Two loci on chromosome 9 control bile acid composition: evidence that a strong candidate gene, *Cyp8b1*, is not the culprit[S]

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Abstract An intercross between C57BL/6J and CASA/Rk mice was used to study the genetics of biliary bile acid composition. In parental strains, male C57BL/6J mice had significantly higher cholic acid (CA; 14%) and lower β-muricholic acid (βMC; 27%) than CASA/Rk mice, whereas females did not differ. However, quantitative trait locus analysis of F2 mice revealed no significant chromosome 9 loci in males but loci in females on chromosome 9 for percentage CA (%CA) at 72 centimorgan (cM) [logarithm of the odds (LOD) 5.89] and %βMC at 54 cM (LOD 4.09). Chromosome 9 congenic and subcongenic strains representing CASA/Rk intervals 38–73 cM (9KK) and 68–73 cM (9DKK) on the C57BL/6J background were made. In 9KK and 9DKK males, %CA was increased and %βMC was unchanged, whereas in 9KK but not 9DKK females, %CA was increased and %βMC was decreased. Sterol 12α-hydroxylase (*Cyp8b1*) channels bile acid precursors into CA and maps at chromosome 9 (73 cM). However, there was no significant difference in *Cyp8b1* mRNA or enzymatic activity between parental mice, parental-congenic-subcongenic mice, or high-low biliary %CA F2 mice. In summary, two chromosome 9 loci control sexually dimorphic effects on biliary bile acid composition: a distal (68–73 cM) major determinant in males, and a more proximal (38–68 cM) major determinant in females. In this intercross, *Cyp8b1*, a strong candidate, does not appear to be responsible.—Sehayek, E., L. R. Hagey, Y-Y. Fung, E. M. Duncan, H. J. Yu, G. Eggertsen, I. Björkhem, A. F. Hofmann, and J. L. Breslow. **Two loci on chromosome 9 control bile acid composition: evidence that a strong candidate gene, *Cyp8b1*, is not the culprit. *J. Lipid Res.* 2006.** 47: **2020–2027.**

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In humans and mice, biliary bile acid composition affects the enterohepatic metabolism of lipids, including the synthesis and transport of bile acids across hepatocytes, the excretion of biliary lipids, and the absorption of sterols from the intestines (1–6). In humans, the two major primary bile acids are chenodeoxycholic acid (CDCA), a dihydroxy bile acid, and cholic acid (CA), a trihydroxy bile acid. The difference between them is hydroxylation of the 12 carbon of the sterol ring, which is carried out by the enzyme sterol 12α-hydroxylase (CYP8B1). As a result, CDCA is more hydrophobic and CA is more hydrophilic. In mice, CA is one of the major primary bile acids; it is formed by CYP8B1-mediated 12 hydroxylation. Muricholic acids comprise the other major murine bile acids, and they are formed from CDCA by 6 hydroxylation and 7 epimerization to generate α- and β-muricholic acid (βMC), with βMC predominating. βMC is even more hydrophilic than CA. Although the mouse does not have CDCA as a major species, it is possible to use this model to identify genes that influence the 12 hydroxylation pathway and uptake of bile acids from the intestines by studying the proportions of CA and βMC in bile. Identifying the genes that influence 12 hydroxylation and bile acid uptake from the intestines is particularly important because they determine the proportions of CA and CDCA and hence the hydrophobicity of the bile acid pool in humans.

In humans, the proportions of CA and CDCA vary between individuals (7, 8), and there is some evidence for genetic control. For example, studies in monozygotic and dizygotic twins found a significant pair-wise correlation of bile acid composition in the former but not in the latter (9). In mice, homozygosity for the *Cyp8b1* knockout trait results in bile devoid of CA, indicating a central role for this gene in CA formation (10). Therefore, it is possible

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Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; cM, centimorgan; CYP8B1, sterol 12α-hydroxylase; LOD, logarithm of the odds; βMC, β-muricholic acid; QTL, quantitative trait locus.

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[S] The online version of this article (available at http://www.jlr.org) contains an additional two tables.

that variation in bile acid composition between humans and between mouse strains is attributable to *Cyp8b1* genetic variation. However, a recent study of two groups of gallstone patients with no overlap in the biliary ratio of CA and CDCA found no group differences in the sequence of *CYP8B1*, and there is no evidence that *Cyp8b1* accounts for strain differences in the proportions of CA and βMC in the mouse (8). In this study, we undertook an intercross between two distantly related mouse strains, C57BL/6J and CASA/Rk, to determine genes that influence bile acid hydroxylation at carbon 12 and bile acid uptake from the intestines, as determined by the relative content of CA and βMC in bile. These genes would then be strong candidates for regulating the CA and CDCA content of human bile. Our analysis revealed two loci on chromosome 9 that control sexually dimorphic effects on biliary percentage CA (%CA) and percentage βMC (%βMC). Although *Cyp8b1* is close to the peak of linkage in one of these loci, further studies of CYP8B1 mRNA and activity in parental mice, CYP8B1 mRNA in F2 mice with high and low %CA, CYP8B1 mRNA in chromosome 9 congenic and subcongenic animals, and interstrain *Cyp8b1* sequence biliary bile acid comparisons indicate that this is not the causative gene at this locus.

MATERIALS AND METHODS

Animals

The inbred mouse strains C57BL/6J, CASA/Rk, BALB/c, and 129SV/J were purchased from Jackson Laboratories (Bar Harbor, ME). CASA/Rk males were mated with C57BL/6J females to generate F1 animals. F1 males were intercrossed with F1 females to generate 369 F2 animals (185 males and 184 females). A speed congenic approach was used to generate congenic 9KK animals, with a 38–73 centimorgan (cM) CASA/Rk interval, and subcongenic 9DKK animals that (as shown in Fig. 2 below) overlap with 9KK congenic animals only at the 68–73 cM CASA/Rk interval (11). Briefly, F1 males were backcrossed to C57BL/6J females (purchased from Jackson Laboratories; stock number 00664) generating ~10–20 N2 male mice. These were genotyped for 11 markers specific for the chromosome 9 interval and for 140 additional markers, with average spacing of 10 cM, across the genome. Male N2 mice heterozygous at the chromosome 9 interval with the highest rate of C57BL/6J genotype for markers across the rest of the genome were selected for the next generation of backcrossing with C57BL/6J females. This selective breeding was repeated for five generations to generate N6 animals that were shown to be heterozygous at the chromosome 9 interval and homozygous for C57BL/6J at all other loci throughout the genome. Male and female N6 mice were intercrossed to generate 9KK congenic and 9DKK subcongenic animals homozygous for the CASA/Rk alleles on the C57BL/6J background. Congenic mice with the C57BL/6J interval on the CASA/Rk background were not generated because the difficulty of breeding mice on this background precluded generating sufficient numbers of animals for the speed congenic technique (11). The experimental design compared either 9KK congenic or 9DKK subcongenic animals with wild-type C57BL/6J mice that were matched for age and sex. The logistical difficulties of breeding the numbers of mice required precluded comparing 9KK, 9DKK, and wild-type mice in the same experiment. All animals were bred and housed in a single humidity- and temperature-controlled room with a 12 h dark/light cycle (light from 6 AM to 6 PM) at the Laboratory of Animal Research Center at The Rockefeller University and fed with a single lot of Picolab Rodent Chow 20 (catalog No. 5053) pellets containing 0.02% (w/w) cholesterol. At the age of 11 weeks, food was removed from the cages at 10 AM (4 h into the light phase) and the animals allowed access to water. At 3 PM (9 h into the light phase), the mice were anesthetized with intramuscular injection of ketamine/xylazine and tail tipped for DNA extraction, the abdominal cavity was exposed, gallbladder bile was aspirated, and liver tissue was harvested and stored in RNA*later* (Ambion, Austin, TX) at −20°C or snap-frozen in liquid nitrogen and stored at −80°C. The Institutional Animal Care and Research Advisory Committee approved all experiments.

Bile acid composition

Bile acid composition was analyzed in 197 F2 animals (99 males and 98 females) by HPLC using a modification of the technique of Rossi, Converse, and Hofmann (12). An octadecylsilane column (RP C-18; Beckman Instruments, Fullerton, CA) was used with an isocratic elution at 0.75 ml/min. The eluting solution was composed of a mixture of methanol and 0.01 M KH_2PO_4 (60.7%, v/v) adjusted to an apparent pH of 5.4 with H_3PO_4. Bile acids were identified by matching their relative retention times with those of known standards. Relative retention times were calculated in relation to that of taurocholic acid, which ran at 32.5 min in this buffer. %CA and %βMC were calculated as the ratio of the area under the peak of these bile acids to the total area of all identified bile acids. Note that in these studies, we made the assumption that the bile acid composition of the gallbladder bile is essentially identical to that in the jejunum and the upper ileum, where the bulk of dietary sterol absorption takes place.

Cyp8b1 expression

For Northern blotting, total RNA was extracted from liver homogenates of five C57BL/6J and five CASA/Rk males and from four F2 females with the lowest and four F2 females with the highest %CA using the Trizol reagent kit (Life Technologies, Gaithersburg, MD). Total RNA (10 μg/lane) was loaded on a 1.5% agarose formaldehyde gel and transferred overnight onto a positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN). For measurements of *Cyp8b1* expression levels, total RNA was reverse-transcribed into cDNA with poly(dT) primers and Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). A *Cyp8b1* PCR-amplified template was prepared using gene-specific primers (forward, 5′-TTGGCCCCATCATTAAGAACACAG-3′; reverse, 5′-GGAGGCCCAGAGCATCATAAAGT-3′), and the probe was radiolabeled with [^{32}P]dATP (NEN Life Science, Boston, MA) according to the DECAprime II Klenov-Decamer protocol (Ambion). For Northern blotting, the nylon membrane was hybridized with CYP8B1-radiolabeled probe diluted in ExpressHyb Hybridization Solution (Clontech, Palo Alto, CA) according to the manufacturer's instructions, processed, and exposed to Biomax MS film (Eastman Kodak Co., Rochester, NY) for autoradiography. Autoradiographs were developed after a 16 h exposure and scanned, and band intensities were quantified using the Image-Pro Plus program (Media Cybernetics, Inc., Silver Spring, MD) and normalized to those of 18S rRNA bands. Real-time quantitative PCR was performed as described with slight modifications using the Applied Biosystems 7900 Sequence Detection System (13). Primers and probe were specific for the mouse *Cyp8b1* gene: forward primer, 5′-GAACTCAACCAGGCCATGCT-3′; reverse primer, 5′-

GGCACCCAGACTCGAACCT-3′; probe, 6-FAM-ACAGCCTATCCTTGGTGATGCTAGGGCC. Cyclophilin was used as a housekeeping gene: forward primer, 5′-GGCCGATGACGAGCCC-3′; reverse primer, 5′-TGTCTTTGGAACTTTGTCTGCAA-3′; probe, 6-FAM-TGGGCCGCGTCTCCTTCGA. *Cyp8b1* expression is displayed as the ratio of CYP8B1 to cyclophilin message level.

CYP8B1 activity

CYP8B1 activity was measured in C57BL/6J, CASA/Rk, BALB/c, and 129SV/J animals. Liver tissue was snap-frozen and stored at −80°C. Liver microsomes were isolated and assayed for 12α-hydroxylase activity as described previously using a mass spectrometric determination of the ratio between the substrate, 7α-hydroxy-4-cholesten-3-one, and the product, 7α,12α-dihydroxy-4-cholesten-3-one (14).

Genotyping

Tail tips from parental, F1, F2, and N2–N6 animals were digested with proteinase K, DNA-precipitated with ethanol, and PCR genotyped for 255 microsatellite markers polymorphic between C57BL/6J and CASA/Rk using fluorescently labeled primers as described previously (15). Allele scores were analyzed using ABI Genotyper 3.6 NT software. Throughout this paper, marker positions in cM correspond to mapping data found in the Mouse Genome Informatics Database at http://www.informatics.jax.org.

Statistical analyses

Differences in percentage of bile acids and *Cyp8b1* expression between C57BL/6J and CASA/Rk, between F2 males and F2 females, and between chromosome 9 congenic or subcongenic and C57BL/6J controls were examined using two-tailed unpaired Student's *t*-test, and results are expressed as means ± SD. Differences in bile acid composition and CYP8B1 activity between C57BL/6J, CASA/Rk, BALB/c, and 129SV/J animals and comparisons of %CA and %βMC for F2 mice with the various combinations of genotypes at D9Mit136 and D9Mit151 were analyzed using one-way ANOVA with Tukey's posttest. Linkage and interval mapping (using the maximum likelihood algorithm) were analyzed with the R/qtl software package version 0.94-17 (16). The C57BL/6J and CASA/Rk allele effects at chromosome 9 for %CA and %βMC were assessed for additivity and dominance using Map Manager QTXb10 version 0.19 software.

RESULTS

The %CA and %βMC in the bile of male and female C57BL/6J and CASA/Rk mice are displayed in **Fig. 1**. In males, C57BL/6J mice had 14% more %CA (39.3 ± 3.8% vs. 24.9 ± 1.8%; $P < 0.0001$) and 27% less %βMC (38.0 ± 4.9% vs. 64.9 ± 6.9%; $P = 0.002$) compared with CASA/Rk mice. In females, in contrast, C57BL/6J and CASA/Rk mice did not differ significantly in %CA (33.0 ± 2.6% vs. 38.0 ± 4.1%) or %βMC (43.5 ± 4.9% vs. 48.0 ± 1.5%). The biliary bile acid composition of less abundant bile acid species is shown in supplementary Table I for C57BL/6J and CASA/Rk males. The CA-derived secondary bile acid, deoxycholic acid, was ~4-fold higher in C57BL/6J than in CASA/Rk, compatible with what was seen for CA. However, two bile acids without 12 hydroxylation, CDCA and the CDCA-derived α-muricholic

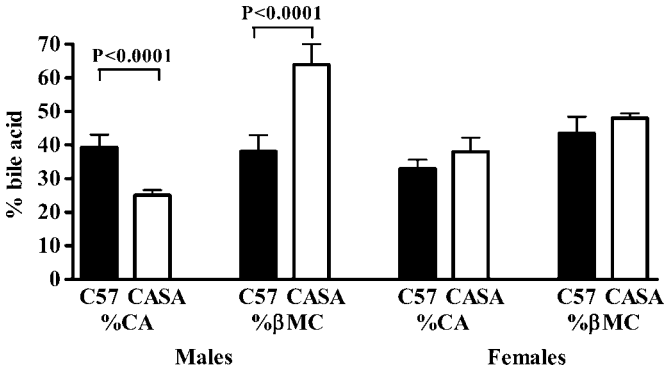

Fig. 1. Percentage cholic acid (%CA) and percentage β-muricholic acid (%βMC) in male and female C57BL/6J and CASA/Rk mice. Animals at 11 weeks of age were fasted for 5 h, gallbladder bile was aspirated, and bile acid composition was analyzed as described in Materials and Methods. Data represent means ± SD of five animals in each group.

acid, were also higher in C57BL/6J than in CASA/Rk, incompatible with what was seen for βMC. Because the main goal of this study was to determine the gene(s) that control the 12 hydroxylation of bile acids, we chose to base our quantitative trait locus (QTL) mapping analysis on the measurement of biliary %CA and %βMC in F2 mice.

The QTL mapping analysis was done in 99 F2 males and 98 F2 females in which gallbladder bile was analyzed for bile acid composition. In F2 mice, males and females had significantly different bile acid composition. For example, in males versus females, the %CA was decreased (44.3 ± 11.3% vs. 54.9 ± 11.3%; $P < 0.0001$) and the %βMC was increased (44.9 ± 14.3% vs. 36.2 ± 14.3%; $P < 0.0001$). Therefore, the QTL analysis was carried out separately for males and females using the R/qtl program.

The results of the QTL mapping for %CA and %βMC are shown in **Table 1**. In females, the only significant linkages were for chromosome 9, which for %CA had a logarithm of the odds (LOD) score of 5.89 at 72 cM and for %βMC had a LOD score of 4.09 at 54 cM. In females, there was a suggestive locus for %CA on chromosome 4 at 77 cM. In males, there was a suggestive locus on chromosome 9 for %CA with no linkage at all for %βMC. However, in males, significant loci were found on chromosome 11 for %CA (LOD 3.66, 15 cM) and %βMC (LOD 3.93, 15 cM). In view of the strongest linkages on chromosome 9, further analysis was focused on this chromosome.

The chromosome 9 interval maps for %CA and %βMC are shown in **Fig. 2**. In females, although the map for %CA

TABLE 1. Linkage of %CA and %βMC in F2 females and F2 males

	F2 females			F2 males		
Variable	Chromosome	Position (cM)	LOD	Chromosome	Position (cM)	LOD
%CA	9	72	5.89	11	15	3.66
	4	77	2.67	9	65	2.85
%βMC	9	54	4.09	11	15	3.93

%CA, percentage cholic acid; cM, centimorgan; LOD, logarithm of the odds; %βMC, percentage β-muricholic acid.

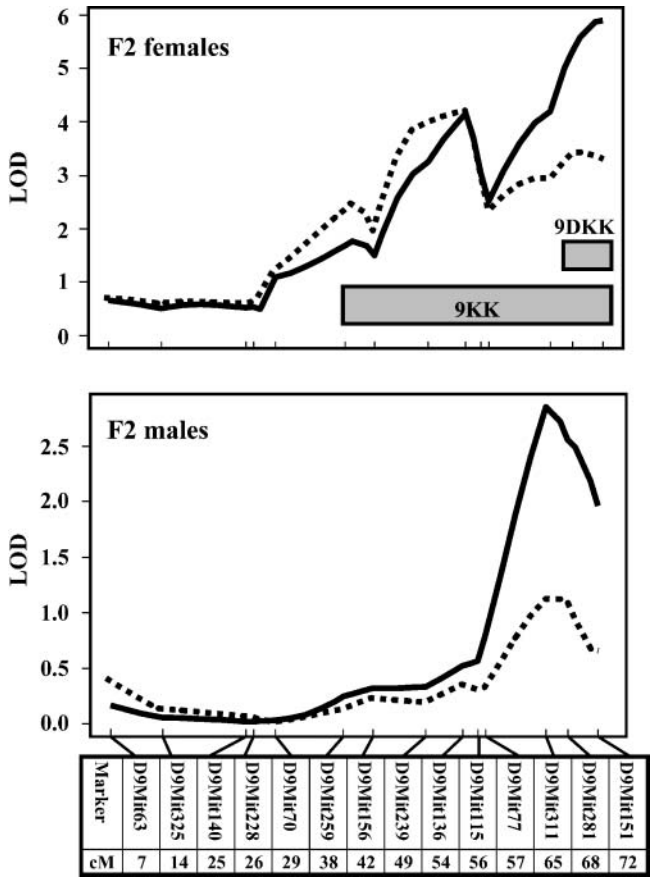

Fig. 2. Chromosome 9 interval maps for %CA (solid lines) and %βMC (dashed lines) in F2 females and F2 males and chromosome 9 intervals for 9KK congenic and 9DKK subcongenic animals. F2 animals were euthanized at 11 weeks of age, gallbladder bile was aspirated, and bile acid composition was analyzed as described in Materials and Methods. Linkage analysis was carried out separately in F2 females and F2 males using the R/qtl program. Congenic 9KK and subcongenic 9DKK animals were generated as described in Materials and Methods. Boxed intervals correspond to CASA/Rk intervals introduced onto the C57BL/6J background. Marker positions correspond to mapping data found in the Mouse Genome Informatics database. cM, centimorgan; LOD, logarithm of the odds.

displays two peaks, one with maximum LOD at 72 cM and the other with a lower LOD at 54 cM, the peak for maximum LOD for %βMC is at 54 cM. In contrast, in male mice, the scale for the whole chromosome 9 interval map is reduced: there is no suggestion of the 54 cM peak for any of the traits and weak but suggestive evidence for a single peak for %CA more distally. These data suggest that the chromosome 9 locus that influences biliary bile acid composition may be complex, with two loci on the distal part of the chromosome. The more proximal of these appears to have an effect only in females.

The genotypic means for F2 female mice of %CA and %βMC are shown in **Table 2** for the marker on chromosome 9 that is closest to the maximum LOD score for that trait. At the marker D9Mit151 at 72 cM, there appears to be a significant gene dosage effect, with the presence of one and two CASA/Rk alleles increasing %CA in a step-wise manner, suggesting codominant inheritance of a CASA/Rk allele capable of increasing %CA. At the marker D9Mit136 at 54 cM, there appears to be a significant effect, with the presence of two but not one allele of the CASA/Rk allele decreasing %βMC, suggesting a recessive effect of a CASA/Rk allele capable of decreasing %βMC. In F2 females, the chromosome 9 locus at 72 cM appears to explain 23% of the variance in %CA, whereas the locus at 54 cM explains 14% of the variance in %βMC.

To confirm that the locus on chromosome 9 affects the composition of bile acids, we generated 9KK congenic and 9DKK subcongenic strains, with different overlapping chromosome 9 CASA/Rk intervals introgressed onto the C57BL/6J background. As shown in Fig. 2, 9KK congenic mice carry 35 cM of a CASA/Rk interval, from 38 to 73 cM, that covers the peak for %CA at 72 cM as well as the peak for %βMC at 54 cM. In contrast, 9DKK subcongenic mice carry only 5 cM of a CASA/Rk interval, from 68 to 73 cM, that covers only the distal peak for %CA at 72 cM. As shown in **Table 3**, 9KK and 9DKK males had significant 15% and 17% higher %CA but showed no significant differences in %βMC compared with control C57BL/6J males. Similar to 9KK males, 9KK females had significant 15% higher %CA, but unlike 9KK males, they had a small but significant 4% lower %βMC. In contrast, and opposite to what was seen for 9KK females, 9DKK females had significant 7% lower %CA and 22% higher %βMC. Therefore, in agreement with the genotypic means in F2 females, 9KK and 9DKK males as well as 9KK females recapitulated the chromosome 9 effect on %CA, whereas 9DKK females displayed an opposite effect on this trait.

The mouse genome database indicates that the *Cyp8b1* gene is located on chromosome 9 at 73 cM, very close to the linkage peak for %CA at 72 cM. This gene encodes CYP8B1, which converts bile acid precursors to CA, making it an obvious candidate gene at this locus. Several experiments were undertaken to test this hypothesis. If

TABLE 2. Genotypic effect of chromosome 9 on %CA and %βMC in F2 females

Locus	Locus Closest Marker	F2 Females			
Phenotype	Peak (cM)	Name	BB	BC	CC
CA[a]	72	D9Mit151	48.3 ± 9.3	56.1 ± 10.7	63.2 ± 11.4
βMC[b]	54	D9Mit136	41.2 ± 11.7	38.5 ± 13.8	26.3 ± 14.0

[a] Overall $P < 0.0001$: BB versus BC, $P < 0.01$; BB versus CC, $P < 0.001$; BC versus CC, $P < 0.05$ by one-way ANOVA.
[b] Overall $P = 0.0001$: BB versus CC, $P < 0.001$; BC versus CC, $P < 0.01$ by one-way ANOVA.

TABLE 3. Bile acid composition in C57BL/6J mice and chromosome 9 congenic and subcongenic animals

Strain (n)	Gender	CA[a]	Deoxycholic Acid[b]	Chenodeoxycholic Acid[c]	Ursodeoxycholic Acid[d]	α-MC[e]	β-MC[f]	ω-MC[g]
C57BL/6J (6)	Males	58.0 ± 5.0	1.2 ± 0.6	1.5 ± 0.5	0.7 ± 0.1	6.9 ± 0.9	16.5 ± 6.0	15.2 ± 2.0
9KK (6)		72.9 ± 4.2	3.0 ± 0.7	0.4 ± 0.2	0.3 ± 0.2	2.0 ± 0.3	16.1 ± 2.7	5.3 ± 1.6
C57BL/6J (6)	Females	61.9 ± 4.0	1.2 ± 0.5	1.9 ± 0.4	1.2 ± 0.2	7.1 ± 1.2	18.4 ± 2.6	8.4 ± 1.4
9KK (5)		75.5 ± 3.1	2.0 ± 0.5	0.6 ± 0.1	0.6 ± 0.2	2.3 ± 0.4	14.2 ± 3.5	4.8 ± 0.5
C57BL/6J (6)	Males	64.2 ± 6.8	0.9 ± 0.2	0.7 ± 0.3	0.6 ± 0.1	4.4 ± 1.1	16.1 ± 5.6	13.2 ± 2.2
9DKK (6)		81.3 ± 4.0	1.6 ± 0.5	0.2 ± 0.1	0.2 ± 0.1	2.5 ± 3.1	13.9 ± 3.2	2.4 ± 0.9
C57BL/6J (6)	Females	63.0 ± 3.0	1.3 ± 0.7	1.1 ± 0.1	1.3 ± 0.6	6.2 ± 1.3	17.1 ± 5.5	10.1 ± 2.4
9DKK (6)		55.8 ± 5.5	ND	0.5 ± 0.2	1.4 ± 0.4	3.1 ± 0.9	39.2 ± 4.4	ND

ND, not detectable. Values shown are % ± SD.
C57 versus 9KK males: [a]$P < 0.0003$; [b]$P < 0.002$; [c]$P < 0.0006$; [d]$P < 0.02$; [e]$P < 0.0001$; [f]$P = 0.88$; [g]$P < 0.0001$.
C57 versus 9KK females: [a]$P < 0.0001$; [b]$P < 0.02$; [c]$P < 0.0001$; [d]$P < 0.0002$; [e]$P < 0.0001$; [f]$P = 0.03$; [g]$P < 0.0001$.
C57 versus 9DKK males: [a]$P < 0.0003$; [b]$P < 0.02$; [c]$P < 0.004$; [d]$P < 0.0002$; [e]$P = 0.174$; [f]$P = 0.51$; [g]$P < 0.0001$.
C57 versus 9DKK females: [a]$P < 0.04$; [b]$P < 0.006$; [c]$P < 0.002$; [d]$P = 0.8$; [e]$P = 0.003$; [f]$P < 0.0002$; [g]$P < 0.0001$.

this locus contributed to the increased %CA in parental C57BL/6J versus CASA/Rk males, one might expect the former to have higher levels of hepatic CYP8B1 mRNA. However, as shown in **Fig. 3A**, no difference was found by Northern blot analysis. In addition, if in this cross *Cyp8b1* influenced %CA, one might expect the %CA to be directly proportional to hepatic CYP8B1 mRNA levels, especially in female mice. This was tested by comparing in Northern blot analysis CYP8B1 mRNA levels in the livers of F2 female mice with the lowest and highest %CA levels. As shown in Fig. 3B, no significant difference was found; in fact, the trend was in the opposite direction, with the lowest %CA F2 mice having the higher mRNA levels. Furthermore, if *Cyp8b1* is responsible for increased %CA in 9KK congenic and 9DKK subcongenic males, one would expect increased expression of this gene in these animals. Instead, as shown in **Fig. 4**, compared with control C57BL/6J mice, 9KK and 9DKK males showed either no significant difference or even a decreased CYP8B1 mRNA level. Finally, if *Cyp8b1* is responsible for the differences in %CA in 9DKK subcongenic males and females, one would expect decreased expression of this gene in 9DKK females. Instead, as shown in Fig. 4, 9DKK females did not differ from 9DKK males in *Cyp8b1* expression.

Another possibility was that a coding sequence difference between the strains in *Cyp8b1* might explain the chromosome 9 locus effect. Therefore, the open reading frame of *Cyp8b1* was sequenced from both strains and, as shown in supplementary Table II, eight base differences were found. Seven of these did not change the amino acid sequence, and the eighth changed residue 242 from C57BL/6J lysine to CASA/Rk glutamic acid (lys242glu). Gafvels et al. (17) previously described this polymorphism between 129SV/J (242lys) and BALB/c (242glu) mice. If this polymorphism influenced bile acid composition, one might expect C57BL/6J and 129SV/J mice with 242lys to have a consistently higher %CA or lower %βMC than CASA/Rk and BALB/c mice with 242glu. Biliary bile acid composition was determined in five males of each strain, and the results shown in **Table 4** do not show such a consistent difference. Furthermore, CYP8B1 enzymatic activity was measured in livers of CASA/Rk, BALB/c, C57BL/6J, and 129SV/J animals, and no significant differences between strains were found, as shown in **Fig. 5**. Thus, it is unlikely that *Cyp8b1* is the responsible gene at the chromosome 9 distal locus.

DISCUSSION

In this study, we used two inbred mouse strains, C57BL/6J and CASA/Rk, that differ in %CA and %βMC in bile,

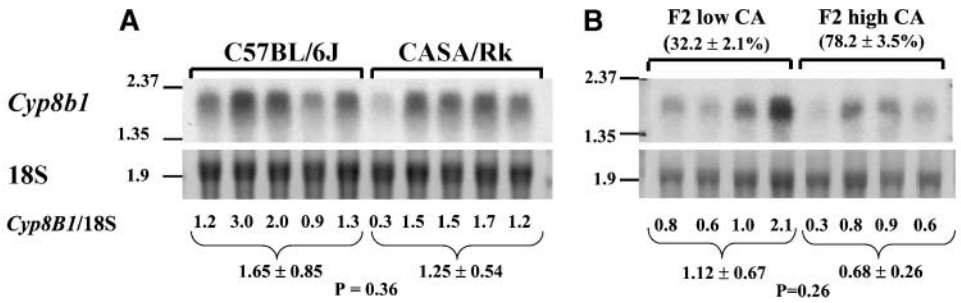

Fig. 3. Liver sterol 12α-hydroxylase (CYP8B1) mRNA levels in parental C57BL/6J and CASA/Rk males (A) and in F2 females with the lowest and highest %CA in bile (B). Animals at 11 weeks of age were euthanized, liver tissue was harvested, and Northern blotting of CYP8B1 mRNA was performed as described in Materials and Methods. Values represent ratios of CYP8B1 mRNA to 18S rRNA in each animal, means ± SD are averages of the ratios in each group, and *P* values are from group comparisons using two-tailed unpaired Student's *t*-test.

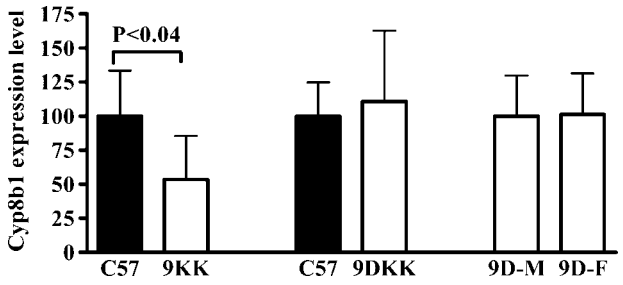

Fig. 4. Liver *Cyp8b1* expression levels in congenic 9KK, subcongenic 9DKK, and control C57BL/6J animals. Results are from three different experiments: 9KK versus age-matched C57BL/6J males (C57), 9DKK versus age-matched C57BL/6J males, and 9DKK males (9D-M) versus age-matched 9DKK females (9D-F). Animals were euthanized, liver tissue was harvested, and real-time PCR was performed as described in Materials and Methods. Values represent means ± SD of arbitrary *Cyp8b1* expression units after normalization to cyclophilin. Expression levels in control C57BL/6J males and in 9DKK males are 100%. n = 5–6 animals per group.

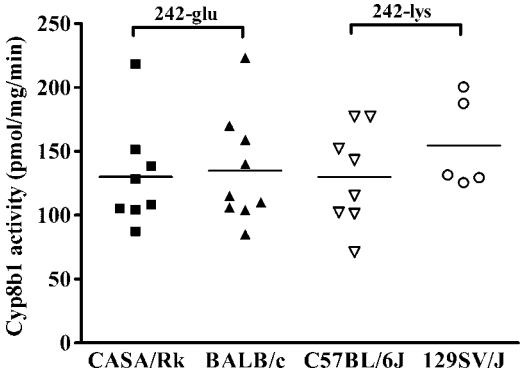

Fig. 5. Activity of CYP8B1 in CASA/Rk, BALB/c, C57BL/6J, and 129SV/J livers. Animals at 11 weeks of age were euthanized, liver microsomes were isolated, and CYP8B1 activity was measured as described in Materials and Methods. 242glu and 242lys designate strains with polymorphisms at amino acid 242. Each symbol represents the activity in a single animal.

and through a genetic cross mapped two loci on chromosome 9 that control these traits. Our findings indicate that *i*) these loci control sexually dimorphic effects on bile acid composition, and *ii*) although *Cyp8b1* is very close to one of these loci, studies of mRNA and enzyme activity indicate that this gene does not control these traits in this cross.

In mice, bile acid composition plays an important role in regulating the excretion of lipids into bile, the synthesis and transport of bile acids across hepatocytes, and the absorption of sterols from the intestines. In studies in which bile acids were fed to mice, it has been reported that CA, the major hydrophobic primary bile acid in this species, stimulates the excretion of cholesterol and phospholipids into bile, whereas the major hydrophilic bile acid, βMC, fails to influence or decrease excretion (5, 6). Bile acid species also vary in their effects on bile acid synthesis and hepatobiliary transport. For example, bile acids differ markedly in their binding affinity to farnesoid X receptor, a transcriptional factor involved in the feedback regulation of bile acid synthesis and transport (1, 2). Moreover, *Cyp8b1* knockout mice, which lack CA, have decreased expression of the farnesoid X receptor target gene small heterodimer partner. This causes increased expression of cholesterol 7α-hydroxylase, the rate-limiting enzyme in the classic bile acid synthesis pathway, and this can be reversed by CA feeding (10). In addition, feeding various bile acid species to mice has differential effects on cholesterol absorption. CA increases the absorption of cholesterol, whereas βMC has the opposite effect (6). Thus, bile acid composition plays a major role in the enterohepatic metabolism of bile acids and cholesterol.

In humans, biliary bile acid composition has also been shown to have important effects. Obese subjects fed CDCA had decreased biliary cholesterol saturation, whereas CA had no effect (18). The CDCA effect was attributable to decreased hepatic biliary excretion of cholesterol (3). CDCA, but not CA, feeding decreased the activity of the rate-limiting enzyme in cholesterol biosynthesis, hepatic 3-hydroxy-3-methylglutaryl CoA, by 40% (19). In primary human hepatocyte culture, CDCA decreased CA formation by 56%, but CA had no effect on CDCA synthesis. CDCA was also shown to alter mRNA levels of cholesterol 7α-hydroxylase, sterol 27-hydroxylase, CYP8B1, and small heterodimer partner (4). Finally, feeding CA to humans increased the absorption of cholesterol from the intestine (20). Therefore, biliary bile acid composition is important in both humans and mice.

Studies in humans indicate the presence of interindividual variation in bile acid composition (7, 8) and provide evidence for genetic components that explain part of the variance (9). Furthermore, a gender effect on bile acid composition has been reported in human studies that examined the proportions of CA and CDCA in the gallbladder bile and bile acid pool of normal males and females (21, 22). However, the molecular mechanisms that underlie the interindividual variation and gender effect on bile acid composition are largely unknown. In this study, we used an intercross between two inbred mouse strains and discovered two loci on chromosome 9 that control sexually dimorphic effects on %CA and %βMC. Compelling evidence for the role of these loci is provided by studies in chromosome 9 congenic and subcongenic strains. In 9KK and 9DKK males, a similar

TABLE 4. Relation of sterol 12α-hydroxylase lys242glu polymorphism to %CA and %βMC in males

Mice	Residue 242	%CA	%β-MC
C57BL/6J (n = 5)	Lysine	40.2 ± 5.5	33.9 ± 6.2
129SV/J (n = 5)	Lysine	35.8 ± 4.3	44.0 ± 3.9
CASA/Rk (n = 5)	Glutamic acid	25.8 ± 3.3[a]	52.1 ± 6.5[b]
BALB/c (n = 5)	Glutamic acid	33.5 ± 3.9	40.3 ± 4.2

C57BL/6J versus CASA: $^{a,b}P < 0.001$ by one-way ANOVA.
C57BL/6J versus BALB/c: NS by one-way ANOVA.
129SV/J versus CASA: $^{a}P < 0.05$ by one-way ANOVA.
129SV/J versus BALB/c: NS by one-way ANOVA.

effect for these two strains on bile acid composition indicates that a common interval, between 68 and 73 cM, is in control of %CA. In females, the regulation of bile acid composition is more complex. In 9KK females, the increase in %CA further supports a role for the 68–73 cM interval in controlling the proportion of CA in bile. However, unlike 9KK males, 9KK females present a small but significant decrease in %βMC. One possible interpretation of these findings is that in females, but not in males, the 38–68 cM CASA/Rk interval increases the proportion of CA through channeling of bile acid precursors to CA synthesis and/or preferential uptake of CA from the intestines. This hypothesis gains strong support from our studies in 9DKK females. In these females, replacement of the 38–68 cM CASA/Rk interval with a C57BL/6J interval led to the complete reversal of what was seen for 9KK females, with a decrease in %CA and an increase in %βMC. These findings indicate that whereas the 68–73 cM interval harbors gene(s) that control the proportion of CA in males and females, the 38–68 cM interval has an exclusive and critical role in modifying the proportions of βMC and CA in females.

The fact that *Cyp8b1* knockout mice are deficient in CA indicates that this enzyme is crucial for CA formation. Therefore, it is possible that mouse strain variation in the proportions of CA and CDCA-derived bile acids is attributable to genetic differences in the *Cyp8b1* gene, genes that control CYP8B1 activity, or genes that influence substrate availability. The *Cyp8b1* gene is a logical candidate at chromosome 9, between 68 and 73 cM, to control the proportion of CA in bile. However, we showed the following: *i*) male parental mice that differ in %CA had similar hepatic CYP8B1 activity and mRNA levels; *ii*) F2 females with the highest and lowest %CA did not differ in CYP8B1 mRNA levels; *iii*) chromosome 9 congenic and subcongenic animals with increased %CA had either no effect or even decreased CYP8B1 mRNA levels; and *iv*) 9DKK males and females with differences in %CA displayed no differences in CYP8B1 mRNA levels. Finally, there was only one coding difference between C57BL/6J and CASA/Rk CYP8B1 (lys242glu), and strains with 242lys did not show a consistent difference in bile acid composition or CYP8B1 activity compared with those with 242glu. Therefore, we concluded that variation in the *Cyp8b1* gene or genes that influence its activity does not explain the variance in the proportions of CA and CDCA-derived bile acids in our cross. In conclusion, it is possible that the chromosome 9 locus gene modifies CYP8B1 substrate availability. Human studies also challenge the notion that variations in the *Cyp8b1* gene or activity are responsible for interindividual differences in bile acid composition. For example, liver CYP8B1 activity was not decreased in patients with decreased CA synthesis (23), and assessment of CYP8b1 activity in vivo using radioisotopes did not correlate with the %CA and %CDCA in bile (24).

The uptake of bile acids from the intestines is another important process that controls the proportions of CA and CDCA-derived bile acids in bile. For example, in humans, the ratio of CA to CDCA synthesis is ~2:1, whereas the two bile acids have equimolar proportions in bile because of the more efficient intestinal uptake of CDCA (25). Reclaiming of bile acids from the intestines is a complex process and involves Na-dependent and Na-independent uptake as well as passive diffusion of unconjugated bile acids across the brush border membrane of enterocytes. The Na-dependent uptake involves the ileal apical bile acid transporter ASBT, the most important transporter in humans and mice (26, 27). This transporter has different affinities to different bile acid species and operates as a homotetramer that interacts with bile acid transport protein (28). The Na-independent uptake involves the Oatp3 transporter, also with different affinities to different bile acid species, which localizes to the apical membrane of jejunal enterocytes (29). Neither ASBT nor Oatp3 maps to our loci on chromosome 9, and a search through the list of genes at the chromosome 9 locus revealed no obvious candidate gene that is known to be or is potentially involved in either bile acid synthesis or bile acid transport across enterocytes.

In summary, a cross between two inbred mouse strains revealed novel loci on chromosome 9 that control the %CA and %βMC in bile and the gender effect on these traits. Although *Cyp8b1* is very close to one of these loci, our studies reject a role for this enzyme as the most important modifier of bile acid composition in this cross. It is expected that studies in additional subcongenic animals will narrow these intervals and ultimately lead to the discovery of gene(s) that influence the 12 hydroxylation of bile acids, a crucial step in determining the proportions of CA and CDCA and hence the hydrophobicity of bile in humans.

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