	MNS > WKY > S
<i>FGG</i>	K01337	CAGTAAGTGCTCTTCAGAAGCC TGTAAGTGTGGGAGAAAAA	136	MNS = WKY > S
<i>NEP*</i>	U01225	GGGCACTCATAGATGAAGATGG CCGTCCTTTTAATGATC	333	S > WKY = MNS
<i>NEP*</i>	U01226	AAGGCTCCAAACTACAAACCCG TTGGCTCAGTCAGGATCTAAGG	241	R > S = MNS = WKY
<i>CPB</i>	M23951	TCCCTTCTAAGCATACTTTCCA TGAAGAGACAACCCTAGAGACC	149	WKY > MNS > S
<i>AT1B*</i>	U01223	CTCTCCATCCACAAACATCC ACTGTCCTGTCTGACTAG	192	S > MNS = WKY
<i>AT1B*</i>	U01224	TTTCTGAACCAACCAACC AAGTGGTCTCTAACCTG	276	S > MNS = WKY
<i>PRLR</i>	M19304	ATCTTGATCCCTCAGTCTCACG CAAGCCAGAAAAGCATGAAGC	209	WKY = MNS > S

* Indicates newly developed and newly assigned loci. The two primer sets for *AT1B* and *NEP* are for different SSR at these loci.

by 2 d, and these three separate session pressures were averaged as the final blood pressure.

Genotyping. Rats were killed by an overdose of sodium pentobarbital, and pieces of liver were frozen at -70°C . Liver DNA was extracted by the method of Blin and Stafford (22).

All genotyping was done with DNA amplification by PCR around simple sequence repeats (SSR) (so-called microsatellites). The methods were given in detail previously (6). Table I gives the primer sets used for PCR at each locus. Although the selection of 7 out of the 10 loci (an anonymous locus [*D2N35*], calmodulin-dependent protein kinase II-delta [*CAMK*], Na^+ , K^+ -ATPase $\alpha 1$ [*NAK α 1*], 3β -hydroxysteroid dehydrogenase/delta 5 isomerase [*HSD*], fibrinogen gamma [*FGG*], carboxy peptidase B [*CPB*], and prolactin receptor [*PRLR*]) used is based on previous chromosomal localization and mapping data (15–17), all of the primers, except *HSD* (17), *NAK α 1* (16), and *FGG* (15), were redesigned based on GenBank sequences. *GCA* primers were those we used previously (6) based on sequence data for *GCA* in the literature (23). Primers for neutral endopeptidase/enkephalinase (*NEP*, EC 3.4.24.11) and angiotensin II receptor AT1b (*AT1B*) are based on our new sequence data.

Developing new SSR markers. A cDNA probe of 1,340 bp for *NEP* was obtained by PCR from a cDNA lambda gt11 library made from S kidneys. The primers used for amplification were based on a published sequence (24) and consisted of a forward primer of 5'-CAAATATGCTTGTGGAGGCTGG-3' and a reverse primer of 5'-GTCCTGGAGGTTTCATAATGG-3'. A genomic DNA probe of 241 bp for *AT1B* was based on a published sequence (25) and was amplified by PCR from rat genomic DNA with a pair of primers that amplified a region specific for *AT1B* as opposed to another angiotensin II receptor, *AT1A*. The primers were forward 5'-ACTACCGAGGAGTGTTCACCAACC-3' and reverse 5'-TACGTTTCTGCTAGTACACC-3'. After PCR amplification, these probes were separated on a 1.5% agarose gel, excised, purified

using glass milk (BIO 101, Inc., La Jolla, CA), subcloned into a PCR TA II vector (Invitrogen, San Diego, CA), and sequenced using a Sequenase kit (US Biochemical, Cleveland, OH) to confirm that the sequence of the products obtained agreed with the published sequences.

For genomic library screening the *NEP* or *AT1B* DNA probes were labeled using a random priming kit (Boehringer Mannheim, Indianapolis, IN), purified using a Bio-6 column (BIO-RAD, Melville, NY), and used to screen a rat genomic library made from Dahl salt-resistant rats in lambda phage GEM-11 (Promega Corp., Madison, WI). Standard procedures for growing phage, filter lifting onto nylon filters (Schleicher and Schuell, Inc., Keene, NH), treatment of DNA on the filters, and hybridization were used (26).

Phage DNA from purified clones containing *NEP* or *AT1B* was prepared after growth in NZCYM liquid medium by a standard method (26). The cloned DNA inserts were cut with a series of restriction enzymes that cut the vector only once. The digested fragments were then separated on a 1% agarose gel, transferred to a sheet of Duralon-UV membrane (Stratagene, La Jolla, CA), fixed by UV cross-linking, and probed with a mixture of $(\text{CA})_{15}$ and $(\text{CT})_{15}$ oligonucleotides that were labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and T4 polynucleotide kinase and purified on a Sep-Pak C_{18} column (Waters Associates, Milford, MA).

The genomic fragments recognized by the $(\text{CA})_{15}$ or $(\text{CT})_{15}$ oligonucleotides were then subcloned into a plasmid vector pT7T3 18U (Pharmacia LKB Biotechnology, Piscataway, NJ). The subcloned genomic fragments containing $(\text{CA})_n$ or $(\text{CT})_n$ for either *NEP* or *AT1B* were repeatedly digested with different restriction enzymes followed by another round of subcloning until the fragment containing the repeated elements was < 1 kb. These clones were then sequenced, and the sequence information was used to design primers that would amplify a product containing the $(\text{CA})_n$ or $(\text{CT})_n$ repeated elements.

Statistical analysis. Analysis of variance was done using SPSS programs (SPSS Inc., Chicago, IL). Oligonucleotide primers for PCR were

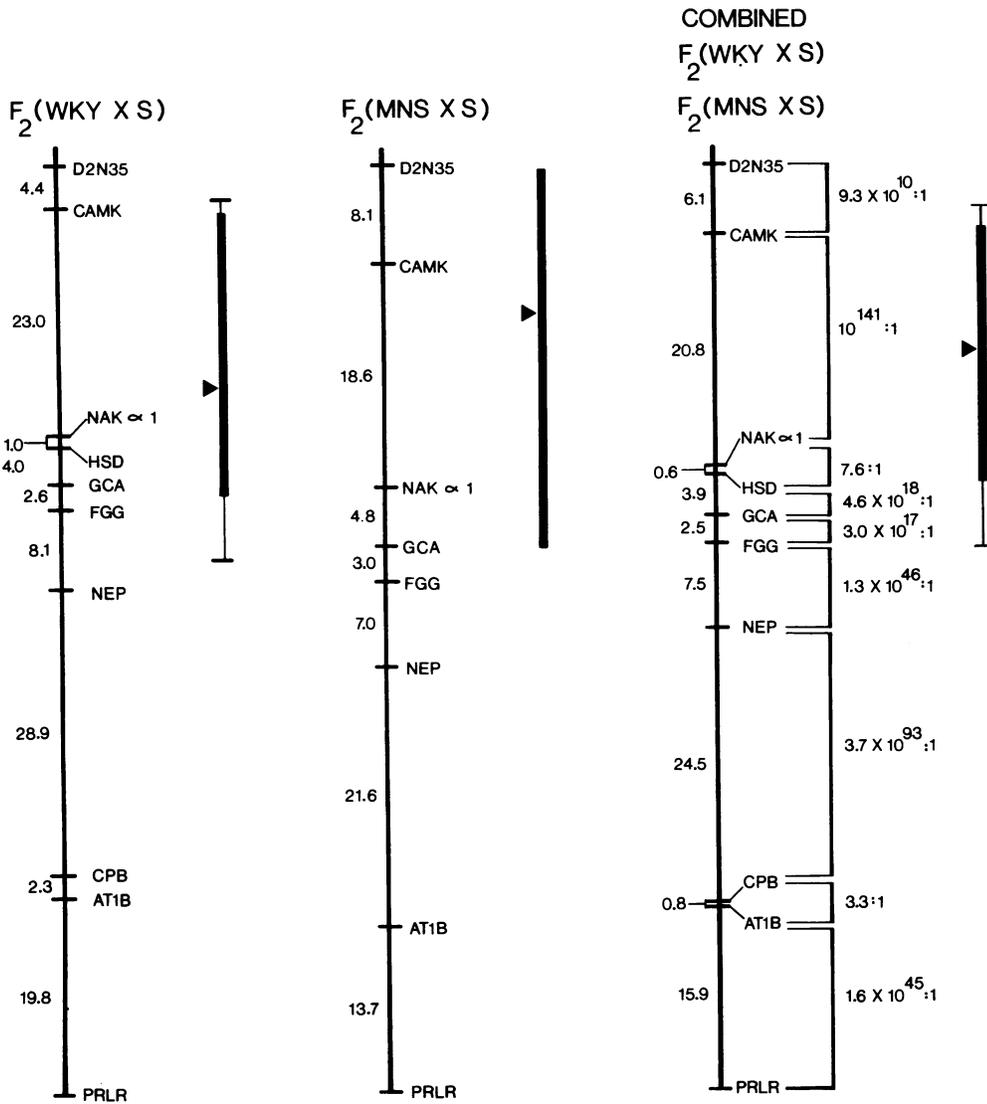


Figure 1. Linkage maps for rat chromosome 2 determined on F_2 (WKY \times S) (left) as F_2 (MNS \times S) (center) populations and a map combining both populations (right). The numbers on the left of each map are map units in centi-Morgans using the Haldane correction. To the right of the maps, the arrowhead indicates the computer-predicted position of the QTL for blood pressure (i.e., maximum LOD score), the heavy vertical bar is ± 1 LOD unit, and the thin bar is ± 2 LOD units. There is no thin bar for the F_2 (MNS \times S) population because of interference from a possible second weak QTL in this population in the lower part of the map around *AT1B*. The combined map represents data on 330 F_2 rats. The numbers to the right of the combined map are the odds for a map with the order of loci as given to an order with each pair of markers reversed. The position of the centromere relative to the map is unknown.

designed using the Primer Detective Program (Clontech, Palo Alto, CA). Linkage maps and QTL localization were done with MAPMAKER/QTL programs (27, 28) for IBM-compatible personal computers available from Eric Lander (Whitehead Institute, Cambridge, MA).

In QTL analysis of blood pressure in the rat, Jacob et al. (5) suggested that an LOD score of 3 should be set as the significance level to reasonably establish the existence of a QTL. Based on their analysis (5), we feel that it is also reasonable to further investigate loci with LOD scores between 2 and 3 by trying to find more flanking markers and/or to increase the size of the population.

Results

Fig. 1 gives the linkage maps for rat chromosome 2 generated for the F_2 (WKY \times S) and F_2 (MNS \times S) populations and a composite map combining both populations. Blood pressure data by genotype and analysis of variance data are given for the chromosome 2 loci in Table II for F_2 (WKY \times S) and Table III for F_2 (MNS \times S). In Table II for the F_2 (WKY \times S) population of 159 rats, it is obvious that the blood pressure effect cosegregating with the chromosomal markers between

D2N35 and *NEP* is at a maximum at the *NAK α 1* locus and falls off substantially on either side of *NAK α 1*. The MAPMAKER/QTL program localizes the QTL (Fig. 1) close to *NAK α 1*, between *NAK α 1* and *CAMK* with a maximum LOD score of 3.42. The QTL accounted for 10.9% of the total variance and $\sim 31\%$ of the genetic variance.

In Table III for the F_2 (MNS \times S) population of 171 rats, the blood pressure effect of the chromosomal region from *D2N35* to *NEP* is less pronounced than for the F_2 (WKY \times S) population. The maximum LOD score was 2.62 which localized the QTL closer to *CAMK* than *NAK α 1* (Fig. 1). The QTL accounted for 8% of the total variance and $\sim 23\%$ of the genetic variance. A QTL on chromosome 10 in the region of angiotensin-converting enzyme was previously shown (6) also to cosegregate with blood pressure in the F_2 (MNS \times S) population. The two QTL on chromosome 10 and 2 together accounted for 19% of the total variance (or $\sim 55\%$ of the genetic variance) as estimated by using the MAPMAKER/QTL program. The blood pressure effect and statistical significance decrease going down the loci as listed in Table III to *NEP* and then increase again toward the bottom of the table in the region around *AT1B*. The maxi-

Table II. Systolic Blood Pressure by Genotype for Loci on Rat Chromosome 2 in the F_2 (WKY \times S) Population Fed High Salt Diet

Locus	Blood pressure by genotype				D	Probability one-way ANOVA
	WW	WS	SS			
<i>D2N35</i>	169.0 \pm 4.21	172.8 \pm 2.33	181.6 \pm 4.31		+12.6	0.055
<i>n</i>	44	75	40			
<i>CAMK</i>	165.8 \pm 3.81	173.6 \pm 2.57	183.4 \pm 4.02		+17.6	0.0038
<i>n</i>	44	73	42			
<i>NAKα1</i>	166.0 \pm 3.28	173.1 \pm 2.44	186.7 \pm 4.86		+20.7	0.0005
<i>n</i>	49	74	36			
<i>HSD</i>	166.8 \pm 3.37	172.3 \pm 2.45	186.5 \pm 4.73		+19.7	0.0007
<i>n</i>	47	75	37			
<i>GCA</i>	167.6 \pm 3.35	172.3 \pm 2.44	185.3 \pm 4.81		+17.7	0.0027
<i>n</i>	48	73	38			
<i>FGG</i>	167.8 \pm 3.39	173.2 \pm 2.60	183.6 \pm 4.69		+15.8	0.014
<i>n</i>	46	77	36			
<i>NEP</i>	169.4 \pm 3.47	174.0 \pm 2.56	179.9 \pm 5.11		+10.5	0.18
<i>n</i>	44	82	33			
<i>CPB</i>	170.4 \pm 3.90	174.2 \pm 2.65	176.3 \pm 4.22		+5.9	0.60
<i>n</i>	32	87	40			
<i>ATIB</i>	170.7 \pm 3.83	174.2 \pm 2.63	176.3 \pm 4.39		+5.6	0.63
<i>n</i>	33	88	38			
<i>PRLR</i>	171.0 \pm 4.77	173.7 \pm 2.41	176.7 \pm 4.39		+5.7	0.64
<i>n</i>	29	89	41			

Blood pressure data are means \pm SE in millimeters of mercury for each genotype. W, Allele for WKY rats; S, allele for S rats; D, blood pressure differences homozygous SS minus homozygous WW; n, number of rats in each genotypic class.

imum LOD score in this region is at *ATIB* and equals 1.83.

The population blood pressure means for the F_2 (WKY \times S) and F_2 (MNS \times S) populations were 174.0 and 173.9 mmHg, respectively. The two populations were combined, and QTL interval mapping was performed on the combined data ($n = 330$). The combined data gave an LOD score of 5.66 for the existence of a blood pressure QTL at about the midpoint between *NAK α 1* and *CAMK* (Fig. 1). In the combined analysis, the QTL accounted for 9.2% of the total variance and $\sim 26\%$ of the genetic variance.

The blood pressure distributions of both F_2 populations were skewed to the right. A logarithmic transformation partially corrects for this. Analysis on the log of blood pressure did not alter any of the conclusions given above. Use of a nonparametric test (Kruskal-Wallis) in place of the one-way analysis of variance in Tables II and III also did not alter the conclusions.

Discussion

The data show that a QTL for blood pressure response to high salt diet is located on rat chromosome 2 between the *NAK α 1* and *CAMK* loci. Essentially, the same localization was obtained in both the F_2 (WKY \times S) and F_2 (MNS \times S) populations, which strengthens the result. The region likely to contain the QTL spans about 30 centiMorgans (cM), which is too large to work with for positional cloning. Computer simulations suggest that QTL localization is dependent mainly on the size of the experiment and the magnitude of the effect of the QTL (29). The effect of the QTL is not under our control, and the size of these experiments (a total of 330 rats in the two populations) has reached a practical limit. Even with a population of n

= 1,000 and a QTL of large effect, simulations show that QTL localization cannot be better than 10 cM (29).

An obvious way to proceed for better QTL localization is to next produce a congenic strain with a large segment of chromosome to prove (or disprove) the existence of the QTL. Then from this a series of congenic substrains containing small overlapping regions of chromosome from the region now known to contain the QTL can be constructed. Regions transferred between strains containing the QTL should influence blood pressure, and those regions not containing the QTL should not influence blood pressure. Provided a dense genetic map is available allowing the manipulation of small regions, the QTL localization could be further narrowed to an even smaller chromosomal fragment. This should map the QTL to within 1–2 cM.

The data for the presence of a second blood pressure QTL on chromosome 2 near *ATIB* in the F_2 (MNS \times S) population are merely suggestive. The significance levels associated with this region are too weak to establish the existence of a second QTL. It has, however, been reported (8) in an F_2 population derived from a cross of Lyon hypertensive and Lyon normotensive rats that systolic blood pressure (as well as pulse pressure) cosegregates highly significantly with *CPB*. *CPB* is located very close to *ATIB*. The fact that there was clearly no evidence for a second QTL in the F_2 (WKY \times S) population at the *CPB*–*ATIB* region can be explained by assuming that WKY and S do not carry functionally contrasting alleles in this region. Alternatively, there may, in fact, be no QTL in this region.

It is of interest that there are several candidate genes for blood pressure regulation on rat chromosome 2. These are *NAK α 1*, *HSD*, *GCA*, *NEP*, and *ATIB* with *NAK α 1* being the closest to the position of the maximum LOD score for the

Table III. Systolic Blood Pressure by Genotype for Loci on Rat Chromosome 2 in the F₂(MNS × S) Population Fed High Salt Diet

Locus	Blood pressure by genotype				Probability one-way ANOVA
	MM	MS	SS	D	
<i>D2N35</i>	171.8±2.51	171.5±2.18	181.9±4.22	+10.1	0.032
<i>n</i>	45	89	37		
<i>CAMK</i>	169.0±2.24	171.8±2.27	182.9±3.86	+13.9	0.0031
<i>n</i>	48	79	44		
<i>NAKα1</i>	168.1±2.48	174.7±2.16	180.3±4.53	+12.2	0.026
<i>n</i>	52	84	35		
<i>HSD</i>	Not tested				
<i>GCA</i>	168.2±2.57	173.9±2.12	181.0±4.41	+12.8	0.024
<i>n</i>	47	88	36		
<i>FGG</i>	168.6±2.44	174.1±2.15	181.3±4.78	+12.7	0.028
<i>n</i>	51	88	32		
<i>NEP</i>	168.8±2.60	175.7±2.24	176.4±4.14	+7.6	0.13
<i>n</i>	50	84	37		
<i>CPB</i>	Not tested				
<i>AT1B</i>	166.8±2.35	177.6±2.30	175.1±4.29	+8.3	0.014
<i>n</i>	52	87	32		
<i>PRLR</i>	168.1±2.32	175.7±2.40	178.6±4.16	+10.5	0.036
<i>n</i>	56	80	35		

Blood pressure data are means±SE in millimeters of mercury for each genotype. *M*, Allele from MNS rats; *S*, allele from S rats; *D*, blood pressure difference homozygous *SS* minus homozygous *MM*; *n*, number of rats in each genotypic class.

QTL. Since sodium balance is generally thought to be intimately related to chronic regulation of blood pressure, any genes involved in sodium transport or regulation are obvious candidates for influencing blood pressure. Because various isoforms of NaK-ATPase are involved not only in sodium handling by the kidney but also in regulation of vascular smooth muscle tone and cardiac contractility, NaK-ATPase genes are candidates for influencing blood pressure. HSD could alter adrenal steroid production and could thereby affect blood pressure. Atrial natriuretic peptide is a hormone secreted from heart atria in response to blood volume expansion and causes the kidney to excrete sodium and water. Thus, *GCA*, one of the receptors for atrial natriuretic peptide, is potentially important in blood pressure regulation. *NEP* is a peptidase that is important in metabolizing atrial natriuretic peptide and has been a target of antihypertensive drug therapy. *AT1B* is a receptor for angiotensin II. Since the renin-angiotensin system is intimately involved in blood pressure regulation, *AT1B* is an obvious candidate gene.

NAKα1 has been studied in segregating populations derived from a cross of Dahl S and Dahl salt-resistant (R) rats. It did not cosegregate with blood pressure in this cross (30), indicating that S and R do not carry a functionally different blood pressure QTL in this region of chromosome 2. *NAKα1* was thought to have a single base change in the coding region of S and R rats which would alter enzyme structure (31). This DNA variation, however, could not be confirmed and could be the result of a cloning artifact (32). *NEP* was also tested in an F₂(R × S) population and also did not cosegregate with blood pressure (Deng, A. Y., and J. P. Rapp, unpublished observations).

There is no reason to suppose that the region of rat chromosome 2 is unique with regard to the density of candidate genes for blood pressure regulation. On the contrary, it is our recent

experience that many regions where blood pressure QTLs are found also contain multiple rational candidates and that the identity of the QTL could be none of the obvious candidates. A possible example of a failed candidate is the renin locus where alleles present in Dahl S and R rats cosegregate strongly with blood pressure in F₂ (3) and backcross populations (33) and in a duplicated F₂ population (34). There was, however, no DNA sequence difference in the coding or regulatory regions of S and R renin alleles (35), and there is no logical physiological correlate, since S rats have low plasma and renal renin (36, 37), whereas high renin would make more sense as a cause of elevated blood pressure. An example of a possibly successful candidate is 11β-hydroxylase in Dahl S and R rats where there is a logical physiological correlate (altered steroidogenic pattern resulting in high production of the mildly hypertensinogenic steroid, 18-hydroxy-11-deoxycorticosterone) (1, 2, 38), the alleles in S and R rats cosegregate with blood pressure (1, 12), and there are base changes in the coding region causing five amino acid substitutions (12, 39). In vitro production of chimeric 11β-hydroxylase genes between S and R was able to narrow the causative lesion to a subset of three of these five amino acid changes (39).

In summary the data clearly point to the existence of a blood pressure QTL between *NAKα1* and *CAMK* on rat chromosome 2 that accounts for 9.2% of the total variance and 26% of the genetic variance for rats on a high salt diet.

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