Congenic Substitution Mapping Excludes *Sa* as a Candidate Gene Locus for a Blood Pressure Quantitative Trait Locus on Rat Chromosome 1

Norbert Hübner, Young-Ae Lee, Klaus Lindpaintner, Detlev Ganten, Reinhold Kreutz

Abstract—Previously, linkage analysis in several experimental crosses between hypertensive rat strains and their contrasting reference strains have identified a major quantitative trait locus (QTL) for blood pressure on rat chromosome 1 (Chr 1) spanning the *Sa* gene locus. In this study, we report the further dissection of this Chr 1 blood pressure QTL with congenic substitution mapping. To address whether the *Sa* gene represents a candidate gene for the Chr 1 blood pressure QTL, congenic strains were developed by introgressing high blood pressure QTL alleles from the stroke-prone spontaneously hypertensive rat (SHRSP) into the normotensive Wistar-Kyoto (WKY-1) reference strain. Congenic animals carrying a chromosomal segment from stroke-prone spontaneously hypertensive rats between genetic markers *Mt1pa* and *D1Rat200* (including the *Sa* gene locus) show a significant increase in basal systolic and diastolic blood pressure compared with their normotensive Wistar-Kyoto progenitors ($P<0.001$, respectively), whereas congenic animals carrying a subfragment of this Chr 1 region defined by markers *Mt1pa* and *D1Rat57* (also spanning the *Sa* gene) do not show elevated basal blood pressure levels ($P=0.83$ and $P=0.9$, respectively). Similar results were obtained for NaCl-induced blood pressure values. Thus, the blood pressure QTL on Chr 1 is located centromeric to the *Sa* gene locus in a region that is syntenic to human chromosome 11p15.4-p15.3. This region excludes the *Sa* as a blood pressure–elevating candidate gene locus on the basis of congenic substitution mapping approaches. **(*Hypertension*. 1999;34:643-648.)**

Key Words: hypertension, essential ■ rats, inbred SHR ■ rats, inbred WKY ■ genetics ■ complex traits ■ crosses, genetic

Primary hypertension is one of the most common chronic diseases. It represents a major risk factor for cardiovascular morbidity and mortality. Hypertension shows a significant degree of heritability and is commonly recognized as a complex, polygenic disorder with the exception of rare monogenetic forms.[1,2] The nature of this complex disease makes it difficult to identify contributing genes.[2] A method to reduce the complexity of hypertension is the use of inbred animal models in the genetic analysis of this disorder. Information on genetic factors identified in such experimental systems may provide insights into disease mechanisms that can subsequently be applied to study human hypertension.

The identification of the *Sa* gene by Iwai and Inagami[3] and the observation of its differential expression pattern in kidneys from hypertensive and normotensive rat models has generated considerable interest over the past few years.[4] Subsequent to its identification, several investigators have shown that the *Sa* gene locus cosegregates with blood pressure and is localized within a major blood pressure quantitative trait locus (QTL) on rat chromosome 1 (Chr 1).[5–12] Furthermore, cosegregation of an *Sa* gene polymorphism and differential *Sa* gene expression was demonstrated.[7] The *Sa* gene therefore represents an attractive candidate to explain the effect of the Chr 1 QTL on blood pressure. Congenic experimentation recently lead to the verification of the Chr 1 blood pressure QTL spanning the *Sa* locus.[13–15]

Despite successful QTL detection and isolation of a large congenic region around the *Sa* gene locus, only fine mapping of the QTL to a small chromosomal fragment will ultimately facilitate positional cloning or positional candidate gene identification. The basis of single QTL–oriented fine mapping is similar to that of Mendelian-gene fine mapping, that is, analysis of recombinants within a congenic interval previously found to account for the phenotypic variation.

The current set of experiments was aimed to evaluate whether the *Sa* gene explains the effect of the Chr 1 QTL on blood pressure by congenic substitution mapping approaches.

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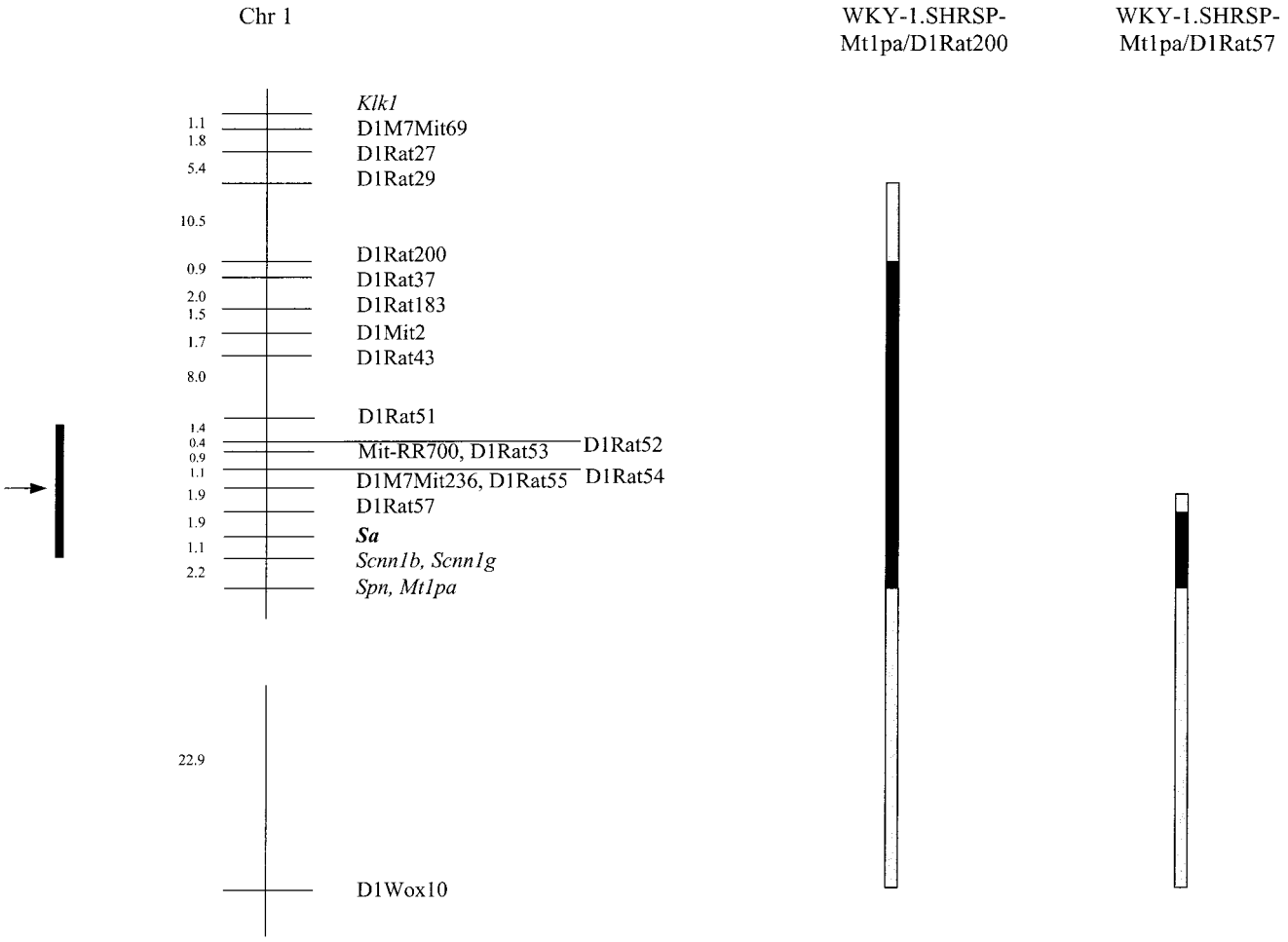

Figure 1. Schematic of rat Chr 1 and regions introgressed in the congenic strains (not drawn to scale). Numbers next to the chromosome and congenic segments indicate the genetic distance in cM. Bar to the left of Chr 1 represents the 100:1 odds interval for localization of the blood pressure QTL identified in F_2 (WKY-1×SHRSP) cross. The arrow indicates the obtained maximum LOD score of 5.0 at marker *D1M7Mit236*.[12] *D1M7Mit236* denotes a mouse marker that also amplifies the homologous rat locus; terminology for loci used is in accordance with the guidelines of the committee on rat gene nomenclature.[29] The black and shaded areas show the minimal and potentially maximal segments introduced into WKY-1.SHRSP-Mt1pa/D1Rat200 and WKY-1.SHRSP-Mt1pa/D1Rat57, respectively.

Congenic strains were developed by introgressing high blood pressure QTL alleles from the stroke-prone spontaneously hypertensive rat (SHRSP) into the normotensive Wistar-Kyoto (WKY-1) reference strain. Blood pressures in congenic animals were determined by radiotelemetry at baseline and after dietary sodium loading. Our current results provide definitive evidence that the *Sa* gene cannot be considered a candidate within the investigated QTL on Chr 1. This would currently allow the localization of the Chr 1 blood pressure QTL centromerically to the *Sa* gene locus, possibly, as recently suggested,[16] guiding the genetic analysis for human essential hypertension.

Methods

Animals

All animals were obtained from our original Heidelberg (indicated by the suscript HD) colonies of $SHRSP_{HD}$ and $WKY\text{-}1_{HD}$ (subsequently referred to as SHRSP and WKY-1) at the Max-Delbrück-Center for Molecular Medicine, Berlin, Germany, and represented the same colonies that were used in our previous F_2 experimentation that lead to the identification of the blood pressure QTL on Chr 1.[12] The WKY-1 strain has been characterized elsewhere.[17] Experimental procedures for animal housing and breeding have been reported previously.[17] All animal experiments were conducted in accordance with institutional guidelines for the care and use of laboratory animals.

Breeding

We have previously reported on a blood pressure QTL on Chr 1 that included the *Sa* gene locus.[12] This locus was identified by marker *D1M7Mit236* in an F_2 (WKY-1×SHRSP) intercross and was mapped to a chromosomal region flanked by markers *D1 Mit2* and *Mt1pa* (Figure 1). The SHRSP blood pressure allele between *Mt1pa* and *D1 Mit2* was transferred onto the WKY-1 background by 8 successive backcrosses starting from F_1 animals. This was accomplished by breeding a male SHRSP with a female WKY-1. Each subsequent backcross was performed by mating male rats that have genotypically been confirmed to be heterozygous for the SHRSP-Mt1pa/D1 Mit2 allele with female WKY-1 rats. The identification of breeder animals within each backcross generation was accomplished by analyzing multiple simple-sequence length polymorphisms (SSLP) within the region of interest, including markers exceeding the 100:1 odds support interval for the localization of the QTL on either side (Figure 1). Before homozygous animals were bred, female rats that were heterozygous for the Chr 1 QTL were mated with male WKY-1 animals. Male littermates again were mated with WKY-1 female rats, and their offspring was brother×sister mated to produce

Blood Pressures in Parental and Congenic Rats Obtained by Radiotelemetry

	Blood Pressure, mm Hg±SD					
Phenotype	WKY-1	WKY-1.SHRSP-Mt1pa/D1Rat200	*P* (ANOVA)	WKY-1	WKY-1.SHRSP-Mt1pa/D1Rat57	*P* (ANOVA)
Systolic basal	124.0±4.5	134.0±3.9	<0.001	130.7±3.1	131.0±4.4	0.833
Diastolic basal	85.9±2.3	94.3±2.4	<0.001	89.5±2.6	89.7±3.8	0.900
MAP basal	103.5±3.0	112.3±2.9	<0.001	108.2±2.9	108.0±4.0	0.910
Systolic NaCl	129.2±5.5	138.3±4.6	<0.001	134.1±3.4	135.2±4.8	0.560
Diastolic NaCl	89.8±3.4	97.4±3.1	<0.001	91.5±3.0	91.7±4.7	0.899
MAP NaCl	107.9±4.1	115.9±3.6	<0.001	110.7±3.1	110.9±4.8	0.901

Phenotype comparison between WKY-1 (n=11) vs WKY-1.SHRSP-Mt1pa/D1Rat200 (n=9) and WKY-1 (n=12) vs WKY-1.SHRSP-Mt1pa/D1Rat57 (n=11). All blood pressures were obtained at 16 weeks of age, one day before dietary NaCl loading was initiated (basal), and 12 days after NaCl loading (NaCl), respectively, and represented the identical time points of blood pressure determinations that were analyzed in F_2 (WKY-1×SHRSP) animals. MAP, mean arterial blood pressure.

homozygous WKY-1.SHRSP-Mt1pa/D1 Mit2 congenic animals. This breeding scheme ensured that, on average, >99.8% of the background genome that included both gender chromosomes were derived from the WKY-1 recipient. Congenic sublines were established by mating heterozygous WKY-1.SHRSP-Mt1pa/D1 Mit2 with WKY-1, the offspring was genotyped to identify recombinations with the use of *SSLP* markers. Appropriate animals were subsequently bred to homozygosity. This led to the establishment of the congenic subline WKY-1.SHRSP-Mt1pa/D1Rat57.

Blood Pressure Measurements

For all experiments a radiotelemetric method (Data Sciences International), which allows highly accurate and reproducible blood pressure determinations, was used as previously reported[17] to characterize WKY-1 progenitor and congenic animals. Animals were operated under anesthesia at 12 weeks of age and were allowed to recover for 14 days. Hemodynamic measurements were performed from week 14 to week 16 after birth at baseline and during the following 12 days of dietary sodium loading (1% NaCl in drinking water with free access).

Genotype Determination

DNA was extracted according standard procedures from tail tips. SSLP were obtained from a panel we used previously[18,19]; additional oligonucleotide sequences for published microsatellites were obtained from public databases (http://www.genome.wi.mit.edu/rat/public or http://www.well.ox.ac.uk/~bihoreau/). PCR was performed on 50 ng of genomic DNA in a final volume of 10 μL, which contained 100 nmol/L of each primer, 200 μmol/L dNTPs, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100, and 0.25 U of Taq DNA Polymerase (Promega). The forward primer was labeled with [γ^{32}P]ATP by T4 polynucleotide kinase. PCR products were processed and subsequently analyzed by autoradiography after PAGE as described.[17]

Statistical Analysis

Differences in blood pressure between progenitor and congenic/subcongenic strains were evaluated by ANOVA. Linkage analysis of Chr 1 and blood pressure in the F_2 (WKY-1×SHRSP) has been reported[12] and was calculated by MAPMAKER/EXP and MAPMAKER/QTL[20] with blood pressure values adjusted to male levels and the first reciprocal cross.[18] All blood pressure values are expressed as mean±SD.

Results

Genetic Characterization of Chr 1 Congenic/Subcongenic Animals

The transferred segments in the original congenic strain and a congenic substrain are presented in Figure 1. Additionally, Figure 1 shows the localization of the 100:1 odds support interval of the previously published blood pressure QTL on Chr 1 spanning the *Sa* gene locus in the F_2 (WKY-1×SHRSP) intercross[12] for comparison.

Further genotype analysis with newly available markers for rat Chr 1 was performed in the original WKY-1.SHRSP-Mt1pa/D1 Mit2 congenic strain. The results indicated that this strain inherited a fragment derived from SHRSP that spanned at least the chromosomal segment between markers *D1Rat200* and *Mt1pa*. Genotyping analysis in the subcongenic line confirmed the successful transfer of a subsegment from SHRSP onto the WKY-1 genetic background, which defined WKY-1.SHRSP-Mt1pa/D1Rat57 as a congenic substrain of the WKY-1.SHRSP-Mt1pa/D1Rat200 strain (Figure 1). In the WKY-1.SHRSP-Mt1pa/D1Rat57 congenic substrain, the chromosomal region centromeric from the *Sa* gene was substituted by WKY-1 parental alleles (Figure 1). SSLP analysis with the use of 73 genetic markers throughout the genome confirmed the congenic status of the animals designated WKY-1.SHRSP-Mt1pa/D1Rat200 and WKY-1.SHRSP-Mt1pa/D1Rat57 (data not shown).

Phenotype Characterization of Congenic Animals

Blood pressures were determined in WKY-1.SHRSP-Mt1pa/D1Rat200 and WKY-1. Systolic and diastolic blood pressure values were significantly higher in WKY-1.SHRSP-Mt1pa/D1Rat200 as compared with WKY-1 at baseline (16 weeks of age, day before NaCl loading was initiated; $P<0.001$ and $P<0.001$, respectively) and after 12 days of NaCl exposure ($P<0.001$ and $P<0.001$, respectively, Table). The strain differences in blood pressure persisted consistently over the whole time period studied (Figure 2A) and mirrored the punctual observations made at baseline and on day 12 of sodium exposure. The difference in the increase of blood pressure between the 2 strains after NaCl loading was not statistically significant.

Phenotype Characterization of Subcongenic Animals

Measurements of the congenic substrain WKY-1.SHRSP-Mt1pa/D1Rat57 and WKY-1 progenitors did not show a difference in either systolic or diastolic blood pressure at

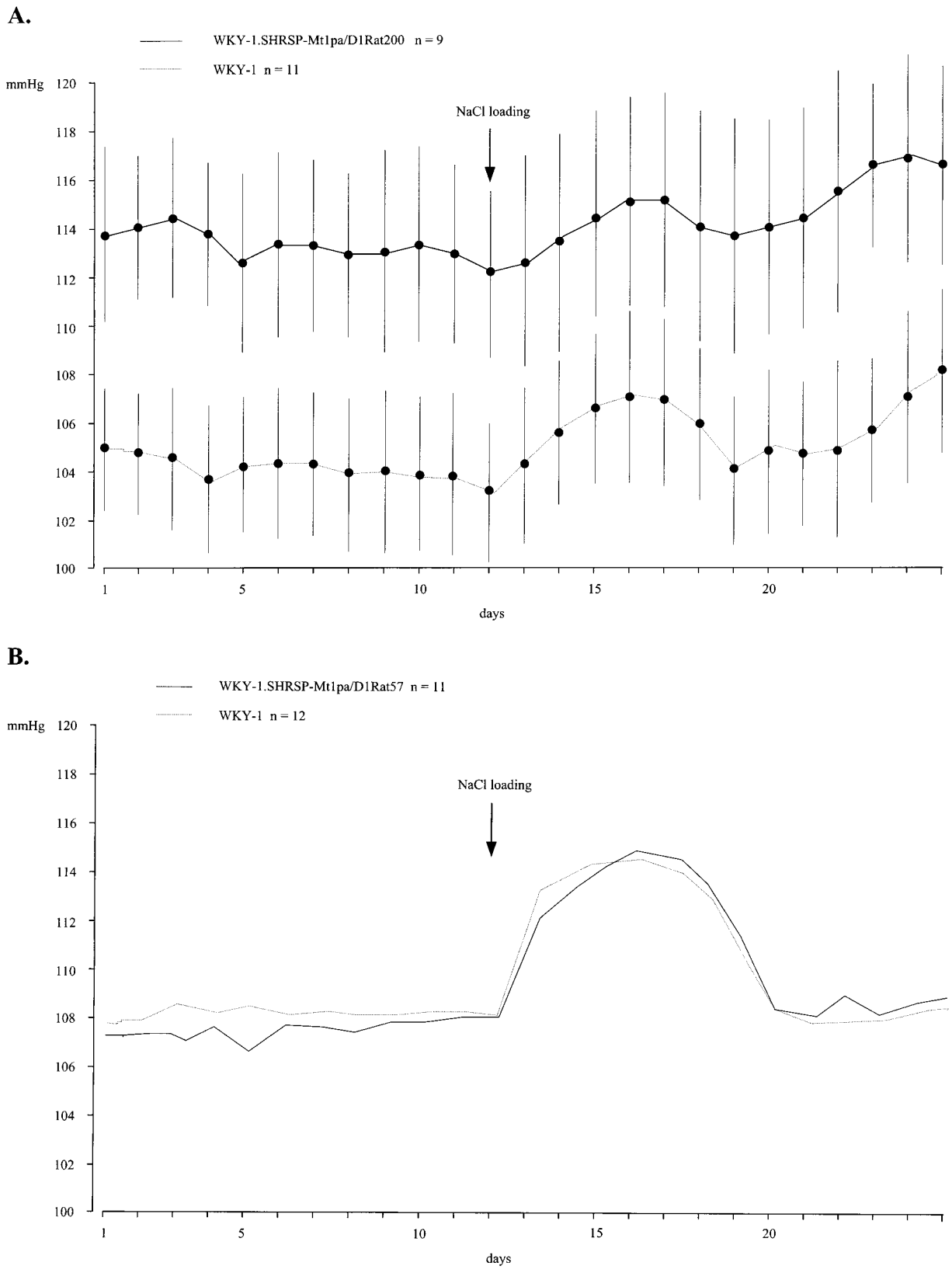

Figure 2. Twenty-four-hour average mean arterial blood pressure determined by radiotelemetry over a period of 12 days before and 12 days after dietary sodium loading. Animals were operated on at 12 weeks of age and were subsequently allowed to recover for 2 weeks before blood pressure measurements were taken. Blood pressure values were averaged at each 24-hour period and represented ≈300 individual measurements in each rat. Subsequently group averages were calculated. A, Comparison between WKY-1.SHRSP-Mt1pa/D1Rat200 (n=9) and WKY-1 (n=11). Vertical bars indicate SD of group averages. B, Comparison between WKY-1.SHRSP-Mt1pa/D1Rat57 (n=11) and WKY-1 (n=12). For clarity, graphical representation of SDs was omitted.

baseline ($P=0.83$ and $P=0.90$, respectively) or after NaCl loading ($P=0.56$ and $P=0.89$, respectively; Table). No difference in blood pressure was detected at any time point investigated (Figure 2B). There was a significant but equal increase in blood pressure in response to NaCl exposure in both WKY-1.SHRSP-Mt1pa/D1Rat57 and WKY-1, which showed no differences between strains (Figure 2B, Table).

Discussion

In this paper, we describe a series of studies conducted to further define our previous findings of a blood pressure relevant locus on Chr 1 that segregates among the SHRSP and WKY-1 strains. In our present studies, we combined congenic experimentation with the development of subcongenic animals, with only a fraction of the initial congenic segment for the QTL region on Chr 1, to initiate fine mapping within this region. The present investigations demonstrate that this blood pressure QTL on Chr 1 is localized centromeric to the *Sa* gene locus. Therefore, the *Sa* gene does not represent the gene that explains the observed blood pressure variation expressed by this QTL on Chr 1.

The hypothesis that this Chr 1 QTL contains a gene or genes that influence blood pressure is supported by the isolation of this QTL in congenic animals. Introgressing a large chromosomal region from SHRSP into WKY-1 animals resulted in the establishment of the congenic strain WKY-1.SHRSP-Mt1pa/D1Rat200 (Figure 1). This congenic strain encompassed a region of Chr 1 that exceeded the 100:1 odds support interval for the initial localization of this QTL in F_2 (SHRSP×WKY-1) animals and spanned the *Sa* gene locus. The increase in blood pressure of ≈10 mm Hg in WKY-1.SHRSP-Mt1pa/D1Rat200 congenic animals compared with WKY-1 progenitors is in agreement with recent reports on blood pressure effects in Chr 1 congenic strains with SHR and WKY or Brown Norway rats as model strains.[13–15] A more pronounced blood pressure response after NaCl loading in congenic WKY-1.SHRSP-Mt1pa/D1Rat200 compared with WKY-1 reference animals, although theoretically expected from data obtained in the F_2 (WKY-1×SHRSP) cross,[12] was absent, which suggested a less marked phenotypic effect of this QTL on the NaCl-induced blood pressure in the absence of overall higher blood pressure attributed to many QTLs in the F_2 cohort. The overall effect of this blood pressure QTL accounted for ≈40% of the blood pressure variance observed after NaCl loading in the F_2 (WKY-1×SHRSP) cross.

The relevance of the *Sa* gene as a candidate for blood pressure variation was further investigated by initiating congenic substitution mapping approaches. Our study was designed to differentiate between blood pressure effects of the *Sa* gene locus and the genetic marker *D1M7Mit236*, which gave rise to the maximal LOD score in our F_2 (SHRSP×WKY-1) cohort (Figure 1) detecting the QTL. We identified a recombinant congenic substrain that carried SHRSP alleles defined by markers *Mt1pa* and *D1Rat57* and exhibited wild-type alleles centromeric from marker *D1Rat57* (Figure 1). The fact that there was no detectable blood pressure effect in the congenic substrain WKY-1.SHRSP-Mt1pa/D1Rat57 compared with the WKY-1 progenitor strain rules out the *Sa* gene as a positional candidate for hypertension. Therefore, the QTL is localized centromeric with reference to the *Sa* gene locus (Figure 1), possibly between markers *D1Rat57* and marker *D1 Mit2* although the centromeric confines of the transferred SHRSP alleles are likely to exceed the latter. These data are in agreement with the maximum placement of this QTL in our F_2 (WKY-1×SHRSP) intercross that is 3.8 cM centromeric to the *Sa* gene locus (Figure 1) at marker *D1M7Mit236*.

Our data rule out 2 additional genes, the β- and γ-subunits of the epithelial sodium channel (*Scnn1b* and *Scnn1g*, respectively) as positional candidates that represent this Chr 1 QTL. Mutations in these genes have been identified in patients with Liddle's syndrome, which leads to the formation of constitutively activated epithelial sodium channels thus increasing renal sodium reabsorption and causing hypertension.[21,22]

Moreover, *Scnn1b* and *Scnn1g* have recently been implicated in possibly playing a role as basic mechanotransducers in baroreceptor nerve terminals.[23] Both genes were previously shown to be closely linked to the *Sa* gene locus, just on the border of the 100:1 odds support interval of this blood pressure QTL (Figure 1).[12] Although earlier investigations could not reveal any relevant coding sequence mutations in different models of genetic hypertension, more subtle mutations, eg, in regulatory elements, could not be excluded in these studies.[12] The choice to use WKY-1 as the recipient strain for constructing Chr 1 congenic lines stems from our observation that the Chr 1 QTL exhibits a significant blood pressure effect only in F_2 (WKY-1×SHRSP) animals[12] but not in the F_2 (WKY×SHRSP) cohort[24] according to stringent statistical criteria.[25]

The Chr 1 blood pressure QTL has been confined to a region centromeric of the *Sa* gene locus. In contrast to the adjacent region telomeric of the *Sa* gene (11p15.5), this region is syntenic to human chromosome 11p15.4-p15.3, which has not previously been investigated in human hypertension. This provides a possible explanation why the findings on rat Chr 1 could only partly be corroborated in human hypertension thus leading to conflicting results.[26–28,30,31]

The present data warrant a note of caution on potential pitfalls of focusing on candidate genes at early stages in the analysis of complex disease phenotypes. Although the *Sa* gene exemplifies a prime candidate gene because of its cosegregation with the disease phenotype, expression in the kidney, and differential expression pattern between hypertensive and normotensive parental strains, it should be observed as a marker in linkage studies. Only after a more rigorous approach, such as congenic experimentation and confinement to a small chromosomal region, can a candidate gene be viewed seriously as a bona fide candidate gene.

Given the marked consistency of the effect of this Chr 1 QTL on blood pressure variation of ≈10 mm Hg within our and several other congenic strains,[13–15] it should be emphasized that results from congenic experimentation with the use of different parental strains might be integrated. If indeed the same QTL alleles are operative in different strains, this would significantly advance the fine mapping of the QTL locus by comparing blood pressure effects and genetic breakpoints within several congenic/subcongenic strains.

Despite major successes in QTL detection, fine mapping has been difficult to achieve. This difficulty is presently a major obstacle to both positional cloning and positional candidate gene identification. Further work on the establishment of additional congenic lines that carry smaller subfractions of the Chr 1 blood pressure QTL defined in this study will facilitate both goals.

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