Genetic analysis of intestinal cholesterol absorption in inbred mice

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Abstract **A genetic mapping strategy was employed to identify chromosomal regions harboring genes that influence the absorption of intestinal cholesterol in the mouse. Analysis of seven inbred strains of male mice (129P3, AKR, BALB/c, C3H/He, C57BL/6, DBA/2, and SJL, all from Jackson Laboratories) revealed substantial differences in their abilities to absorb a bolus of cholesterol delivered by gavage. Crosses between high (AKR, 129) and low (DBA/2, SJL) absorbing strains revealed evidence for the presence of dominant genes that increase and decrease cholesterol absorption. Backcrosses between F_1 offspring and parental strains (DBA/2xAKD2F_1 and 129xSJL129F_1) followed by linkage analyses revealed four quantitative trait loci that influenced cholesterol absorption. Analyses of recombinant inbred strains identified an additional three loci affecting this phenotype. These seven quantitative trait loci, which map to different chromosomes and are termed *Ch*olesterol *ab*sorption *1–7* (*Chab1–7*) loci, together influence the absorption of intestinal cholesterol in mice and are likely to be involved in different steps of this complex pathway.**—Schwarz, M., D. L Davis, B. R. Vick, and D. W. Russell. **Genetic analysis of intestinal cholesterol absorption in inbred mice.** ***J. Lipid Res.*** **2001.** 42: **1801–1811.**

Supplementary key words quantitative trait mapping • lipid metabolism • bile acids

Cholesterol is an essential constituent of mammalian cell membranes and is further metabolized to many important molecules, including bile acids, steroid hormones, and vitamins. In the whole animal, the demand for cholesterol is met by de novo sterol synthesis and by the absorption of intestinal cholesterol. The amount of cholesterol obtained from these sources depends on the composition of the diet and the species in question. For example, in humans, approximately equal amounts of cholesterol are obtained from each pathway (1), whereas in the laboratory mouse, ~70% of the cholesterol demand is met through synthesis and 30% through absorption (2). These percentages are not absolute because some of the cholesterol absorbed in the gut is derived from cholesterol synthesized in the liver and secreted into the bile. Whereas the genes encoding many enzymes in the biosynthetic pathway are well characterized, less is known about those involved in cholesterol absorption.

The absorption of dietary cholesterol proceeds in a series of steps, starting in the small intestine and progressing to the lymphatic circulation and liver (3). Both dietary and biliary cholesterol are solubilized initially by bile acids and phospholipids to form mixed micelles (4). This cholesterol is taken up by enterocytes by a poorly defined physiological process and thereafter transported to the endoplasmic reticulum, where it is esterified by the enzyme ACAT. The cholesteryl esters are packaged into chylomicron particles and exported from the enterocyte into the lymphatic circulation (5). After delipidation in peripheral tissues, chylomicron remnants are delivered to the liver, internalized via cell surface receptors, and degraded to their constituents in the lysosomal compartment. The released cholesterol has several possible fates, including assembly into new lipoprotein particles, esterification via ACAT, secretion into bile, or catabolism to bile acids. The overall process of dietary cholesterol utilization is sometimes divided into an uptake phase, defined as the entry of cholesterol into the enterocyte, and an absorption phase, defined as the transfer of cholesterol from the intestinal lumen into the lymph (6). In this and the accompanying study (7), we do not make a distinction between these two phases and refer to the process of uptake and transfer as absorption.

Utilization of dietary cholesterol requires the coordinated actions of many genes that control these different steps, and disruption of a gene in the pathway often leads to a decrease in the utilization of intestinal cholesterol. For example, mutations in cholesterol 7α-hydroxylase and sterol 27-hydroxylase, two key enzymes in the bile acid synthesis pathway, cause a reduction in the solubilization of

Abbreviations: ABC, ATP binding cassette; Chab, cholesterol absorption; cM, centimorgan; LOD, logarithm of the odds; QTL, quantitative trait loci; RI, recombinant inbred.

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intestinal cholesterol and a concomitant decrease in the contribution of this pathway to the whole body pool (8–10). In these cases, there are corresponding increases in the amounts of cholesterol synthesized de novo in the liver and other tissues that maintain cholesterol homeostasis. Drugs that block cholesterol uptake from the intestinal lumen (11–13) or that inhibit the subsequent intracellular esterification of the sterol by ACAT (14) also reduce the utilization of intestinal cholesterol and increase reliance on the biosynthetic pathway. Inactivation of the mouse ACAT2 gene reduces intestinal absorption of cholesterol by preventing esterification (15), and mutation of the apolipoprotein B gene, which disrupts the assembly of chylomicrons in the enterocyte, also decreases the use of intestinal cholesterol (16).

Several indirect lines of evidence suggest that additional genes may participate in the metabolism of gut cholesterol. First, the step in which cholesterol is taken up by enterocytes exhibits selectivity; cholesterol is preferentially absorbed, but related plant sterols are not [as reviewed in ref. (6)]. Second, this selectivity is lost in individuals with the genetic disease sitosterolemia, in whom sistosterol and other plant sterols are readily absorbed due to mutations in the ATP binding cassette G5 (ABCG5) or ABCG8 transport proteins (17, 18). Third, indirect experiments suggest that cholesterol uptake in the mouse is confined to the proximal segment of the small intestine, indicating that the gene products responsible for absorption exhibit region-specific expression patterns (8, 19). Fourth, clinical studies in humans reveal a large degree of interindividual variation in the amount of cholesterol that is absorbed from a standardized diet (20, 21). Fifth, ligands that activate retinoid X or liver X receptors decrease cholesterol absorption by increasing the expression of ABC transport proteins that export cholesterol from the enterocyte back into the lumen of the small intestine (17, 22).

These observations support the existence of multiple genes that function in the utilization of intestinal cholesterol. The identification of new genes in this pathway is hampered by its complexity and the absence of in vitro systems that faithfully reconstitute the many steps. In this and an accompanying paper (7), we use linkage analysis in inbred strains of mice to identify genetic loci that determine how much cholesterol is absorbed from the intestine and the extent to which this cholesterol accumulates in the liver. The results confirm and extend earlier studies on the genetics of cholesterol absorption in the mouse (23–26).

MATERIALS AND METHODS

Animals and diets

Male inbred mice (129P3/J, AKR/J, BALB/cJ, C3H/HeJ, C57BL/6J, DBA/2J, and SJL/J, n = 8–10 per strain), F_1 hybrids ($AKD2F_1$/J, $D2AKF_1$/J, $SJL129F_1$/J, $129SJLF_1$/J, and $B6129F_1$/J, n = 30 per strain), and the recombinant inbred (RI) set AKxD (n = 8 for each of 21 available strains) were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed individually in plastic colony cages containing wood shavings in a humidity-controlled room with alternating periods of light (11 AM to 11 PM) and dark (11 PM to 11 AM). Mice had free access to water at all times and were fed a cereal-based, pelleted rodent diet ad libitum (Teklad No. 7001; Harlan Teklad, Madison, WI). This diet consisted of crude protein 24% (minimum), crude fat 4% (minimum), and crude fiber 5% (maximum). The basal cholesterol content was 0.016%, and the fatty acid composition, expressed as a percentage of total fat, was as follows: oleic acid (31.0%), linoleic acid (30.2%), palmitic acid (19.4%), stearic acid (8.6%), linolenic acid (2.9%), palmitoleic acid (1.7%), myristic acid (0.9%), other (8.2%).

Backcross offspring (N_2 mice) were generated in Dallas. The mating schemes performed were $DBA/2xAKD2F_1$ and $129xSJL129F_1$. In each case, 15 F_1 males were repeatedly mated to 40–60 inbred females until the total number of offspring reached 250–300. During mating and pregnancy and until weaning of offspring, all mice were fed a Teklad No. 7002 chow, which was similar in composition to the Teklad No. 7001 chow except that it contained 6% fat. After weaning (21–28 days of age), male N_2 mice (n = 132 and n = 168 for the two backcrosses, respectively) were housed individually and fed the Teklad No. 7001 diet as described above. The cholesterol absorption phenotypes of the N_2 animals were determined at 2.5–3 months of age.

Intestinal cholesterol absorption

Intestinal cholesterol absorption was measured in vivo by a fecal dual-isotope ratio method (27). At least 3 days before the actual experiment, mice were left undisturbed (no exchange of cages), and maintenance activities in the mouse room were kept to a minimum. The experiment was routinely performed at 8:00–9:00 AM (second half of dark cycle). Mice were anesthetized for ~10 s with diethylether and were dosed intragastrically with a mixture of 2 μCi [5,6-^{3}H]sitostanol (American Radiolabeled Chemicals, Inc., St. Louis, MO) and 1 μCi [4-^{14}C]cholesterol (NEN Life Science Products, Boston, MA) in MCT oil (Mead Johnson & Company, Evansville, IN). The total volume of the dosing mixture was adjusted to the body weight of each mouse (4 μl/g body weight). After gavage, mice were caged individually and provided with fresh food. Stools were collected 72 h after dosing and weighed. The ratios of ^{14}C to ^{3}H in the stool and dosing mixture were determined, and the percentage of cholesterol absorption and SEM were calculated as described (27). In each experiment, a strain of mice known to absorb cholesterol at a high rate was used as a positive control and a strain lacking cholesterol 7α-hydroxylase was used as a negative control. The latter mice have a small bile acid pool size and consequently fail to absorb cholesterol (8, 10).

To examine the time dependence of absorption, measurements were performed after a 24-h stool collection and compared with those obtained after a 72-h stool collection. Absorption was slightly higher (74 ± 3 and 42 ± 5%, respectively) in the 129 and DBA/2 strains at 24 h than at 72 h (67 ± 3 and 35 ± 4%, respectively); however, the relative difference in absorption between the two strains was maintained. The effect of coprophagy during a 72-h collection period has been examined previously in hamsters (27) and found to be of little significance to the percentage absorption determined by this method. The fecal dual-isotope ratio method described here yielded reproducible results in different sets of the same strain of mice and produced values that differed by ±10–15% when absorption measurements were repeated in the same animals at 3-week intervals.

Intestinal cholesterol absorption was measured in parallel using both the fecal dual-isotope ratio method and the plasma dual-isotope ratio method (28) in 129 and AKR mice (high-absorbing strains) and in DBA/2 and SJL mice (low-absorbing strains). The results obtained with the plasma method were consistently 15–25% higher than those obtained with the fecal

method: 85 ± 6 versus 66 ± 4% for 129 mice; 77 ± 8 versus 59 ± 8% for AKR mice; 45 ± 7 versus 29 ± 4% for DBA/2 mice; and 42 ± 7 versus 37 ± 3% for SJL mice. As the rank order of cholesterol absorption among the strains was the same with both assays, the fecal ratio method was used in the current studies for logistical reasons.

Bile acid pool size and composition

The total bile acid content of the small intestine, gallbladder, and liver was measured by extracting the tissues in ethanol after addition of a recovery standard ([24-^{14}C]taurocholic acid; NEN Life Science Products). Lipids were analyzed by HPLC (29), and bile acids were detected by measurement of the refractive index and identified by comparison with standards.

Fecal bile acid excretion

Collected stools were dried, weighed, and ground to a fine powder. The bile acid content of a 1-g aliquot of the ground material was determined by an enzymatic method (29). The daily excretion rate was expressed as μmol/day/100 g body weight.

Biliary lipid composition

Gallbladder bile was harvested from mice that had been fasted for 6 h. The absolute concentrations of bile acid, phospholipid, and cholesterol were measured by HPLC (30, 31) and expressed as mole percent of total lipid content per sample.

Protein measurements

Cholesterol 7α-hydroxylase enzyme activity was measured in liver microsomes prepared by sequential centrifugation as described (8, 10). Immunodetection of this enzyme was performed after separation of proteins by electrophoresis through polyacrylamide gels containing SDS (10). Detection of the ileal bile acid transporter in intestinal brush border membranes by immunoblotting was accomplished using a polyclonal antibody supplied by Dr. Paul A. Dawson (Wake Forest University School of Medicine, Winston-Salem, NC).

DNA preparation

Genomic DNA was isolated from mouse tails by phenol/chloroform extraction (32) and quantitated by spectrophotometry.

Genotype analysis

A total of 100 informative polymorphic markers[4] were analyzed in selected mice from each backcross using PCR and oligonucleotide primers obtained from Research Genetics (Huntsville, AL). PCR reactions (final volume = 10 μl) were performed in 96-well plates in a Gene Amp PCR System thermocycler (Perkin Elmer–Applied Biosystems, Foster City, CA). The final concentrations or amounts of reagents in the reactions were 3.0 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 2 ng/μl genomic DNA, 1× RediLoad buffer (Research Genetics), 0.22 units thermostable DNA polymerase (AmpliTaq; Perkin Elmer), and 0.2 μM of each primer. Reaction mixtures were denatured at 95°C for 2 min followed by 30 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min, and a final elongation step of 72°C for 7 min. When marker amplification was not achieved by this protocol, AmpliTaq polymerase was replaced with Platinum Taq polymerase (0.22 units; GIBCO BRL, Gaithersburg, MD). PCR products were electrophoresed on 2.5–4% (w/v) MetaPhor agarose gels (BioWhittaker Molecular Applications, Rockland, ME) and visualized by ethidium bromide staining. Individual gels were melted and reused up to five times.

[4] The Mouse Genome Database accession number for the set of 100 polymorphic loci used in genotyping animals from the 129xSJL cross is J71432.

Linkage analysis and quantitative trait loci (QTL) mapping

Linkage analysis was performed in the AKxD recombinant inbred set using the MapManager computer program (33, 34). Linkage of phenotypes to previously typed genetic markers (n = 351) in this set was found using interval mapping by linear regression after the elimination of strains RI 2, 7, 9, 10, 13, 16, 20, 21, and 24, which were uninformative (i.e., mean ± SEM overlapped the 40% mean absorption value of parental strains).

Initially, linkage of phenotype to genotype was determined in the N_2 progeny using a Chi-squared test with one degree of freedom (Bymarker program, version 0.2, www.infosci.coh.org/jal). Groups of N_2 mice exhibiting low and high cholesterol absorption were analyzed following a selective genotyping strategy (35). Chromosomal regions defined by markers that showed significant linkage ($P < 0.05$) were analyzed subsequently in the entire progeny. Statistical significance of linkage was re-evaluated by two-point analysis, and several regions that initially showed significant linkage of genotype and phenotype based on skewed genotype ratios in the selected animal population were excluded from further analysis. For the remaining regions, statistical significance of linkage was increased 1.5- to 2-fold by analysis of the complete N_2 progeny. A genetic map of linked chromosomal regions was generated using the Mapmaker/Exp (version 3.06) program based on recombination rates. Using this map, multipoint analyses of phenotypes were performed using the MapMaker QTL program (version 1.1.b). Logarithm of the odds (LOD) scores of 1.9 and 3.3 were used as thresholds for statistically suggestive linkage and significant linkage, respectively (36).

Statistical methods

Concordance correlations between cholesterol absorption measurements in two different sets of RI strains were tested using a procedure described elsewhere (37). Reproducibility in the rank order of RI strain means was determined using a top-down correlation test (38). Frequency distributions of cholesterol absorption values in N_2 populations were tested for symmetry and kurtosis using the D'Agostino-Pearson K^2 test (39). The data fit closely to a normal distribution and were used directly in QTL analyses without further manipulation.

RESULTS

Cholesterol absorption in inbred mice

To determine if the amount of cholesterol absorbed from the diet was under genetic control, cholesterol absorption was determined using a dual-isotope fecal assay in 3-month-old male animals (n = 15–18) from seven inbred strains of laboratory mice. The mean percent cholesterol absorption and SEM were calculated for each strain using data obtained from two separate experiments. The mean cholesterol absorption varied 3-fold and ranged from a high of 66% in 129 mice to a low of 28% in SJL mice (**Fig. 1**). The rank order of absorption in the strains was 129 > AKR > C3H/He > BALB/c > C57BL/6 > DBA/2 > SJL. The mean cholesterol absorption values in high- and low-absorbing strains were between 3 and 4 SD apart, providing the statistical power to identify genes that contribute to this phenotypic trait.

Parameters of bile acid metabolism in inbred mice

The amount of dietary cholesterol absorbed is directly correlated with the size and composition of the bile acid

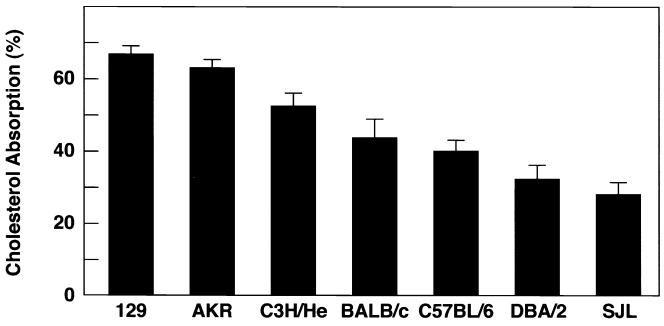

Fig. 1. Cholesterol absorption in inbred strains of mice. Fifteen to 18 male animals (age = 3 months) from each of the indicated strains were assayed for cholesterol absorption in two separate experiments. Measurements from individual animals of each strain were used to calculate a mean cholesterol absorption value (y-axis) and SEM (vertical lines on histogram bars). Strains are arranged on the x-axis by descending cholesterol absorption values from left to right.

pool (8–10). To determine if the observed differences in cholesterol absorption between inbred strains were due to alterations in these parameters, we compared the size and composition of the bile acid pool in two high-absorbing strains (129 and AKR, Fig. 1) and two low-absorbing strains (DBA/2 and SJL, Fig. 1). Cholesterol absorption did not correlate with the size of the bile acid pool (**Table 1**), because one of the highest absorbing strains (AKR) had the smallest pool size per gram body weight. Similarly, no marked differences were found in the composition of the bile acid pool; the ratios between the two predominant bile acids cholate and β-muricholate were similar in the four strains (Table 1). The concentrations of biliary cholesterol, bile acids, and phospholipids in the four strains of mice are also reported in Table 1. Low-absorbing DBA/2 mice had a higher molar ratio of cholesterol in gallbladder bile than did the high-absorbing AKR mice (P = 0.034), but biliary cholesterol saturation was similar in the high-absorbing 129 versus low-absorbing SJL mice.

Hepatic bile acid synthesis was estimated by quantifying fecal bile acid excretion rates in the steady state, and again, no correlation was observed between this parameter and cholesterol absorption (Table 1). The AKR mice had the highest, and 129 mice the lowest, bile acid excretion rates, and yet these two strains had similar cholesterol absorption (Fig. 1). The differences in bile acid synthesis rates correlated with the levels of cholesterol 7α-hydroxylase mRNA, which were highest in AKR mice and lowest in 129 mice (data not shown).

The four strains of mice did not exhibit differences with respect to the distributions in the intestine or the expression levels of the ileal bile acid transporter (data not shown), suggesting similar rates of bile acid absorption and pool recycling. Baseline parameters such as body weight, food intake, and stool output varied from strain to strain but did not correlate with absorption (Table 1). Together, these experiments suggested that genes other than those specifying bile acid pool size, composition, and recycling affected cholesterol absorption in the mouse.

Genetic analysis of cholesterol absorption

To determine whether the cholesterol absorption phenotype was controlled by a small number of genes with major effects or by a large number of genes with minor effects, we performed crosses between three pairs of strains with disparate absorption values, and this parameter was measured in the resulting F_1 offspring. In a cross between AKR females and DBA/2 males, the $AKD2F_1$ males exhibited the high-absorbing trait of the AKR parental strain (**Fig. 2A**). The data from this cross suggested the existence of dominant high-absorbing traits in the AKR strain.

Similarly, when high-absorbing 129 males were crossed with C57BL/6 females that exhibited a medium-absorption trait, the $B6129F_1$ males inherited the high-absorption phenotype of the 129 parental strain (Fig. 2B). In contrast, when high-absorbing 129 males were crossed with low-absorbing SJL females, the $SJL129F_1$ males exhibited the low-absorption trait of the SJL parental strain (Fig. 2C). These results indicated the possible existence of one or more dominant low-absorbing traits in the SJL genome.

Comparison of the distribution of cholesterol absorption values measured in the F_1 offspring with those of the parental strains revealed slight differences (Fig. 2), which suggested either incomplete penetrance or that more than one gene contributed to cholesterol absorption.

TABLE 1. Bile acid parameters in inbred strains of mice

	129	AKR	DBA/2	SJL
Bile acid pool size (μmol/day/100 g body weight)	99.5 ± 7.8	75.4 ± 3.2	102.5 ± 10.6	96.4 ± 16.2
Cholic acid/β-muricholate ratio	1.34 ± 0.14	1.07 ± 0.10	0.95 ± 0.07	0.84 ± 0.20
Fecal bile acid (μmol/day/100 g body weight)	8.7 ± 1.0	13.5 ± 0.9	11.3 ± 0.9	10.2 ± 1.0
Body weight (g)	23.2 ± 1.1	34.7 ± 1.6	26.1 ± 0.9	27.2 ± 0.9
Food intake (g/day/100 g body weight)	16.3	18.7	19.8	13.6
Stool output (g/day/100 g body weight)	3.0 ± 0.2	3.1 ± 0.1	3.6 ± 0.2	2.7 ± 0.2
Biliary cholesterol (μmol/ml)	2.1 ± 0.1	2.6 ± 0.3	4.0 ± 0.4	1.9 ± 0.4
Biliary bile acids (μmol/ml)	151.7 ± 11.1	174.2 ± 16.7	200.1 ± 8.0	172.1 ± 15.7
Biliary phospholipids (μmol/ml)	17.8 ± 1.2	24.6 ± 2.7	26.9 ± 2.3	18.0 ± 3.1
Molar ratio of cholesterol in bile (%)	3.2 ± 0.2	2.9 ± 0.1	4.3 ± 0.3	2.5 ± 0.3

Measurements of the indicated parameters were performed in 8–10 male animals (3 months of age) from each strain as described in Materials and Methods and are expressed as the mean ± SEM. Food intake was measured in a single experiment involving five animals per strain.

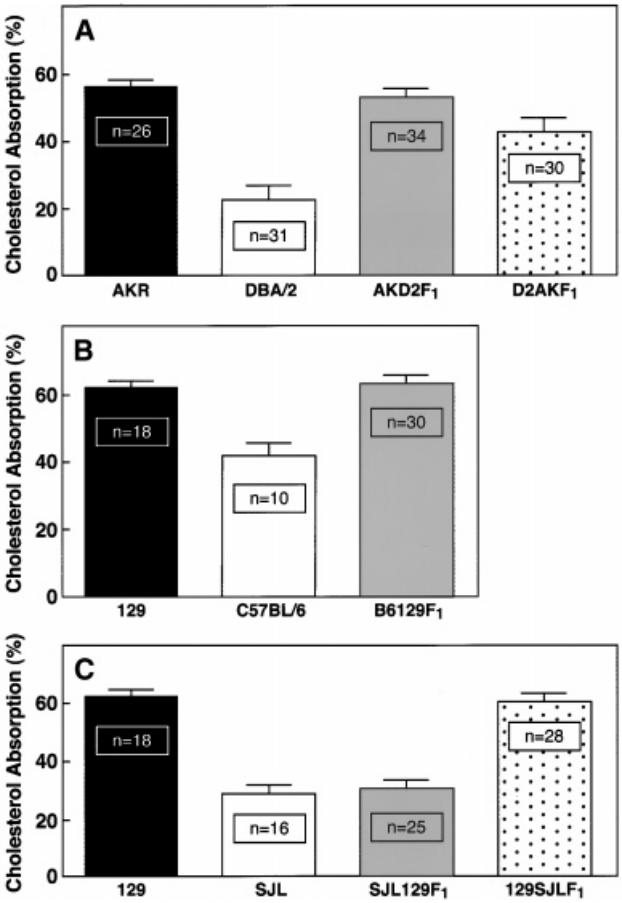

Fig. 2. Cholesterol absorption in parental strains and F_1 hybrids. Inbred strains of mice that exhibited high (AKR, 129), intermediate (C57BL/6), or low (DBA/2, SJL) cholesterol absorption phenotypes were crossed to produce the indicated F_1 generations. The phenotypes of male mice in the F_1 populations were determined by the dual-isotope fecal absorption assay and compared with those of the parental strains. Absorption values were expressed as means of individual measurements (histogram bars) with SEMs (vertical lines on histogram bars). The number of animals (n) assayed for each inbred strain and F_1 generation are indicated inside the histogram bars. A: Crosses were carried out between AKR females and DBA/2 males to produce the $AKD2F_1$ population and between DBA/2 females and AKR males to produce the $D2AKF_1$ population. B: Crosses between C57BL/6 females and 129 males produced the $B6129F_1$ generation. C: Crosses between SJL females and 129 males produced the $SJL129F_1$ population, and crosses between 129 females and SJL males produced $129SJLF_1$ mice.

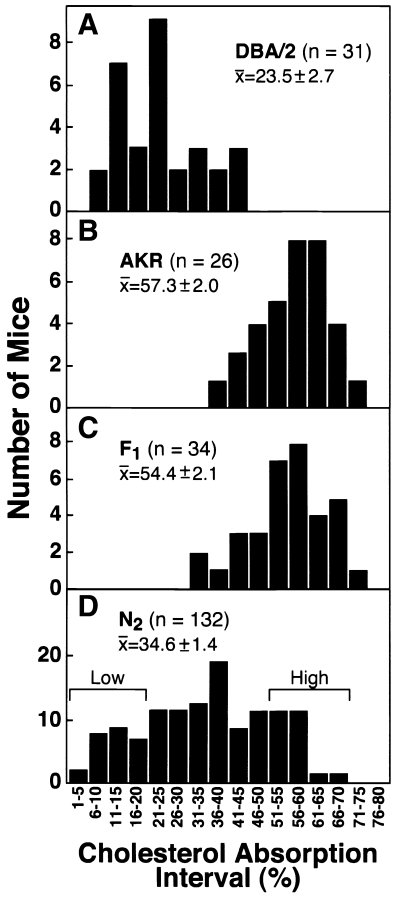

Fig. 3. Distributions of cholesterol absorption values in parental, F_1 hybrid, and N_2 backcross mice. A: Distribution of cholesterol absorption values in 31 DBA/2 male mice and calculated mean (x) and SEM of the population. B: Distribution of cholesterol absorption values in 26 AKR male mice and calculated mean and SEM of the population. C: Distribution of cholesterol absorption values in 34 male offspring (F_1 generation) arising from a cross between AKR females and DBA/2 males and the calculated mean and SEM of the population. D: Distribution of cholesterol absorption values in 132 male offspring (N_2 generation) from a cross between $AKD2F_1$ males and DBA/2 females and calculated mean and SEM of the population. Twenty animals in the N_2 population (15%) that absorbed cholesterol in the low range (<30%) and 23 animals in the N_2 population (17%) that absorbed cholesterol in the high range (>60%) were selected for genotype analysis.

The cholesterol absorption phenotype of the F_1 offspring further depended on which parent exhibited the dominant trait. Thus, when high-absorbing AKR males were crossed with low-absorbing DBA/2 females, the $D2AKF_1$ males absorbed cholesterol in the medium range (Fig. 2A). This effect was even more dramatic when high-absorbing 129 females were crossed with low-absorbing SJL males; the $129SJLF_1$ males showed the high-absorption trait characteristic of the mother (Fig. 2C). We concluded from these data that imprinting or other sex-specific genetic phenomena influenced the expression of genes determining cholesterol absorption (10).

Two approaches were taken to identify genes in the AKR strain that contribute to increased cholesterol absorption. First, $AKD2F_1$ males were crossed to low-absorbing DBA/2 females to generate male offspring representing an N_2 backcross generation (n = 132 mice), and cholesterol absorption was measured in each of these animals. These data were plotted as a frequency distribution and compared with those obtained from the parental strains and the F_1 animals (**Fig. 3**). If a major dominant and highly penetrant gene was responsible for the differences in cholesterol absorption between the two parental strains, then a bimodal distribution of absorption values would be expected in the N_2 generation, however, this distribution was not observed. Instead, cholesterol absorption in the N_2 mice exhibited a continuous distribution that extended

to the extremes of the parental strains (Fig. 3). The absorption values in the N_2 animals ranged from a low of 1–5% to a high of 66–70%, with a mean value of 34.6%, which was intermediate between the means of the two parental strains.

Estimating number of genes that specify high absorption in AKR mice

The normal distribution ($K^2 = 3.45$, $P = 0.19$) of cholesterol absorption values in the N_2 mice suggested that more than one gene influenced how much cholesterol was absorbed. The minimum number of genes contributing to the variance in cholesterol absorption was estimated to be three using Wright's polygene equation (40). This estimate is based on a number of assumptions, including that all loci involved are unlinked, make equal contributions to the phenotype, and are active in a semidominant fashion (41). If several of these assumptions are incorrect, then the method underestimates the number of contributing loci (42).

Linkage analysis of DBA/2xAKD2F$_1$ N_2 backcross animals

Selective genotyping was employed to map the chromosomal locations of these putative genes (35). Genomic DNA was prepared from 20 animals from the N_2 generation exhibiting low cholesterol absorption values (1–20%) and from 23 animals exhibiting high cholesterol absorption (51–70%) (Fig. 3D). Genotype analysis was performed using a panel of 100 polymorphic microsatellite markers distributed at an average distance of one every 15 centimorgans (cM). The resulting genotypic data were analyzed with computer programs (MapMaker/Exp 3.0, MapMaker/QTL 1.16) to identify genetic loci that segregate with the high cholesterol absorption trait.

When a free genetics model with no fixed QTLs was assumed, two statistically significant ($P < 0.05$) linkages were identified in the analysis (**Fig. 4**). A locus on chromosome 2 ($P = 0.0002$) located 4 cM distal to D2Mit305 (LOD score = 3.5) accounted for 9.9% of the variance observed in cholesterol absorption between parental strains. A second locus on chromosome 10 ($P = 0.002$), which was 8 cM proximal to D10Mit61 (LOD score = 1.9), accounted for 6.9% of the genetic variance. We termed these loci *Ch*olesterol *ab*sorption (*Chab*) 1 and 2, respectively. When an additive genetic model was assumed, the LOD scores for D2Mit305 and D10Mit6 increased to 4.0 and 3.5, respectively. In the latter analysis, one locus was fixed to remove its contributed variance, and the chromosomal region was again scanned by multipoint analysis.

Cholesterol absorption in AKxD RI strains

RI strains were used in a second approach to map genes contributing to the cholesterol absorption phenotype. These animals are homozygous at all loci, and the presence or absence of a DBA or AKR region in a chromosome can be correlated with a cholesterol absorption phenotype (41). Cholesterol absorption was measured in eight male mice from 21 AKxD RI strains. A single, fully penetrant gene with a major effect on absorption would be expected

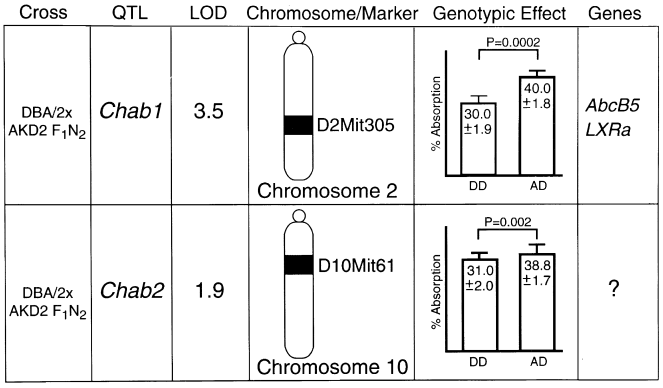

Fig. 4. Chromosomal locations of the *Chab1* and *Chab2* loci identified in DBA/2xAKD2F$_1$ N_2 backcross animals. Idiograms of two chromosomes are shown together with the locations of markers linked to the *Chab1* and *Chab2* quantitative trait loci (QTL). The distributions of the DBA/2 (D) and AKR (A) alleles in the N_2 backcross population animals in the low and high cholesterol absorption intervals are indicated by histogram bars. Candidate genes that map in the identified intervals are shown at right. LOD, logarithm of the odds.

to yield a discrete high-absorbing or low-absorbing phenotype in each RI strain. The distribution of cholesterol absorption values in these strains, however, was continuous, ranging from 23% to 67% (**Fig. 5**). The experiment was repeated in eight additional animals from each of the informative RI strains (numbers 1, 3, 6, 14, 18, 22, 23, 25, 27, and 28). The correlation between the two experimental measurements was high ($r = 0.94$, $P < 0.001$), and there was good agreement between them with respect to the rank order of strain means ($r = 0.74$, $P = 0.016$), indicating reliability of genetic variance. Together, these data suggested incomplete penetrance of a single major gene and/or that the differences in cholesterol absorption between the two strains were due to the effects of multiple genes.

The heritability (h^2) of a quantitative trait indicates the extent to which variation in phenotypes is determined by genetic variation in a shared environment (43). For RI strains, in which genetic variation due to dominance is eliminated, h^2 is expressed as the ratio of additive genetic variance (V_A) and the sum of additive genetic variance and environmental variance (V_E). The V_A and V_E terms for the cholesterol absorption trait in the AKxD RI set were determined as outlined by Plomin and McClearn (44) and were 120.1 and 196.5, respectively. An intermediate heritability value of $h^2 = 0.38$ was obtained from these calculations, indicating that the genes influencing the absorption trait in these two strains do not cause a high degree of variance. The observed phenotypic differences among individual RI strains were therefore due more to environmental variation than to genetic variation.

Linkage analysis in AKxD RI strains

To map the chromosomal locations of genes in the RI strains that contribute to the high-absorption phenotype, the known genotypes were correlated with the cholesterol absorption value using the Map Manager computer pro-

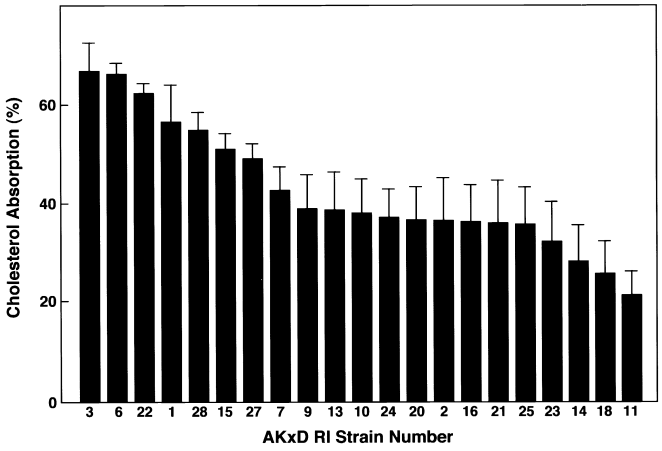

Fig. 5. Cholesterol absorption in AKxD recombinant inbred (RI) strains of mice. Four to eight male animals from each of the indicated strains were assayed for cholesterol absorption in two separate experiments. Measurements from individual animals of each strain were used to calculate a mean cholesterol absorption value (y-axis, histogram bars) and SEM (vertical lines on histogram bars). Strains are arranged on the x-axis by descending cholesterol absorption values from left to right.

gram. This analysis revealed four genetic loci that segregated in a statistically significant ($P < 0.05$) manner with the high cholesterol absorption trait and that were different from those identified in the DBA/2xAKD2F$_1$ N$_2$ backcross (Fig. 4). As summarized in **Fig. 6**, a locus termed *Chab3*, which was located on chromosome 6 closely linked to the *rhodopsin* (*Rho*) gene, exhibited a probability value of $P = 0.003$, a LOD score of 2.0, and accounted for

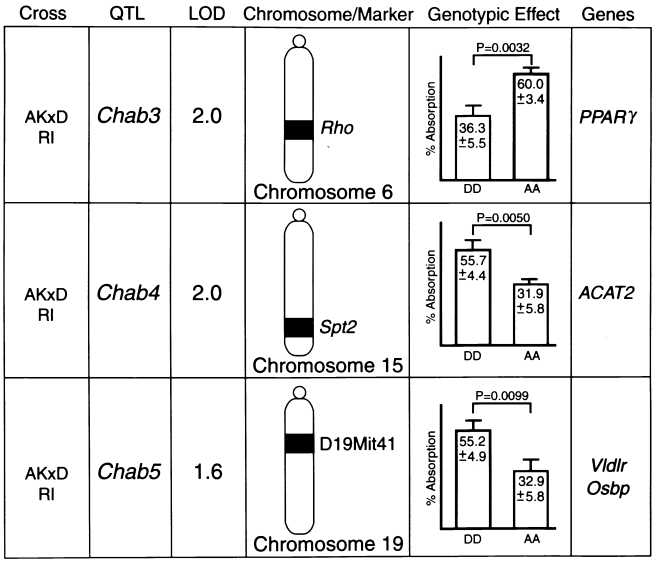

Fig. 6. Chromosomal locations of the *Chab3*, *Chab4*, and *Chab5* loci identified in AKxD RI strains. Idiograms of three chromosomes are shown together with the locations of markers linked to the individual QTL. Histogram bars indicate the distributions of the DBA (D) and AKR (A) alleles in the high- and low-absorbing RI strains. Candidate genes that map in the identified intervals are shown at right.

~50% of the variance observed in cholesterol absorption in the AKxD RI mice. Similarly, the *Chab4* locus on chromosome 15 ($P = 0.005$), which was located close to the *salivary protein 2* (*Spt2*) gene, exhibited a LOD score of 2.0 and accounted for 49% of the genetic variance, whereas the *Chab5* locus on chromosome 19 ($P = 0.009$, LOD = 1.6) that was linked to the D19Mit41 marker generated 42% of the variance. The sum of the variances contributed by the *Chab3*, *Chab4*, and *Chab5* loci was >100%, which suggested that interactions between these loci affected absorption values.

Linkage analysis of cholesterol absorption in SJL mice

As indicated by the data shown in Fig. 2C, the F$_1$ mice arising from a cross between high-absorbing 129 males and low-absorbing SJL females exhibited the low-absorption trait of the SJL strain. To identify genes in these two strains that contributed to cholesterol absorption, we crossed SJL129F$_1$ males to high-absorbing 129 females to generate male offspring representing an N$_2$ backcross generation (n = 168). Cholesterol absorption was measured in each N$_2$ animal, and the results were plotted as a frequency distribution. This distribution showed no significant deviation from normality ($K^2 = 1.948$, $P = 0.39$). The data shown in **Fig. 7** compare the cholesterol absorption values obtained in the parental SJL strain (Fig. 7A), the parental 129 strain (Fig. 7B), the F$_1$ generation (Fig. 7C), and the N$_2$ generation (Fig. 7D). The amounts of cholesterol absorbed in N$_2$ mice varied from a low of 6–10% to a high of 76–80% and spanned the ranges of the parental SJL and 129 strains (Fig. 7). The calculated N$_2$ mean value (46.8%) was intermediate between those of the parental strains. The distribution of absorption values in the N$_2$ mice suggested multiple genetic influences on the amount of cholesterol absorbed. The number of involved genes was estimated using the Wright equation (40) and yielded a minimum estimate of 2.5 genes that contributed to the dominant low-absorbing trait in the SJL strain.

Linkage analysis of 129xSJL129F$_1$ N$_2$ backcross animals

Selective genotyping was employed to map the chromosomal locations of these putative genes. Genomic DNA was prepared from 40 low-absorbing (6–30%) and 40 high-absorbing (61–80%) animals from the N$_2$ generation (Fig. 7D). Genotype analysis was performed using a panel of 100 informative microsatellite markers[4] distributed at a mean distance of every 15 cM. From these data, two genetic loci were identified that segregated with the low cholesterol absorption trait. The *Chab6* locus on chromosome 1 ($P = 0.003$) was located 6 cM proximal to D1Mit90 (LOD score = 2.1) and accounted for 12.3% of the observed genetic variance between parental strains. The *Chab7* locus on chromosome 5 ($P = 0.0006$) was located 4 cM proximal to D5Mit317 (LOD score = 3.3) and accounted for 14.1% of variance. When an additive genetic model was considered in the calculations, the LOD scores for D1Mit90 and

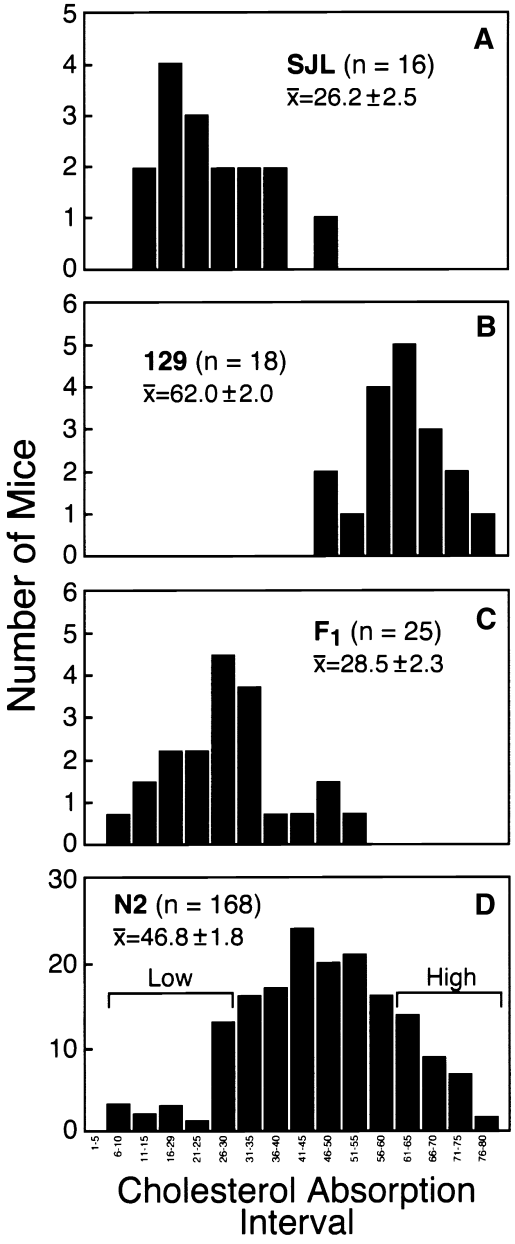

Fig. 7. Distributions of cholesterol absorption values in parental, F_1 hybrid, and N_2 backcross mice. A: Distribution of cholesterol absorption values in SJL male mice (n = 16) and calculated mean (x) and SEM of the population. B: Distribution of cholesterol absorption values in 129 male mice (n = 18) and calculated mean and SEM of the population. C: Distribution of cholesterol absorption values in male offspring (F_1 generation, n = 25) arising from a cross between SJL females and 129 males and calculated mean and SEM of the population. D: Distribution of cholesterol absorption values in male offspring (N_2 generation, n = 168) arising from a cross between SJL129F_1 males and 129 females and calculated mean and SEM of the population. Forty animals in the N_2 population that absorbed cholesterol in the low range (<30%) and 40 animals in the N_2 population that absorbed cholesterol in the high range (>60%) were selected for genotype analysis.

D5Mit317 increased to 2.9 and 4.3, respectively. Again, these loci were different from those identified in the DBA/2xAKD2F_1 backcross (Fig. 4) and in the analysis of the AKxD RI strains (Fig. 6).

DISCUSSION

The experiments described here reveal that cholesterol absorption in inbred strains of mice, as defined by a dual-isotope fecal absorption assay, is a multigenic quantitative trait with a complex inheritance pattern. The genetic factors controlling cholesterol absorption are strain specific, influenced by sex-specific genetic phenomena, and interact with varying degrees of dominance. These findings confirm previous studies on the genetic control of cholesterol absorption in inbred mice (23–26), and they provide new insights into the genetic bases of this quantitative trait.

Five QTL were identified in the AKR genome that segregated with a dominant high cholesterol absorption phenotype in crosses with the low-absorbing DBA/2 strain. Two of these loci were identified through the analysis of N_2 backcross animals, whereas three loci were identified in RI strains. These putative cholesterol absorption (*Chab*) genes mapped to distinct chromosomes. Similar genetic analyses identified loci on chromosomes 1 and 5 that segregate with a dominant low-absorbing phenotype in crosses between 129 and SJL mice. The chromosomal locations of these genes are different from those that give rise to the high-absorption phenotype present in the AKR strain. The large number of QTL identified here that influence intestinal lipid absorption in inbred strains of mice underscores the complexity of the absorption process, which involves dozens of different genes. In addition, statistical analyses of linkage data from both backcrosses, which assumed a free genetic versus an additive model of inheritance, indicated the presence of epistatic interactions between the identified loci. Thus, the cohort of genes involved in absorption may encode both structural and regulatory proteins.

The loci in the AKR mice that influence cholesterol absorption map to regions of the genome that have been shown by other investigators to contain genes participating in lipid metabolism (Figs. 4 and 6). The *Chab1*-containing region identified on chromosome 2 has a significant LOD score (3.5) and includes the genes for the ABC transporter protein B5 and for the liver X receptor α, which encodes a transcription factor that regulates the expression of many lipid-metabolizing genes (45), including ABC transporters that influence biliary cholesterol secretion and intestinal cholesterol absorption (17, 18, 22, 46). This QTL does not overlap with a locus conferring resistance to diet-induced hypercholesterolemia localized to proximal chromosome 2 in studies using C57BL/6ByJ mice (47).

The *Chab2* QTL on chromosome 10 has a LOD score of 1.9 that increases to 3.5 when an additive model of gene contribution is assumed. There were no obvious genes involved in lipid metabolism within this region. The region on chromosome 6 harboring *Chab3* has a suggestive LOD score (2.0) and encompasses the peroxisome proliferator activator receptor γ gene, which encodes another member of the nuclear hormone receptor family that modulates the transcription of lipid-metabolizing genes (48).

Chab4 on chromosome 15 has a suggestive LOD score (2.0) and is linked to the ACAT2 gene (49), which is essential for the esterification and absorption of intestinal cholesterol (15, 50). This region of chromosome 15 also contains a locus that influences plasma triglyceride levels (51). The *Chab5* QTL on chromosome 19 has the lowest LOD score (1.6) and includes several potential candidate genes that may participate in cholesterol absorption, including the VLDL receptor gene (*Vldlr*), which binds lipoproteins and influences neuronal migration in the brain (52, 53), and the oxysterol binding protein gene (*Osbp*), which binds oxysterols within the cell (54). None of these loci overlap with a dominant QTL mapped to chromosome 4 that confers sensitivity to diet-induced obesity in AKR/J mice (55) or with the *ald* locus on chromosome 1, which encodes a truncated but active ACAT1 enzyme in AKR/J mice (56). Thus, the high cholesterol absorption phenotype in the AKR/J mice does not appear to be caused by the peculiarities in lipid metabolism previously described in this strain.

The chromosomal regions identified in the DBA/2xAKD2F_1 N_2 backcross animals (Fig. 4) are different from those identified in the AKxD RI strains (Fig. 6). This outcome may be due to the fact that RI and backcross analyses examine different genotypes. All loci are homozygous in the RI strains but either homozygous or heterozygous in the N_2 backcross animals. If, for example, the AKR alleles of *Chab3*, *Chab4*, or *Chab5* must be present in homozygous form to convey high absorption, then linkage to these loci may not be detected in a backcross analysis. Rather, genetic study of the AKRxDBA F_2 generation would be needed to further dissect the involvement of these loci in the absorption phenotype. Conversely, the *Chab1* and *Chab2* loci identified in the backcross population may have too small effects to be detected in the RI analysis, the power of which is limited by the small number of inbred strains (n = 21) available for the AKxD set.

Two different chromosomal regions containing cholesterol absorption QTL were identified in backcrosses between SJL and 129 mice (**Fig. 8**). Unlike the loci found in the DBA/2xAKD2F_1 N_2 and AKxD RI strains, these two genes segregate with a dominant low-absorption phenotype (Fig. 2). The *Chab6* QTL with a suggestive LOD score of 2.1 maps to chromosome 1 in a region that contains the sterol 27-hydroxylase gene (*Cyp27*) and a gene encoding an HDL binding protein (*Hdlp*). Sterol 27-hydroxylase participates in the synthesis of bile acids (57) and the metabolism of triglycerides (9). No overt differences were found between the SJL and 129 strains in various parameters of bile acid metabolism (Table 1), which suggests either that the *Chab6* QTL is linked to but distinct from the sterol 27-hydroxylase gene or that extra-hepatic expression of this enzyme may be influencing the amount of cholesterol absorbed from the small intestine (58). The *Hdlp* gene on chromosome 1 (59) encodes a protein that has the capacity to bind HDL (60), however, a direct role for this protein in lipid metabolism has not been demonstrated (61). The *Chab7* QTL has a significant LOD score (3.3) and is located in the same region of chromosome 5

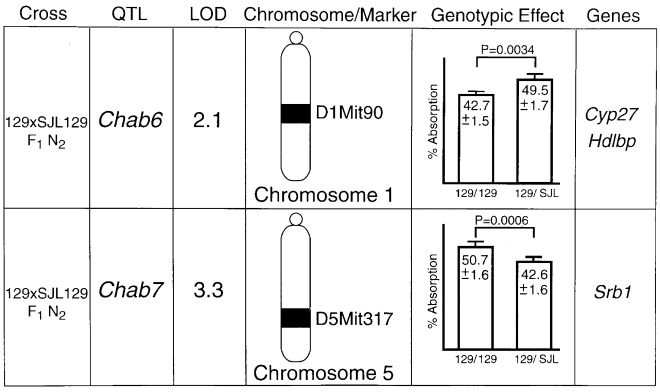

Fig. 8. Chromosomal locations of the *Chab6* and *Chab7* loci identified in 129xSJL129F_1 N_2 backcross animals. Idiograms of two chromosomes are shown together with the locations of markers linked to the *Chab6* and *Chab7* QTL. The distributions of the 129 and SJL alleles in the N_2 backcross population animals in the low and high cholesterol absorption intervals are indicated by histogram bars. Candidate genes that map in the identified intervals are shown at right.

as the SR-BI gene, which has been directly implicated in cholesterol absorption (62). However, studies in mice lacking *Srb1* do not support a role for this cell surface receptor in intestinal cholesterol absorption (63).

The list of candidate genes included in the cholesterol absorption QTL is also distinguished by what it does not contain. Several genes that may play direct roles in the utilization of intestinal cholesterol, including apolipoprotein E (64), the ABCA1, ABCG5, and ABCG8 transporters (17, 22), and ACAT-1 (65), do not map to the absorption gene intervals identified here, nor do genes that encode known components of the absorption pathway, such as enzymes that synthesize bile acids (hydroxylases encoded by the *Cyp7a1*, *Cyp7b1*, and *Cyp39a1*) or that transport bile acids (*Ibat*, *Ibabp*, etc.) or those that package cholesterol in the gut (apolipoprotein B, microsomal triglyceride transport protein genes). The genes predisposing to cholesterol gallstone formation (*Lith* genes) also map outside the intervals identified here (66, 67).

There are several possible explanations for these exclusions. First, the dual-isotope fecal absorption assay used here may not detect the quantitative influences of all genes that participate in the utilization of intestinal cholesterol. Second, the relative contribution of a particular gene may be too low to be detected in the phenotypic assay or in the genetic backgrounds tested here. Third, we have screened for absorption genes in just four strains of inbred mice, which may contain a limited subset of absorption QTL. Fourth, there may be little or no interstrain contribution of these genes to the amount of cholesterol absorbed. We are currently designing more specific tests to determine the contributions of the QTL identified in this study and of these various candidate genes.

It will be straightforward to test the potential role in cholesterol absorption of each candidate gene by examining their tissue-specific and strain-specific expression pat-

terns in the various parental, RI, and backcross animals generated in this study. If these studies eliminate candidate genes that map to the intervals recognized here, then the next step will be the identification of the actual gene or genes in each chromosomal region that influence cholesterol absorption. Achieving this goal will entail cloning these regions of DNA, a task largely accomplished in the mouse genome effort (68), and the analysis of coding or regulatory regions within the DNAs. The latter undertaking will be accelerated by knowledge of the DNA sequence of the mouse genome and by deriving congenic sublines that contain smaller regions of the target chromosomal regions.

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