Quantitative Trait Loci Influencing Hepatic Copper in Rats

I.D. DE WOLF,*[,1] A.C.M. BONNÉ,* X.M. FIELMICH-BOUMAN,* B.A. VAN OOST,†'†'§ A.C. BEYNEN,*'¶ L.F.M. VAN ZUTPHEN,* AND H.A. VAN LITH*

*Department of Laboratory Animal Science, †Department of Clinical Sciences of Companion Animals, †Department of Equine Sciences, §Department of Farm Animal Health, and ¶Department of Nutrition, Faculty of Veterinary Medicine, Graduate School of Animal Health, Utrecht University, P.O. Box 80.166, NL-3508 TD Utrecht, The Netherlands

Significant differences in liver copper content have been observed between rat inbred strains. To define loci controlling this trait, the offspring (n = 190) from an (LEW/OlaHsd × BC/CpbU) F_2-intercross was genetically analyzed. From each F_2 animal, liver copper content was determined and genomic DNA was screened with polymorphic DNA markers. We found a major quantitative trait locus (QTL) for liver copper content in females on chromosome 2 and in males on chromosome 10. Both QTLs accounted for approximately 20% of the genetic variance. In addition, suggestive linkage for liver copper content was found on rat chromosomes 1, 8, 10, 12, 14, and 19. The regions on these chromosomes contain genes that are responsible for 9.0–15.5% of the genetic variance of liver copper content.

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Strain differences in rat liver copper content have been described by Yu *et al.* (1), Hayashi *et al.* (2), Schilsky *et al.* (3), and De Wolf *et al.* (4). Previously, we have searched for the genetic components associated with liver copper content using a set of recombinant rat inbred strains derived from SHR/OlaIpcv and BN-*Lx*/Cub (4) since no QTL analyses, except for the Long-Evans Cinnamon (LEC) mutant rat, have been carried out with strains that differ in liver copper content. It was suggested that at least two regions, one on chromosome 10 and one on chromosome 16, are associated with liver copper content in male rats (4). However, the limited power of recombinant inbred strains for detecting QTLs prompted us to perform a total genome scan of an F_2 population to search for additional genetic factors controlling liver copper content. Furthermore, the previous study in which we used recombinant inbred strains included male rats only. In this study, we included both male and female rats to study whether gender-related differences in QTLs for liver copper concentration or liver copper content exist. The F_2 intercross from the LEW/OlaHsd and BC/CpbU inbred strains, previously used for testing the genetic basis of the differences in susceptibility for cholesterol, was available for this study.

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[1] To whom reprints requests and correspondence should be addressed at Department of Laboratory Animal Science, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 2, P.O. Box 80.166, NL-3508 TD Utrecht, The Netherlands. E-mail: wolfid@wish.net

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Materials and Methods

The research project was approved by the Animal Experimentation Committee of the Utrecht Faculty of Veterinary Medicine.

Animals and Housing. All animals were kept under SPF conditions and a 12 hr per day light regimen (0700–1900). The other laboratory conditions, temperature and humidity, were also controlled. From four males and four females of the parental strains LEW/OlaHsd ("LEW," obtained from Harlan, U.K.) and BC/CpbU ("BC," obtained from the Central Laboratory Animal Institute of the Utrecht University, The Netherlands), the liver copper content was determined as described in the experimental protocol (see below). The F_1 generation consisted of 17 males and 15 females and was derived by reciprocal matings of LEW and BC animals. The F_1 hybrids were intercrossed, producing F_2 progeny. From 90 F_2 males and 100 F_2 females, the liver copper content was determined.

Experimental Protocol. The animals were housed as pairs or as groups of three animals in wire-topped Macrolon type III cages with a layer of sawdust as bedding. The rats had free access to food and tap water. After weaning up to an age of 7 weeks, the animals were fed a commercial, pelleted diet (RMH-B®, Hope Farms BV, Woerden, The Netherlands). The chemical composition of this commercial diet has previously been described (5). The rats then received a commercial diet supplemented with 5.0% (w/w)

olive oil and 2.0% (w/w) cholesterol. This diet had been fed for 4 weeks. In a previous experiment, we found that this diet did not influence the strain difference in liver copper content.[2]

At the age of 11 weeks, the body weight of the rats was determined. The animals were anesthetized with diethyl ether and exsanguinated via aorta punction, and the livers and spleens were removed and weighed. For each animal, two pieces of liver (0.5 g) were immediately frozen. The spleen was used for DNA isolation.

Chemical Analyses. Copper in the liver was determined by drying the pieces of the liver overnight at 105°C, after which the dry weights were measured. Subsequently, the samples were ashed at 200°C for 1 hr, 300°C for 2 hr, 400°C for 3 hr, and 500°C for 10 hr. The remaining ash was dissolved in 1 ml of concentrated $HClO_4$, which was then evaporated at 225°C. This step was repeated until the ash was completely white. The ash was then dissolved in 1 ml of 6 *M* HCl. Copper was measured by using flame atomic absorption spectrophotometry on a Varian-AA275 (Varian, Springville, Australia). The copper concentration of the two liver pieces was averaged. Previously, it has been described that the analysis of a portion of rat liver can be representative of the elemental composition of the whole organ (6).

Genome Scan. DNA of each F_2 animal and the (grand-) parents was extracted from the spleen using a standard procedure. A total of 256 microsatellite (SSLP) markers, one RFLP (for the Lcat gene, coding for lecithin cholesterol acyl transferase) and a 17-bp repeat (detected in the Mvpd gene, coding for mevalonate pyrophosphate decarboxylase), polymorphic between the LEW and BC strain, were used for screening of the F_2 progeny.[3] These markers were dispersed throughout the rat genome. Primers flanking the rat microsatellite markers were obtained from Research Genetics Inc. (MapPairs™, Huntsville, AL). The primers for the Lcat gene and those flanking the 17-bp repeat (Mvpd gene) were synthesized by Amersham Biosciences (Buckinghamshire, U.K.). When polymorphisms between BC and LEW differed less than 10 bp, the forward primer was 5′ end-labeled with [γ-^{32}P]dATP (Amersham Biosciences, 10 μCi/μl). Twenty nanograms of genomic DNA was used in the PCR mixture. After the PCR reaction, the products were heated for 5 min at 95°C and then separated on a standard 5% (w/v) polyacrylamide gel. If the polymorphism of the microsatellites between BC and LEW differed more than 10 bp, then 100 ng genomic DNA was used for the PCR reaction. The non-radioactive PCR products were separated on a 3% (w/v) agarose gel and visualized by ethidium bromide staining (0.5 μg/ml PCR products). The PCR reaction was performed under standard conditions.

All genotype scoring was done independently by two persons. After one turn of genotype scoring, all differences between the independent reads were checked. All indications of a double-recombinant event were re-scored and, if necessary, re-typed.

Map Construction. The segregation ratio for the individual markers was checked by means of the Chi-squared goodness-of-fit test. None of these markers had a significant segregation distortion. The genetic map distance for the markers was computed with the computer package JoinMap™, version 3.0 (7). For the establishment of linkage groups we used a critical minimal Lod score of 3.0. For calculation of map distances and estimating most likely gene orders, we used a critical Lod score of 0.05. Recombination frequencies were converted to map distances in centiMorgans with the Kosambi function. The generated map spans a sex-averaged genetic length of 1790 cM with an average spacing of consecutive markers of 7.7 cM. The largest gap is 26.1 cM (on the X chromosome). It was estimated that this genetic map is linked to about 90% of the DNA in the rat genome[3].

Statistical and QTL Analyses. Both for the parental strains and for the F_2 intercross rats, all statistical analyses were carried out according to Petrie and Watson (8) using a SPSS PC$^+$ computer program (9). The probability of a Type I error < 0.05 was taken as criterion for significance.

Parental Strains. The Kolmogorov–Smirnov one-sample test was used to check normality of the measured phenotypic characteristics of the BC and LEW rats. The significance of the differences between groups was calculated by a two-way analysis of variance (ANOVA) with strain and gender as main factors. Homogeneity of the variances was tested using Bartlett's test. When necessary, the variances were equalized by logarithmic transformation of the data (8). After transformation the variances were similar and the transformed within-group data were still normally distributed. Thus, application of an analysis of variance on the (transformed) data is then straightforward.

F_2 Animals. The location of the QTLs affecting the measured quantitative traits and the variance explained by each locus was determined using the MapQTL computer package (10). The interval-mapping module was used (11). Results were expressed as Lod scores. Lod score thresholds to achieve the genome-wide significance levels of 5% (significant linkage) were, as proposed by Lander and Kruglyak (12), 4.3 when no model of gene action was specified ("free" genetics), 3.4 when gene action was restricted to recessive or dominant models, and 3.3 when gene action was restricted to an additive model. For suggestive linkage, Lod score values were 2.8 ("free" genetics), 2.0 (recessive or dominant), and 1.9 (additive), respectively.

For selected microsatellite markers, comparison of the

[2] De Wolf ID, Fielmich-Bouman XM, Lankhorst Æ, van Oost BA, Beynen AC, Křen V, Pravenec M, van Zutphen LFM, van Lith HA. Liver copper content of rats hypo- or hyperresponsive to dietary cholesterol. 2002. (Submitted)

[3] Bonné ACM, den Bieman MGCW, Gillissen GF, van Zutphen LFM, van Lith HA. A rat linkage map based on BCxLEW intercross. Folia Biologica (Praha), 47:119–122, 2002.

Table I. Body Weight, Liver Weight, and Liver Copper Content of BC/CpbU and LEW/OlaHsd Rats[a]

Measure	BC/CpbU		LEW/OlaHsd		Sig[b]
	Males ($n = 4$)	Females ($n = 4$)	Males ($n = 4$)	Females ($n = 4$)	
Final body weight (g)	220 ± 21	177 ± 5	352 ± 34	224 ± 12	S,G,SxG
Liver wet weight					
Absolute (g)	10.0 ± 1.2	9.3 ± 0.4	13.4 ± 1.8	8.1 ± 0.4	G,SxG
Relative (g/kg body wt)	45.2 ± 2.6	52.2 ± 1.3	38.0 ± 1.8	36.3 ± 0.7	S,G,SxG
Liver copper concentration					
(μg/g wet wt)	8.7 ± 2.0	8.1 ± 2.4	6.3 ± 0.4	7.9 ± 2.0	—
(μg/g dry wt)	18.9 ± 3.6	18.7 ± 5.7	15.2 ± 1.2	18.0 ± 4.5	—
Liver copper store					
(μg/whole liver)	85.3 ± 10.0	75.2 ± 23.5	83.3 ± 6.3	64.0 ± 14.4	—
(μg/100 g body wt)	39.3 ± 8.0	42.4 ± 13.0	23.7 ± 0.7	28.8 ± 7.8	S[c]

[a] Values are means ± SD; n is the number of animals per group. All results within groups were normally distributed (Kolmogorov–Smirnov one-sample test, $P > 0.05$).
[b] Significance ($P < 0.05$) based on two-way ANOVA with main factors strain and gender. S, effect of strain; G, effect of gender; SxG, interaction.
[c] ANOVA after logarithmic transformation of the data.

liver copper traits of the F_2 animals after grouping by genotype of a microsatellite marker was performed. If a microsatellite marker and the trait of interest are segregating independently, then the values of the trait will be equally distributed among the homozygote and heterozygote genotypes. This type of analysis was only done for the markers flanking a significant QTL and for the gender in which the significant QTL was detected. These analyses were restricted to the traits that showed a significant association with these markers in the interval mapping approach. The Kolmogorov–Smirnov one-sample test was used to check normality of these data. The co-segregation of phenotypes with alleles at (selected) marker loci was evaluated by comparing the values between different genotypes by one-way ANOVA.

Results

Parental Strains. At the end of the test period, the BC and LEW rats were of the same age, but LEW rats had a higher body weight than BC rats. The strain effect on body

Table II. Summary of QTLs for Hepatic Copper Content in Rats[a]

Phenotypic trait	Chromosome	Gender	Model[b]	Peak Lod[c]	Location[d]	% Variance[e]
Liver copper concentration						
(μg/g wet wt)	2	Females	Recessive	*2.44*	D2Rat234 (41.8 cM)	10.6
	10	Males	Dominant	*2.37*	D10Rat45 (84.0 cM)	11.4
(μg/g dry wt)	1	Females	Dominant	*2.23*	D1Rat185-D1Rat29 (91.9 cM)	10.3
	2	Females	Recessive	**3.87**	D2Rat185-D2Rat241 (19.2 cM)	21.2
	10	Males	Recessive	**4.15**	D10Rat27-D10Rat98 (37.3 cM)	20.3
	14	Males	Dominant	*2.59*	D14Rat1 (75.6 cM)	12.5
	19	Females	Additive	*2.36*	D19Rat98 (51.6 cM)	10.3
Liver copper store						
(μg/whole liver)	1	Males	Dominant	*2.02*	D1Rat185 (90.4 cM)	9.9
	8	Males	Recessive	*2.67*	D8Rat156 (74.4 cM)	13.3
	10	Males	Dominant	*2.30*	D10Rat27-D10Rat98 (38.3 cM)	11.3
	12	Males	Dominant	*2.59*	D12Rat2-D12Rat56 (55.8 cM)	15.5
(μg/whole liver/100 g body weight)	1	Females	Additive	*1.97*	D1Rat196-D1Rat19 (116.7 cM)	9.0
	2	Females	Recessive	*2.48*	D2Rat234 (41.8 cM)	10.9
	8	Males	Dominant	*2.19*	D8Rat71-D8Rat6 (0.5 cM)	10.6
	8	Males	Recessive	*2.35*	D8Rat156 (74.4 cM)	12.1
	12	Males	Dominant	*2.30*	D12Rat2-D12Rat56 (56.8 cM)	14.3
	14	Males	Dominant	*2.59*	D14Rat1 (75.6 cM)	12.7

[a] QTLs were investigated using the MapQTL software on data collected from 190 (LEW/OlaHsd x BC/CpbU) F_2 intercross rats. Within each gender all liver copper traits were normally distributed (Kolmogorov–Smirnov one-sample test, $P > 0.05$).
[b] Additive or dominant or recessive was defined with respect to the LEW/OlaHsd parent's allele.
[c] Data were shown only when significant or suggestive results were found. Thresholds for suggestive and significant linkage were those of Lander and Kruglyak (12). Significant results are indicated in boldface type, and suggestive results are in italic type.
[d] Location on the chromosome where the Lod score peaked is given in parentheses.
[e] Percentage of the genetic variance explained by the QTL.

weight was more pronounced in the male rats. As would be expected males were significantly heavier than females (Table I).

For both strains male rats have higher absolute liver weights than female rats. This gender effect was higher in LEW than in BC rats. Relative liver weight was similar in male and female LEW rats. In contrast, group means for relative liver weight of male BC rats were about 13% lower when compared with those of female BC rats (Table I).

Group means of hepatic copper concentration (μg/g liver dry weight) and hepatic copper store (μg/whole liver) were higher in BC when compared with LEW rats. However, only if liver copper content was expressed relative to body weight (μg/whole liver/100 g body weight), the difference reached statistical significance (Table I).

Genetic Mapping of Quantitative Traits. The liver copper of F_2 rats was expressed in four different units, as μg/g wet weight, μg/g dry weight, μg/whole liver, and μg/whole liver/100 g body weight. Within each gender, all liver copper traits were normally distributed (Kolmogorov–Smirnov one-sample test, $p>0.05$). Whenever a (suggestive) QTL was found using the MapQTL software, a one-way ANOVA with *post-hoc* Student's *t*-test was performed for the markers flanking the peak of the QTL or at the peak of the QTL. The mode of inheritance was chosen as free, additive, dominant, or recessive according to the significance of differences in the mean values of the traits between rats that were homozygous LEW, heterozygous LEW:BC, and homozygous BC. Results are shown in Table II. For females, we found a significant QTL on chromosome 2 and

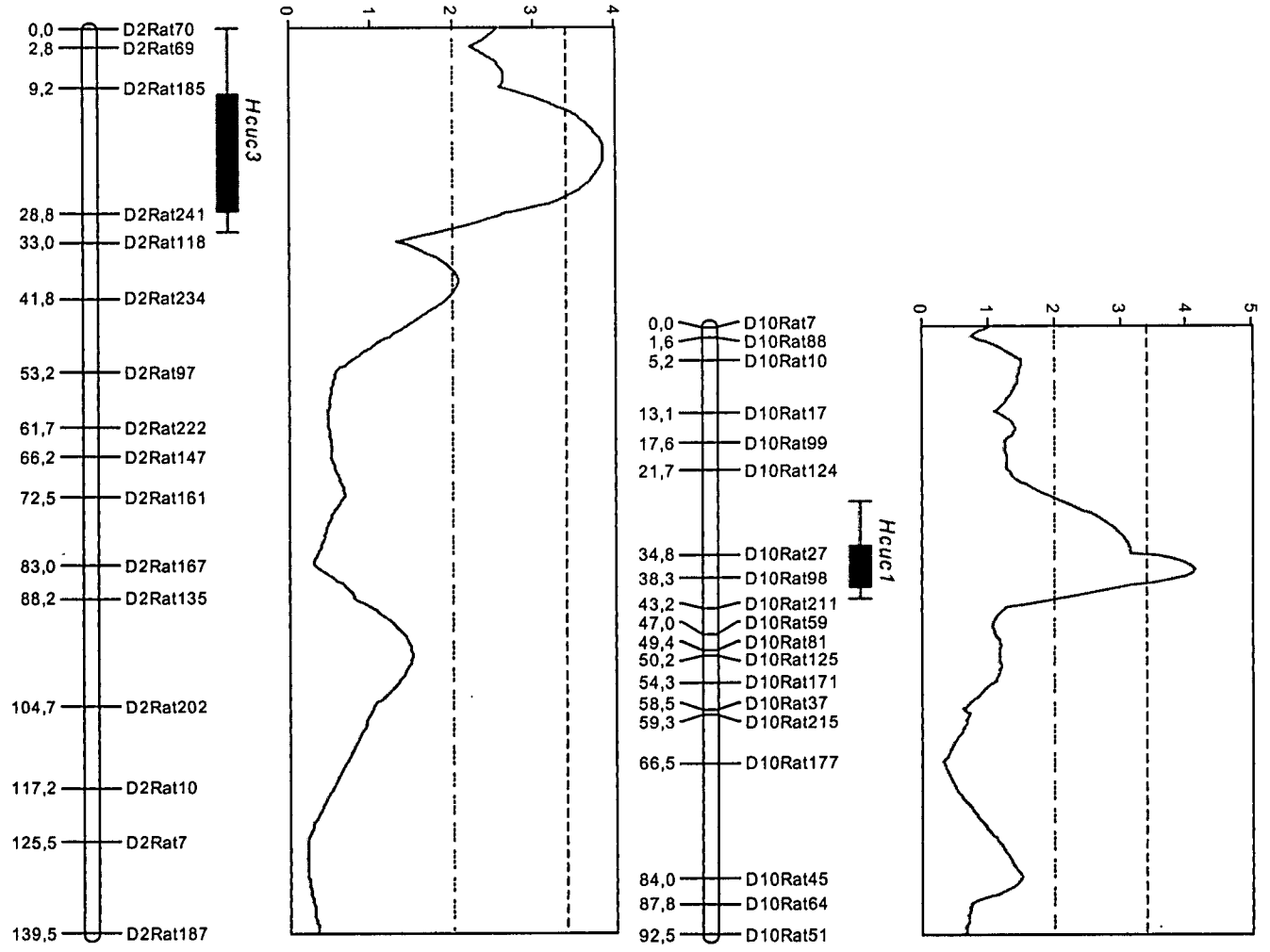

Figure 1. Lod score plots of chromosomes exhibiting significant linkage of quantitative trait to microsatellite markers. Quantitative trait linkage analysis was performed by interval mapping using MapQTL on data collected from 190 (LEW/OlaHsd × BC/CpbU) F_2 intercross rats investigated in this study. (A) Chromosome 2, females: hepatic copper concentration (μg/g dry weight). (B) Chromosome 10, males: hepatic copper concentration (μg/g dry weight). The distances are indicated in cM. The positions of selected marker loci genotyped in the F_2 progeny are indicated on the *x* axis of each panel. The most likely position for each QTL, determined by its 1.0-Lod support interval, is indicated by a solid, thick bar under the plot. The thin lines at both ends of this bar represent the 2.0-Lod support interval. Thin and thick dotted lines represent threshold value of the Lod score considered as suggestive and significant for linkage, respectively, in the model of inheritance chosen according to the Student's *t*-test analysis (dominant/recessive model).

Table III. Co-segregation Analysis Results for Hepatic Copper Concentration (μg/g dry weight) in F_2 Progeny of LEW/OlaHsd and BC/CpbU Rats[a]

Marker	Gender	Genotype[b]			Lod score[c]	*P* (one-way) ANOVA
		LL	LB	BB		
Chromosome 2						
D2Rat185	Females	15.8 ± 3.2 (21)	13.6 ± 2.6 (58)	13.3 ± 2.3 (20)	2.60	0.0031
D2Rat241	Females	15.6 ± 2.6 (25)	13.5 ± 2.7 (54)	13.3 ± 2.5 (20)	2.69	0.0025
Chromosome 10						
D10Rat27	Males	17.6 ± 4.0 (13)	13.8 ± 4.1 (43)	14.9 ± 4.2 (32)	3.17	0.0198
D10Rat98	Males	17.9 ± 4.5 (14)	13.7 ± 3.7 (45)	14.8 ± 4.4 (30)	4.02	0.0053

[a] Values are means ± SD; number of rats is given in parentheses. All results within genotype groups were found to be normally distributed (Kolmogorov–Smirnov one-sample test, $P > 0.05$). Some DNA samples failed to give a conclusive genotype, hence the number of rats typed varied slightly with each locus.
[b] L = LEW/OlaHsd allele, B = BC/CpbU allele.
[c] Lod scores reported are at the marker indicated. The Lod score between markers is higher (see Table II).

suggestive QTLs on chromosomes 1 and 19. For males, a significant QTL was found on chromosome 10 and suggestive QTLs were detected on chromosomes 1, 8, 10, 12, and 14. Figure 1 shows the Lod score curve across chromosome 2 (females) and 10 (males) for hepatic copper concentration (μg/g dry weight). As shown in Table III, the LEW alleles in the *D2Rat185-D2Rat241* region increased liver copper concentration in female rats in a recessive manner. In male rats, the LEW alleles in the *D10Rat27-D10Rat98* region also increased the liver copper concentration in a recessive manner.

Discussion

In the present study, genome wide scanning for associations between marker genotypes and liver copper content resulted in the localization of two significant (on rat chromosomes 2 and 10) and eight suggestive QTLs (on rat chromosomes 1, 2, 8, 10, 12, 14, and 19; Table II, Fig. 1). There is evidence that some QTLs are recessive and other QTLs are dominant with respect to the LEW allele (Tables II and III).

Previous genetic analysis of a large set of recombinant inbred strains derived from BN and SHR revealed that a QTL on the central part of chromosome 10, tentatively indicated as *Hcuc1*, influences hepatic copper concentration in male rats (4). In the present (LEW × BC) F_2 intercross, liver copper concentration expressed as μg/g dry weight in males was also found to be associated with the central part of chromosome 10 (Table II). The *Hcuc1* (2-Lod) support interval is very narrow (about 2 cM): *D10Mit4* plus about 2.0 cM telomeric from this marker (4). In the present study, the 2-Lod support interval is about 15.5 cM and contains the markers *D10Rat27* and *D10Rat98* (Fig. 1b). The estimated distance between the marker *D10Mit4* and *D10Rat27* is about 30 cM, whereas the distance between *D10Rat45* (location of the suggestive QTL on rat chromosome 10, Table II) and *D10Mit4* is about 25 cM (13). Taken together, we therefore conclude that the QTL position in De Wolf *et al.* (2001) (4) is different from the rat chromosome 10 QTLs detected in the present study. As in our previous results (4) we hypothesized that the rat *Atox1* (antioxidant protein 1) gene might be a positional candidate for the liver copper QTL in the central part of the chromosome. We suggest that in the liver the antioxidant protein 1 binds and delivers cytosolic copper to the Wilson disease ATPase protein (ATP7B) in the trans-Golgi network. This ATPase is required for incorporation of copper into ceruloplasmin (the major copper binding protein in the circulation) during its formation and folding or to release hepatic copper into bile (14). The present results support the presence of a candidate gene on chromosome 10.

The region of rat chromosome 2 that shows in female rats linkage to hepatic copper (Tables II and III, Fig. 1) contains the gene *Hsd3b* coding for the enzyme 3β-hydroxysteroid dehydrogenase-δ5-δ4 isomerase (15). This enzyme plays a crucial role in the biosynthesis of all classes of active steroids (16). It is known that glucocorticoids can stimulate synthesis of the copper-containing protein ceruloplasmin in the liver. Copper, absorbed through the intestine, is transported to and taken up by the liver. In part it is incorporated into newly synthesized ceruloplasmin that is excreted into the plasma. Besides incorporation of copper in copper-containing proteins, part is stored as metallothionein. The remaining copper is excreted into the bile (17). Thus, once ceruloplasmin is synthesized, there might be a decrease in liver copper content and an increase in plasma copper concentration. As a result of differential activity of the enzyme 3β-hydroxysteroid dehydrogenase-δ5-δ4 isomerase more or less circulating glucocorticoids are produced resulting in more or less ceruloplasmin synthesis. This in turns leads to less or more copper in the liver. BC rats when compared with LEW rats have higher circulating concentrations of aldosterone and corticosterone.[4] Thus, it could be anticipated that rats with a BC allele for *Hsd3b* have a lower hepatic copper content when compared with rats homozygous for the LEW allele (Table III). We propose to use the

[4] Bonné ACM, Den Bieman MG, Gillissen GF, Lanuhorst Æ, Kenyon CJ, Van Zutphen BFM, Van Lith HA. Quantitative trait loci influencing blood and liver cholesterol concentration in rats. (Submitted)

symbol *Hcuc3* (hepatic copper content 3) for the QTL on chromosome 2.

In summary, the present study supports that chromosome 10 contains a QTL (*Hcuc1*) that plays a role in controlling the hepatic copper content in male rats and indicates that a QTL (tentatively indicated as *Hcuc3*) for liver copper content of female rats is located on chromosome 2. There is some evidence that the *Atox1* and *Hsd3b* genes are the candidate loci for *Hcuc1* (rat chromosome 10) and *Hcuc3* (rat chromosome 2), respectively. Furthermore, there was evidence that rat chromosomes 1, 8, 12, 14, and 19 also contain QTLs involved in hepatic copper content. Further experiments including the development of congenic sublines of BC are necessary to confirm and precisely map the QTLs on rat chromosomes 2 and 10.

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