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High-Resolution Mapping of the Blood Pressure QTL on Chromosome 7 Using Dahl Rat Congenic Strains

George T. Cicila,[1] Michael R. Garrett,[1] Soon Jin Lee, Jun Liu, Howard Dene, and John P. Rapp[2]

Department of Physiology and Molecular Medicine, Medical College of Ohio, Toledo, Ohio 43614

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It was previously shown using Dahl salt-sensitive (S) and salt-resistant (R) rats that a blood pressure quantitative trait locus (QTL) was present on rat chromosome 7. In the present work, this QTL was localized to a region less than 0.54 cM in size on the linkage map using a series of congenic strains. This region was contained in a single yeast artificial chromosome that was 220 kb long. This small segment still contained the primary candidate locus *Cyp11b1* (11β-hydroxylase), but the adjacent candidate genes *Cyp11b2* (aldosterone synthase) and *Cyp11b3* were ruled out. It is concluded that 11β-hydroxylase, through its known genetic variants altering the production of 18-hydroxy-11-deoxy corticosterone, is very likely to account for the blood pressure QTL on chromosome 7 in the Dahl rat model of hypertension. This QTL accounts for about 23 mm Hg under the condition of 2% NaCl diet for 24 days.

INTRODUCTION

Human essential hypertension is a complex, multifactorial disorder resulting from the interplay of multiple environmental and genetic factors. While several environmental factors contributing to elevated blood pressure have been identified (e.g., excess dietary NaCl), less progress has been made in unraveling the genetic determinants of blood pressure control. Identifying the genetic determinants of this cardiovascular disorder in humans is difficult. Studying rat models of hypertension is one way to circumvent this difficulty.

The Dahl salt-sensitive (S) and salt-resistant (R) rat strains comprise the most widely studied genetic model of blood pressure salt-sensitivity and salt-resistance. In the S strain, supplemental dietary NaCl increases blood pressure, whereas in the R strain, supplemental dietary NaCl has little or no effect (Dahl *et al.*, 1962; Rapp and Dene, 1985). The Dahl S rat is also a genetically hypertensive strain, developing high blood pressure over time, even when maintained on low or normal levels of dietary NaCl. Thus, Dahl rats can model the influence of genetic factors, as well as genetic–environmental interactions influencing blood pressure, cardiac mass, and other related phenotypic traits. Segregating populations and congenic strains derived from these inbred strains can be used to screen for and confirm quantitative trait loci (QTLs) responsible for heritable differences in blood pressure, as well as loci underlying the gene × environment (dietary NaCl intake) interactions responsible for blood pressure salt-sensitivity and salt-resistance.

In 1971 it was shown that adrenal mitochondria of S rats convert a higher proportion of exogenous 11-deoxycorticosterone (DOC) into 18-hydroxy-11-deoxycorticosterone (18-OH-DOC) compared to adrenal mitochondria of R rats (Rapp and Dahl, 1971). This was found to be due to genetic variants in 11β-hydroxylase (*Cyp11b1*) (Rapp and Dahl, 1976), an adrenal steroidogenic enzyme of the cytochrome P450 superfamily. This enzyme catalyzes the conversion of DOC into corticosterone (also called compound B) and 18-OH-DOC by its 11β- and 18-hydroxylation activities, respectively. The steroidogenic patterns characteristic of S and R rats were found in 1972 to cosegregate with blood pressure (Rapp and Dahl, 1972a), and this represents one of the earliest suggestions of a blood pressure QTL. Plasma 18-OH-DOC is higher in S than R rats (Rapp and Dahl, 1971, 1972b; Matsukawa *et al.*, 1993; Rapp *et al.*, 1978), and 18-OH-DOC is a weak mineralocorticoid (Rapp and Dahl, 1972b; Carroll *et al.*, 1981) that could account for a component of salt-sensitivity in the S rat (Rapp and Dahl, 1972b).

Five nucleotide differences leading to amino acid substitutions were identified in the 11β-hydroxylase gene of S rats compared to R rats (Matsukawa *et al.*, 1993; Cicila *et al.*, 1993). These mutations cosegregated with the adrenal capacity to synthesize 18-OH-DOC and blood pressure in a population bred from

[1] These authors contributed equally to this work.

[2] To whom correspondence and reprint requests should be addressed at Department of Physiology and Molecular Medicine, Medical College of Ohio, Block Health Science Building, 3035 Arlington Avenue, Toledo, OH 43614. Telephone: (419) 383-4297. Fax: (419) 383-6168. E-mail: jrapp@mco.edu.

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Dahl S and R rats (Cicila *et al.*, 1993). However, this analysis did not prove that 11β-hydroxylase was the causative locus for its linked QTL. Indeed, aldosterone synthase, which is linked to, and shares significant sequence homology with, 11β-hydroxylase, also has amino acid substitutions in the alleles carried by S and R rats (Cover *et al.*, 1995).

A congenic strain (S.R-Cyp11b) carrying the portion of the R-rat chromosome 7 containing the 11β-hydroxylase locus was previously bred onto the S-rat genetic background to confirm that a blood pressure QTL was present. As expected, S.R-Cyp11b congenic rats had decreased blood pressure compared to S rats when fed a high-salt diet. Furthermore, S.R-Cyp11b congenic rats had a markedly increased survival compared to S rats, in the context of a high dietary NaCl intake (Cicila *et al.*, 1997).

The introgressed portion of R-rat chromosome 7 in the original congenic strain, S.R-Cyp11b (Cicila *et al.*, 1997), was selected by retention of R-rat alleles for the *Cyp11b* gene family. The congenic region of S.R-Cyp11b spans up to 20.2 cM as defined by the linkage map used in the present work. This amount of DNA potentially encodes hundreds of genes, any of which could be responsible for the blood pressure effect of the congenic strain. The present study used a set of congenic substrains to define better the blood pressure QTL-containing interval to a small region of $\leq$220 kb that still contains 11β-hydroxylase (*Cyp11b1*).

MATERIALS AND METHODS

Inbred rat strains and genetic crosses. The inbred Dahl salt-hypertension-sensitive (SS/Jr) and salt-hypertension-resistant (SR/Jr) rat strains were developed (Rapp and Dene, 1985) from outbred stock originally obtained from Dr. L. Dahl (Dahl, *et al.*, 1962) and will be referred to by their generic designations of S (sensitive) and R (resistant). S and R rats used to make the genetic crosses and develop congenic strains were from the colony at the Medical College of Ohio. Lewis rats (LEW/NCrlBR) were obtained from Charles River Laboratories (Wilmington, MA) and are referred to as LEW. The Milan normotensive strain (MNS) originated from the Veterinary Resources Branch at the National Institutes of Health (Bethesda, MD), and Albino Surgery (AS) rats were from the National Institute for Medical Research (Mill Hill, UK). Spontaneously hypertensive rats (SHR/NHsd), Brown Norway rats (BN/SsNHsd), and Wistar–Kyoto rats (WKY/NHsd) were obtained from Harlan Sprague–Dawley (Indianapolis, IN) and will be referred to as SHR, BN, and WKY, respectively.

Two backcross $F_1(S \times R) \times S$ populations ($n = 150$ rats) were used to construct a detailed linkage map of chromosome 7. The breeding and phenotyping of these populations have been previously described in detail (Cicila *et al.*, 1997, 1999). A large population ($n = 643$) of F_2 rats derived from a cross of S rats and the congenic substrain 11bx4 described below was also made. The purpose of this cross was to collect recombinant chromosomes for future congenic substrain construction, but it served here also to provide a detailed linkage map of our subsequently reduced target region.

Development of congenic substrains of S.R-Cyp11b. Congenic substrains were developed by crossing the previously described S.R-Cyp11b congenic strain (Cicila *et al.*, 1997) with S rats to yield $F_1(S \times$ S.R-Cyp11b) animals heterozygous for the introgressed region of R-rat derived chromosome 7. $F_1(S \times$ S.R-Cyp11b) rats were intercrossed to obtain a population of $F_2(S \times$ S.R-Cyp11b) ($n = 113$) rats, which was screened to identify rats having crossovers within this region of introgressed chromosome. Rats containing three different recombinant chromosomes were identified and used to develop congenic substrains carrying smaller portions of R-rat chromosome 7 as follows. Rats carrying the appropriate recombinant chromosomes were crossed with S rats to duplicate the recombinant chromosome, and the resulting heterozygous progeny were intercrossed. Intercross progeny were genotyped to identify those carrying two copies of the same recombinant chromosome. Rats homozygous for a recombinant chromosomal segment were crossed to fix the recombinant chromosome and establish the new congenic substrains 11bx1, 11bx2, and 11bx3. During construction of these congenic substrains, progeny containing additional crossovers within the introgressed region of chromosome 7 were identified. These rats were crossed with S rats to duplicate the recombinant chromosome, which was then fixed as described above. In this manner, substrains 11bx4 and 11bx5 were derived from substrain 11bx1, and substrain 11bx6 was derived from substrain 11bx2.

Cardiovascular phenotyping. Blood pressure and heart weight were measured for male rats of each congenic substrain and a group of age- and weight-matched control S rats. Each set of congenic substrain rats ($n = 20$) and control S rats ($n = 20$) was bred, housed, and studied concomitantly to minimize environmental effects. Rats were weaned at 30 days of age and placed on a low-salt diet (0.3% NaCl, Harlan Teklad diet TD7034). Four animals (two of each strain) were randomly assigned to each cage. At 40–42 days of age, rats were fed 2% NaCl diet (Harlan Teklad diet TD94217) for 24 days, and their blood pressure was determined.

Systolic blood pressure was measured using the tail-cuff microphonic method (Buñag and Butterfield, 1982) on conscious restrained rats warmed to 28°C. Operators were unaware of the identity of the rat during these measurements. The blood pressure of each rat was measured for 4 consecutive days. Blood pressure values for each day were the means of three to four consistent readings. The final blood pressure value used was the mean of the four daily blood pressure values. Rats were sacrificed by CO_2 inhalation, and body and heart weights were measured.

Genotyping. DNA for genotyping rats during congenic substrain development was extracted from tail biopsy material using the QIAamp Tissue DNA Kit (Qiagen, Chatsworth, CA). PCR amplification and gel electrophoresis were performed as previously described (Cicila *et al.*, 1997).

Additional microsatellite markers were placed on the rat chromosome 7 linkage map to identify markers useful in defining the extent of R-rat chromosome introgressed into the congenic strain and substrains. Markers were selected from the following sources: (1) Massachusetts Institute of Technology (Cambridge, MA) (http://www.genome.wi.mit.edu), available from Research Genetics (Huntsville, AL); (2) Wellcome Trust Centre for Human Genomics (Oxford, UK) (http://www.well.ox.ac.uk), available from GenoSys (Cambridge, UK); and (3) Medical College of Ohio (http://www.mco.edu/depts/physiology/research/rat/marker.html). Markers were placed by genotyping a combined $F_1(S \times R) \times S$ backcross population (described above), and a linkage map was developed using the Map Manager QTX program (Manly and Olson, 1999) obtained from Dr. Kenneth F. Manly (State University of New York, Buffalo, NY). Potential errors in typing, i.e., loci involved in double-recombination events, were retyped to confirm or correct the results.

A new marker was developed to distinguish between the 11β-hydroxylase (*Cyp11b1*) alleles of S and R rats, using the direct mismatch method (Haliassos *et al.*, 1989; Davidow, 1992). A primer set (*D7Mco7*) was designed to amplify across the single basepair strain difference between S and R rats at nucleotide 1141 of the cDNA sequence as numbered by Nonaka *et al.* (1989). These primers, 5′-CGGAACCCAGATGTTCAG and 5′-GGTCTGAGTGTACGATTCTCTGT, amplify a 411-bp PCR product from genomic DNA, which extends from exon 6 to exon 7 of the *Cyp11b1* gene. The penultimate "G" of the second (reverse) primer, hybridizing in exon 7, represents a mismatch with the genomic sequence. This in combination with the strain difference between S and R rats at nucleotide 1141 creates an

*Rsa*I restriction site (GTAC) in the S-rat *Cyp11b1* PCR product but not in the R-rat product, where the sequence produced is TTAC. PCR products that could conceivably be amplified from all other known Cyp11b gene family members using these primers would also lack this artificial *Rsa*I site. Digestion of the *Cyp11b1* PCR product from the S allele with *Rsa*I results in a 388-bp product while the uncut R allele product is the full 411 bp in length.

Steroid ratio analysis. The adrenal steroid biosynthetic phenotype was determined by methods similar to those described previously (Rapp and Dahl, 1972a). Whole adrenal homogenates were incubated with excess DOC and NADPH for 10 min at 37°C. Samples were immediately extracted with cold methylene dichloride and concentrated by evaporation of the solvent.

Under these conditions, the main steroid products are 18-OH-DOC and corticosterone (compound B, referred to as B). 18-OH-DOC, B, and DOC were separated by high-pressure liquid chromatography (HPLC) using a Nova-Pak C18 reversed-phased column (3.9 mm × 300 mm column, HPLC-Dionex AI450 Model 2; Waters Associates, Milford, MA). Samples were dissolved in 100% acetonitrile, and prior to loading, a dilution of each sample was made in a 10% acetonitrile solution. The steroid peaks were automatically integrated (Dionex Software, Waters Associates), and the "steroid ratio" was calculated by dividing the amount of 18-OH-DOC by the sum of the amounts of 18-OH-DOC and compound B. The steroid ratio for each strain was determined by averaging the ratio calculated from five animals. In previous studies, this steroid ratio readily differentiated S and R rats (Rapp and Dahl, 1971, 1972a, 1976; Cicila *et al.,* 1993).

Development of new microsatellite markers. The WI/MIT rat yeast artificial chromosome (YAC) library (Haldi *et al.,* 1997) (Research Genetics) was screened by PCR amplification of DNA pools to identify clones containing *D7Rat131* and *D7Mco7*. PCR amplification and Southern filter hybridization analysis confirmed that these markers were present in the YAC clones. The smallest YAC clone (220 kb) containing both *D7Rat131* and *D7Mco7* was clone 152G06, which was used to develop new microsatellite markers.

YAC DNA from clone 152G06 was separated from yeast chromosomal DNA by pulsed-field gel electrophoresis (CHEF DR-II; Bio-Rad, Hercules, CA) as follows. Agarose plugs containing YAC clone 152G06 DNA were loaded across the entire width of a 1% low-melt agarose gel (Bio-Rad) and electrophoresed at 14°C for 32 h in 0.5× TBE with a voltage gradient of 6 V/cm, a reorientation angle of 120°, and a switch time of 21 s. The YAC DNA band was excised from the gel, equilibrated in gelase buffer, melted, and digested with gelase (Epicentre Technologies, Madison, WI). Isolated YAC DNA was then ethanol-precipitated and resuspended in TE (pH 8.0).

Purified YAC DNA was digested with *Rsa*I (New England Biolabs; Beverly, MA), ligated into the pCR-Blunt vector (Zero Blunt PCR cloning kit; Invitrogen, Carlsbad, CA), and transformed into competent cells (TOP 10, Invitrogen). Clones containing dinucleotide repetitive elements were detected by hybridization to ^{32}P-labeled $(CA)_{15}$ or $(CT)_{15}$. Inserts in plasmid clones were cycle-sequenced using a ^{33}P-labeled dideoxynucleotide terminator kit (ThermoSequenase kit; USB, Cleveland, OH). Primer sets were designed to PCR-amplify across these dinucleotide repeats to develop novel genetic markers.

Newly developed microsatellite markers were mapped by genotyping DNA from a rat radiation hybrid panel (Watanabe *et al.,* 1999) (Research Genetics). The RHMAPPER program (Stein *et al.,* 1995), present on the Medical College of Wisconsin server (http://ares.ifrc-.mcw.edu/index.html), was used to analyze the marker-retention pattern for each clone and to determine the most likely map placement. A lod score >15 was selected as the criterion for linkage.

Sequence analysis of cDNAs of Cyp11b gene family. cDNA was synthesized from total adrenal RNA as previously described (Cai *et al.,* 1993). Total RNA (5 μg) was heat-denatured for 10 min at 68°C and incubated at 37°C for 1 h with 0.5 μg oligo$(dT)_{20}$, a 0.5 mM concentration of each dNTP, 50 mM Tris–HCl (pH 8.3), 10 mM $MgCl_2$, 20 units of placental ribonuclease inhibitor (RNAsin; Promega, Madison, WI), and 200 units of M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) in a 20-μl volume. cDNA was amplified with primers specific for portions of three different *Cyp11b* family members using a modified protocol of Cai *et al.* (1993) in a 25-μl reaction consisting of 20 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg/ml bovine serum albumin, a 250 μM concentration of each dNTP, 20 pmol of each primer, and 0.5 units *Taq* DNA polymerase (Promega). Following an initial denaturation of 94°C for 5 min, PCR was performed for 25 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 1 min, and primer extension at 72°C for 2 min. A final extension at 72°C for 5 min was included to ensure that reactions went to completion.

A 1724-bp portion of rat *Cyp11b1,* containing most of the coding region, was PCR-amplified from adrenal cDNA of S, R, and substrains 11bx4 and 11bx5 using a previously described primer set (Cicila *et al.,* 1993). A 1554-bp portion of rat *Cyp11b2* was PCR-amplified from adrenal cDNA of S, R, and substrains 11bx4 and 11bx5 using two previously described primer sets (Cover *et al.,* 1995). All but the first 18 nucleotides of the *Cypb2* coding region could be compared among the four strains. A 1510-bp portion of rat *Cyp11b3* was PCR-amplified from S and R rat adrenal cDNA using two previously described primer sets (Zhou *et al.,* 1995) as well as a newly designed primer set, 5′-AGAGAACTCCGTGGCCTGAG and 5′-AAGGTCTCTTTAAGGGCAGC. All but 47 nucleotides of the *Cyp11b3* coding region could be compared between S and R rats.

PCR products were electrophoretically size-fractionated on a 2.0% low-melt agarose gel, and bands were excised with a razor. Amplified bands were cycle-sequenced using a ^{33}P-labeled dideoxynucleotide terminator kit (ThermoSequenase kit, USB). All regions of *Cyp11b1* and *Cyp11b2* cDNA containing known nucleotide differences between Dahl S-rat and R-rat alleles (Matsukawa *et al.,* 1993; Cicila *et al.,* 1993; Cover *et al.,* 1995) were sequenced.

Statistical analysis. Analysis of variance (ANOVA) was calculated using the SPSS (Chicago, IL) and SuperANOVA (Abacus Concepts, Mountain View, CA) programs. The Tukey HSD post hoc test was used to evaluate the steroid ratio data for significant differences in mean values among the rat strains. Where significant differences in final body weight were observed between a congenic substrain and concomitantly raised, control S rats, the strong linear relationship between heart weight (HW) and body weight (BW) allowed regression analysis to remove the influence of body weight from heart weight.

RESULTS

The introgressed portion of R-rat chromosome 7 present in the original congenic strain, S.R-Cyp11b (Cicila *et al.,* 1997), contains a low-blood-pressure QTL allele as well as R-rat alleles for the *Cyp11b* gene family and many other genes. Substitution mapping was used to delimit the introgressed region of R-rat chromosome containing the gene(s) responsible for this blood pressure QTL. This was accomplished by constructing congenic substrains.

The congenic substrains constructed included four with lower blood pressures and heart weights than the parental S strain (11bx1, 11bx2, 11bx4, and 11bx5) and two congenic substrains (11bx3 and 11bx6) that were like S rats (Fig. 1 and Table 1). The overlap between substrains 11bx4 and 11bx5 defined a minimum region of 0.7 cM containing the blood pressure QTL (Fig. 1). Also note in Fig. 1 that the two strains without a blood pressure effect, 11bx6 and 11bx3, flank the QTL-containing region from either side. Substrain 11bx5 carried R-rat alleles for both *Cyp11b1* and *Cyp11b2,* as determined by the *D7Mco7* and *D7Wox19* markers,

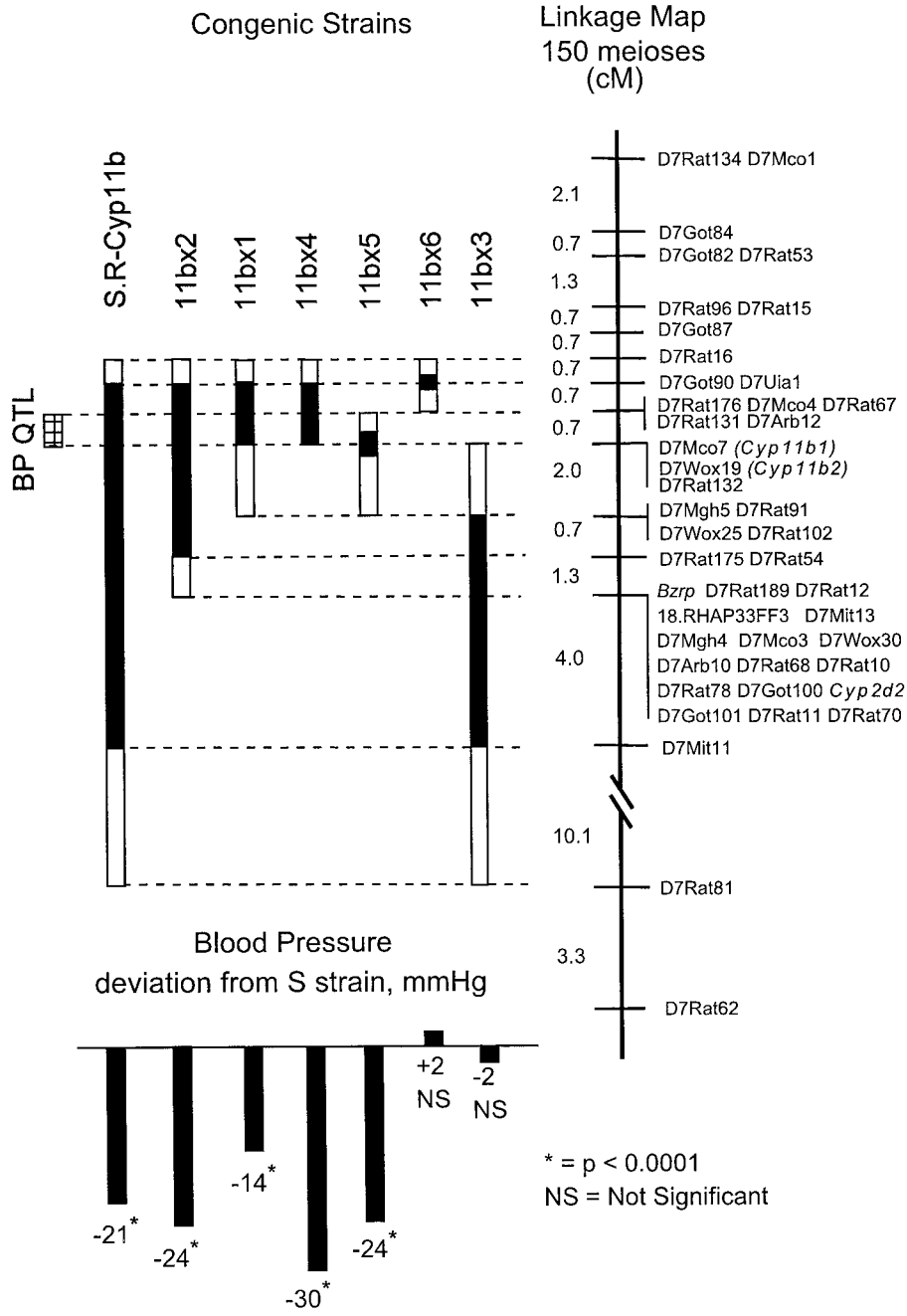

FIG. 1. Map of rat chromosome 7 showing locations of congenic segments. A linkage map of selected markers on rat chromosome 7 using an [F_1(S × R) × S] backcross population of 150 rats was drawn using the Map Manager QTX program (Manly and Olsen, 1999). Distances between loci are expressed in centimorgans and were corrected using the Kosambi function (Kosambi, 1944). The map is oriented with the centromere toward the top (Gauguier *et al.*, 1999). The known extent of the R-rat-derived portion of chromosome 7 carried by each congenic strain and substrain is designated by the filled portion of the bars to the left of the linkage map. Markers defining the ends of these filled bars and all markers spanned by the filled bars were homozygous for the R genotype. The open ends of the congenic bars designate intervals containing the recombinant end-points. The portion of donor strain chromosome carried by the initial congenic strain, S. R-Cyp11b, is shown by the left-most bar, with the portions carried by the six new congenic substrains (11bx2, 11bx1, 11bx4, 11bx5, 11bx6, and 11bx3) shown by adjacent bars. Each of the substrains 11bx5 and 11bx6 is defined by only a single marker location homozygous for the R genotype. These strains are depicted by a short filled bar encompassing the single R-derived marker location. The distal end of 11bx4 is defined in detail in the text. The lower part of this figure is a bar graph indicating in mm Hg the deviation of blood pressure of the original congenic strain and its substrains from concomitantly raised control S rats. A significant negative deviation indicates the presence of the R-rat blood pressure QTL allele in the congenic region. Data for the original congenic strain S. R-Cyp11b are from Cicila *et al.* (1997). The average blood pressure effect of the five strains that lowered blood pressure was −23 mm Hg. The minimal blood pressure QTL region is defined by the overlap between substrains 11bx4 and 11bx5, both of which retain the QTL allele lowering blood pressure.

TABLE 1

Comparison of the Chromosome 7 Congenic Substrains with the S Strain

	N	Heart weight (mg)	Blood pressure BP (mm Hg)	Body weight (g)
11bx1 strain	20	1109 ± 8.9	210.5 ± 2.8	292.1 ± 2.2
S strain	20	1221 ± 17.1	224.9 ± 4.8	298.2 ± 4.2
Difference[a]		−112	−14.4	−6.1
P value		<0.0001	0.014	0.21
11bx2 strain	20	1181 ± 15.3	219.0 ± 3.4	296.3 ± 3.0
S strain	20	1279 ± 16.1	243.4 ± 4.0	289.4 ± 2.0
Difference		−98	−24.4	+6.9
P value		<0.0001	<0.001	0.063
11bx3 strain	20	1229 ± 15.8	228.8 ± 2.4	302.4 ± 2.3
S strain	20	1253 ± 20.5	230.6 ± 4.7	297.1 ± 2.3
Difference		−24	−1.8	+5.3
P-value		0.36	0.74	0.12
11bx4 strain	20	1148 ± 10.0	205.0 ± 1.8	300.2 ± 2.6
S strain	20	1271 ± 28.4	235.1 ± 4.6	289.8 ± 3.2
Difference		−123	−30.1	+10.4
P value		<0.0001	<0.0001	0.016
11bx5 strain	20	1158 ± 14.2	209.0 ± 2.2	299.4 ± 2.8
S strain	20	1243 ± 14.8	232.7 ± 3.2	297.7 ± 2.5
Difference		−85	−23.7	+1.7
P value		<0.0001	<0.0001	0.65
11bx6 strain	19	1312 ± 14	223.7 ± 4.0	306.4 ± 1.7
S strain	19	1289 ± 18	221.8 ± 2.9	302.3 ± 3.5
Difference		+23	+1.9	+4.1
P value		0.33	0.71	0.30

Note. Values presented are the mean ± standard error of the mean.
[a] Difference = congenic-rat value minus S-rat value. Comparisons were made between matched sets of S and congenic substrain rats. Congenic substrains are defined in Fig. 1. Male S and congenic substrain rats were maintained on a low-salt diet to age 37 days and were then fed a 2% NaCl diet for 24 days prior to testing. Statistical significance was analyzed using a *t* test.

respectively. In contrast, substrain 11bx4 carried the R-rat allele for *Cyp11b1* and the S-rat allele for *Cyp11b2*, when genotyped with the above two markers.

Congenic substrains 11bx1, 11bx2, 11bx4, and 11bx5 had decreased heart weight, compared to the parental S strain (Table 1). This was interpreted to be a consequence of the decreased blood pressure observed in these four substrains. With the exception of substrain 11bx4, no significant differences in body weight were observed between the congenic substrains and the concomitantly raised control S rats (Table 1). Body weight-adjusted heart weights were also lower for substrain 11bx4 than for S rats (1140 mg vs 1253 mg, respectively; $P < 0.0001$). Thus, the lower blood pressure associated with substrain 11bx4 was corroborated by either the decreased heart weight or the decreased adjusted heart weight compared to the parental S strain.

Congenic Substrains and 18-OH-DOC Synthesis

Cyp11b1 catalyzes both the synthesis of corticosterone (compound B) by 11β-hydroxylation of DOC and the synthesis of 18-OH-DOC by 18-hydroxylation of DOC. The steroid ratio 18-OH-DOC/(18-OH-DOC + B) is a normalized measure of the *in vitro* capacity of adrenal homogenates to synthesize 18-OH-DOC and was determined for S and R rats, S.R-Cyp11b, and five congenic substrains derived from S.R-Cyp11b. The steroid ratio divided these eight strains into three distinct phenotypic groups: (1) R rats, S.R-11b, and substrains 11bx1, 11bx2, and 11bx5 with low mean steroid ratios of between 0.13 and 0.14; (2) substrain 11bx4 with a slightly higher mean steroid ratio, 0.16, than the group 1 strains; and (3) S rats and substrain 11bx6 with a higher mean steroid ratio of 0.38 (Fig. 2). While the steroid ratios of group 1 and 11bx4 were statistically different, Fig. 2 shows that substrain 11bx4 obviously has a steroid ratio similar to that of group 1 strains, which carry the R-rat *Cyp11b1* allele, as defined by the *D7Mco7* marker.

Sequencing Cyp11b Gene Family cDNAs

The above congenic mapping and steroid ratio results suggested that a crossover occurred in the construction of congenic substrain 11bx4, in the region containing the *Cyp11b1* and *Cyp11b2* loci. The coding regions of the S and R rats are expected to contain five strain-specific nucleotide differences in *Cyp11b1* (Matsukawa *et al.*, 1993; Cicila *et al.*, 1993) and seven such

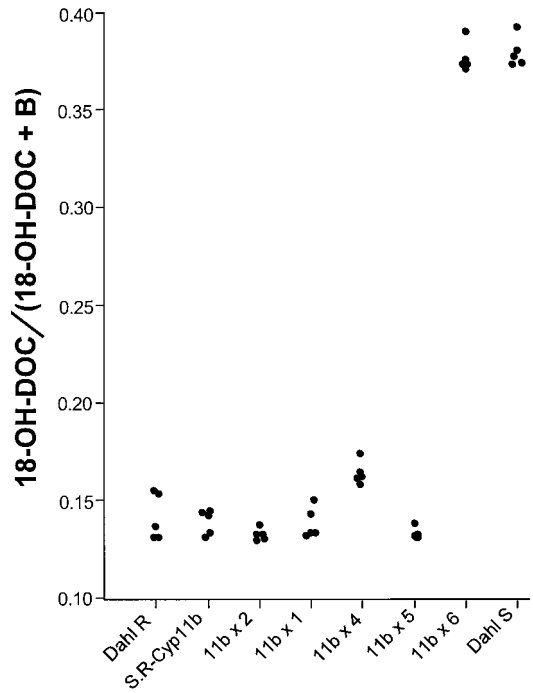

FIG. 2. Scatterplot of the steroid ratio of 18-OH-DOC/ (18-0H-DOC + B) for rat adrenal incubates. Each point denotes the steroid ratio of a single rat. Note that S and R rats have distinct steroid ratios, and with the exception of substrain 11bx4, the original congenic strain (S. R-Cyp11b) and its substrains had mean steroid ratios identical to either the S (i.e., 11bx6) or the R (i.e., S. R-Cyp11b, 11bx1, 11bx2, 11bx5) strains. Substrain 11bx4 was unique because it contains a chimeric 11β-hydroxylase gene (see text). B is compound B, i.e., corticosterone.

TABLE 2

Nucleotide and Amino Acid Differences in the Cyp11b1 (11-Hydroxylase) Genes of S, R, and Congenic Substrains 11bx4 and 11bx5

Mutation	1	2	3	4	5
Location					
Exon	2	6	7	7	8
Nucleotide	379	1052	1141	1150	1327
Residue	127	351	381	384	443
S rat					
Codon	**C**GT	G**T**T	**G**TA	**A**TC	**G**TG
Amino acid	Arg	Val	Val	Ile	Val
11bx4					
Codon	**C**GT	G**C**T	**T**TA	**C**TC	**A**TG
Amino acid	Arg	Ala	Leu	Leu	Met
11bx5					
Codon	**T**GT	G**C**T	**T**TA	**C**TC	**A**TG
Amino acid	Cys	Ala	Leu	Leu	Met
R rat					
Codon	**T**GT	G**C**T	**T**TA	**C**TC	**A**TG
Amino acid	Cys	Ala	Leu	Leu	Met

Note. Shown are the sequences for the five codons in the *Cyp11b1* gene of S, R, 11bx4, and 11bx5 rats that were previously shown to differ between Dahl S and R rats (Matsukawa *et al.*, 1993; Cicila *et al.*, 1993). The codon and deduced amino acid residue are shown for the four strains. Nucleotide and amino acid locations are numbered as in Nonaka *et al.* (1989). The boxes enclose the components of the chimeric 11bx4 allele in common with the S or R alleles. The variant bases are shown in boldface type.

differences in *Cyp11b2* (Cover *et al.*, 1995). Sequencing of cDNAs from *Cyp11b1* (Table 2) and *Cyp11b2* (Table 3) showed that substrain 11bx5 carried R-rat alleles for both *Cyp11b1* and *Cyp11b2* and that substrain 11bx4 carried a chimeric *Cyp11b1* allele and an S-rat allele for *Cyp11b2*. For the five known nucleotide differences in the *Cyp11b1* coding region between S and R rats, substrain 11bx4 carried a *Cyp11b1* allele encoding a C at nucleotide 379 in exon 2, consistent with an S-rat allele, but also carried four nucleotide differences in exons 6, 7, and 8 consistent with an R-rat allele (Table 2). This chimeric sequence indicates that the crossover that occurred in forming substrain 11bx4 was between exons 2 and 6 of *Cyp11b1*; this accurately defines the distal limit of the chromosomal region containing the blood pressure QTL. The sequences of Dahl rat *Cyp11b2* cDNAs were as described by Cover *et al.* (1995), except that no variant was seen at amino acid residue 191 (Table 3). Sequencing of *Cyp11b3* cDNA revealed no nucleotide differences between inbred Dahl S and R rats.

Fine-Mapping of the Chromosomal Region Containing the Blood Pressure QTL

Having established that one end-point of the congenic region in substrain 11bx4 was within the *Cyp11b1* gene, we developed a physical map of the region and identified additional genetic markers better defining the extent of the QTL interval. We identified 10 YAC clones containing both *D7Rat131* and *D7Mco7* (*Cyp11b1*), which are the markers defining the QTL interval in Fig. 1. The smallest YAC clone, 152G06, was approximately 220 kb, suggesting that the QTL-containing region is no larger than this. Ten novel microsatellite markers were developed from YAC clone 152G06, 9 of which were polymorphic between Dahl S and R rats (Table 4). Radiation hybrid mapping placed these markers on rat chromosome 7 near the blood pressure QTL region (Fig. 3). Two markers, *D7Mco8* (both H and L forms) and *D7Mco9*, mapped between the QTL-delimiting markers *D7Rat131* and *D7Mco7* (*Cypb11b1*) (Fig. 3). Figure 3 also shows an improved linkage map of the QTL-containing region based on 1286 meioses derived from an F_2[(substrain 11bx4) × S] population of 643 rats. The YAC-derived marker *D7Mco8H* essentially bisects the QTL-containing interval and defines the chromosomal region containing the crossover at the centromeric end of substrain 11bx5 to a 0.31-cM region (Fig. 3). The other 7 polymorphic markers developed from the YAC (D7Mco10, 11, 12, 13, 14, 15, and 17) are shown on the radiation hybrid map in Fig. 3, but are omitted from the linkage map for clarity. These markers all clustered on the linkage map with *D7Rat131*.

The QTL-containing interval is defined by the over-

TABLE 3

Nucleotide and Amino Acid Differences in the Cyp11b2 (Aldosterone Synthase) Genes of S, R, and Congenic Substrains 11bx4 and 11bx5

Mutation	1	2	3	4	5	6	7
Location							
Exon	1	3	3	3	4	4	6
Nucleotide	75	405	408	573	750	752	978
Residue	25	135	136	191	250	251	326
S rat							
Codon	GG**C**	GC**T**	GA**A**	AG**T**	AC**C**	C**A**G	AC**A**
Amino acid	Gly	Ala	**Glu**	Ser	Thr	**Gln**	Thr
11bx4							
Codon	GG**C**	GC**T**	GA**A**	AG**T**	AC**C**	C**A**G	AC**A**
Amino acid	Gly	Ala	**Glu**	Ser	Thr	**Gln**	Thr
11bx5							
Codon	GG**T**	GC**A**	GA**C**	AG**T**[a]	AC**T**	C**G**C	AC**C**
Amino acid	Gly	Ala	**Asp**	Ser	Thr	**Arg**	Thr
R rat							
Codon	GG**T**	GC**A**	GA**C**	AG**T**[a]	AC**T**	C**G**C	AC**C**
Amino acid	Gly	Ala	**Asp**	Ser	Thr	**Arg**	Thr

Note. Shown are the sequences for seven codons in the *Cyp11b2* gene of S, R, 11bx4, and 11bx5 rats that were previously found to differ between Dahl S and R rats (Cover *et al.*, 1995). The codon and deduced amino acid residue are shown for the four strains, with the variable nucleotides and amino acids in boldface type. The boxes enclose the codons that are identical among the strains. Nucleotide and amino acid locations are numbered as in Cover *et al.* (1995), beginning with the initiating methionine codon and amino acid residue.

[a] R-rat allele sequence for mutation 4 was AGC in Cover *et al.* (1995), but both AGC codons, or AGT as found here, encode serine.

TABLE 4

New Rat Microsatellite Markers for Rat Chromosome 7 Developed from YAC 152G06

Marker	GenBank Accession No.	Size[a] (bp)	Primers	Polymorphism
D7Mco8[b]	AF271630	205	ACCGACCAAAGGCTGTAGAGAC TTGACTGTGCTGCCTTTAGAGG	AS>MNS=SHR>S=LEW=WKY>BN>R (H) R>S=LEW=WKY=AS=MNS=SHR>BN (L)
D7Mco9	AF271631	181	CCATCTCTATCATAAATACGCTGCT GGCAGTAAAATCAGACTCAGCACA	S=LEW=WKY=AS=MNS=BN=SHR>R
D7Mco10	AF271622	330	AAAGCCTGGACACACGAGAG TGTATATCTCTGGGTGCTGTGG	LEW=WKY=SHR>BN>S=AS=MNS>R
D7Mco11	AF271623	222	GAGGCCATGGGATTCCATTC CTGTTCCACCAAGGTCGCTG	BN>R>S=LEW=WKY=MNS=AS=SHR
D7Mco12	AF271624	205	CAAGTGCCAGCTGAGCTATG TCACACATTTATGCTCACACCTAC	R>MNS=SHR>S=LEW=WKY=AS>BN
D7Mco13	AF271625	292	GCCCTTTGACTTCCACACACG ATGGCTCGTCTCATTGTCTCCC	S=LEW=WKY=AS=MNS=BN=SHR>R
D7Mco14	AF271626	283	GGGCAATCCAGGGATCTCAG GAACCCGTCTGGGCATCTTC	LEW=R>S=WKY=AS=MNS=BN=SHR
D7Mco15	AF271627	358	TGAGTGGTTCCGAAGGTGGGAG TCCAGATGTCCAGCAGCTGTGC	S=LEW=WKY=AS=MNS=SHR>R=BN
D7Mco16	AF271628	215	CCTGGATCTTCCTATTGCATGG AAGCACCTCTCGTTGTTTGGAG	S=LEW=WKY=R=AS=MNS=SHR=BN
D7Mco17	AF271629	267	AGCTGCATAATAGCATCTTGCC CCAGCTCCGTGATGGATATAAG	BN>R>S=LEW=WKY=MNS=AS=SHR

Note. Strain polymorphism column shows the relative sizes of PCR products for the eight rat strains: S, Dahl salt-sensitive; R, salt-resistant; AS, albino surgery; BN, Brown Norway; MNS, Milan normotensive strain; WKY, Wistar–Kyoto; SHR, spontaneously hypertensive rat; LEW, Lewis.

[a] PCR product sizes given are from sequencing data obtained from clones derived from YAC clone 152G06 DNA.

[b] *D7Mco8* amplifies two PCR products of different sizes, a higher (H) and a lower (L) migrating form. While these two PCR products differ in their strain distributions of polymorphisms, both map to the same chromosomal location on the radiation hybrid map. Subsequent sequencing showed that the two PCR products arise from a tandemly duplicated region containing the (duplicated) microsatellite (Garrett and Rapp, unpublished results).

lap of substrains 11bx4 and 11bx5. This overlap is now seen in Fig. 3 to be at most 0.54 cM. The actual size of the overlap depends on position of the crossover point of the centromeric end of substrain 11bx5 in the interval between *D7Mco9* and *D7Mco8H*.

DISCUSSION

Genome scans have proven extremely useful for determining the rough chromosomal location of QTLs in segregating rodent populations. However, the imprecision with which genome scans localize QTLs presents difficulties in further studying the gene(s) responsible for the phenotypic effects. Theoretical studies suggest that QTLs can be mapped with confidence intervals of only about 10–35 cM (Darvasi *et al.*, 1993; Hyne *et al.*, 1995; van Ooijen, 1992) using standard genetic crosses. Such large chromosomal regions are likely to contain thousands of different genes, several of which would undoubtedly be considered candidate genes for the QTL. Congenic strains offer a potentially much more precise QTL localization, establishing definitive limits for a QTL-containing region. These limits can be narrowed further by constructing substrains retaining the QTL within progressively smaller amounts of donor chromosome (Rapp and Deng, 1995). This strategy has proven effective in confirming and localizing blood pressure QTL locations in hypertensive rat models (reviewed in Rapp, 2000).

In this study we showed how additional substrains, containing progressively smaller regions of introgressed donor chromosome, narrowed the chromosomal region known to contain the QTL. Figure 1 shows how substrains developed from S.R-Cyp11b localized the blood pressure QTL to a 0.7-cM region defined by the overlap of substrains 11bx4 and 11bx5. These substrains both had significantly lower blood pressure and cardiac mass than the parental S strain. The simplest interpretation of these data is that the gene(s) responsible for the blood pressure QTL lies in the region of R-derived chromosome 7 common to these two substrains. Both substrains had the low 18-OH-DOC/(18-OH-DOC + B) ratio characteristic of the R-rat *Cyp11b1* allele. The presence of *Cyp11b1* in the overlap between the congenic regions of 11bx4 and 11bx5 confirms this gene as a strong candidate to explain the chromosome 7 blood pressure QTL, as suggested by previous, less precise genetic studies (Rapp and Dahl, 1972a, 1976; Cicila *et al.*, 1993, 1997). The critical QTL-containing region was further localized to an interval of less than 0.54 cM using new YAC-derived genetic markers and a higher resolution linkage map (Fig. 3).

Another use of congenic strains is to eliminate candidate loci from further consideration. Substrain 11bx4 carried the S-rat allele at *Cyp11b2* (aldosterone synthase), but still retained a strong blood pressure effect.

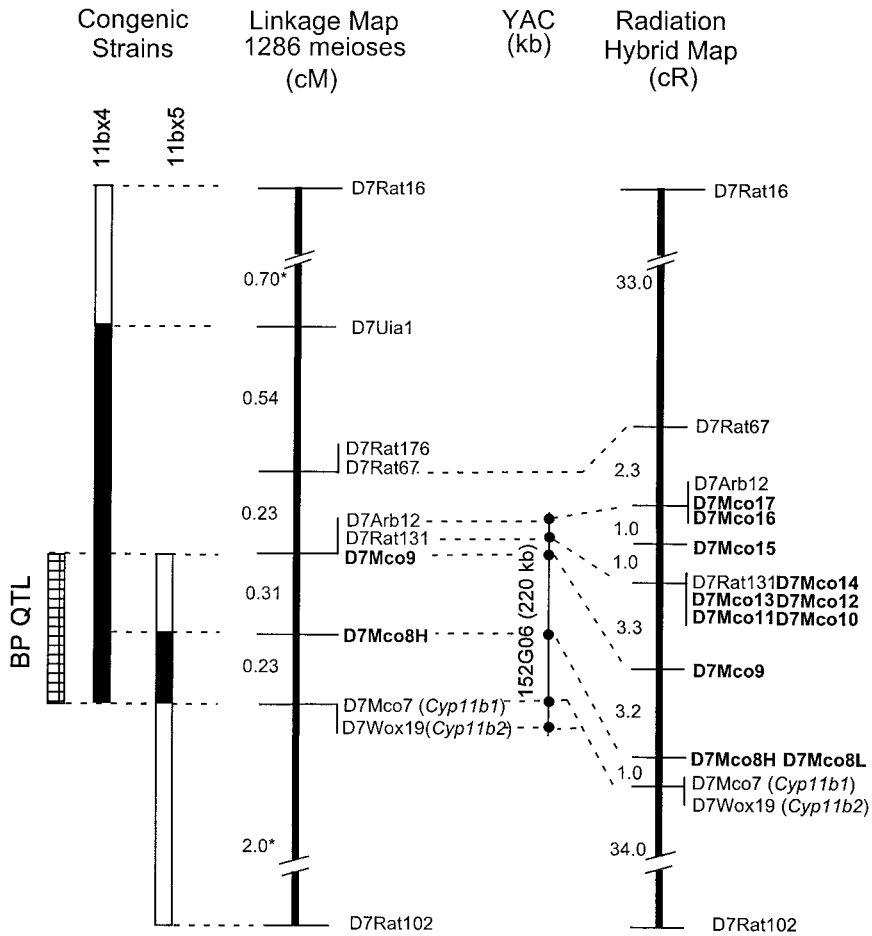

FIG. 3. Maps of the critical part of rat chromosome 7 showing improved resolution of the blood pressure QTL-containing region. The left side shows a linkage map based on an intercross population of 643 rats (1286 meioses) derived from substrain 11bx4 and S rats. Distances are in centimorgans. The map is oriented with the centromeric end toward the top (Gauguier *et al.*, 1999). The position of YAC clone 152G06 is shown relative to the linkage map. The overlapping congenic substrains 11bx4 and 11bx5 that define the QTL-containing region are also presented relative to the new, higher resolution linkage map. The extent of the R-rat-derived portion of chromosome 7 carried by each congenic substrain is designated by the filled portion of the bars to the left of the linkage map. Markers defining the ends of the filled bars and all markers spanned by the filled bars were homozygous for the R genotype. The open ends of the congenic bars designate intervals containing the recombinant end-points. The right side shows a radiation hybrid (RH) map of a portion of rat chromosome 7 drawn using the RHMapper program (Stein *et al.*, 1995) that gives the positions of framework markers and microsatellite markers derived from YAC clone 152G06 (shown in boldface type). Distances in the RH map are expressed in centirays. Markers present in both the linkage and the RH maps are joined with a dashed line. *Indicates linkage distances from Fig. 1.

This eliminated *Cyp11b2* as a candidate locus. We were unable to find strain-specific differences in the locus *Cyp11b3*, which would have been useful in placing it in or out of the QTL region. *Cyp11b3* can, however, be logically eliminated as a candidate based on its deduced position relative to *Cyp11b1* and *Cyp11b2*. Nomura *et al.* (1993) showed that *Cyp11b2* and *Cyp11b3* were in close physical proximity, and it is clear from their work that *Cyp11b1* is not between *Cyp11b2* and *Cyp11b3*. Thus the most logical order of these loci is shown in Fig. 4, which reasonably eliminates *Cyp11b3* as a candidate. Preliminary sequencing of this region (Garrett and Rapp, unpublished results) is compatible with Fig. 4. Other examples where congenic strains have been used to eliminate candidate loci are *Nos2* (Dukhanina *et al.*, 1997) on chromosome 10, β and γ subunits of the epithelial sodium channel and the S_A gene (Saad *et al.*, 1999) on chromosome 1, and renin (DiPaola *et al.*, 1997; Zhang *et al.*, 1997) on chromosome 13.

The crossover occurring within the *Cyp11b1* gene of substrain 11bx4 produced a chimeric gene containing an exon 2 S-rat sequence and exon 6, 7, and 8 R-rat sequences (Table 2, Fig. 4). The resulting chimeric 11β-hydroxylase protein had an altered capacity to synthesize 18-OH-DOC *in vitro* compared to Dahl S and R rats, resulting in an 18-OH-DOC/(18-OH-DOC+B) steroid ratio that was slightly, but significantly, higher than that of R rats (Fig. 2). These data are consistent with the enzymatic activity of artificially constructed chimeric S and R *Cyp11b1* alleles studied *in vitro*. Matsukawa *et al.* (1993) and Nonaka *et al.* (1998) showed that the main determinants of the steroid ratio characteristic of Dahl S and R rats are differences at amino acid residues 381 and 384 of *Cyp11b1*. Substitutions at both positions are required

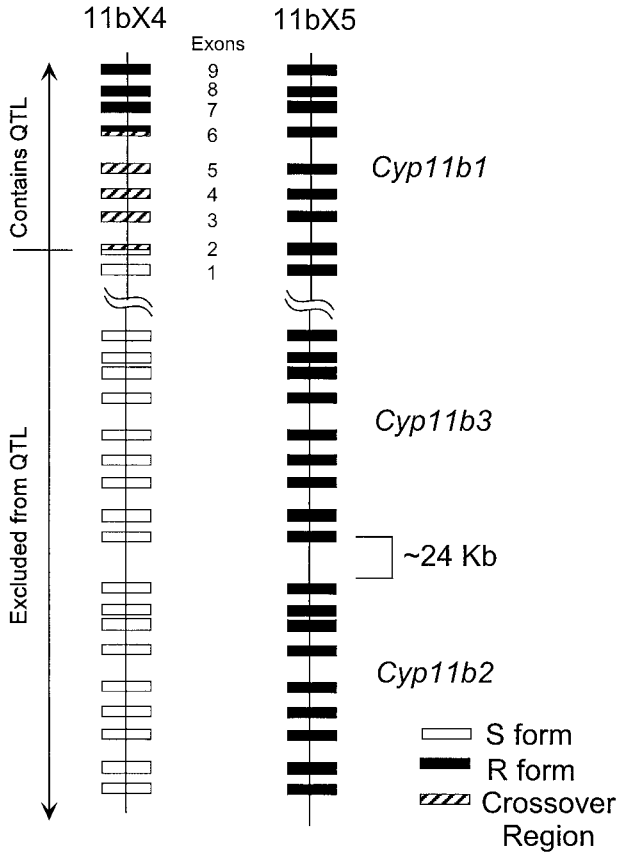

FIG. 4. Diagram showing the deduced order of Cyp11b loci on rat chromosome 7. This diagram (not to scale) shows the most likely spatial relationship of the *Cyp11b1*, *Cyp11b2*, and *Cyp11b3* loci and describes the alleles at these loci carried in substrains 11bx4 and 11bx5. The three loci have similar exon–intron structure. The orientation and distance between *Cyp11b2* and *Cyp11b3* are from Nomura *et al*. (1993). The centromere is at the top and the q-terminus is at the bottom. This figure demonstrates how *Cyp11b2* and *Cyp11b3* are eliminated as candidate genes for the blood pressure QTL in the Dahl S and R model based on how the end of the substrain 11bx4 congenic region was defined by a crossover within *Cyp11b1* and the likely orientation of the three *Cyp11b* loci. While it is possible that the orientation of the segment containing *Cyp11b2* and *Cyp11b3* could be reversed based only on published work, the orientation shown is compatible with our preliminary sequencing of this region (Garrett and Rapp, unpublished results).

for full expression of the S and R phenotypes (Nonaka *et al.*, 1998). This is compatible with the chimeric *Cyp11b1* gene in substrain 11bx4 having R-rat amino acids at positions 381 and 384 and a steroid ratio essentially similar to that of R rats. The small difference in the 18-OH-DOC/(18-OH-DOC + B) ratio seen in 11bx4 rats is almost certainly due to substitution of the amino acid at position 127 in the chimeric *Cyp11b1* gene. Nonaka and co-workers (1998) also found a small effect on the steroid ratio due to this amino acid substitution in their *in vitro* expression study with artificial chimeric *Cyp11b1* genes.

There is a discrepancy between the size of the QTL-containing region of 0.54 cM on the linkage map between *D7Rat131* and *D7Mco7* (*Cyp11b1*) and the approximately 220-kb YAC insert that spans this same region. The rat genome is about 1895 cM in size, which is the average of three estimates of 1938 cM (Garrett *et al.*, 1998), 1998 cM (Bihoreau *et al.*, 1997), and 1749 cM (Brown *et al.*, 1998). Since genomes of eutherian mammals are approximately 3×10^9 bp (Silver, 1995) in length, 1 cM in the rat is about $3 \times 10^9/1895 = 1.58 \times 10^6$ bp. Thus, a 0.54-cM chromosomal interval is expected to be equal to 853 kb. However, the 0.54-cM region between *D7Rat131* and *D7Mco7* (*Cyp11b1*) is $\leq$220 kb, suggesting that this chromosome 7 region may be a recombination "hot-spot," with a crossover rate three to four times that of the rat genome as a whole.

In summary, substitution mapping localized a blood pressure QTL to a small region of rat chromosome 7 ($\leq$220 kb). This chromosomal interval was defined by the overlap between two congenic substrains, 11bx4 and 11bx5. *Cyp11b1* (11β-hydroxylase) was contained in this interval and remained a strong candidate locus to account for the QTL. *Cyp11b2* (aldosterone synthase) was unequivocally outside this interval and was eliminated as a candidate. *Cyp11b3* was also eliminated based on its deduced position relative to *Cyp11b1* and *Cyp11b2*.

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