Lith6: a new QTL for cholesterol gallstones from an intercross of CAST/Ei and DBA/2J inbred mouse strains1,2,5

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Abstract  A complex genetic basis determines the individual predisposition to develop cholesterol gallstones in response to environmental factors. We employed quantitative trait locus/loci (QTL) analyses of an intercross between inbred strains CAST/Ei (susceptible) and DBA/2J (resistant) to determine the subset of gallstone susceptibility (Lith) genes these strains possess. Parental and first filial generation mice of both genders and male intercross offspring were evaluated for gallstone formation after feeding a lithogenic diet. Linkage analysis was performed using a form of multiple interval mapping. One significant QTL colocalized with Lith1 [chromosome (chr) 2, 50 cM], a locus identified previously. Significantly, new QTL were detected and named Lith10 (chr 6, 4 cM), Lith6 (chr 6, 54 cM), and Lith11 (chr 8, 58 cM). Statistical and genetic analyses suggest that Lith6 comprises two QTL in close proximity. Our molecular and genetic data support the candidacy of peroxisome proliferator-activated receptor γ (Pparγ) and Slc21a1, encoding Pparγ, and the basolateral bile acid transporter SLC21A1 (Slc21a1/Oatp1), respectively, as genes underlying Lith6. Lyons, M. A., H. Wittenburg, R. Li, K. A. Walsh, M. R. Leonard, R. Korstanje, G. A. Churchill, M. C. Carey, and B. Paigen. Lith6: a new QTL for cholesterol gallstones from an intercross of CAST/Ei and DBA/2J inbred mouse strains. J. Lipid Res. 2003. 44: 1763–1771.

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Studies of model (1–3), animal (4–6), and human biles (7–9) have greatly contributed to the elucidation of the pathophysiology of cholesterol gallstone formation (cholelithiasis). Investigation of both human (10) and mouse models (11) demonstrated that a complex genetic basis determines the individual predisposition to develop cholesterol gallstones in response to environmental factors (12). Advancement of our understanding of cholesterol homeostasis and of the mechanisms of bile formation enables us to identify the primary genetic determinants of cholesterol gallstone formation (13) and may lead to improved management and ultimately prevention of this prevalent and costly disorder (14). To this end, we employed quantitative trait locus/loci (QTL) analysis to define genomic regions associated with cholelithiasis in inbred mice. Our ultimate goal is to identify genes that carry polymorphisms determining cholesterol gallstone susceptibility (i.e., Lith genes) (15, 16). Including this report, 11 major QTL for cholelithiasis, named Lith1 through Lith11 (11, 16–20), have been reported. Each gallstone-susceptible inbred mouse strain carries only a subset (21) of all gallstone susceptibility alleles. Therefore, to confirm loci from previous studies and to identify the entire ensemble of Lith loci in inbred mice, we are conducting a further series of QTL crosses between cholesterol gallstone-susceptible and -resistant inbred mouse strains that were selected on the basis of a large strain survey (22).

Abbreviations: Aheb11, bile salt export pump (Bsep); Apobec1, apolipoprotein B mRNA editing complex 1; CAST, CAST/Ei; Cav, caveolin; Cav2, caveolin 2; Cfb, cystic fibrosis transmembrane conductance regulator; ChMC, cholesterol monohydrate crystal; chr, chromosome; CI, confidence interval; CSI, cholesterol satu ration index; Cyp7a1/CYP7A1, cholesterol 7α-hydroxylase; D2, DBA/2J; F2, first filial generation; F2 second filial generation or intercross; Lith, lithogenic locus; LOD, logarithm of the odds ratio; Lrp2/LRP2, low density lipoprotein receptor-related protein 2; Nh4h3/NR1H3, nuclear oxysterol receptor, (Lxra/ LXRα); Pparγ/Pparγ, peroxisome proliferator-activated receptor γ; QTL, quantitative trait locus/loci; Rara, retinoid X receptor α; Slc21a1/SLC21A1, solute carrier family 21A, common name Oatp/OATP, organic anion transporting polypeptide.

1 Presented in part at the Digestive Diseases Week 2002, San Francisco, CA, and published as an abstract in Gastroenterology 2002; 122: A626.
2 DNA sequences cited in this manuscript have been submitted to GenBank with the following accession numbers: AY236532, AY236533, AY236534, AY236535, AY236539, AY236531, AY236528, AY236529, AY195869, AY195868, AY236536, and AY236537.
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7 The online version of this article (available at http://www.jlr.org) contains three supplemental tables.
This intercross between the gallstone-susceptible inbred strain CAST/Ei (CAST) and the gallstone-resistant inbred strain DBA/2J (D2) confirmed the previously identified locus Lith1 and detected three new Lith loci named Lith6 [reported in abstract form (19)], Lith10, and Lith11. We provide evidence that Lith6 represents a complex locus that is likely determined by two closely linked QTL, for which the candidate genes are Pparg and Slc21a1.

MATERIALS AND METHODS

Animals and diet

Animals, breeding protocols, and facilities have been described in detail elsewhere (23). At 6 to 8 weeks of age, mice were transferred from chow to the cholesterol gallstone-promoting [lithogenic (11, 22)] diet. Parental strains and first filial generation (F1) mice were fed the diet for 8 weeks, whereas second filial generation (F2) progeny consumed the diet for 10 weeks. Animals were allowed free access to food and water. All animals fasted 4 h prior to sacrifice by cervical dislocation. The Institutional Animal Care and Use Committees of The Jackson Laboratory and Harvard University approved all protocols.

Phenotyping of hepatic and gallbladder biles

To define the physical-chemical sequences of cholesterol gallstones, we collected both gallbladder and hepatic biles from each strain. The gallbladders of the two strains were very small (D2, 4.5 ± 0.5 mg, n = 45; CAST, 6.5 ± 0.3 mg, n = 45 μL). Parental male mice were fed the lithogenic diet for 4 weeks. Gallbladders were removed, punctured at the fundus, and bile collected. A second group of animals were anesthetized with intraperitoneal injections of a cocktail comprising xylazine (20 mg kg−1; Phoenix Pharmaceutical, Inc., St. Joseph, MO) and ketamine (100 mg kg−1; Fort Dodge Animal Health, Fort Dodge, IA) prior to the introduction of a biliary fistula introduced via the gallbladder fundus (24). Anesthesia was maintained with ketamine alone. Gallbladder bile was allowed to drain (5 min) prior to hepatic bile collection by gravity (60 min, 37 ± 0.5°C). Hepatic bile volume was determined gravimetrically (assuming a density of 1 g ml−1). Both gallbladder and hepatic biles were stored at −20°C and lipid analyses conducted as described (24).

Cholesterol gallstones

To determine prevalence rates, mice of the parental strains CAST and D2 (n = 10 per gender and strain) and the reciprocal F1 progeny (n = 10 per lineage male; n = 5 per lineage female) and male F2 mice (n = 278) were phenotyped for cholesterol gallstones using standard methods (principally polarizing light microscopy) (5, 25).Opaque solid cholesterol gallstones were counted, air dried overnight, and weighed (5). In addition to the gallstone score (18, 24) assigned to bile samples, aggregated cholesterol monohydrate crystals (ChMCs) were semiquantified using a 0–4 scale, where 0 represented absence of aggregated crystals and 4 represented a gallbladder full of aggregated crystals. Livers were removed, frozen in liquid nitrogen, and stored at −80°C for isolation of mRNA.

Genotyping and QTL analyses

Genotyping and parameters for QTL analyses have been previously described for this intercross (23). QTL analyses were performed using the multistage analysis of Sen and Churchill (26), whose application was described recently in detail (24). To determine the likelihood that a QTL comprised more than one locus linked to the respective phenotype, the QTL on chromosome (chr) 2 and chr 6 were fit with models comprising one, two, or three QTL and a maximum logarithm of the odds ratio (LOD) score calculated for each. Employing permutation testing, we determined that increases of 2.0, 1.6, and 1.4 in the LOD score (∆LOD) are the thresholds that define multiple QTL at the 95%, 90%, and 80% confidence levels, respectively.

Candidate gene mapping and sequencing

From the mouse genome database (ENSEMBL), two candidate genes encoding Na+−independent solute (organic anion) carriers were identified on distal chr 6. Slc21a1 was mapped to chr X using fluorescent in situ hybridization (27), whereas Slc21a5 was mapped to its cytogenetic band on chr 6. Therefore, the T31 Mouse Radiation Hybrid Panel (Research Genetics, Huntsville, AL) was employed to map the positions of Slc21a1 (Outpl) and Slc21a5 (Outp2) following the recommendations of The Jackson Laboratory T31 Mouse Radiation Hybrid Database (http://www.jax.org/resources/documents/cmdata/rhmap/rh.html). As described (23), primer pairs that discriminated between the mouse and hamster genes were identified (Slc21a1, 5′-agt tat gag cta gtc aag gaa gag c-3′ (forward) and 5′-gag gat cta ttc acc cta tgt c-3′ (reverse); Slc21a5, 5′-tic agg ccc ttc ttt cat tac tcc-3′ (forward) and 5′-gga cta gtt ttc ttc cct tga-3′ (reverse). We sequenced cDNA and ∼750 to 1,000 nucleotides proximal to the transcription start site of Pparg and Slc21a1, two key candidate genes for Lith6, using previously described methods (23).

mRNA expression analyses

We evaluated hepatic mRNA expression profiles of candidate genes using quantitative (real-time) PCR. Expression differences between the parental strains (n = 5 per strain) could be regulated by elements lying within the QTL or by elements located elsewhere in the genome. To determine which was true, we selected F2 progeny (n = 10–15 per genotype), which possessed identical alleles in the locus of interest but random combinations of alleles at all other loci. If differential expression between genotypes was confirmed in these F2 groups, we concluded that regulatory elements must be located within the QTL region. As described elsewhere (23), primers were designed and verified and analyses performed. (Primer sequences are presented as supplementary data.)

General statistical analyses

Data are presented as means ± SEM and were analyzed using Graphpad Prism (Windows v3.00, GraphPad Software, San Diego, CA). Continuous data (e.g., biliary secretion rates, mRNA expression) were analyzed using Student’s t-test. Bonferroni post-test was applied to the analyses of mRNA expression data. P < 0.05 was considered significant.

RESULTS

Gallstone prevalence and distribution

Gallstone prevalence rates are presented in Fig. 1. Mice from the parental strains developed sandy (slucent) cholesterol gallstones, but no solid (opaque) gallstones (n = 40). In F1 mice, females were gallstone resistant, but males were gallstone susceptible (0 vs. 100% prevalence); three F1 males developed solid cholesterol gallstones. These results induced us to do the following with regard to the F2 animals: 1) only males were phenotyped (the sexual dimorphism was not pursued); 2) the feeding regimen was prolonged to 10 weeks; 3) both sandy and solid stones
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were included to determine prevalence. Because no difference was observed between male reciprocal F1 progeny, cholesterol gallstone susceptibility in this cross was not inherited by maternal or imprinted genetic factors.

The distributions of the gallstone phenotypes for the F2 population are depicted in Fig. 2. Of those mice that developed solid gallstones (n = 58; Fig. 2A, B), the mean mass was 0.74 ± 0.67 mg (mean ± SD, range 0–3.2 mg) and the mean number was 5.4 ± 5.3 (mean ± SD, range 0–28). The gallstone score phenotype resembled a binary distribution (i.e., absence/presence) with approximately twice as many animals developing cholesterol gallstones (score = 2) as those not displaying phase separation (score = 0; Fig. 2C). Similar numbers of F2 mice displayed no aggregated ChMCs (n = 117) or varying degrees of aggregated ChMCs (n = 154) (Fig. 2D).

**Biliary lipid analyses**

**Hepatic bile.** Strains CAST and D2 displayed no differences in bile salt or phospholipid secretion rates over 60 min (Fig. 3A) or in total bile salt or phospholipid compositions (Fig. 3B), but CAST demonstrated significantly more cholesterol in hepatic bile compared with strain D2 (P < 0.01; Fig. 3A, B). Male CAST mice displayed an increased rate of bile flow compared with D2 males (137.3 ± 4.3 vs. 88.9 ± 6.4 μl min⁻¹ kg⁻¹, respectively; n = 7 per group, P < 0.0001). Because CAST mice do not differ from D2 in total bile salt or phospholipid secretion rates, we inferred that the increased bile flow was most likely bile salt independent (28). Indeed, when we calculated the bile salt-independent bile flow by extrapolating bile salt output to a theoretical value of zero, CAST displayed greater flow than did D2 (Fig. 3C). This conclusion was supported further by the significantly lower total lipid concentration of hepatic bile displayed by strain CAST compared with strain D2 (1.85 ± 0.14 vs. 2.41 ± 0.16 g dl⁻¹, respectively; n = 9–12 per group, P < 0.0005). The calculated cholesterol saturation indices (CSIs) of hepatic bile (3) showed that CAST exhibited a significantly greater CSI compared with D2 (P < 0.0005; Fig. 3D), thus explaining its greater propensity to form cholesterol gallstones.

**Gallbladder bile.** Using individual D2 gallbladder bile (n = 3) and a pooled sample of CAST gallbladder bile, we confirmed that the CAST gallbladder bile CSI was greater than that of strain D2, in agreement with the hepatic bile CSI (Fig. 3D). Although the hepatic biles of both strains exhibited a CSI > 1, only the gallbladder bile of CAST mice ex-
Hepatic biliary lipid analyses of parental strains CAST (open) and D2 (filled) after 4 weeks' consumption of the lithogenic diet. An acute biliary fistula was fashioned and hepatic bile collected via the gallbladder for 60 min. Strain CAST displayed a significantly greater cholesterol secretion rate compared with strain D2 (A). The lipid composition of hepatic bile (B) indicated that CAST differed significantly from D2 only in cholesterol composition. CAST displayed higher bile salt-independent bile flow (C) compared with D2. The cholesterol saturation indices (CSIs, D) were calculated from the critical tables for taurocholate-rich bile (40). CAST displayed greater cholesterol saturation in hepatic bile compared with strain D2. This finding was confirmed for gallbladder bile (D) using individual D2 biles and a single sample of pooled bile from CAST mice. Note that the CSI < 1 for D2 gallbladder bile accounts for the gallstone resistance of this strain, whereas CSI >> 1 for strain CAST reflects its gallstone susceptibility. Despite all efforts to the contrary, the very high CSI of CAST gallbladder bile suggests the admixture of microscopic ChMCs in the sample. However, this does not affect the observation that CAST gallbladder bile was cholesterol supersaturated, whereas D2 gallbladder bile was not supersaturated. Data represent mean ± SEM; n = 8–12 per strain except D, gallbladder bile (n = 1, CAST; n = 3, D2). aP < 0.005; bP < 0.01; cP < 0.0005.

QTL analyses

Three gallstone phenotypes (score, gallstone presence, stone weight) were used for genome-wide scans. Single QTL are presented in Fig. 4, but no gene interactions (epistasis) were detected. Suggestive (LOD > 2.2, P < 0.10) and significant (LOD > 3.3, P < 0.05) LOD scores were determined by permutation testing (29). Because the genome-wide scans for gallstone score and gallstone presence were similar, we present data for gallstone presence only. The gallstone presence scan (Fig. 4A) detected a significant QTL (D2Mit94, peak 52 cM) that confirmed the previously detected QTL, Lith1 (11, 16), and a suggestive QTL on chr 6 (D6Mit86, peak 0 cM); the latter locus became significant.

Fig. 3. Hepatic biliary lipid analyses of parental strains CAST (open) and D2 (filled) after 4 weeks' consumption of the lithogenic diet. An acute biliary fistula was fashioned and hepatic bile collected via the gallbladder for 60 min. Strain CAST displayed a significantly greater cholesterol secretion rate compared with strain D2 (A). The lipid composition of hepatic bile (B) indicated that CAST differed significantly from D2 only in cholesterol composition. CAST displayed higher bile salt-independent bile flow (C) compared with D2. The cholesterol saturation indices (CSIs, D) were calculated from the critical tables for taurocholate-rich bile (40). CAST displayed greater cholesterol saturation in hepatic bile compared with strain D2. This finding was confirmed for gallbladder bile (D) using individual D2 biles and a single sample of pooled bile from CAST mice. Note that the CSI < 1 for D2 gallbladder bile accounts for the gallstone resistance of this strain, whereas CSI >> 1 for strain CAST reflects its gallstone susceptibility. Despite all efforts to the contrary, the very high CSI of CAST gallbladder bile suggests the admixture of microscopic ChMCs in the sample. However, this does not affect the observation that CAST gallbladder bile was cholesterol supersaturated, whereas D2 gallbladder bile was not supersaturated. Data represent mean ± SEM; n = 8–12 per strain except D, gallbladder bile (n = 1, CAST; n = 3, D2). aP < 0.005; bP < 0.01; cP < 0.0005.

Fig. 4. Genome-wide QTL analyses for single cholesterol gallstone susceptibility loci in the F2 population derived from strains CAST and D2. Chromosomes (chrs) 1 through X are represented numerically on the ordinate. The relative width of the space allotted for each chr reflects the number of simple sequence length polymorphism markers on each chr. The abscissa represents the logarithm of the odds ratio (LOD) score, the traditional metric of genetic linkage. The significant (LOD > 3.3, P < 0.05) and suggestive (LOD > 2.2, P < 0.10) levels of linkage were determined by permutation testing (29). Gallstone presence (0–1) is presented in A, gallstone number in B, aggregated ChMCs in C. Fine-mapping of the QTL for aggregated ChMCs on chr 2 (D) and chr 6 (E) are presented (solid line). The posterior probability density (broken curved line) is a likelihood statistic that gives rise to the 95% confidence intervals that are indicated by the gray bars (26). These data suggest that a third QTL exists on chr 2 and that at least two QTL exist on chr 6.
using gallstone number as the phenotype (peak 4 cM; Fig. 4B). The gallstone number analysis detected a significant QTL on distal chr 8 (D8Mit88, peak 58 cM). Finally, the genome-wide scan for aggregated ChMCs detected two suggestive QTL on chr 2 (proximal D2Mit79, peak 8 cM; distal D2Mit94, peak 46 cM) and one significant QTL on distal chr 6 (D6Mit62, peak 54 cM; Fig. 4C). However, the QTL fine-mapping of these loci suggests the presence of three QTL on chr 2 (Fig. 4D) and two to three QTL on chr 6 (Fig. 4E). Details of the QTL are presented in Table 1. We named the significant loci as follows: proximal chr 6, Lith10; distal chr 6, Lith6; and distal chr 8, Lith11.

Application of statistical models indicated that the QTL on chr 2 for gallstone presence was best fit by a two- or three-QTL model (95% and 90%, respectively; Table 2). The additional QTL likely reside at the proximal to mid region of chr 2 (Fig. 4D). Consistent with the QTL fine-mapping (Fig. 4E), the QTL on chr 6 for aggregated ChMCs suggested the existence of two closely linked QTL (80% confidence; Table 2), possibly accounting for the relatively large 95% confidence interval (CI) (40–70 cM) of Lith6.

At each simple sequence length polymorphism (SSLP) marker linked to a QTL, we determined the allele effect that shows which strain contributed the gallstone-susceptibility allele, the mode of inheritance, and the magnitude of the effect (Fig. 5). Gallstone susceptibility was determined by recessive D2 alleles at the QTL on proximal chr 2 (Fig. 5A), Lith10, (Fig. 5C) and Lith11 (Fig. 5E), by a dominant D2 allele at Lith1 (Fig. 5B), and by a dominant CAST allele at Lith6 (Fig. 5D).

**Candidate gene analyses**

QTL analysis detects fundamental genetic differences in genes encoding key regulatory proteins (13). The mutations or polymorphisms may be in regulatory or coding regions of the genome, or both, resulting in altered transcription levels, mRNA stability, or amino acid sequences. To initiate our search, we identified positional candidate genes within the 95% CIs of the QTL that possessed a direct or indirect role in lipid metabolism. We stipulate that our conclusions are based on mRNA expression data, but definitive exclusion of candidate genes requires cDNA sequencing.

**Candidate gene mapping**

Both Slc21a1 and Slc21a5 mapped to chr 6, both with the highest anchor LOD to D6Mit58 (67.0 cM). Therefore, we confirmed experimentally the localization of these genes on distal chr 6 in the Slc21a cluster.

**mRNA expression**

Candidate genes were evaluated by determining whether their hepatic mRNA expression profiles (Fig. 6) were consistent with their known activities and allele effects. The QTL on distal chr 2 colocalized with Lith1, for which Abcb11, encoding the canalicular bile salt export pump (Bsep, 38.4 cM), is a strong candidate gene. Abcb11 was located outside of the 95% CI of the QTL detected in this intercross, and, not surprisingly, we detected no difference in Abcb11 mRNA (Fig. 6A) or in hepatic bile salt secretion rate (Fig. 3A). Nr1h3 (Lxra, 40.4 cM), encoding the nuclear oxysterol receptor LXRα, lay within the 95% CI. After adjustment for multiple comparisons, the difference in Nr1h3 expression between the parental strains was nonsignificant (Fig. 6A). Furthermore, genes whose transcription is activated by NR1H3, Cyp7a1 and Abeg5/Abeg8, were not up-regulated in CAST (data not shown), as expected if Nr1h3 determined Lith1. We concluded that both Abcb11 and Nr1h3 were not likely responsible for the QTL on distal chr 2 in this intercross. An association in humans was found recently between gallstones and a poly-

**TABLE 1. QTL for cholesterol gallstone formation identified in the CAST × D2 intercross**

| Locus (cM) | QTL Name | Phenotype | chr | LODe | Peak (cM) (95% CI) | Variance (%) | Susceptible Allele, Inheritancea | Overlapping QTL (Ref:;) | Candidate Genes (cM) |
|-----------|----------|-----------|-----|------|-------------------|--------------|---------------------------------|----------------------|-------------------|
| D2Mit79 (10.0) | D2Mit94 (48.3) | Agg. ChMC | 2 | 3.0 | 8 (0–20) | 4.8 | D2, Rec | Gel (16), Bsep (17) |
| D2Mit86 (6.5) | Lith10 | Stone presence | 6 | 2.8 | 0 (0–6) | 4.6 | D2, Rec | Cav (3.2), Cyp2a (2.3), Cyp2a (3.1) |
| D6Mit88 (58.0) | Lith11 | Stone number | 8 | 3.5 | 58 (48–60) | 5.2 | CAST, Dom | Ppar (52.7), Apo1m (54.5), Slc21a (67) |

chr, chromosome; CI, confidence interval; cm, centimorgan; LOD, logarithm of the odds ratio; QTL, quantitative trait locus.

a Using permutation analyses, suggestive QTL LOD > 2.2, significant QTL LOD > 3.3.

b Susceptible allele and mode of inheritance: Rec, recessive; Dom, dominant.

c Reference.

d Carnlinus ester lipase.

e Retinoid X receptor.

f Reference 16 and unpublished data, Wittenburg, Carey, and Paigen.

References

1. Nuclear oxysterol receptor, common name Lxra.

2. Canalicular bile salt export pump, common name Bsep.

3. Caveolin.

4. Caveolin 2.

5. Cystic fibrosis transmembrane conductance regulator.

6. Peroxisome proliferator activated receptor γ.

7. Apolipoprotein B mRNA editing complex 1.

8. Solute carrier family 21a gene cluster encoding members 1 (Oatpl), 5 (Oatp2), 7 (Oatp3), and 10 (Oatp4).

9. QTL detected with second filial generation (F2) lineage as covariate.


**TABLE 2. Determination of number of loci on chrs 2 and 6 using models describing one, two, and three QTL.**

| Phenotype | chr | LOD1 | LOD2 | LOD3 | LOD3 - LOD1 | Number of QTL |
|-----------|-----|------|------|------|-------------|---------------|
| Stone presence | 2   | 3.1  | 5.2  | 2.1  | 7.0         | 1.8           | 5             |
| Agg. ChMC | 6   | 3.7  | 5.1  | 1.4  | 5.6         | -             | 2             |

ChMC, cholesterol monohydrate crystal. Using permutation analyses for an intercross of 278 mice, we determined that increases of 2.0, 1.6, and 1.4 or greater in the LOD score between models are the thresholds used to declare multiple QTL at the 95%, 90%, and 90% confidence levels, respectively. The data indicate that three QTL likely exist on chr 2 that determine the gallstone presence phenotype. Similarly, the data suggest the existence of two QTL for the aggregated ChMC phenotype.

+ LOD1 is the LOD score calculated for the one-QTL model; LOD2 refers to the two-QTL model, and LOD3 refers to the three-QTL model. ΔLOD is the difference between the respective models.

**DISCUSSION**

Our biliary lipid analyses indicated that the physical-chemical explanation for cholesterol gallstone susceptibility of male CAST mice was cholesterol hypersecretion. Using an intercross between strains CAST and D2, we detected three new, significant QTL (Lith6, Lith10, and Lith11) and one suggestive QTL (proximal chr 2) for cholesterol gallstone susceptibility. In addition, a significant, fifth QTL coincided with Lith1, a QTL identified previously (11, 16). We used three different phenotypes to evaluate gallstone susceptibility (aggregated ChMCs, gallstone number, and gallstone presence). Not surprisingly, these alternative methods of trait evaluation resulted in identification of the same QTL. Unexpectedly, the susceptible strain CAST contributed one susceptibility allele, Lith6, whereas the resistant strain D2 contributed the susceptibility alleles at the four remaining QTL. These observations account for the increased gallstone prevalence in F1 intercrosses.
Seven studies conducted previously in our laboratories indicated suggestive genetic linkage between cholesterol gallstone formation and distal chr 6 using a variety of different strains, which yielded eight QTL [(16–18) and M. C. Carey and B. Paigen, unpublished observations]. Four QTL clustered between 54 cM and 56 cM, whereas the remaining four QTL clustered between 65 and 71 cM (see supplementary data). Pparg and Slc21a1 are located on chr 6 at 52.7 cM and 67.0 cM, respectively. Furthermore, the 95% CIs are generally broad, often an indicator of multiple QTL, in our experience. In this study, both QTL fine-mapping (Fig. 4E) and modeling (Table 2) suggested that two QTL were present on distal chr 6. In addition to Pparg and Slc21a1, potential candidate genes for this locus included Apobec1 and a further three Na⁺-independent bile acid and bile salt transporters (32, 33). We confirmed that Slc21a1 mapped to chr 6 using radiation hybrid mapping, a position consistent with the ENSEMBL mouse genome assembly, the Mouse Genome Database (http://www.informatics.jax.org/), and the position of human SLC21A3 on chr 12p12 (34), which is orthologous to mouse distal chr 6. We can only conclude that the original report that mapped Slc21a1 to mouse chr X was an artifact. Overall, we determined that Apobec1, Slc21a5, Slc21a7, and Slc21a10 did not underlie Lith6. However, all data were consistent with Pparg and Slc21a1 being candidate genes for Lith6. The major finding from the present data is the identification of the new cholesterol gallstone susceptibility QTL, Lith6, which is likely to result from two QTL in close proximity.

We postulate that Pparg and Slc21a1 might affect intracellular bile salt concentrations and the availability of cholesterol for biliary secretion. It is now recognized that PPARγ is expressed at low levels in the liver, particularly in cases of obesity, type 2 diabetes, and steatosis (35, 36). One putative role of PPARγ in cholesterol gallstone formation is its derepression of CYP7A1 activity (37). Additionally, troglitazone, a synthetic PPARγ agonist, increased Cyp7a1 expression when fed to mice (38), although we did not observe differences in Cyp7a1 mRNA (data not shown). However, because PPARγ is a transcription factor, it probably regulates many more genes than Cyp7a1 alone. Hepatic basolateral bile salt uptake is predominantly mediated via the Na⁺-dependent taurocholate cotransporting polypeptide, encoded by Slc10a1 (Ntcp), and the Na⁺-independent solute (organic anion) carriers of the Slc21a family (32, 33). We observed nucleotide changes in Slc21a1 that caused four changes in the amino acid sequence of SLC21A1 from CAST compared with D2 (T475S, C538S, V660F, V8I; supplementary data). The alignment of sequences from mouse, rat, and human indicated that within this subfamily, valine and isoleucine are interchangeable at residue 8 and phenylalanine is present in one human member at residue 660, suggesting that such amino acid substitutions may not be important for function. However, threonine and cysteine are conserved at residues 475 and 538, respectively (33), suggesting that these changes might cause a functional variation in protein activity. We speculate that increased CAST SLC21A1 activity (rather than decreased as implied by the CAST mRNA expression data) might increase the availability of bile salts, resulting in a concomitant decrease in cholesterol catabolism [via NR1H4/farnesoid X receptor (FXR)-mediated inhibition], thereby increasing the availability of cholesterol for biliary secretion.

A comment is required regarding our conclusion that...
Nrlh3 was unlikely to determine the QTL that coincided with Lith1 and the possibility of confounding effects caused by cholic acid (in the lithogenic diet) and NR1H4 (FXR) activation. Previously, it was demonstrated that NR1H4-mediated repression of Cyp7a1 transcription was able to overcome NR1H3 (LXRα)-mediated activation of Cyp7a1 transcription (39). However, these data do not support the candidacy of Nrlh3 for the following reasons: 1) because no QTL was detected in any of the regions harboring Nrlh4, Nrlh02 (Shp), or Nrlha2 (Lith), we inferred that the effect of NR1H4 activation was similar in all F2 mice; 2) for the locus coincident with Lith1 to be detected by QTL analysis, it must have affected gallstone susceptibility; and 3) no sequence differences causing amino acid changes were observed in Nrlh3 (23). Therefore, because NR1H4 activation overcomes NR1H3 activation, and NR1H3 did not display altered function due to inherent amino acid changes, gallstone susceptibility was not affected by NR1H3.

In summary, this study demonstrated that gallstone susceptibility of strain CAST was primarily due to cholesterol hypersecretion, and identified new Lith loci on proximal chr 6 (Lith10), distal chr 6, (Lith6) and distal chr 8 (Lith11). Lith6 is likely a complex locus comprising two linked QTL. However, none of the candidate genes we tested fully accounted for the cholesterol hypersecretion of strain CAST. Elucidation of the underlying mechanisms awaits completion of the construction of overlapping congenic strains, which will enable us to resolve the multiple QTL, to narrow the individual QTL regions, and importantly, to test the hypotheses that were generated in this study. This work further demonstrates the complexity of the genetic origins of cholesterol gallstone formation and its pathobiology and likely will illuminate new approaches toward understanding this very common human disease.

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