Quantitative trait loci that determine lipoprotein cholesterol levels in an intercross of 129S1/SvImJ and CAST/Ei inbred mice

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Lyons, Malcolm A., Henning Wittenburg, Renhua Li, Kenneth A. Walsh, Ron Korstanje, Gary A. Churchill, Martin C. Carey, and Beverly Paigen. Quantitative trait loci that determine lipoprotein cholesterol levels in an intercross of 129S1/SvImJ and CAST/Ei inbred mice. Physiol Genomics 17: 60–68, 2004. First published December 30, 2003; 10.1152/physiolgenomics.00142.2003.—To identify genetic determinants of lipoprotein levels, we are performing quantitative trait locus (QTL) analysis on a series of mouse intercrosses in a “daisy chain” experimental design, to increase the power of detecting QTL and to identify common variants that should segregate in multiple intercrosses. In this study, we intercrossed strains CAST/Ei and 129S1/SvImJ, determined HDL, total, and non-HDL cholesterol levels, and performed QTL mapping using Pseudomarker software. For HDL cholesterol, we identified two significant QTL on chromosome (Chr) 1 (Hdlq5, 20–60 cM), and Chr 4 (Hdlq10, 20–60 cM). For total cholesterol, we identified three significant QTL on Chr 1 (Chol7, 20–60 cM), Chr 4 (Chol8, 12 cM), and Chr 17 (Chol9, 54 cM). For non-HDL cholesterol, we identified significant QTL on Chr 8 (Nhdlq1, 34 cM), and Chr X (Nhdlq2, 6 cM). Hdlq10 was the only QTL detected in two intercrosses involving strain CAST/Ei. Hdlq5, Hdlq10, Nhdlq1, and two suggestive QTL at D7Mit246 and D15Mit115 coincided with orthologous human lipoprotein QTL. Our analysis furthers the knowledge of the genetic control of lipoprotein levels and points to the importance of Hdlq10, which was detected repeatedly in multiple studies.

Castaneus; mouse; QTL; HDL; high-density lipopolysaccharide; genetics; Abca1; Lpl

INBRED MOUSE STRAINS ARE GENETICALLY IDENTICAL WITHIN A STRAIN but are genetically diverse between strains. Since different inbred strains display great variation in plasma lipoprotein levels when exposed to an identical environment (31, 33), the determinants of these differences must be the genetic background of the strains. Therefore, the genes carrying alleles that affect lipoprotein levels can be mapped by quantitative trait locus (QTL) analysis (27, 53). Furthermore, it appears that QTL determining high-density lipoprotein (HDL) cholesterol levels in mice and humans are localized in orthologous chromosomal regions (53). This leads to the attractive hypothesis that polymorphisms within the orthologous genes underlie the QTL in each species. Therefore, inbred mice appear to be a powerful model to predict which genes harbor polymorphisms determining lipoprotein levels in human populations.

Based on the results of a recent QTL analysis of an intercross between the wild-derived inbred strain CAST/Ei (CAST) and strain DBA/2J, we postulated that polymorphisms in the common genes carrying mutations causing rare human monogenic disorders of cholesterol metabolism may determine, in part, the quantitative variation of lipoprotein levels in human populations (20). Evidence consistent with roles for Abca1 (encoding a cholesterol/phospholipid efflux transporter) and Ldlr [encoding low-density lipoprotein (LDL) receptor] in the determination of HDL and total cholesterol levels, respectively, was provided (20). HDL deficiency exhibiting monogenic inheritance can be caused by mutations in ABCA1, APOA1, LCAT, and LPL (6). Five disorders of LDL metabolism with monogenic inheritance stemming from mutations in LDLR, APOB, ARH, ABCG5, and/or ABCG8 (14) and CYP7A1 (38) have been identified. Therefore, in addition to identifying genetic determinants of lipoprotein levels, we aim to answer the question of whether polymorphisms in the genes that cause the monogenic lipoprotein disorders also contribute to variation in overall lipoprotein levels. Elucidation of the genetic mechanisms underlying these traits is crucial since an inverse correlation exists between HDL concentrations and atherosclerosis risk (15), whereas atherosclerosis risk is correlated positively with LDL cholesterol concentrations (16).

To confirm our results from the QTL analysis of an intercross between CAST and DBA/2J mice (20) and to determine additional loci carrying polymorphisms that determine lipoprotein levels in CAST mice, we performed an intercross between strain CAST and the inbred strain 129S1/SvImJ (129). Moreover, these intercrosses comprise part of a larger “daisy chain” experimental design, which involves eight genetically diverse inbred mouse strains (Fig. 1), that aims to uncover a substantial fraction of alleles that contribute to the complex lipoprotein traits. The inbred strains were selected based on their genetic diversity (3) and their manifestation of lipoprotein cholesterol levels and cholesterol gallstone susceptibility (31, 33), a second major focus of our investigations. Each strain is crossed to two other strains that each differ in their lipoprotein cholesterol levels from the common strain. This design provides a greater probability of detecting genes that affect lipoprotein cholesterol concentrations carried by that single parental strain (46). Additionally, common variants are likely to segregate in multiple intercrosses (46). Finally, combining data from separate intercrosses will likely enable us to resolve linked QTL and narrow 95% confidence intervals (CI) (46). We aim eventually to combine data from multiple crosses for further QTL analyses in addition to performing haplotype analyses (50) based on crosses either displaying or not displaying certain QTL. Importantly, the experimental design lends itself to

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repeated identification of significant QTL, since reproducibility is central to the search for genetic determinants of complex traits (18).

We report here our investigation of HDL, total, and non-HDL cholesterol concentrations during feeding of an atherogenic diet to intercross (F2) progeny bred from strains CAST and 129. This diet contains high amounts of cholesterol and cholic acid, a bile acid that promotes cholesterol absorption by replacement of the endogenous murine bile salt pool and alters the lipoprotein profile. In total, 10 QTL were detected for lipoprotein cholesterol concentrations, one of which was concordant with a QTL detected in the previous CAST × DBA/2J intercross (20), whereas others were specific to the present intercross between strains CAST and 129.

MATERIALS AND METHODS

Inbred strains. Strains CAST and 129 were selected to intercross because they differed in their lipoprotein cholesterol levels when fed standard diet and the atherogenic diet (31, 33). These findings were confirmed in the present study (see RESULTS, Table 1). These strains differed in their susceptibility to cholesterol gallstone formation also, which was the subject of a previous report (21). Animals, breeding protocols, and facilities were described in detail previously (20, 21). At 6–8 wk of age, mice were transferred from standard diet to the atherogenic diet for 8 wk (parental and F1; n = 9–11 per group) or 10 wk (F2; n = 277). Male mice only were studied and had free access to food and water. All animals fasted 4 h prior to blood sampling and death. The Institutional Animal Care and Use Committees of The Jackson Laboratory and Harvard University approved all experimental protocols.

Lipoprotein cholesterol determination. Phenotyping was performed as described (20). Briefly, blood was collected via the retro-orbital sinus with disodium EDTA as anticoagulant. Plasma was isolated by centrifugation. HDL and total cholesterol concentrations were measured using an automated chemistry analyzer and the manufacturer’s reagents (Synchroin CX-5 Delta; Beckman, Palo Alto, CA). Because mice were fasted before blood was collected and because chylomicrons display very short half-lives (10), non-HDL was presumed to comprise predominantly VLDL and LDL. Non-HDL was calculated as the difference between total and HDL cholesterol concentrations. Strain CAST lipoprotein data were derived from a previous cross between strains CAST and DBA/2J (20). These studies were closely associated (Fig. 1), shared strain CAST and were conducted simultaneously.

QTL analyses. Genotyping was performed on all F2 mice (n = 277) using DNA prepared from tail samples, as described (20, 21). Simple sequence length polymorphisms (MapPairs primers; Research Genetics, Huntsville, AL) that discriminate between CAST and 129 alleles were employed for QTL analyses (n = 100, interval ranges of 1 to 24.4 cM) (21).

To identify single and interacting QTL associated with lipoprotein cholesterol concentrations, the multistage analysis of Sen and Churchill (42) was employed using Pseudomarker software, as detailed previously (21, 55). Pseudomarker uses an explicit multiple-QTL model. Its mechanism of QTL detection is a free genetics model. Significance thresholds were determined by experiment-wide permutation testing (n = 1000 permutations) (8). 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). This method contrasts with the universal thresholds suggested by Lander and Kruglyak (18), since the resultant thresholds are dependent upon the input data. Therefore, the permutation-derived thresholds may be higher or lower than those suggested previously (18) and now represent the preferred method for determination of significance thresholds (11). Furthermore, our stringency for the suggestive threshold of genetic linkage is higher than suggested previously (18). The 95% CI were calculated as described (42). Results are expressed as logarithm of the odds ratio (LOG) scores. Significant QTL were named immediately, but suggestive QTL were named only when confirmed by two or more independent breeding crosses, consistent with proposed guidelines (11, 18). For each QTL, we determined the allelic effect by calculating the phenotype mean for each of the three possible genotypes and determined which strain contributed the allele that increased lipoprotein cholesterol concentrations.

In the parental strains of mice, CAST and 129, and selected F2 progeny, hepatic levels of mRNA expression were determined for genes that colocalized with QTL and were known to have a direct or indirect role in lipid metabolism (positional candidate genes). These putative candidate genes were identified from the genome sequence databases. Assuming a steady state for the expression of genes

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**Table 1. Plasma lipoprotein cholesterol concentrations in male parental, F1 and F2 populations**

<table>
<thead>
<tr>
<th>Mice Population</th>
<th>Lipoprotein Cholesterol, mg/dl</th>
<th>Total</th>
<th>HDL</th>
<th>Non-HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAST</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>300±23</td>
<td>110±9</td>
<td>191±16</td>
</tr>
<tr>
<td>CAST × 129F1F2</td>
<td>11</td>
<td>247±13</td>
<td>96±4</td>
<td>150±13</td>
</tr>
<tr>
<td>(129 × CAST)F1</td>
<td>9</td>
<td>278±20</td>
<td>80±8</td>
<td>199±25</td>
</tr>
<tr>
<td>Combined F1</td>
<td>20</td>
<td>261±12</td>
<td>89±4</td>
<td>172±14</td>
</tr>
<tr>
<td>F2</td>
<td>277</td>
<td>245±4</td>
<td>110±3</td>
<td>137±5</td>
</tr>
</tbody>
</table>

Values are means ± SE and were analyzed using one-way ANOVA with the Tukey multiple comparison posttest. Parental and F1 mice were fed the atherogenic diet for 8 wk, whereas F2 mice were fed the diet for 10 wk prior to cholesterol determinations. HDL and total cholesterol were determined using automated enzymatic assays. Non-HDL was calculated as the difference between total and HDL cholesterol concentrations. Reciprocal F1 mice did not differ significantly for any of the lipoprotein cholesterol levels and were grouped to compare with strains CAST and 129. CAST data were derived from Ref. 20. Because it is the distribution and not the mean that is most important for detecting quantitative trait loci (QTL) among the F2 population, we did not test for significant differences between this group and the parental strains or F1 group. *Different from CAST, P < 0.001. †Different from 129, P < 0.05. ‡Different from CAST, P < 0.05. §Different from 129, P < 0.001.
induced by the diet, hepatic tissue was harvested, as described, from male parental mice (5 per strain) fed the atherogenic diet for 4 wk (20). The CAST hepatic cDNA used for expression analyses was identical to that used previously (20); however, all data presented in this study were from independent experiments. Livers were collected from F2 animals at the time of death. Groups of F2 animals were selected based on their genotypes at the QTL peaks and the genetic markers flanking those peaks. At Hdlq10, Chol9, and Nhldq1, eight animals per genotype per locus were selected. mRNA expression levels were determined using quantitative (real-time) PCR as detailed elsewhere (20). Data were expressed per 10^6 molecules of Gapd. Statistical analyses were performed on the normalized data.

**RESULTS**

**Lipoprotein cholesterol levels.** Compared with strain 129, strain CAST displayed significantly lower HDL (Table 1) but significantly higher total (Table 1) and non-HDL (Table 1) cholesterol levels. No differences were observed between male reciprocal F1 progeny, indicating that these traits were not inherited by maternal or imprinted genetic factors in this cross; thereafter, the F1 mice were treated as a single group. Low levels of HDL cholesterol and high levels of total and non-HDL cholesterol were inherited dominantly and resembled strain CAST (Table 1). Surprisingly, the F1 animals exhibited HDL cholesterol levels that were lower than in both strains CAST and 129 (Table 1). These data indicate that either both strains contributed alleles leading to lower HDL cholesterol levels or that some alleles for higher HDL cholesterol levels were inherited in a recessive fashion and, therefore, were obscured in the heterozygous state. Non-HDL and total cholesterol levels correlated markedly (Pearson correlation coefficient, 0.830; \( P < 0.0001 \)), likely reflecting the fact that total cholesterol was composed predominantly of non-HDL cholesterol in mice fed the atherogenic diet. Non-HDL cholesterol correlated negatively with HDL cholesterol (coefficient, \(-0.445; P < 0.0001 \)), but HDL was not correlated with total cholesterol (\( P > 0.05 \)).

To improve the sensitivity of evaluating cholesterol gallstone formation, which was investigated in this intercross but reported elsewhere (21), we extended the feeding period for the F2 population from 8 to 10 wk. However, the distributions of the lipoprotein cholesterol determinations in the F2 animals were consistent with distributions of the parental and F1 mice (Table 1). Furthermore, because it is the distribution and not the mean of the F2 population that is fundamental to QTL analyses, we did not compare this group to the parental strains or to the F1 group.

**QTL analyses.** The genome-wide scans for single QTL are presented in Fig. 2. Details of the QTL detected, including the LOD score, QTL peak, 95% CI, variance, allele conferring higher cholesterol concentration, and candidate genes, are presented in Table 2. Significant QTL for HDL cholesterol were detected on chromosomes (Chrs) 1 and 4 (Fig. 2A and Table 2). Similarly, significant QTL for total cholesterol were detected on Chrs 1 and 4, also (Fig. 2B and Table 2). A third significant QTL for total cholesterol was detected on Chr 17 (Fig. 2B and Table 2). In addition, nine suggestive QTL for HDL cholesterol were identified on Chrs 8 and X, and one suggestive QTL was detected on proximal Chr 15 (Fig. 2C and Table 2). In total, the two QTL for HDL cholesterol accounted for 14.1%, the six QTL for total cholesterol accounted for 30.4%, and the three QTL for non-HDL cholesterol accounted for 15.3% of the variance in the F2 population (Table 2).

The second stage of the QTL analysis was used to detect gene interactions (epistasis). Using our criteria for significance (21), we detected no interacting QTL in this intercross. Therefore, only single QTL are presented. Consistent with our previous report, significant, unnamed QTL for total cholesterol were named Chol, and non-HDL cholesterol QTL were named Nhldq, each followed by an Arabic number. Hence, the significant QTL for total cholesterol on Chrs 1, 4, and 17 were named Chol7, Chol8, and Chol9, respectively, and the significant QTL for non-HDL cholesterol on Chrs 8 and X were named Nhldq1 and Nhldq2, respectively (Table 2). In the previous intercross between strains CAST and DBA/2J, we detected a QTL for HDL cholesterol contributed by CAST, which we named Hdlq10 (Chr 4) (20). Since strain CAST was common to both intercrosses and the CAST allele increased HDL cholesterol in both instances (Fig. 3A), we retained the name Hdlq10 for the HDL cholesterol QTL on Chr 4 in this intercross (Fig. 2A). The significant QTL for HDL cholesterol on Chr 1 overlapped with previously described QTL for HDL cholesterol named Hdlq5 that was detected in multiple crosses...
Table 2. QTL for plasma lipoprotein cholesterol concentrations identified in the CAST × 129 intercrosses

<table>
<thead>
<tr>
<th>Locus</th>
<th>Name</th>
<th>Phenotype</th>
<th>Chr</th>
<th>LOD</th>
<th>Peak, cM (95% CI)</th>
<th>Variance, %</th>
<th>High Allele and Inheritance</th>
<th>Coincident QTL (Reference)</th>
<th>Candidate Genes and Genetic Positions, cM</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1Mit102</td>
<td>Hdlq5</td>
<td>HDL</td>
<td>1</td>
<td>5.1</td>
<td>82 (60–100)</td>
<td>8.1</td>
<td>129, additive</td>
<td>Hdlq5 (52), Hdlq6 (52), Ab1l (32), Chab6 (41), Pcho1 (40)</td>
<td>Apo2 (92.6), Nr5a2 (74.3)</td>
</tr>
<tr>
<td>D4Mit110</td>
<td>Hdlq10</td>
<td>HDL</td>
<td>4</td>
<td>3.7</td>
<td>20 (10–30)</td>
<td>6.0</td>
<td>CAST, additive</td>
<td>Hdlq10 (20), Lch1 (47), Lch2 (48), (22)</td>
<td>Abc1l (23.1)</td>
</tr>
<tr>
<td>D1Mit102</td>
<td>Chol7</td>
<td>Total</td>
<td>1</td>
<td>3.3</td>
<td>74 (65–80)</td>
<td>5.3</td>
<td>129, additive</td>
<td>Hdlq5 (52), Hdlq6 (52), Ab1l (32), Chab6 (41), Pcho1 (40)</td>
<td>Apo2 (92.6), Nr5a2 (74.3)</td>
</tr>
<tr>
<td>D4Mit194</td>
<td>Chol8</td>
<td>Total</td>
<td>4</td>
<td>4.7</td>
<td>12 (0–30)</td>
<td>7.6</td>
<td>CAST, dominant</td>
<td>Hdlq10 (20), Lch1 (47), Lch2 (48)</td>
<td>Abc1l (23.1), Cyp7al (1.3)</td>
</tr>
<tr>
<td>D7Mit246</td>
<td></td>
<td>Total</td>
<td>7</td>
<td>2.4</td>
<td>20 (0–30)</td>
<td>3.9</td>
<td>129, recessive</td>
<td></td>
<td>Abcc6 (24), Apoe (4), Cebpa (12), Nr1h2 (23), Lca9 (53)</td>
</tr>
<tr>
<td>D8Mit248</td>
<td></td>
<td>Total</td>
<td>8</td>
<td>2.9</td>
<td>50 (20–68)</td>
<td>4.7</td>
<td>CAST, recessive</td>
<td>Chab4 (41), Hdlq4 (36, 39)</td>
<td>Soat2 (61.7)</td>
</tr>
<tr>
<td>D15Mit79</td>
<td></td>
<td>Total</td>
<td>15</td>
<td>2.3</td>
<td>68 (60–70)</td>
<td>3.7</td>
<td>CAST, recessive</td>
<td>Chab4 (41), Hdlq4 (36, 39)</td>
<td>Soat2 (61.7)</td>
</tr>
<tr>
<td>D17Mit221</td>
<td>Chol9</td>
<td>Total</td>
<td>17</td>
<td>3.2</td>
<td>54 (20–60)</td>
<td>5.2</td>
<td>CAST, dominant</td>
<td>Hdlq4 (25)</td>
<td>Abcgs5/Abcg8 (55)</td>
</tr>
<tr>
<td>D8Mit248</td>
<td>Nhd1q1</td>
<td>Non-HDL</td>
<td>8</td>
<td>3.8</td>
<td>34 (20–60)</td>
<td>6.1</td>
<td>CAST, additive</td>
<td></td>
<td>Cpe (32.6), Lpl (33)</td>
</tr>
<tr>
<td>D15Mit115</td>
<td></td>
<td>Non-HDL</td>
<td>15</td>
<td>2.8</td>
<td>20 (0–40)</td>
<td>4.6</td>
<td>CAST, recessive</td>
<td></td>
<td></td>
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<tr>
<td>D6Mit81</td>
<td>Xh1q2</td>
<td>Non-HDL</td>
<td>4.6</td>
<td>0.6</td>
<td>18 (6–18)</td>
<td>4.6</td>
<td>129</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significant interaction with lineage. †These QTL are likely identical. ‡suggestive interaction with lineage. §QTL named in this study are given in bold font. ¶Empirically, suggestive QTL LOD > 2.2, significant QTL LOD > 3.1. QTL peak, with 95% confidence interval given in parentheses. ††Allele determining high cholesterol phenotype and likely mode of inheritance. †‡Point mutation from a mutagenesis experiment, not a QTL, which colocalizes with Hdlq10 for the same phenotype (HDL). †§Nuclear receptor subfamily 5, group A, member 2 (liver receptor homolog 1). †‖Apolipoprotein A2. †§§ATP-binding cassette, subfamily A (ABC1), member 1 (cholesterol/phospholipid flippase). †||Cholesterol 7a-hydroxylase. †††ATP-binding cassette, subfamily C (CFTR/MRP), member 6. †‡‡Apolipoprotein C1, C2, C4 gene cluster. †§§Apolipoprotein E. †|||CCAAT/enhancer binding protein (C/EBP), α. †‡‡Nuclear receptor subfamily 1, group H, member 2 (liver X receptor β). †§§|Lechini: cholesterol acyl transferase. †||Sterol O-acyl transferase 2 (acyl-coenzyme A: acyltransferase 2). ††††ATP-binding cassette, subfamily G (WHITE), members 5 and 8 (canicular cholesterol transporter). †|||Carboxypeptidase E. †§§Lipoprotein lipase.

(53). Based on the similarity of the QTL peak location (82 cM) and its large contribution to variance in HDL cholesterol (8%) between this and the previously identified QTL, we reasoned that the QTL in the present study was identical to Hdlq5, although its 95% CI was broad (Table 2), perhaps due to the presence of more than one QTL within one locus.

Since Hdlq5 and Chol7 exhibited discrepant peak locations and dissimilar allele effects (Fig. 3), we concluded that these likely represent distinct QTL despite their colocalization (Table 2). If Hdlq5 described two QTL, then the second locus might coincide with Chol7. However, our test for multiple QTL (21) was inconclusive (data not shown), and we were unable to declare that two QTL were present. Similarly, the suggestive QTL for total cholesterol linked to D8Mit248 and the QTL for non-HDL cholesterol, Nhd1q1, displayed discrepant peak locations and dissimilar allele effects. This caused us to conclude that the QTL are more likely to be distinct, despite linkage to the same genetic marker. Furthermore, the 95% CI for these two QTL were broad, perhaps due to the presence of more than one QTL. However, our tests for multiple QTL did not suggest the presence of multiple QTL (data not shown). Conversely, despite variation in the peak locations, the allele effects for Hdlq10 and Chol8 were consistent with a single gene underlying both QTL.

mRNA transcription. QTL analyses detect polymorphisms in genes encoding regulatory proteins (17). The polymorphisms, or mutations, may affect protein levels via mRNA transcription or stability, or protein function via altered coding sequence. Since the liver is central to lipoprotein synthesis and catabolism (13), we identified positional candidate genes that colocalized with the lipoprotein cholesterol QTL (Table 2) and evaluated their hepatic mRNA expression profiles in a preliminary screening assay. Candidate genes for all QTL, except for the loci on proximal Chr 15 (D15Mit115) and Chr X (DXMit81) for which no obvious candidates were identified, were evaluated in each of the parental strains. Demonstration of differential expression between F2 animals that exhibit a homozygous genotype for one parental strain vs. the other only in the region harboring the candidate gene 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 Hdlq10 (and Chol8), Nhd1q1, and Chol9 in the F2 population. We selected individual samples that were either homozygous CAST or homozygous 129 over these loci on proximal Chr 15, Chr 8, and Chr 17, respectively. Furthermore, this intercross was included for analysis of apolipoprotein A2 (Apo2), a candidate gene for Hdlq5 (X. Wang, R. Korstanje and B. Paigen, manuscript submitted).

Subsequent to Bonferroni adjustment for multiple comparisons, differential mRNA expression was observed between the parental strains for the following candidate genes: Nrs5a2, Abc1l, Cpe, Soat2, Abcg5, Apo2, and Apoc2 (Fig. 5, A and B). Nrs5a2 (Lrh1), encoding nuclear receptor subfamily 5, group A, member 2, was considered a putative candidate gene since it encodes a competence factor whose transcriptional target genes include Tcf1 (35), Cyp7a1, Srb1, and Nr0b2 (Shp1) (12), each encoding proteins involved in cholesterol homeostasis. Cpe, encoding carboxypeptidase E, was considered a candidate gene because mice possessing the fat mutation (a spontaneous mutation in the Cpe gene) exhibited higher non-HDL and total cholesterol concentrations relative to controls after feeding the atherogenic diet (4). Although strain 129 displayed lower Cpe expression (Fig. 4A), strain 129 did not confer the allele that increased total cholesterol at Nhd1q1 (Fig. 3C). Soat2, encoding sterol O-acyl transferase 2, was considered a candidate gene because Soat2 knockout mice displayed lower plasma cholesterol concentrations. Cpe expression (Fig. 4A), strain 129 did not confer the allele that increased total cholesterol at Nhd1q1 (Fig. 3C). Soat2, encoding sterol O-acyl transferase 2, was considered a candidate gene because Soat2 knockout mice displayed lower plasma cholesterol concentrations.
Recently, a comprehensive analysis of APOA2 amino acid substitution is related to the altered expression of Apoa2 mRNA, those data demonstrated that Apoa2 was the underlying cause of Hdlq5.

Four factors prompted our selection of genes for investigation of their mRNA expression in the F2 mice: 1) we wished to concentrate on the significant QTL; 2) all available evidence indicated that Hdlq5 is determined by Apoa2 (X. Wang, R. Korstanje and B. Paigen, manuscript submitted) 3) based on the combination of data from the allele effects and the expression studies, Cpe appeared an unlikely putative candidate for Nhdlq1; and 4) Abca1, Lpl, and Abcg5/Abcg8 were implicated in the monogenic lipoprotein disorders. Therefore, we focused on Abca1, Lpl, and Abcg5/Abcg8 and tested them for differential expression among the F2 population.

The significantly greater expression of Abca1, encoding the cholesterol/phospholipid transporter ABCA1, by both strain CAST (2.6-fold, Fig. 4A) and the F2 progeny bearing the CAST genotype (2.2-fold, Fig. 4C), was consistent with the allele effect of Hdlq10 (Fig. 3A) and Chol8 (Fig. 3B). Because overexpression of ABCA1, albeit the human gene, in mice resulted in significant increases both in HDL and total cholesterol levels (44, 49), our data are in agreement with the notion that the same gene may determine both Hdlq10 and Chol8, if Abca1 indeed underlies Hdlq10.

We observed higher expression of Lpl by strain 129 (2.4-fold), which was consistent with the allele effect of Nhdlq1 (Fig. 3C), but after adjustment for multiple comparisons, the difference was nonsignificant (Fig. 4B). However, since the Bonferroni correction is conservative, and because mutations in LPL can cause monogenic lipoprotein disorders, we investigated the expression of Lpl in the F2 progeny in which the 129 genotype conferred significantly higher expression (1.6-fold, Fig. 4C).

Abcg5 and Abcg8 encode the half-transporters comprising the canalicular sterol transporter, whose putative role is to limit intestinal sterol absorption and facilitate biliary sterol secretion (56, 57), thereby modulating the cholesterol available for lipoprotein assembly. Consistent with the allele effect of Chol9 (Fig. 3B), strain 129 expressed higher levels of Abcg5, but not Abcg8 (Fig. 4A). However, differential mRNA expression was not observed for Abcg5 or Abcg8 in the intercross animals (Fig. 4C), indicating that cis-acting elements were unlikely to affect Abcg5/Abcg8 transcription.

In summary, Abca1 and Lpl exhibited differential expression between both the parental strains and the F2 progeny that possessed either homozygous CAST or homozygous 129 genotypes in the Hdlq10 or Nhdlq1 regions, respectively. These data suggest that the expressions of Abca1 and Lpl are controlled by cis-acting elements within the respective QTL regions.

**DISCUSSION**

Using this intercross between strains CAST and 129, we identified 10 QTL that affected lipoprotein cholesterol concentrations: 1) Hdlq5; 2) colocalizing Hdlq10 and Chol8; 3) Chol7; 4) Chol9; 5) Nhdlq1; 6) Nhdlq2; 7) the suggestive QTL at D17Mit221; 8) D8Mit248; 9) D15Mit79; and 10) D15Mit115. Our experimental diet includes cholic acid, which makes the lipoprotein profile and bile acid pool more similar to humans.
Cholesterol and facilitates cholesterol absorption. Cholic acid decreased HDL levels in some strains of mice (22, 32). Such transcriptional repression of apolipoprotein A1 (Apoa1) was probably mediated by the bile acid-activated transcription factor NR1H4 (FXR) (9). Nevertheless, QTL for HDL phenotypes from studies using standard diet and different high-fat diets tend to colocalize with one another (53). Therefore, the loci described in this study constitute valid contributions to our understanding of the genetic control of lipoprotein metabolism.

Fig. 4. Hepatic mRNA expression analysis of positional candidate genes identified for the QTL on Chr 1, 4, 7, 8, 15, and 17 determined using quantitative PCR. The candidate gene is indicated on the abscissa with the chromosome number following in parentheses. Additionally, for those genes tested in the intercross progeny, the relevant QTL name is given. Male animals of the parental strains (129, open bars; CAST, solid bars) were fed the atherogenic diet for 4 wk prior to collection of liver tissue (A and B). Liver tissue from male F1 animals (C), homozygous for 129 or CAST alleles over the Hdlq10, Nhdlq1, and Chol9 QTL, was collected at 10 wk of feeding. Tissue samples for mRNA expression analyses were prepared and analyzed as described (20). Data are reported as the number of target molecules per 10⁶ Gapd molecules, except Abca1 (A) and Apo2, Apoε, and Abcg8 (B), which are reported per 10⁶ Gapd molecules. Data are means ± SE (A and B, mean of 3 separate determinations, n = 5 animals per parental group; C, mean of 2 separate determinations, n = 8 animals per F2 group). The two groups of animals for each candidate gene were compared using Student’s t-test. *P < 0.005. †P < 0.05. ‡P < 0.001. §After adjustment for multiple comparisons, these data were not significantly different (unadjusted P < 0.01). However, since the Bonferroni correction is conservative, we tested the candidates in the F2 progeny.

Chol8 and Chol9 essentially explained the increase in total cholesterol exhibited by the F1 animals. These two QTL elevated total cholesterol by ~50 and ~35 mg/dl, respectively (Fig. 3B), and accounted for the ~90 mg/dl increase displayed by the combined CAST/F1 group. In contrast, the non-HDL and HDL cholesterol levels in the F1 mice were only partially explained by the discovered QTL. Nhdlq1 increased non-HDL cholesterol via a likely additive CAST allele, one copy of which accounted for ~40 mg/dl (Fig. 3C), but the overall change in non-HDL cholesterol concentrations was 120 to 140 mg/dl (Table 1). The ~50 mg/dl decrease in HDL cholesterol in the F1 mice compared with strain 129 (Table 1) was only partially explained by Hdlq5, which decreased HDL cholesterol by 17 mg/dl (Fig. 3A). This is in agreement with our prediction, based on the lower HDL cholesterol levels exhibited by the F1 mice compared with strain CAST (Table 1), that both strains contributed alleles that decreased HDL cholesterol (Fig. 3A).

Although strain 129 exhibited higher HDL cholesterol levels than strain CAST, Hdlq10 increased HDL cholesterol via a CAST allele. Similarly, Chol7 and the suggestive QTL on Chr 7 each increased total cholesterol via 129 alleles, despite strain CAST exhibiting higher total cholesterol levels. Such a phenomenon of detecting an allele that increases a trait from the parental strain with lower values for the trait is encountered frequently in QTL crosses (27). In this cross, both parental strains carry alleles that influence lipoprotein cholesterol levels in both directions.

To investigate the potential of altered transcription rates, we performed mRNA expression studies on a subset of genes located within the 95% CI of our QTL that we considered likely candidate genes, that is, genes with known roles in lipid metabolism. Furthermore, one hypothesis that was formed from our earlier investigations is that functional but variant forms of the proteins that are mutated and cause monogenic lipoprotein disorders are also involved in determining lipoprotein levels in general (20). Two such genes, Abca1 and Lpl, displayed differential expression between strains (and genotypes in the F2 mice). Importantly, our data are consistent with other data such as those derived from studies of the respective knockout and transgenic mice.

Several lines of evidence support the candidacy of Abca1 for Hdlq10. Hdlq10 was identified in an earlier cross between
strains CAST and DBA/2J in which Abca1 exhibited differential expression, both between parental strains and the alternate homozygous F2 genotypes (20). The present investigation, which confirmed Hdlq10, indicated that the expression of Abca1 was greater in strain CAST than strain 129, which we confirmed using the F2 animals selected for their homozygous genotypes across the Hdlq10 locus (Fig. 4C). These data strongly suggest that local, cis-acting elements control the expression of Abca1 rather than trans-acting elements remote from the gene and QTL. High expression (transgenic; Refs. 44, 49) and low expression (knockout; Refs. 7, 24, 30) of Abca1 resulted in increased and decreased HDL levels, respectively. The evidence indicates that at least one polymorphism exists in the murine Abca1 gene or its regulatory region(s) that affects HDL cholesterol concentrations via higher hepatic expression, since the liver is the predominant source of nascent HDL (2, 28). Hdlq10, detected in intercrosses between strain CAST and both strains 129 (present study) and DBA/2J (20), confirmed an earlier QTL for HDL cholesterol in a cross between strains C3H/HeJ and C57BL/6J (22). Mutagenesis experiments identified independently two mutations (Lch, 26.7 cM; and Lch2, 26.0 cM) with low HDL concentration phenotypes (47, 48). In human studies, HDL cholesterol levels were linked to a QTL harboring ABCA1 (1, 34) and were associated with ABCA1 promoter polymorphisms (19). We postulate that Abca1 comprises an exciting candidate gene for Hdlq10 and, if proven to be the underlying gene, may contribute to the variability of the HDL phenotype in both mice and humans.

Several lines of evidence support the candidacy of Lpl for the co-occident QTL for non-HDL (Nhdlq1) and total (D8Mit248) cholesterol on Chr 8. Importantly, it must be noted that LPL/Lpl is not normally expressed in adult mammalian liver (26) but is expressed under certain conditions including cholesterol feeding (37). Lpl knockout mice demonstrated marked increases in non-HDL cholesterol (45, 54), whereas Lpl transgenic mice exhibited much reduced non-HDL cholesterol levels (43). Consistent with these findings, a CAST allele at Nhdlq1 increased non-HDL cholesterol (Fig. 3C), and the CAST genotype displayed lower Lpl mRNA expression in both parental (Fig. 4B) and F2 mice (Fig. 4C). As the QTL region is narrowed to identify the causative gene, the search may benefit from simultaneously testing the hypotheses generated in this study. For example, it will be key to determine whether differential expression of Lpl mRNA and LPL activity occurs in muscle and adipose tissues, the primary sites of LPL expression (26).

In addition to our interest in genes such as Abca1 and Lpl, recent investigations by our laboratory identified the gene underlying Hdlq5, a QTL for HDL cholesterol that was identified repeatedly in mouse and human studies (53). An analysis of the amino acid sequence of APOA2 among 42 inbred mouse strains indicated that in the 16 crosses that exhibited Hdlq5 (including the present study), a valine (129) to alanine (CAST) amino acid substitution at residue 61 was responsible for this QTL (X. Wang, R. Korstanje, and B. Paigen, unpublished observations). In the present study, it remains to be determined whether Hdlq5 actually represents two QTL, the second of which may coincide with Chol7.

Crossing strain CAST into two genetic backgrounds, i.e., strains DBA/2J and 129, largely resulted in the detection of different QTL in the two studies. Three of the four QTL for HDL cholesterol that were detected in the CAST × DBA/2J intercross were contributed by strain DBA/2J (20), thus providing an explanation for their lack of detection in the present intercross. These data are consistent with the determination of lipoprotein levels either by different loci or by different alleles at the same locus between strains DBA/2J and 129. In contrast, a number of QTL were detected in one study and not the other, despite the fact that all were determined by CAST alleles. In this cross, two QTL increased non-HDL cholesterol via CAST alleles on Chrs 8 (Nhdlq1) and 15 (D15Mit115) (Fig. 3C), whereas in the CAST × DBA/2J intercross, we detected one QTL that increased non-HDL cholesterol (and total cholesterol) via a CAST allele on Chr 9 (Chol6) (20). The final example derived from a comparison of the two studies highlighted the fourth QTL that increased HDL cholesterol, Hdlq10. In both studies, Hdlq10 was contributed by strain CAST and displayed similar allele effects (Fig. 3A and Ref. 20). We can infer from the combined data that complex metabolic interactions are uncovered only in the presence of certain alleles that are present in some strains but not others. The corollary of this is that QTL that are found in multiple crosses, despite the differences in genetic background, are likely to be more important, stronger (e.g., Hdlq10), and caused by the same underlying gene. These observations are testament to the “daisy chain” experimental design that we employed to reveal the full ensemble of lipoprotein regulatory genes. Furthermore, the ability to repeat the detection of QTL such as Hdlq5 and Hdlq10 was considered an important aspect of QTL mapping for complex traits (18). Currently, we are exploring new methods to refine genomic regions such as Hdlq10. One method is to combine data from multiple crosses, e.g., CAST × 129 combined with CAST × DBA/2J, prior to a new QTL analysis. A second method is haplotype analysis of single nucleotide polymorphism data from which instructive data may be extracted both from crosses that do and do not exhibit a certain QTL (50).

Many of the QTL detected in this intercross overlap QTL for lipoprotein cholesterol levels from mice and with orthologous QTL from humans. This is a crucial factor, since our working hypothesis is that we will identify human lipoprotein genes using murine genetics. The QTL for total cholesterol on Chr 7 colocalized with QTL for total and HDL cholesterol in mice fed standard diet (22) and partially overlapped a human QTL for HDL cholesterol (53). This QTL also colocalized with a human QTL for LDL cholesterol (29) that was located near the APOC1/APOC2/APOE gene cluster and CEBPA. The QTL likely harbors the human ABCC6 gene, a polymorphism of which was associated with lower plasma triacylglycerol and higher HDL cholesterol levels (51), but did not display differences in expression between strains CAST and 129 (Fig. 4A). The QTL for total cholesterol on distal Chr 15 (D15Mit79) colocalized with loci affecting cholesterol absorption (Chab4, Ref. 41) and HDL cholesterol levels (Hdlq4; Refs. 36, 39). The QTL for non-HDL cholesterol on proximal Chr 15 (D15Mit115) coincided with an unnamed locus also determining non-HDL cholesterol levels in mice (39). This locus overlapped two human QTL for HDL cholesterol (53). The remaining QTL on Chr 8 and X did not overlap with any reported murine lipoprotein or cholesterol homeostatic QTL. However, the QTL at D8Mit248 colocalized with a human QTL for HDL cholesterol that mapped close to LCAT (23), but
not LPL. Since Ndhq1 was detected for non-HDL cholesterol and not HDL cholesterol, these data imply that there may be multiple lipidoprotein cholesterol QTL in these orthologous regions. We evaluated positional candidate genes for 8 of the 10 QTL. For QTL on proximal Chr 15 and Chr X, no candidate genes were obvious. Our mRNA expression data, combined with supporting evidence from other sources, suggest that Abca1 and Lpl represent intriguing candidate genes for Hdlq10 and Ndhq1, respectively. Other genes remain to be investigated, including the numerous other genes in the QTL region that may possess unrecognized roles in lipidoprotein metabolism. These studies will be facilitated by the completion of the remaining intercrosses in our “daisy chain” (Fig. 1), especially as we explore new methods of refining QTL. We are actively pursuing methods that include combining data from multiple crosses for a single analysis and haplotyping of single nucleotide polymorphisms among various crosses (50). Five of the 10 QTL detected in this study coincided with orthologous QTL for lipidoprotein phenotypes derived from humans [Hdlq1 (Chr 1), Hdlq4 (Chr 4), Ndhq1 (Chr 8), and the QTL on Chr 7 and proximal Chr 15], indicating that these QTL are ideal to pursue in order to predict the corresponding human genes that affect plasma lipidoprotein concentrations.

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