New quantitative trait loci that contribute to cholesterol gallstone formation detected in an intercross of CAST/Ei and 129S1/SvImJ inbred mice

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Submitted 28 April 2003; accepted in final form 25 June 2003

Cholesterol gallstone formation (cholelithiasis) is ultimately derived from multifactorial and heterogeneous hepatobiliary phenomena. Investigation of model (12, 48, 53), animal (20, 54, 55), and human biles (1, 9, 52) contributed greatly to the elucidation of the pathophysiological underpinnings of cholelithiasis. Exploration in vivo of both humans (15) and mouse models (22) demonstrated that a complex genetic basis, comprising multiple genes and their interactions with environmental factors (summarized in Ref. 41), predisposes individuals to cholesterol gallstone formation. As knowledge of cholesterol homeostasis and bile formation progresses, so too does our ability to identify genes that participate in cholesterol gallstone formation (23).

Ideally, the identification of the primary genetic determinants that underlie this disorder should allow a true understanding of the pathophysiology of cholesterol gallstone formation. This should lead to novel means of risk assessment, nonsurgical management, and ultimately prevention of this prevalent and costly disorder (44). Quantitative trait locus/loci (QTL) analysis is a powerful, phenotype-driven experimental approach that associates a trait with a genotype, thereby defining genomic regions that harbor genes conferring the given trait in inbred mice (41, 58). The aim of this approach is to identify the entire complement of genes (Lith genes) that carry polymorphisms (i.e., alleles) determining cholesterol gallstone susceptibility (23, 58) in the mouse model. Based on the conservation of the mouse and human genomes, these data will allow for the prediction and evaluation of the corresponding human genes. Inclusive of this report, 14 major QTL for cholesterol gallstone formation, named Lith1 through Lith14 (22, 24, 25, 29, 41, 42, 56, 59), are now known. Of these, pathophysiologically relevant candidate genes were suggested for Lith1 (25), Lith2 (4), Lith6 (29), Lith7, Lith8, and Lith9 (59).

This study comprises the second intercross between the gallstone-susceptible, wild-derived inbred strain CAST/Ei (CAST) and a gallstone-resistant inbred mouse strain, 129S1/SvImJ (strain 129). The first intercross between CAST and DBA/2J was reported previously (29). In the present QTL analysis, we detected one new QTL on proximal chromosome 5 (Chr 5), which we named Lith13. We confirmed a locus, Lith6, previously identified on distal Chr 6 (29). Two additional loci...
on distal Chr 2 (Wittenburg H, Carey MC, and Paigen B, unpublished observation) and Chr 16 (24), detected below the significance threshold in two previous crosses, were confirmed and named Lith12 and Lith14, respectively.

MATERIALS AND METHODS

Experimental Design

It is apparent that inbred mouse strains each carry only a subset of the complete, heterogeneous set of alleles for a given trait (17), in this instance, cholesterol gallstone susceptibility. Consistent with these premises, our studies, thus far, demonstrated that cholesterol gallstone susceptibility alleles are contributed by both gallstone-susceptible and -resistant parental inbred strains. On the basis of a large strain survey (5) (http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn=projects/details&id=29), we selected eight inbred strains to intercross in a "daisy chain" experimental design. We aimed to maximize the genetic diversity of the eight strains, which comprised four gallstone-resistant and four gallstone-susceptible inbred strains. These strains were alternatively intercrossed such that each gallstone-susceptible strain was intercrossed with two gallstone-resistant strains and vice versa. Because each strain is crossed to two other strains, this design should provide us a greater probability of detecting a Lith gene carried by a single parental strain (50). Additionally, it should enable us to approximate allele frequencies since common variants are likely to segregate in multiple intercrosses (50). Finally, combining data from separate intercrosses will likely enable us to resolve linked QTL and narrow 95% confidence intervals (CI) (50). This intercross comprises two genetically diverse inbred strains that differ in their susceptibility to cholesterol gallstone formation: strain CAST, gallstone susceptible, and strain 129, gallstone resistant (5, 40).

Animals and diet. This study was conducted concurrently with an intercross between strains CAST and DBA/2J. Animals, breeding protocols, and facilities were identical to those described in detail previously (28) except that strain 129 mice were used instead of DBA/2J mice. The male mice displayed greater phenotype variation than female mice, and therefore we evaluated male F2 progeny only because they conferred the greatest statistical power for QTL detection. In addition, the results of our introductory studies (RESULTS, Fig. 2) induced us to prolong the feeding regimen to 10 wk for the F2 mice in an attempt to increase solid gallstone formation. At 6–8 wk of age, the different populations of animals initiated consumption of the cholesterol gallstone-promoting (lithogenic) diet for periods between 4 and 10 wk. Previously, cholesterol gallstone formation was demonstrated to constitute a distribution among different strains of inbred mice when fed the lithogenic diet (5, 22, 40). Animals were allowed free access to food and water. All animals fasted for 4 h prior to death. The Institutional Animal Care and Use Committees of the Jackson Laboratory and Harvard University approved all experimental protocols.

Hepatic and gallbladder bile. To define the physical-chemical sequences of cholesterol gallstone formation in male mice of strains CAST and 129, we collected both gallbladder bile and hepatic bile and performed lipid analyses using our standard methods (54). We demonstrated previously that the biliary lithogenic environment that precedes gallstone formation is rapidly established upon commencement of the lithogenic diet (24, 55, 57). To avoid blocking the free flow of hepatic bile through the cannula and to avoid the introduction of solid cholesterol crystals into gallbladder bile destined for lipid analyses, male mice (6–8 wk old) of each parental strain were fed the lithogenic diet for 4 wk only (n = 6–9 per strain). Gallbladder bile was collected by puncturing the gallbladder fundus. To collect hepatic bile for determination of composition and biliary secretion rates over 1 h, animals were fasted 4 h, anesthetized with an intraperitoneal injection of xylazine (20 mg/kg; Phoenix Pharmaceutical, St. Joseph, MO) and ketamine (100 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA), and then a biliary fistula was introduced via the gallbladder under aseptic conditions. The gallbladder was exposed by a transverse incision below the sternum. Polyethylene tubing (ID 0.28 mm, OD 0.61 mm; Intramedic PE-10 tubing; Becton-Dickinson, Sparks, MD) was inserted into the gallbladder fundus and secured with silk suture. Animals were maintained at 37 ± 0.5°C, and fluid loss was minimized by covering the wound with saline-saturated gauze. Anesthesia was maintained with ketamine alone. Gallbladder bile was allowed to drain (5 min) prior to collection (60 min) of hepatic bile, whose volume was determined gravimetrically (assuming a density of 1 g/ml).

Cholesterol gallstone phenotypes. Mice of the parental strains CAST and 129 (n = 49 per parental strain) and the reciprocal first filial generation [i.e., (CAST × 129)F1 and (129 × CAST)F1] (n = 10–18 male and n = 6–8 female per lineage) were fed the lithogenic diet for 8 wk prior to phenotyping for cholesterol gallstones using standard methods in our laboratories (principally based upon microscopic appearance of bile using polarized light), which included gravimetric determination of gallbladder volume (GBV) (55, 56). The second filial (F2 or intercross) generation comprised males only (n = 277; however, 7 lacked gallbladders). Since the parental CAST mice exhibited intermediate prevalence, and neither the CAST nor the combined F1 mice developed solid gallstones, we elected to extend the feeding period of the F2 mice in an attempt to increase the prevalence of solid stones in that population. Thus the F2 mice were phenotyped for gallstones after 10 wk of consumption of the lithogenic diet. Furthermore, cholesterol gallstone prevalence was defined to include both sandy (translucent) and solid (opaque) cholesterol gallstones.

Gallbladder bile, gallstones, and cholesterol crystals from the F2 mice were collected the same way as the parental animals. One investigator (M. A. Lyons) performed all quantifications. When present, opaque solid cholesterol gallstones were counted, air-dried overnight, and weighed (55). Gallstone weight was abbreviated “GSW.” A semi-quantitative score designated “Solid” was applied according to a simultaneous evaluation of both the number and size of opaque stones (0 = absence, through 4 = most severe, i.e., largest size, greatest number). As described formerly (56), bile samples were assigned a gallstone “Score” according to absence (Score = 0) or presence (Score = 1) of cholesterol monohydrate crystals (ChMC) and the detection of translucent “sandy” or solid gallstones (Score = 2). It should be appreciated that Score is a composite trait rather than a primary phenotype. Aggregated ChMC (AChMC) were semi-quantified using a 0-to-4 scale (0 = absence, through 4 = most severe). Thus we evaluated four cholesterol gallstone phenotypes: GSW, Solid, Score, and AChMC. We also evaluated the related trait GBV.

QTL analyses

Genotyping. DNA was prepared from tail samples and genotyping performed using simple sequence length polymorphisms (SSLP; n = 100; Fig. 1) that discriminate between CAST and strain 129 alleles (MapPairs primers; Research

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Genetics, Huntsville, AL) as described (28). The entire cohort of F₂ mice (n = 277) was genotyped using SSLP markers distributed across the genome (interval range 1–24.4 cM; Fig. 1). Reported genetic map positions were retrieved from the Mouse Genome Database (http://www.informatics.jax.org).

**QTL analyses.** To identify single and interacting QTL associated with the cholesterol gallstone traits GSW, Solid, Score, and AChMC, the multi-stage analysis of Sen and Churchill (47) was employed, the previous application of which was described extensively (56). This software, named PSEUDOMARKER, is available at http://www.jax.org/staff/churchill/labsite/software/index.html. A multiple imputation algorithm was used to account for missing marker genotypes (64 imputations) (47). Each trait was analyzed separately. In the first stage, single loci associated with the traits were detected by a genome-wide scan using 5-cM intervals. Fine mapping of those loci was performed at 2-cM increments throughout the region of interest. Significance thresholds were determined by experiment-wide permutation testing (n = 1,000 permutations) (10), which corrects for multiple comparisons in the genome-wide search. We defined significant loci as those that exceeded the 95th percentile (i.e., P < 0.05) of the permutation distribution, whereas the suggestive loci exceeded the 90th percentile (P < 0.10). Results are expressed as logarithm of the odds ratio (LOD) scores, the traditional measure of genetic linkage (27),
and 95% CI calculated as described (47). All scans were based on a free genetics model that places no constraints on the pattern of allele effects in this F2 population. In the second stage, we searched for genes that affected the trait by interacting with one another (epistasis) using a genome-wide simultaneous search for marker pairs employing a two-way ANOVA model, which included an interaction term. All marker pairs were tested by scanning at 5-cM intervals. We required overall significance of the locus pair at the genome-wide 0.05 level as determined by permutation analysis (10). Furthermore, we required the interaction component of the two-way ANOVA model to be significant at the (unadjusted) 0.01 level. This software uses an explicit multiple QTL model similar to the multiple interval mapping procedure (21).

Tests for multiple QTL. To determine the likelihood that a QTL comprised more than one locus linked to the respective phenotype, we fitted models comprising one, two, or three QTL, and a maximum LOD score was calculated for each. Employing permutation testing for an intercross of 277 mice, we determined that increases (ΔLOD) of 2.0, 1.8, and 1.6 in the LOD score between the one- and two-QTL models and between the two- and three-QTL models are the thresholds that define multiple QTL at the 95%, 90%, and 80% confidence levels, respectively. We regard the 95% level as significant, but report the additional thresholds to provide a scale for near-significant results.

QTL nomenclature. Colocalizing QTL were defined as QTL for which the locus peak fell within the 95% CI of the second QTL. A further criterion was that the 95% CI were substantially overlapping. We interpreted the colocalizing QTL for different gallstone phenotypes to represent identical QTL. Our approach to naming QTL is that significant QTL are named automatically, but suggestive QTL are named only when confirmed by two or more independent breeding crosses. This is in concordance with the recent consensus decision of the Complex Trait Consortium (L. Flaherty et al., unpublished observations).

Allele effects. For each of the QTL, we determined the “allele effect” by calculating the phenotype mean for each of the three possible genotypes. Using the error bars for each of the three genotypes, we determined which strain contributed the gallstone-susceptibility allele and whether that allele caused dominant, additive, or recessive inheritance of the gallstone susceptibility phenotype. A dominant allele was defined as exhibition of a gallstone-susceptible phenotype by the heterozygous genotype F2 population indistinguishable from the gallstone-susceptible homozygous genotype F2 population, whereas a recessive allele was defined as the heterozygous genotype F2 group being indistinguishable from the gallstone-resistant homozygous genotype F2 group. An additive allele was defined as an allele that produced a gallstone phenotype intermediate between each of the homozygous-susceptible and homozygous-resistant F2 genotype populations.

mRNA expression analyses of candidate genes. The utility of QTL analysis lies in its ability to detect fundamental genetic differences in genes encoding crucial regulatory proteins (23), which may lie in either regulatory and/or coding regions of the genome. As such, they can affect transcription efficiency, mRNA stability and/or amino acid sequences. From the 95% CI generated in the QTL analyses, we identified positional candidate genes whose products perform direct or indirect roles in lipid metabolism that colocalized with the QTL (Fig. 1). Our objective was to develop a preliminary screening assay to aid the evaluation of candidate genes’ putative contributions to gallstone formation. We determined hepatic mRNA expression levels of candidate genes in each of the parental strains, CAST and 129. Intercross progeny inherit unique combinations of alleles derived from both parental strains. Therefore, when differential expression of a candidate gene is demonstrated for animals that exhibit a homozygous genotype for one parental strain vs. the other only in the region harboring the candidate gene, it provides strong support for cis-acting elements controlling gene expression rather than trans-acting elements remote from the gene of interest. Hence, we investigated further Lith12, Lith13, and Lith6 in the F2 population. We selected individual samples that were either homozygous CAST or homozygous 129 over these loci on Chr 2, 5, and 6, respectively. To collect tissue for mRNA expression analyses, male mice of strains CAST and 129 (n = 5 per strain) were fed the lithogenic diet for 4 wk, and livers were harvested as described (28). Livers from F2 mice were removed at the time of phenotyping, frozen immediately in liquid nitrogen, and stored at −80°C. As described elsewhere (28), oligonucleotide primers were designed, verified, and expression analyses performed. (Primers and their sequences are available from the corresponding author.)

DNA sequencing and sequence analysis of candidate genes. DNA sequencing was performed as described (28). The cDNA and ~0.7 to 1.1 kb proximal to the transcription start site of Pparg and Slc21a1, two of the key candidate genes for Lith6, were sequenced from strain 129 to investigate putative polymorphisms that may determine mRNA expression differences and the existence of potential amino acid substitutions. These sequences were reported earlier for strain CAST (28). To investigate potential differences in the proximal putative promoter regions of Pparg1 and Pparg2 from the two strains, we compared the mouse Pparg and human PPARG sequences (ENSEMBL) using VISTA software (7, 33). Second, as necessary, we compared our strain sequences using MATCH software (v1.0, public), a matrix-based tool for searching transcription factor binding sites, and included a liver-specific search criterion (32).

General Statistical Analyses

Data are means ± SE and were analyzed using GraphPad Prism (Windows v3.00; GraphPad Software, San Diego, CA). Student’s t-test was used to compare the parental strains, CAST and 129, for continuous data (e.g., biliary secretion rates). GBV was analyzed by ANOVA with Bonferroni adjustment for multiple comparisons. Gallstone prevalence rates of the male reciprocal F1 and allele distributions of the F2 mice (i.e., 1.2:1 ratio for homozygous 129:heterozygous: homozygous CAST genotypes) were analyzed using chi-squared analysis. P < 0.05 was considered significant.

RESULTS

Gallbladder Volume

No significant differences were found in GBV between male and female mice for each respective strain. The F1 progeny did not exhibit significantly different GBV with respect to gender or direction of cross [i.e., (CAST×129)F1, and (129×CAST)F1]. Therefore, male and female mice of each strain were combined for comparison with all F1 animals. The GBV of strain CAST differed significantly from that of strain 129 (6.5 ± 0.8 vs. 40.0 ± 2.8 μl, respectively; P < 0.001, n = 19–20 per strain). The F1 mice (27.9 ± 2.6 μl, n = 41) were intermediate between, and differed significantly (P < 0.05) from, both parental strains.
Cholesterol Gallstone Prevalence

Figure 2 indicates that male and female CAST mice were gallstone susceptible with intermediate prevalence (both 40%, n = 10 per group), but conversely, both male and female strain 129 mice were gallstone resistant (both 0% prevalence, n = 10 per group). These data are consistent with our previous observations (40). Similar to strain 129 females, both groups of female reciprocal F1 animals (n = 6–8 per group) were gallstone resistant (both 0% prevalence; Fig. 2). However, male reciprocal F1 mice (n = 10–18 per group) were gallstone susceptible (67% (CAST×129)F1 and 90% (129×CAST)F1) (Fig. 2). Since the prevalence rates of the reciprocal male F1 mice did not differ significantly, cholesterol gallstone susceptibility in this cross was not inherited by maternal (e.g., mitochondrial) or imprinted (gene expression predominantly from either a maternal or paternal allele) genetic factors, and QTL analyses could detect autosomal regions carrying Lith genes. The F2 population displayed gallstone prevalence (58%, n = 270) intermediate between CAST and the F1 group (Fig. 2).

Biliary Lipid Analyses

Strain CAST displayed a significantly greater (1.9-fold, P < 0.0001) hepatic bile flow rate compared with strain 129 and displayed a trend (P < 0.06) toward a lower total lipid concentration (Fig. 3A). No difference was observed in bile salt secretion rates between the two strains, but CAST secreted significantly more phospholipid (P < 0.05) and cholesterol (P < 0.001) compared with strain 129 during the 60-min collection period (Fig. 3B). When the hepatic biliary lipid data were expressed proportionally, no difference was observed in the total bile salt composition, but it was confirmed that CAST displayed significantly greater (P < 0.0001) biliary cholesterol content (Fig. 3C). However, the phospholipid proportion of CAST hepatic biles was significantly reduced (P < 0.05) compared with that of strain 129 (Fig. 3C), a phenomenon that also contributed to biliary lithogenicity in strain CAST (53). Bile flow is an osmotic response to solutes secreted by the bile canaliculi and is determined by two factors: 1) bile salt-dependent flow, i.e., bile flow caused by bile salts secreted as anions; 2) bile salt-independent flow, i.e., bile flow caused by all other secreted solutes (14). Therefore, as an approximation (because the sample size was too small and the duration was too brief for accurate measurement), we plotted bile salt secretion against bile flow and, extrapolating to a theoretical bile salt concentration of zero, we determined that CAST very likely displayed greater bile salt-independent bile flow (Fig. 3D). These data are consistent with both the increased bile flow (Fig. 3A) displayed by strain CAST and the lack of difference in bile salt secretion rates (Fig. 3B) and bile salt composition (Fig. 3C) between the two strains. Finally, we determined the cholesterol saturation indices (CSIs) of both hepatic and gallbladder biles. Because of the small volumes available, gallbladder biles from strain CAST were pooled. Strain 129 displayed larger GBV, allowing individual determinations from this strain. Strain CAST displayed CSIs greater than strain 129 in both instances (Fig. 3E). Both strains exhibited CSI > 1 in hepatic bile, reflecting the low total lipid concentration of hepatic bile (53) and the presence of cholesterol-carrying vesicles that are more efficient cholesterol-solubilizing agents than mixed micelles (18). However, in gallbladder bile, CAST displayed CSI >> 1, whereas strain 129 displayed CSI < 1 (Fig. 3E). Despite all efforts to the contrary, the very high CSI of CAST gallbladder bile suggests the admixture of microscopic ChMC in the sample. However, this does not affect the observation that CAST gallbladder bile was cholesterol supersaturated, whereas strain 129 gallbladder bile was not supersaturated. These data provide a physical-chemical explanation for the gallstone susceptibility of strain CAST compared with the gallstone resistance of strain 129.

Cholesterol Gallstone Distributions in Intercross Progeny

The distributions of the GSW and gallstone number, Solid, Score, and AChMC phenotypes for the F2 progeny (n = 270) are depicted in Fig. 4. Despite the prolonged feeding period, and similar to both strain CAST and the F1 animals, very few F2 mice developed solid cholesterol gallstones (Fig. 4, A–C). For mice that developed stones, the mean gallstone mass was 0.08 mg (range 0–4.46 mg) and the mean gallstone number was 0.7 (range 0–32). The distributions of the Score...
(Fig. 4D) and AChMC (Fig. 4E) phenotypes indicated that substantial numbers of F2 mice displayed phase separation and cholesterol crystallization, regardless of the low prevalence of solid cholesterol gallstones. Because all animals shared a common environment, the differences in the phenotypic manifestations among F2 progeny are in agreement with complex inheritance of cholesterol gallstone susceptibility, with contributions from alleles at multiple loci derived from both parental strains.

**QTL Analyses**

The genome-wide scans for single QTL for four gallstone phenotypes (GSW, Solid, Score, AChMC) and GBV are presented in Figs. 5 and 6. Experiment-wide suggestive and significant LOD scores were determined to be LOD > 2.2 (P < 0.10) and LOD > 3.1 (P < 0.05), respectively, by permutation testing (10). Details of the individual QTL derived are presented in Table 1.

Analysis of GSW revealed the presence of one suggestive QTL on Chr 5 (3.0 LOD, peak 44.0 cM, D5Mit201; Fig. 5A). Using the Solid phenotype, a coincident, significant QTL was detected also on Chr 5 (4.0 LOD, peak 30.0 cM, D5Mit183; Fig. 5B). Interestingly, we detected a significant QTL for GBV (3.7 LOD, peak 30 cM, D5Mit255) whose peak coincided with the QTL for GSW and Solid (Fig. 5C). Fine mapping of these three QTL suggested the presence of multiple QTL: Solid suggested three loci (Fig. 5D); GSW suggested three loci (Fig. 5E); and GBV suggested two loci (Fig. 5F). The consensus positions of these three peaks on Chr 5 were 30, 36, and 42 cM. GBV is not a primary gallstone trait because it does not necessarily reflect gallbladder stasis. Thus we deemed the QTL for GBV on Chr 5 supportive of the QTL detected using the GSW and Solid traits, and we did not characterize the other suggestive QTL for this phenotype (Fig. 5C).

Analysis of the Score trait yielded five QTL (Fig. 6A). One locus was detected above the significance threshold on distal Chr 2 (3.6 LOD, peak 101 cM, D2Mit113). Additional to the significant QTL, four suggestive QTL for the Score trait were detected on proximal Chr 1 (2.3 LOD, peak 24 cM, D1Mit21), distal Chr 6 (2.5 LOD, peak 54.0 cM, D6Mit44), proximal Chr 14 (2.6 LOD, peak 10 cM, D14Mit98), and on the midsection of Chr 16 (2.3 LOD, peak 42 cM, D16Mit65). The QTL region on Chr 5 showed a peak for the Score phenotype; however, this peak failed to reach the level for a suggestive QTL. Upon analysis of the AChMC phenotype, a semi-quantitative phenotype that is related to the Score phenotype, we detected two suggestive and one significant QTL (Fig. 6B). The significant QTL for AChMC was identified on Chr 6 (5.2 LOD, peak 66 cM, D6Mit14). Suggestive QTL that coincided with QTL for the Score phenotype were detected on Chr 2 (3.0 LOD,

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**Fig. 3.** Hepatic biliary lipid analyses of parental strains CAST (open bars) and 129 (filled bars) after 4 wk of consumption of the lithogenic diet. An acute biliary fistula was fashioned, and hepatic bile was collected via the gallbladder for 60 min. A: hepatic bile flow. B: biliary lipid secretion rates. C: biliary lipid composition (mol%). D: bile salt-independent bile flow. The cholesterol saturation indices (CSI, E) of hepatic and gallbladder biles were calculated from the critical tables for taurocholate-rich bile (8). Data are means ± SE; n = 6–9 per strain except E, gallbladder bile (n = 1 pooled from 5 CAST mice; n = 7, strain 129). *P < 0.0001. †P < 0.05. ‡P < 0.001. §P < 0.0005.
peak 104 cM, \(D2Mit113\)) and Chr 14 (2.7 LOD, peak 8.0 cM, \(D14Mit98\)).

Three of the QTL identified here, on Chrs 2, 5, and 6, were detected above the significance threshold. The QTL on distal Chr 2 colocalized with a previously detected suggestive locus for mucin glycoprotein accumulation, a cholesterol gallstone-related phenotype (24), and with a suggestive QTL for the Score phenotype from another intercross (Wittenburg H, Carey MC, and Paigen B, unpublished observation). According to current nomenclature, we named this significant QTL, \(Lith12\). The significant and novel QTL for Solid trait on Chr 5 was named \(Lith13\), but the suggestive QTL for GSW on Chr 5 remains unnamed. The significant QTL for GBV on Chr 5 was named \(Gbq1\). The QTL on Chr 6 was identified and named \(Lith6\) previously (29). The QTL on Chr 16 was identified previously (24; and Wittenburg H, Carey MC, and Paigen B, unpublished observation) above the suggestive threshold and hence was named \(Lith14\). However, the two remaining QTL, on Chrs 1 and 14, were detected only at the suggestive level and await independent confirmation prior to naming. In summary, using an intercross between CAST and 129 and assuming that colocalizing QTL were identical, we identified seven QTL using traits GSW, Solid, GBV, Score, and AChMC. One

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**Fig. 4.** Frequency distribution of cholesterol gallstone phenotypes in the F2 population after 10 wk consumption of the lithogenic diet. Only 30 F2 mice (\(n = 270\)) developed solid gallstones; the majority of animals did not (gallstone weight (A) and gallstone number (B)). Mice were assigned a gallstone “Solid” rating (C) dependent upon the number and size of opaque stones (0 = absence, 4 = largest size and greatest number of stones). Bile samples were assigned a “Score” (D) according to absence (0) or presence (1) of cholesterol monohydrate crystals (ChMC) or presence of sandy and/or solid stones (2). Aggregated ChMC (AChMC) were semi-quantified (E) based on a 0–4 scale (0 = absence, 4 = most severe presence.)
QTL was new (Lith13), whereas the remaining QTL were suggestive or confirmed QTL detected previously that were both named and unnamed.

The QTL for GSW and the QTL for Solid (Lith13), both on Chr 5 (Fig. 5, D and E), and the QTL for Score and for AChMC, both on Chr 6 (Lith6, Fig. 6, A and B), were each fitted with models comprising one, two, or three QTL to determine the likelihood that any of the QTL comprised more than one locus. The QTL for GSW was best fit by a two-QTL model, with 90% confidence (ΔLOD = 1.8) that at least two QTL existed on Chr 5, but Solid did not suggest multiple loci. These statistical modeling data, at least for GSW, support the fine mapping data (Fig. 5), which suggested the presence of multiple QTL on Chr 5. For Lith6, neither the QTL for the Score nor the QTL for AChMC suggested statistical evidence for the presence of more than one locus. However, the different localization of the QTL peak for the two phenotypes (Table 1) is consistent with the existence of two loci in close proximity, a conclusion that was also drawn from the analysis of an intercross between CAST and DBA/2J (29).

Table 1. QTL for cholesterol gallstone formation identified in the CAST × 129S1 intercross

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<tr>
<th>Locus</th>
<th>Name</th>
<th>Phenotype</th>
<th>Chr</th>
<th>LOD</th>
<th>QTL Peak, cM (95% CI)</th>
<th>Variance, %</th>
<th>Susceptible Allele, Inheritance</th>
<th>Coincident QTL (Reference)</th>
<th>Candidate Genes (cM)</th>
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<td>D5Mit255</td>
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<td>Gbvq1</td>
<td>Solid</td>
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<td>3.7</td>
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</table>

* Chromosome number. † Empirically, suggestive QTL LOD > 2.2, significant QTL LOD > 3.1. ‡ Susceptible allele and mode of inheritance (Rec, recessive; Dom, dominant; Add, additive). § Low-density lipoprotein receptor-related protein associated protein 1. ¶ Peroxisome proliferator-activated receptor γ coactivator 1. ¶± Cholecystokinin A receptor. ¶±QLT detected for Cyp7a1 mRNA level, but showed association with gallstone mass post hoc. ¶± CAST allele confers greater gallbladder volume. ¶±QTL for mucin accumulation. ¶±Phospholipid transfer protein. ¶±Hepatocyte nuclear factor 4. ¶±CCAAT/enhancer binding protein (C/EBP), β. ¶±Wittenburg H, Carey MC, and Paigen B, unpublished observation. ¶± Peroxisome proliferator-activated receptor γ. ¶± Nuclear receptor subfamily 1, group I, member 1 (common name pregnane X receptor). ¶±Solute carrier family 21 (organic anion transporter), member 1 (common name organic anion transporting polypeptide 1).

Fig. 5. Genome-wide QTL analyses for cholesterol gallstone susceptibility single loci and fine mapping for QTL on Chr 5. QTL analyses were performed using the male F2 population derived from strains CAST and 129. Genome-wide scans for GSW (A), Solid (B), and GBV (C). Chrs 1 through X are represented numerically on the ordinate. The relative width of the space allotted for each chromosome reflects the relative number of SSLP markers on each chromosome. The abscissa represents the LOD score. Fine mapping on Chr 5 for GSW (D), Solid (E), and GBV (F). The LOD score is indicated by the 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 horizontal gray bars (47). These data suggest that three QTL exist in close proximity on Chr 5. The experiment-wide significant (LOD > 3.1; P < 0.05) and suggestive (LOD > 2.2; P < 0.10) levels of linkage were determined by permutation analyses (10).
The second stage of the QTL analysis was used to detect gene-gene interactions (epistasis). Using our strict criteria for significance, we detected no interacting QTL in this intercross. Therefore, only single QTL are presented.

**Allele Effects**

The QTL for Score on Chr 1 (D1Mit21) was determined by a dominant CAST susceptibility allele (Fig. 7A). Lith12 (Chr 2, D2Mit113), the QTL detected using Score (Fig. 7A) and AChMC (Fig. 7B), was contributed by a recessive CAST allele. In agreement with previous data (29), Lith6 (Chr 6) was conferred by a dominant CAST susceptibility allele (D6Mit44, Fig. 7A; and D6Mit14, Fig. 7B). The QTL for Score (Fig. 7A) and AChMC (Fig. 7B) on Chr 14 (D14Mit98) was contributed by a recessive CAST susceptibility allele. Lith14 (Chr 16, D16Mit65) was determined by an additive CAST susceptibility allele (Fig. 7A). Lith13 (Chr 5) and the QTL for GSW (Chr 5) were conferred by recessive 129 susceptibility alleles (Fig. 7C). Gbvq1 was determined by a recessive CAST allele dictating larger GBV (Fig. 7C). Because cholesterol gallstone susceptibility alleles were contributed by both the gallstone-resistant (CAST) and gallstone-resistant (129) parental strains, these data are consistent with a complex mode of inheritance of cholesterol gallstone formation. Furthermore, they are consistent with the increased prevalence demonstrated by the male F1 mice compared with CAST, the gallstone-susceptible parental strain. However, because Lith13 and the QTL linked to D5Mit201 were determined by recessive 129 susceptibility alleles, the data suggest the presence of other 129-derived gallstone susceptibility QTL or modifier genes that were not detected using this intercross, but contributed to the increased prevalence of the male F1 mice.
Candidate Gene mRNA Expression Analyses and Sequencing

We determined hepatic mRNA expression levels of the parental strains for 8 candidate genes: three genes for Lith12 (Chr 2), two for Lith13 (Chr 5), two for Lith6 (Chr 6), and one gene for Lith14 (Chr 16) (Fig. 8A). Lith12 candidate genes encoded CCAAT/enhancer binding protein (C/EBPβ; Cebpβ), hepatic nuclear factor 4 (Hnf4α), and phospholipid transfer protein (Pltp). Between the parental strains, CAST demonstrated higher expression of Cebpβ amounting to a fourfold difference (Fig. 8A); neither Hnf4α nor Pltp were differentially expressed (Fig. 8A). Evaluation of Cebpβ expression in the F2 population confirmed that the CAST genotype conferred increased Cebpβ expression (Fig. 8B). C/EBPβ is a transcription factor that is highly expressed in the liver and is likely involved in the transcription of Pparg (49), a candidate for Lith6 (29). CAST contributed the susceptibility allele at Lith12 (D2Mit113, Fig. 7), suggesting that increased expression of Cebpβ might affect the lithogenicity of bile and hence cholesterol gallstone formation.

Lrpap1 and Ppargc1 were considered positional candidate genes for Lith13 (Chr 5). Lrpap1 was expressed at a higher level by strain 129 (15-fold) than strain CAST (Fig. 8A); however, differential expression was not observed in the F2 population (Fig. 8B), indicating regulation of this gene by other elements remote from Lrpap1 itself. Therefore, assuming that amino acid substitutions do not exist in LRPAP1 between strains CAST and 129, we conclude that Lrpap1 is unlikely to be responsible for Lith13. No difference was observed between the strains for the second candidate gene, peroxisome proliferator-activated receptor-γ, coactivator 1 (Ppargc1; Fig. 8A). CAST contributed the allele dictating a larger GBV for the QTL peak that colocalized with Lith13 (Fig. 7C). The gene encoding cholecystokinin receptor A (Cckar, 34.0 cM; Table 1), which mediates gallbladder constriction, was a strong positional candidate gene for this QTL, but we did not determine expression levels due to a lack of gallbladder tissue, nor did we pursue this QTL for GBV further in the present study. Additionally, we detected no differential expression of the Lith14 candidate gene, nuclear receptor subfamily 1, group I, member 2 (Nr1I2/Pxr; Fig. 8A).

Consistent with our earlier findings (29), the CAST genotype determined decreased expression of the Lith6 candidate gene Pparg, encoding peroxisome proliferator-activated receptor-γ (PPARγ), compared with the 129 genotype in both the parental (CAST, 2-fold less; Fig. 7A) and F2 (CAST, 7-fold less; Fig. 7B) populations. However, the second Lith6 candidate gene, solute carrier family 21 (organic anion transporter) member 1 (Slc21a1), was expressed at a higher level by CAST (2-fold), contrary to our prediction based on an intercross between CAST and DBA/2J in which DBA/2J exhibited greater expression than strain CAST (29). Furthermore, differential expression of Slc21a1 was not driven by genotype at Lith6 as determined from expression levels in the F2 population (Fig. 8B).

DNA Sequencing and Sequence Analyses

We aimed to sequence 1 kb of the proximal putative promoter regions and the coding regions of Pparg and
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Slc21a1 for comparison of the sequences derived from strains 129 and CAST (see Supplemental Table 2, available at the Physiological Genomics web site).1 Pparg1 and Pparg2 represent alternative transcripts of Pparg (62). The two transcripts possess different initiation codons but share six common exons at the 3′ end (62). Pparg1 comprises exons A1 and A2, which do not code for amino acids, plus the six common exons. Both PPARY1 and PPARY2 proteins appear to be expressed in mouse and human liver, although PPARY2 appears to predominate (3, 16). Pparg2 comprises exon B1 plus the six common exons and encodes 30 amino acids additional to Pparg1. The coding region is comprised entirely by Pparg2 in which sequence variations were observed but with none causing amino acid substitutions (see Supplemental Table 2). The gene structure of mouse Pparg and human PPARG and their corresponding amino acid sequences are highly conserved (99% similarity, 95% identity) (16). However, VISTA (7, 33) sequence comparisons revealed little similarity between species of exon A1, exon A2, and the 5 kb immediately proximal to exon A1 (data not shown). VISTA (7, 33) analyses of the 1.1-kb promoter sequences of Pparg2 from strains 129 and CAST revealed two regions that displayed greater than 75% identity with the human sequence (data not shown); however, no differences in predicted transcription binding sites were observed between the strains CAST and 129 using the MATCH software (data not shown).

Slc21a1, the second candidate gene underlying the dual-QTL Lith6, displayed many sequence variations (see Supplemental Table 2). The coding region exhibited four polymorphisms that caused amino acid substitutions and potentially might affect protein activity. Two of the substitutions [T1769A (nucleotide), C538S (amino acid residue) and C1581G, T475S] resulted in the replacement of conserved residues (19) (see Supplemental Table 2). These data are consistent with the candidacy of Slc21a1 as a genetic determinant of cholesterol gallstone susceptibility but require functional analysis to test our hypothesis directly.

DISCUSSION

Using this intercross between CAST and strain 129 inbred mice, we identified seven QTL for cholesterol gallstone formation (Table 1). A single QTL that was both new and significant was detected on proximal Chr 5 that we named Lith13. The analysis confirmed Lith6, a previously identified locus on Chr 6. Two additional loci from previous crosses were confirmed on distal Chr 2 and on Chr 16. According to standard nomenclature, these QTL were named Lith12 and Lith14, respectively. Furthermore, we detected new QTL on Chr 1, 5, and 14 that await confirmation in independent crosses prior to naming because their LOD scores fell below the significance threshold. In addition, a locus on Chr 17 was detected just below the suggestive threshold (Fig. 6B). This putative QTL colocalized with Lith9, a locus detected previously. The outstanding candidate genes for Lith9 are the gene pair Abcg5 and Abcg8, which encode the half-transporters comprising the ATP-dependent canalicular cholesterol transporter (60, 61). This is our second QTL analysis employing the wild-derived cholesterol gallstone-susceptible strain CAST. Surprisingly, in the previous cross with the gallstone-resistant strain DBA/2J, only Lith6 was derived from the susceptible strain CAST (29). This QTL was confirmed in the current cross, further underlying the importance of this locus. Interestingly, after crossing CAST mice with strain 129, and therefore into a different genetic background, the QTL analysis revealed five additional significant or suggestive QTL whose susceptible alleles were derived from strain CAST (Table 1). These data clearly demonstrate the importance of the genetic background with presence or absence of modifier genes (39). Knowledge of the subset of resistance and/or susceptibility alleles carried by these strains will aid substantially the evaluation of candidate genes and their sequences.

The goal of QTL analyses of complex phenotypes is the identification of the genes that carry polymorphisms that determine susceptibility or resistance to a given trait. After performing a breeding cross, this objective is hampered often by the large 95% CI of the QTL that harbor myriad genes. Therefore, we were guided in our identification and assessment of positional candidate genes by knowledge of genes with putative roles in cholesterol gallstone formation (23) and by a rigorous physical-chemical characterization of the parental strains of our intercross (Fig. 3). Biliary lipid analyses of male mice of strains CAST and 129 indicated that CAST gallstone susceptibility was primarily the result of cholesterol hypersecretion (Fig. 3B) and increased biliary cholesterol content (Fig. 3C). CAST gallstone susceptibility also was partly due to decreased biliary phospholipid composition (Fig. 3C), but not bile salt hyposcretion (Fig. 3B) or reduced bile salt composition (Fig. 3C). Cholesterol supersaturation in CAST biles was likely exacerbated by the increased bile salt-independent bile flow (Fig. 3D) and resultant lower total lipid concentration (Fig. 3A, P < 0.06), because low total lipid concentrations (53) and choles-terol-solubilizing vesicles (18) each contribute to in-creased CSIs typically exhibited by hepatic biles. A preliminary examination of selected genes (Abcc2/Mrp2, Cjfr, and Slc4a2/Ae2) that contribute to bile salt-independent bile flow did not elucidate any under-lying mechanisms, and in fact, strain 129 exhibited ~2-fold greater expression of Abcc2 (data not shown). Strains CAST and 129 both displayed CSI > 1 in hepatic bile (Fig. 3E). However, CAST displayed a significantly greater CSI compared with strain 129, reflecting the cholesterol hypersecretion of strain CAST (Fig. 3E). Analysis of gallbladder bile from parental strains confirmed that CAST exhibited a mark-edly increased gallbladder CSI compared with 129,
which displayed CSI < 1 (Fig. 3E). Therefore, these data provide a physical-chemical explanation of gallstone susceptibility in strain CAST compared with the gallstone resistance of strain 129. Based on this information and the known genetic map positions of the positional candidate genes (Fig. 1, Table 1), we tested for differential mRNA expression between the parental strains among the candidate genes for loci comprising Lith12, Lith13, Lith6, and Lith14. For those putative candidate genes that exhibited differential hepatic expression between the two parental strains (Fig. 8A), mRNA expression levels were examined further in the F2 population (Fig. 8B). Our aim is to complement the genotype and phenotype data such that we can provide a molecular genetic mechanism for the observed gallstone susceptibility traits.

We performed preliminary, prospective evaluations of mRNA expression of positional candidate genes that lay within the 95% CI of our QTL and were involved in lipid metabolism. Two candidate genes demonstrated differential expression between the parental strains, but not between the two genotypic groups of the F2 population (Fig. 8). These findings validate our investigation of mRNA expression in the F2 population. Since the parental strains were identical at all loci, but the F2 mice were identical only at the selected locus, the data suggest that either or both of the parental strains possessed modifier alleles or epistatic interactions that were not present, or were not detected, in the F2 mice. However, this approach and the criteria we employed are consistent with the concept that only highly significant QTL should be reported so that false positives are minimized (26).

We considered three positional candidate genes for Lith12 (Chr 2): Cebpb, Hnf4, and Pltp. We observed no difference in either Hnf4 or Pltp expression (Fig. 8A). Based on our mRNA expression criteria in this study, these were considered poor candidate genes and were not investigated further. The CAST genotype determined increased expression of Cebpb in the parental mice (Fig. 8A) and the F2 progeny (Fig. 8B), which strongly supports the notion that its expression was determined locally and not by an element outside the QTL region. It is not likely that C/EBPβ influenced the expression of Pparg in this intercross, because these expression levels were inversely related (Fig. 8). Evaluation of mice possessing targeted mutation of Cebpb revealed little with regard to cholesterol homeostasis (49). Without sequencing the coding regions of these candidate genes, we cannot exclude definitively any of the three alternatives. This locus requires more investigation to elucidate the underlying mechanism that influences cholesterol gallstone formation and to identify the responsible gene, but Cebpb represents an ideal starting point in this endeavor.

Lith13 (Chr 5) was contributed by a recessive 129 susceptibility allele (Fig. 7C) and included Lrhap1, Ppargc1, and Ccak as positional candidate genes (Table 1). We concluded, however, that Lrhap1 and Ppargc1 were unlikely to be responsible for Lith13. Lith13 colocalized with a QTL for GBV (Table 1), whose peak (36 cM) was in close proximity to Ccak (34 cM), an attractive candidate gene for both this phenotype and for cholesterol gallstone formation directly (23). Since the QTL for GBV was determined by a recessive CAST allele that increased volume (Fig. 7C), this locus is consistent with the knowledge that gallbladder stasis contributes to cholesterol crystallization and gallstone formation (41). Although it is not definite that GBV accurately reflects gallbladder stasis, GBV does represent a convenient surrogate indicator of gallbladder contraction, and the QTL for this phenotype provides supporting evidence for Lith13. The gene encoding CCKAR is one of the few examples of genes that were linked to cholesterol gallstone formation in humans (36, 46). In mice, both the CCKAR knockout mouse (38, 45) and the mouse with dysfunctional carboxypeptidase E (fat mutation; the enzyme that hydrolyzes the procholecystokinin to cholecystokinin, the active ligand for the receptor) (6) displayed increased susceptibility to gallstone formation. The pathophysiology of gallstone formation due to dysfunctional cholecystokinin or its receptor most likely involves both noncontraction of the gallbladder and reduced intestinal transit leading to enhanced intestinal cholesterol absorption (6). We did not investigate the candidacy of Ccak in this cross, but we believe that this gene will be interesting to pursue in future as a viable candidate for Lith13. Furthermore, the likely existence of dual QTL on Chr 5 will be investigated.

Previously, we determined that Lith6, detected using an intercross between strains CAST and DBA/2J, comprised two closely linked QTL and that Ppargc and Slc21a1 were likely candidates for the two underlying loci (29). Consistent with that conclusion, we detected colocalizing QTL for the Score and AChMC phenotypes (Table 1) whose peaks at 54 cM and 66 cM, respectively, coincided with each of these two candidate genes (Ppargc, 52.7 cM; Slc21a1, 67.0 cM). PPARγ is a ligand-activated transcription factor. In one study in vivo, PPARγ activation upregulated CYP7A1 activity (37), and in another, it increased Cyp7a1 expression (43). Conversely, we infer that lower Ppargc expression and/or PPARγ activation could increase the availability of intracellular free cholesterol for biliary secretion due to decreased catabolism of cholesterol into bile salts via CYP7A1. Consistent with this hypothesis, strain CAST exhibited biliary cholesterol hypersecretion (Fig. 3) and contributed the susceptible Lith6 allele (Fig. 7), and the CAST genotype dictated decreased Ppargc expression in the F2 mice (Fig. 8). We detected no DNA sequence variations that would cause amino acid substitutions, thereby eliminating the possibility of altered protein function. Our preliminary analyses of the Ppargc promoter regions revealed little in terms of transcriptional regulation. Indeed, this might be predicted since Lander’s group concluded recently that due to the difficulty in identifying regulatory elements, research efforts would be more productive by searching for regulatory variation rather than for specific regulatory variants (11). It is interesting to note that despite the overall dissimilarity in the
5-kb region upstream of the two orthologs, *Pparg1* (62) and *PPARG1* (16), each possess substantial promoter activity in the respective 3-kb upstream region and display similar tissue distribution (3, 16).

**Slc21a1**, the second positional candidate gene for *Lith6*, is a transporter on the basolateral membrane of hepatocytes responsible for hepatocellular uptake of bile acids and bile salts (51). Since >80% of conjugated taurocholate but <50% of unconjugated cholate uptake is mediated via the Na+-dependent mechanism, i.e., SLC10A1/NTCP (51), it was suggested that unconjugated bile acids might be transported preferentially by the Na+-independent mechanism (2), i.e., SLC21A1. Given that the lithogenic diet included cholic acid (22), *Slc21a1* may well be a genetic determinant of cholesterol gallstone susceptibility in the present in vivo model. We hypothesized that higher basolateral uptake of bile acids in CAST mice, due to functional differences or differences in expression, could inhibit (via NR1H4/FXR) cholesterol catabolism to bile salts, thereby increasing the availability of cholesterol for canalicular secretion and thus the lithogenicity of bile. The alignment of sequences from mouse, rat, and human indicated that within the subfamily to which *Slc21a1* belongs, 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. However, threonine and cysteine are conserved at residues 475 and 538, respectively (19), suggesting that these changes might cause a functional variation in protein activity. We speculated that SLC21A1 derived from strain CAST might display heightened transporter activity due to the loss of two conserved amino acids (T475 and C538, also observed between CAST and DBA/2J; Ref. 29). Since the expression profiles of Slc21a1 between the two studies involving strain CAST were inconsistent, and the sequences of CAST and DBA/2J were identical in the promoter region, we infer that the amino acid changes might be important for cholesterol gallstone susceptibility, rather than the nucleotide variations in the regulatory regions. Congenic strains are under construction to confirm and narrow the individual loci comprising *Lith6*. Biochemical evaluation of SLC21A1 expressed in vitro also appears a powerful approach to further authenticate the contribution of SLC21A1 to cholesterol gallstone formation.

Using an intercross between strains CAST and 129, we generated genetic and molecular data that further support the likelihood that *Lith6* represents two QTL in close proximity, and candidate genes include *Pparg* and *Slc21a1*. Both genes provide putative molecular explanations for one of the key features of gallstone susceptibility in this cross, biliary hypersecretion of cholesterol via decreased biliary degradation into bile salts. The second physical-chemical principle of cholesterol gallstone formation in strain CAST, higher bile salt-independent bile flow, and therefore lower total lipid concentrations that lead to higher CSI values, remains elusive from this analysis. Similar to *Lith6*, the new QTL for cholesterol gallstone formation on Chr 5 likely represents a complex locus comprising two QTL in close proximity, one of which we named *Lith13*. Lower expression of *Cckar*, a candidate gene within this complex region, by strain 129 might contribute to its higher total lipid concentration of gallbladder bile compared with strain CAST, since gallbladder stasis results in water resorption and concentration of gallbladder lipids. QTL on Chrss 2 and 16, detected in previous crosses, were confirmed, thereby allowing us to name these loci *Lith12* and *Lith14*, respectively. From our daisy chain experimental design, it appears that the number of new cholesterol gallstone susceptibility QTL that we detect from each cross is decreasing, but we are confirming many previously detected QTL using both independent crosses and strains. By combining data from different crosses displaying similar QTL, it is probable that we will resolve complex, closely linked QTL, such as those in the vicinity of *Lith6* and *Lith13*, into their individual QTL components (50). Furthermore, at the completion of all eight intercrosses, and combined with previous crosses, it is likely that identification of all *Lith* loci will be achieved. The focus of such endeavors can then shift entirely to characterizing these primary genetic determinants of cholesterol gallstone formation.

DNA sequences cited in this manuscript were submitted to GenBank with the following accession numbers: AY243579, AY243580, AY243581, AY243582, AY243583, AY243584, and AY243585.

We are indebted to Dr. Jason Stockwell and Jennifer Smith (Jackson Laboratory) for consultation on general statistical methods and assistance with graphics, respectively. We thank David Schultz, Harry Whitmore, and Eric Taylor (Jackson Laboratory) for colony management.

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**DISCLOSURES**

This work was supported in part by National Institutes of Health Grants DK-51568 (to B. Paigen), CA-34196 (Core Grant to the Jackson Laboratory), DK-36588, DK-52911 (to M. C. Carey), and DK-54854 (Core Grant to Brigham and Women's Hospital). M. A. Lyons was supported by the American Physiological Society and the American Liver Foundation. H. Wittenburg was supported by the Deutsche Forschungsgemeinschaft (WI 1905/1-1).

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