A locus conferring resistance to diet-induced hypercholesterolemia and atherosclerosis on mouse chromosome 2

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Abstract Dietary cholesterol is known to raise total and low density lipoprotein cholesterol concentrations in humans and experimental animals, but the response among individuals varies greatly. Here we describe a mouse strain, C57BL/6ByJ (B6By), that is resistant to diet-induced hypercholesterolemia, in contrast to the phenotype seen in other common strains of mice including the closely related C57BL/6J (B6J) strain. Compared to B6J, B6By mice exhibit somewhat lower basal cholesterol levels on a chow diet, and show a relatively modest increase in absolute levels of total and LDL/VLDL cholesterol in response to an atherogenic diet containing 15% fat, 1.25% cholesterol, and 0.5% cholate. Correspondingly, B6By mice are also resistant to diet-induced aortic lesions, with less than 15% as many lesions as B6J. Food intake and cholesterol absorption are similar between B6By and B6J mice. To investigate the gene(s) underlying the resistant B6By phenotype, we performed genetic crosses with the unrelated mouse strain, A/J. A genome-wide scan revealed a locus, designated *Diet1*, on chromosome 2 near marker D2Mit117 showing highly significant linkage (lod = 9.6) between B6By alleles and hyporesponse to diet. Examination of known genes in this region suggested that this locus represents a novel gene affecting plasma lipids and atherogenesis in response to diet.—Mouzeyan, A., J. Choi, H. Allayee, X. Wang, J. Sinsheimer, J. Phan, L. W. Castellani, K. Reue, A. J. Lusis, and R. C. Davis. **A locus conferring resistance to diet-induced hypercholesterolemia and atherosclerosis on mouse chromosome 2. *J. Lipid Res.* 2000.** 41: **573–582.**

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Elevated plasma cholesterol concentration is an established risk factor for atherosclerosis and may also play a role in gall stone formation, stroke, and obesity. Individuals in the human population exhibit a wide range of cholesterol levels as a result of differences in numerous genetic and dietary factors (1–6). Genetic variations in absolute cholesterol levels as well as responsiveness to dietary cholesterol have also been studied in animal models including non-human primates (rhesus, squirrel, cynomolgus, and African green monkey, baboon, and marmoset), dogs, pigs, rabbits, rats, hamsters, and mice (reviewed in 7 and 8). The metabolic basis for variability in response to dietary cholesterol likely represents a combination of factors, cholesterol absorption, lipoprotein production and metabolism, and bile acid excretion, that are individually affected by numerous genetic and environmental factors. In any case, it is clear that cholesterol levels and changes that occur in response to diet are each determined by multiple genes, most of which have yet to be identified. Thus, a better understanding of observed inter-individual variability in cholesterol levels and response to dietary cholesterol awaits the identification of the underlying genes.

Inbred mouse strains have long been a valuable source of genetic variation, and the mouse is unmatched among experimental animal models in the wealth of genetic information available to facilitate gene mapping. We, and others, have investigated genetic differences between inbred mouse strains in several aspects of lipid metabolism and susceptibility to atherosclerosis (9–18). The C57BL/6J strain has been identified as responsive to a cholesterol-enriched atherogenic (Ath) diet in terms of both elevated plasma lipid levels and occurrence of aortic lesions (11–14). When fed the Ath diet, C57BL/6J mice characteristically exhibit a 2- to 3-fold increase in total cholesterol levels, with a 6- to 8-fold increase in LDL/VLDL cholesterol and a 50% decrease in HDL cholesterol levels (14).

Abbreviations: Ath diet, Atherogenic diet; B6By, C57BL/6ByJ; B6J, C57BL/6J; EM, expectation maximization; QTL, quantitative trait locus.

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C57BL/6J mice also develop aortic lesions with characteristics of human atherosclerotic plaques, including smooth muscle cell proliferation, fibrous caps, and calcification (15). These qualities of the C57BL/6J mouse strain have led to its adoption as a useful genetic background in which to express transgenes, and to identify novel genes, that may have a role in lipid metabolism and atherosclerosis (16, 17).

In 1951, a substrain of C57BL/6J was established by separating and breeding independently from the original C57BL/6J strain (19). This strain, known as C57BL/ByJ (referred to here as B6By), is therefore nearly genetically identical to C57BL/6J (B6J) with the only expected differences being the result of de novo mutation occurring since the separation of the two substrains. Despite their common genetic origins, the C57BL/6 substrains B6J and B6By exhibit marked differences in response to the Ath diet. In striking contrast to the B6J strain, B6By mice show relatively modest changes in total cholesterol (ΔTC) or in LDL/VLDL cholesterol (ΔLDL/VLDL) on the Ath diet. Given the extensive genetic identity between B6By and B6J, it is likely that only one or a few genes determine this difference in diet responsiveness.

Kåre Berg (20, 21) has suggested that the genetic factors for atherosclerosis risk may be subdivided into "level" genes, those that establish absolute risk factor levels, and "variability" genes, those that determine variation of risk factors in response to environment including differences in lifestyle and nutrition. In this study, we describe differences in both level and variability factors between the closely related B6J and B6By strains that are manifested in differences in TC and LDL/VLDL cholesterol levels and susceptibility to atherosclerosis. In addition, using a cross between B6By and an unrelated strain, we identify a locus, *Diet1*, on proximal chromosome 2 that has a major effect on ΔLDL/VLDL and ΔTC in response to the atherogenic diet. The identification of such loci may be useful in predicting risk for developing hypercholesterolemia and atherosclerosis in response to the Ath diet.

METHODS

Mouse husbandry and diets

C57BL/6J, C57BL/6ByJ, and A/J mice obtained from the Jackson Laboratory (Bar Harbor, ME) were maintained on a 12-h light/dark cycle and fed ad libitum. For studies of cholesterol absorption and food intake determinations, mice were housed individually to avoid variation associated with social interactions. Mice were maintained on a chow diet containing ~4% fat and 0.03% cholesterol (Ralston Purina Co., St. Louis, MO), or fed an atherogenic diet (Ath) consisting of 75% Purina chow plus 15% fat (primarily cocoa butter), 1.25% cholesterol, and 0.5% sodium cholate (TD 90221, Teklad Research Diets, Madison, WI).

Plasma lipid measurements

Blood was obtained from mice fasted for 16 h by retroorbital bleeding under isoflurane anesthesia. Enzymatic assays for total cholesterol, HDL cholesterol, unesterified cholesterol, triglyceride, and free fatty acids were performed using a Biomek 2000 Automated Laboratory Workstation (Beckman Instruments, Inc., Fullerton, CA) as described (22). LDL/VLDL cholesterol was calculated by subtracting HDL cholesterol from total cholesterol.

Lipoprotein fractionation by gel filtration chromatography

Plasma lipoproteins were fractionated using a fast performance liquid chromatography (FPLC) system with two Superose 6 columns connected in series at a flow rate of 0.5 ml/min (23). Plasma pooled from 3 mice (400 μl total volume) was applied and 0.5 ml fractions were collected. Cholesterol and triglycerides were measured in each fraction by enzymatic assay as described above.

Food intake

Six female B6J and B6By animals were housed individually in metabolic cages and maintained on a 12-h light/dark cycle. Mice were initially maintained on a chow diet and then switched to a powdered atherogenic diet (Ath) for 7 weeks. Access to the food cup was via a narrow (2.5 cm), wire-bottomed channel, 8.5 cm in length, with a shelf beneath to catch any spilled powder. The daily food intake was measured by weighing the food cup after recovering any spilled food from the tray and removing any feces. Consumption data for the individual animals was used to calculate the weekly average for daily food intake for each substrain. Repeated measures analysis (24) was carried out using the xtgee program of the statistical package STATA Version 5.0 (1997) (Stata Corporation, College Station, TX 77840).

Intestinal cholesterol absorption

Absorption of dietary cholesterol was carried out using a variation of the dual isotope method (25). Animals were maintained in metabolic cages with ad libitum access to either chow or Ath diet. After an overnight fast, a feeding tube was used to administer a 100 μl bolus of safflower oil containing a mixture of [^{14}C]cholesterol and [^{3}H]sitostanol, a plant sterol that is poorly absorbed (26, 27). The excreted ^{14}C and ^{3}H were monitored in feces collected over the next 4 days and absorption was calculated by comparing the ratio of cholesterol to sitostanol in feces versus the same ratio in the safflower bolus. Homogenates of the feces were prepared for each animal and the cholesterol/sitostanol was extracted with chloroform–methanol. ^{3}H and ^{14}C were quantitated by scintillation counting. Percent cholesterol absorption was calculated according to the equation:

$$\%\ \text{absorbed} = ((1 - [(^{14}C/^{3}H)_{fecal}/(^{14}C/^{3}H)_{diet}]) \times 100)\,.$$

Aortic lesion analysis

Atherosclerotic lesion area was measured in serial sections in the proximal aorta as previously detailed (15). Briefly, the mice were killed and hearts and proximal aortas were washed in phosphate-buffered saline and embedded in OCT compound (Tissue-Tek). Serial cryosections (10 μm thickness) were collected on poly-d-lysine-coated slides and stained with oil red O and hematoxylin and counterstained with fast green. Average fatty lesion area (square micrometers per section) was normalized to 40 sections in the aortic sinus.

Gene mapping studies

To assess the number of genetic differences between B6By and B6J animals that affect dietary response of plasma cholesterol, a backcross [(B6By × B6J) × B6By] N_2 (n = 66) was performed. Analysis of the population distribution for dietary response (ΔLDL/VLDL) was used as an indicator of the number of genetic differences between substrains that influence the trait.

To map genes responsible for hypo-response to diet in B6By animals, both an F_2 cross (B6By × A/J) F_2 (n = 185) and a backcross [(B6By × A/J) × B6By] N_2 (n = 725) were performed. A/J

was chosen for breeding because its ΔLDL/VLDL closely matches B6J and because DNA of the two strains is readily discernible by microsatellite genotyping. Plasma lipids were measured in animals maintained on a chow diet and again after 5 weeks on the Ath diet. Animals were then killed and tissues were collected for DNA.

To map genetic loci associated with hypo-response to diet in the B6By mice, we used a DNA pooling strategy. We created two DNA pools from [(B6By × A/J) × B6By] N_2 animals representing the extremes of diet response in terms of ΔLDL/VLDL: the hypo-responsive pool (0–5th percentile) and the hyper-responsive pool (95–100th percentile). These pooled DNA samples along with samples from B6By, B6J, A/J, and (B6ByxA/J) F_1 controls were typed for 80 microsatellite markers spanning the whole mouse autosomal genome at intervals of 15 to 20 cM. (A map showing the full set of markers and their locations is posted at http://www.med.ucla.edu/ora/Richard%20Davis/Bailey5+.htm.) Radiolabeled primers for each marker were used to amplify the control or pooled DNAs using polymerase chain reaction (PCR). The PCR products were detected by autoradiography after polyacrylamide gel electrophoresis and marker alleles for B6By and A/J were distinguished on the basis of PCR fragment size. Markers near loci potentially contributing to the diet response were visually identified by the non-random distribution of alleles between DNA pools from hypo- and hyper-responsive animals.

After the linked locus was determined, DNA from the hypo- and hyper-responsive animals plus an additional 330 randomly selected N_2 animals were typed individually for microsatellite markers that flanked the linked locus, and linkage of each marker to diet response was calculated using the Map Manager QT program (28, 29).

RESULTS

C57BL/6ByJ mice exhibit reduced basal cholesterol levels and resistance to diet-induced hypercholesterolemia

In a survey of mouse strains for response to an atherogenic diet, we noted marked differences in the plasma lipid levels between B6By and B6J substrains of C57BL/6 mice on both chow and Ath diets. As shown in **Table 1**, on a chow diet, B6By mice exhibit significantly lower basal levels of total, LDL/VLDL, and HDL cholesterol, and lower triglyceride levels. Furthermore, a striking difference is observed in response to the Ath diet, as measured by the absolute change in plasma total cholesterol (ΔTC) and in LDL/VLDL cholesterol (ΔLDL/VLDL). Both ΔTC and ΔLDL/VLDL are smaller for B6By than for B6J. Although, for females, the reduced ΔLDL/VLDL is only a trend for the set of 5–6 animals shown in Table 1, this reduced response to Ath diet is consistently seen for B6By mice of both sexes. Thus it appears that genes controlling both level and variability in plasma cholesterol levels are different in this substrain. **Figure 1** shows cholesterol concentrations in female B6By and B6J plasma lipoproteins fractionated by FPLC. On a chow diet (Fig. 1A), B6By shows modestly lower HDL and slightly elevated LDL. After 5 weeks on Ath diet (Fig. 1B), B6By shows lower cholesterol levels in all lipoprotein fractions. Difference graphs (Fig. 1C) show that B6By experiences smaller increases in VLDL cholesterol and greater decreases in HDL cholesterol than B6J.

Because female mice are more susceptible to atherosclerosis, experiments to measure impact of this phenotype on atherosclerosis susceptibility and to characterize the phenotype's metabolic basis were carried out in females.

B6By and B6J animals are similar in Ath diet consumption and cholesterol absorption

The observed differences in plasma cholesterol might derive from differences in levels of consumption of the diet or from differences in cholesterol absorption. To test for the differences in consumption, B6By and B6J mice that had been maintained on a chow diet were transferred to individual metabolic cages with ad libitum access to powdered Ath diet. A repeated measures random effects model was fitted to the mouse consumption data (**Fig. 2**) using general estimating equations approach (24). There was a significant linear decrease in consumption in both groups over the 7 weeks (P value = 0.01). The difference was not significant between strains although the B6By mice tended to consume slightly more than the B6J animals (P value = 0.08). While the B6By animals showed a small but significantly increased weight gain after seven weeks, the food consumed per gram body weight was not different between strains (data not shown). Thus, the reduced plasma cholesterol levels observed in B6By do not result from reduced food intake.

TABLE 1. Fasting plasma lipid levels in B6J and B6By mice fed chow and atherogenic diets

	Total Cholesterol		HDL Cholesterol		LDL/VLDL Chol.		Unesterified Chol.		Triglycerides		Free Fatty Acids	
	Chow	Ath	Chow	Ath	Chow	Ath	Chow	Ath	Chow	Ath	Chow	Ath
	mg/dL		*mg/dL*		*mg/dL*		*mg/dL*		*mg/dL*		*mg/dL*	
Male												
B6J	85 ± 7.0	189 ± 56	73 ± 5.3	47 ± 8.4	12 ± 2.3	122 ± 51	14 ± 1.8	36 ± 17	22 ± 4.5	12 ± 3.1	50 ± 2.7	49 ± 5.5
B6By	56 ± 8.3	82 ± 16	52 ± 8.2	39 ± 11	3.4 ± 2.0	46 ± 14	7.4 ± 1.5	14 ± 2.5	6.0 ± 1.2	7.0 ± 8.8	57 ± 3.3	54 ± 5.7
P	0.0003	0.008	0.001	0.001	0.0003	0.02	0.0002	0.04	0.00006	NS	NS	NS
Female												
B6J	79 ± 4.0	158 ± 14	61 ± 4.8	30 ± 3.6	18 ± 0.9	128 ± 15	11 ± 0.8	36 ± 5.3	67 ± 6.2	22 ± 1.1	86 ± 4.1	36 ± 2.1
B6By	55 ± 3.1	115 ± 16	43 ± 3.2	23 ± 2.4	11 ± 1.8	93 ± 16	3.7 ± 0.8	26 ± 6.0	23 ± 1.9	28 ± 3.9	56 ± 3.0	36 ± 3.0
P	0.0004	0.08	0.007	0.06	0.01	0.15	0.0001	0.16	0.0001	NS	0.0001	NS

All values are expressed as mg/dL and represent the mean ± SD of determinations from 5–6 mice. For chow determinations, plasma was obtained from 8-week-old mice fed the chow diet. Mice were then fed the atherogenic diet (Ath) for 3 weeks and plasma was obtained for the Ath determinations. Differences between the B6J and B6By strains were evaluated using the Student's t-test, and P-values are indicated; NS, not significant.

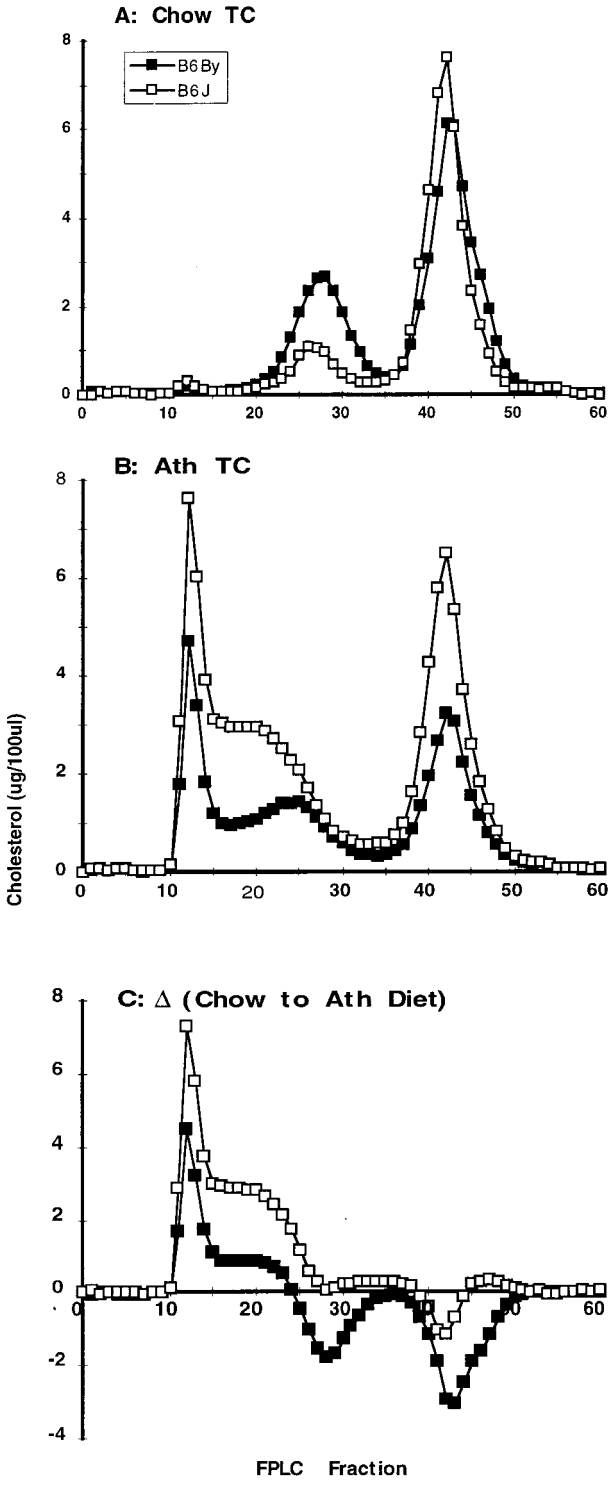

Fig. 1. Cholesterol concentrations in plasma lipoprotein fractions of B6By and B6J mice. Female mice (n = 3 per group) from each substrain were measured for plasma cholesterol first on a chow diet (A) and then after 5 weeks on the Ath diet (B). Plasma pools from each group were fractionated by FPLC and cholesterol concentrations in each fraction were measured. VLDL/LDL elutes in fractions 20–35 and HDL elutes in fractions 38–50. Difference graphs (C), show smaller increases in VLDL cholesterol and greater decreases in HDL cholesterol for B6By mice.

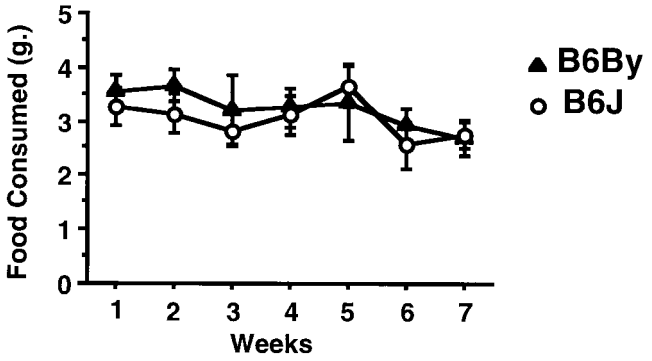

Fig. 2. Food intake by B6By and B6J mice. Female mice from each substrain (n = 6), initially maintained on chow diet, were individually housed in metabolic cages and given powdered Ath diet ad libitum. The amount consumed daily by each animal was monitored over a 7-week period and the weekly average for daily consumption was calculated for each substrain (gm/animal/day ± SD).

To determine whether differences in cholesterol absorption were important in the relative plasma cholesterol levels, the fraction of absorbed cholesterol was measured by a variation of the dual isotope method (25). Oral doses of [^{14}C]cholesterol were administered as a bolus including the plant sterol [^{3}H]sitostanol as a non-absorbed standard and absorption calculated by comparing the ratio of those two labeled sterols in the feces. While absorption in animals on a chow diet was significantly higher than that seen for animals on the Ath diet, there were no significant absorption differences between B6By and B6J substrains on either diet (**Fig. 3**).

B6By animals are resistant to aortic lesion formation

The B6J substrain of C57BL/6 is known for susceptibility to atherosclerotic lesion development in response to the Ath diet (12, 13). We hypothesized that the hyporesponse of B6By to dietary elevation of plasma cholesterol would protect this substrain from lesion develop-

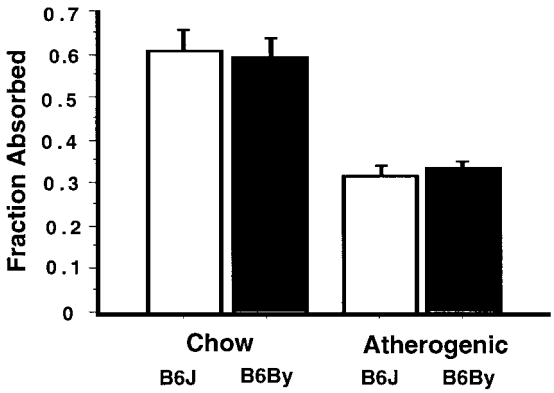

Fig. 3. Intestinal cholesterol absorption in B6By and B6J mice. Fraction of cholesterol absorbed by the intestine (fraction absorbed ± SEM for n = 6) was determined for female B6By and B6J mice on Ath and chow diets using a modification of the dual isotope method. See Methods. Although the Ath diet significantly reduced absorption in both substrains ($P < 0.0001$), no differences were seen between B6By and B6J mice on either diet.

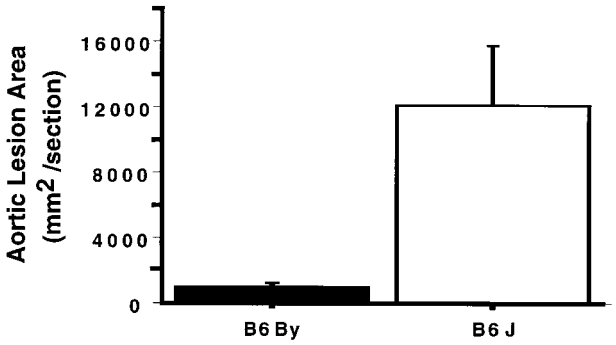

Fig. 4. Atherosclerosis susceptibility in B6By and B6J mice. Female animals from each substrain (B6By: n = 10; B6J: n = 7) were given an Ath diet ad libitum. After 15 weeks, the animals were killed and the aortic lesion area (mm^2 /section ± SEM) was determined (15).

ment. To test this hypothesis, we examined aortic lesion area in mice after 15 weeks of ad libitum access to Ath diet. The B6By mice showed marked resistance to lesion development with lesion areas < 15% of those seen in B6J mice (**Fig. 4**). This confirms that the hypo-response of plasma cholesterol to diet is also reflected in lower fatty streak lesion formation and suggests that the responsible genetic variation is an important marker of atherosclerosis susceptibility.

The above results show that: *i*) the B6By mice are relatively resistant to the elevations of plasma cholesterol induced by the Ath diet, *ii*) that this resistance is associated with a marked decrease in atherosclerosis susceptibility, and *iii*) that resistance is not due to differences in consumption or absorption of dietary cholesterol. In order to identify and characterize genetic loci that might contribute to this phenotype, we carried out several crosses between B6By mice and other inbred strains. In all these experiments we monitored ΔLDL/VLDL and ΔTC after the mice had consumed the Ath diet for 5 weeks.

Genetic characterization of diet hypo-response in B6By mice

The common origin of B6By and B6J mice suggested that the strong differences in response to Ath diet must stem from a limited set of genetic modifications. In particular, our working hypothesis was that the predominant difference in diet response between B6By and B6J mice derives from a mutation in a single gene locus, which we designated *Diet1*. To test this, we measured the diet response in a cross between B6By and B6J mice. We found

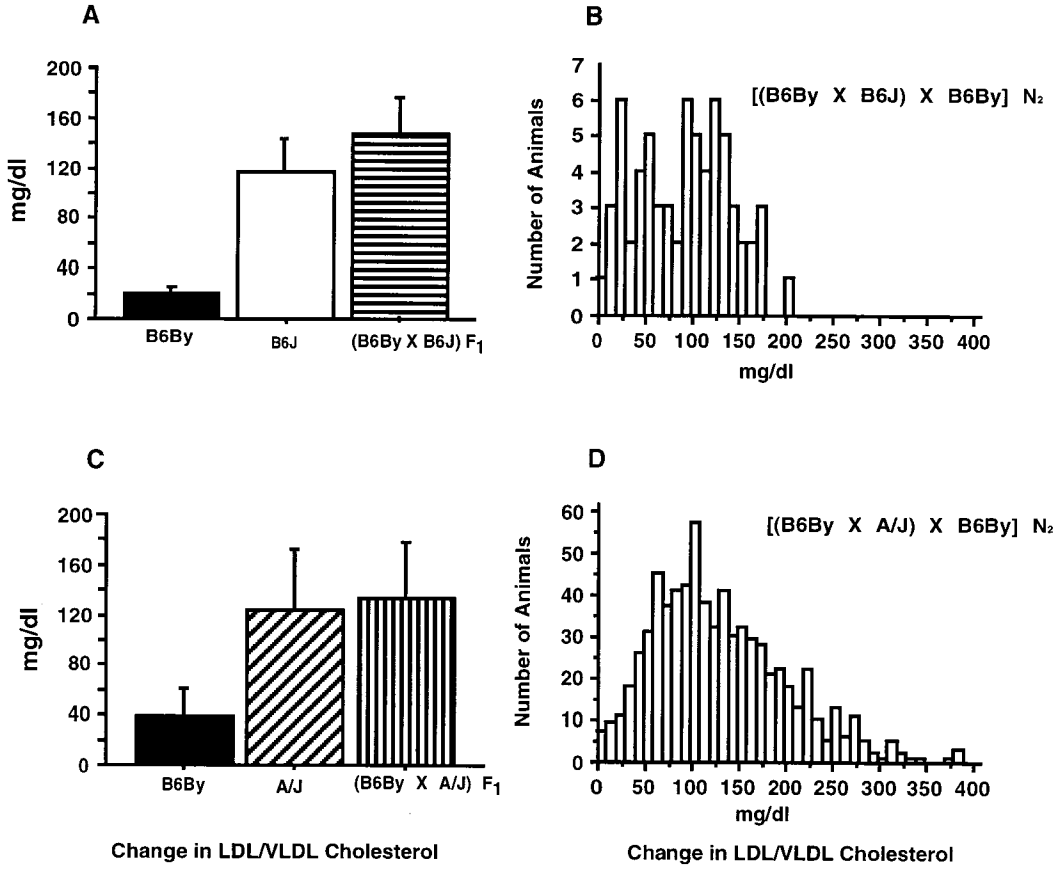

Fig. 5. Genetics of *Diet 1* locus. (A): ΔLDL/VLDL levels (± SEM) were determined for female (B6By × B6J) F_1 (n = 20), B6By (n = 10), and B6J (n = 10) mice after 5 weeks on the Ath diet. (B): ΔLDL/VLDL levels were determined for 66 [(B6By × B6J) × B6By] N_2 mice (male and female) after 5 weeks on the Ath diet. (No significant differences in ΔLDL/VLDL were observed between male and female N_2 mice; $P = 0.48$.) (C): ΔLDL/VLDL levels were determined for female (B6By × A/J) F_1 (n = 17), B6By (n = 10), and A/J (n = 6) mice after 5 weeks on the Ath diet. (D): ΔLDL/VLDL levels were determined for 725 [(B6By × A/J) × B6By] N_2 mice (male and female) after 5 weeks on the Ath diet. (No significant differences in ΔLDL/VLDL were observed between male and female N_2 mice; $P = 0.34$.)

that (B6By × B6J) F_1 mice showed a response similar to that of B6J mice (**Fig. 5A**), suggesting that the B6By allele of *Diet1* is recessive. Furthermore, if the diet response phenotype in B6By mice represents a single recessive gene modification, then, in a backcross experiment, we expect a bimodal distribution of phenotypes with 50% of [(B6By × B6J) × B6By] N_2 animals showing the B6By phenotype and 50% showing the B6J phenotype. In contrast, the random assortment of multiple contributing genes would produce a broader range of phenotypes. Thus, if two genes contribute to a phenotype, backcross progeny will exhibit a tri- or tetramodal distribution, and as the number of contributing genes increases, the individual allele-combinations become difficult to resolve and the observed phenotype distribution quickly tends toward a broad unimodal distribution (30).

Our analysis of 66 [(B6By × B6J) × B6By] N_2 progeny for diet-induced elevations in LDL/VLDL cholesterol is shown in Fig.5B. We combined data from both sexes as there was no difference in ΔLDL/VLDL between male and female backcross animals ($P = 0.48$). Analysis of ΔLDL/VLDL in these mice using an expectation–maximization (EM) algorithm (31) shows best support for a bimodal distribution versus a single normal distribution. (The Chi-square statistic with 2 degrees of freedom is 6.33, P value = 0.04). The best approximation by EM algorithm suggests that the backcross mice resolve into two normally distributed populations, one group representing 45% of the population and showing a mean change in LDL/VLDL cholesterol of 48 mg/dL similar to that seen in B6By mice and the remainder showing a change in LDL/VLDL of 128 mg/dL similar to B6J mice (with the algorithm estimating a common standard deviation of 28 mg/dL). This result is consistent with our hypothesis that the *Diet1* phenotype in B6By mice represents a single gene difference from B6J mice.

To map the *Diet1* locus, we carried out crosses between B6By mice and the inbred strain A/J. The A/J strain was selected because its ΔLDL/VLDL (117 mg/dL) in response to Ath diet is similar to that seen in B6J mice but it has a sufficiently different genetic background to allow typing of DNA microsatellite polymorphisms. While the A/J strain most likely differs from B6By at a number of genetic loci affecting dietary response, we anticipated that quantitative trait locus mapping would most effectively reveal loci with strong effect such as that shown by *Diet1*. As shown in Fig. 5C (B6By × A/J) F_1 mice exhibit a ΔLDL/VLDL of 130 mg/dL after 5 weeks on the Ath diet, suggesting that the B6By allele of *Diet1* is also recessive to the A/J allele. As expected, it also became apparent that the A/J strain introduces alleles of other genes that influence diet response as the ΔLDL/VLDL distribution in [(B6By × A/J) × B6By] N_2 mice is broad and unimodal (Fig. 5D). Again, the distribution combines data from both sexes as there was no difference in ΔLDL/VLDL between male and female backcross animals ($P = 0.34$).

The *Diet1* locus is localized to proximal chromosome 2

To map the gene responsible for low response of plasma cholesterol to the Ath diet, we used a DNA pooling strategy and hypothesized that the most hypo-responsive animals would be homozygous for the B6By allele of *Diet1*. We created two DNA pools from [(B6By × A/J) × B6By] N_2 animals representing the extremes of diet-induced ΔLDL/VLDL. In creating the DNA pools we selected DNAs based on ΔLDL/VLDL rather than ΔTC because we hypothesized that the larger variation seen in ΔLDL/VLDL would make it more sensitive for QTL mapping. The hypo-responsive pool (0–5th percentile) and the hyper-responsive pool (95–100th percentile), along with control DNA samples from B6By, B6J, A/J, and (B6By × A/J) F_1 mice were used for a genome wide screen with microsatellite markers having an average spacing of 15 to 20 cM. Among these markers, those on proximal chromosome 2 showed a non-random distribution between the DNA pools (see **Fig. 6**) In particular,

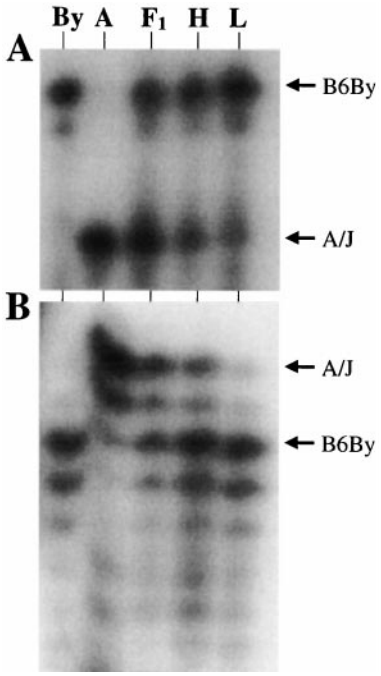

Fig. 6. Quantitative trait locus mapping using DNA pooling. To identify loci affecting ΔLDL/VLDL in [(B6By × A/J) × B6By] N_2 mice, DNAs were pooled from animals showing the highest and lowest ΔLDL/VLDL. The hypo-responsive (L) pool (0–5th percentile) and the hyper-responsive (H) pool (95–100th percentile) were compared with control DNAs from B6By (By), A/J (A), and [By × A] F_1 (F_1) animals. Microsatellite markers spaced at 15–20 cM intervals across the genome were used to type each DNA or DNA pool. The PCR products were detected by autoradiography after polyacrylamide gel electrophoresis, and marker alleles for B6By and A/J were distinguished on the basis of PCR fragment size. (A) Unlinked marker. For markers at loci not contributing to ΔLDL/VLDL, the relative band intensities for B6By and A/J alleles are essentially similar between the H- and L-pools. For both pools, these band intensities simply represent the expected random assortment of alleles: N_2 animal DNA-pools should display B6By alleles and A/J alleles in a ratio of about 3:1 while F_1 animals carry these alleles in the ratio of 1:1. The marker shown is D16Mit50. (B) Linked marker. For markers near the *Diet1* locus, L-pool DNA is enriched in B6By alleles and depleted in A/J alleles compared to H-pool DNA. The marker shown (D2Mit151) is approximately 10 cM distal to the peak lod score for *Diet1* (See Fig.7).

there was strong enrichment of B6By alleles and depletion of A/J alleles in the hypo-responsive pool in marked contrast to the allele distribution in the hyper-responsive pool. This result is consistent with a nearby locus on chromosome 2 affecting dietary response of plasma cholesterol. More specifically, this result reveals a locus at which a high preponderance of animals in the hypo-responsive pool carry homozygous B6By alleles while backcross animals in the hyper-responsive pool are preponderantly heterozygous. A similar non-random distribution of alleles on proximal chromosome 2 was observed in DNA pools from hypo- and hyper-responsive animals in a (B6By × A/J) F_2 cross (data not shown).

To confirm and better localize the *Diet1* locus, a set of chromosome 2 microsatellite markers was typed in individual DNA samples from 400 randomly selected [(B6ByXA/J) × B6By] N_2 mice. The diet-induced change in plasma cholesterol levels for these animals, separated by genotype at the D2Mit177 locus, are shown in **Table 2**. Clearly, animals that are homozygous for the B6By allele of D2Mit177 have ΔLDL/VLDL that is significantly less (by 40–50 mg/dl) than mice that are heterozygous. This is true for both males and females.

Quantitative trait linkage analysis between markers near D2Mit177 and ΔLDL/VLDL in response to the Ath diet (**Fig. 7A**) further confirmed the presence of the locus. The Lod score peaks near D2Mit117 (Lod 9.6) with the 99% confidence interval between D2Mit355 (2 cM) and D2Mit60 (20 cM). These results show that *Diet1* localizes approximately 6 cM distal to the centromere of mouse chromosome 2 and that alleles of *Diet1* carried by B6By mice confer resistance to diet-induced elevations of plasma cholesterol. Separate analysis of males and females in the backcross confirms that the QTL for ΔLDL/VLDL localizes to the same chromosomal region in both sexes. Interestingly, *Diet1* appears to be even more strongly linked to ΔTC (Lod > 12) despite the fact that changes in total cholesterol are quantitatively smaller than changes in LDL/VLDL cholesterol (Fig. 7B). Thus, the *Diet1* locus likely represents a gene important to overall plasma cholesterol metabolism and atherosclerosis susceptibility.

DISCUSSION

In a survey of inbred mouse strains, we have identified a striking variation in cholesterol metabolism between the substrains B6J and B6By. B6By animals are relatively resistant to absolute elevations of plasma cholesterol induced by the Ath diet even though B6By and B6J mice consume and absorb similar levels of dietary cholesterol. Correspondingly, B6By mice show markedly less atherosclerosis in response to the diet. Mapping experiments in crosses between B6By mice and another inbred strain, A/J, identified a locus on chromosome 2 (*Diet1*) that appears to contribute strongly to this phenotype.

Inbred mouse strains vary widely in their susceptibility to diet-induced atherosclerosis (15), with B6J mice being a well-characterized susceptible strain. As recently as 1987, the B6By and B6J substrains exhibited similar phenotypes in response to the Ath diet, both in terms of plasma lipids and in atherosclerosis susceptibility (12). The fact that the resistant B6By strain arose relatively recently in a breeding isolate of B6J mice suggests that the resistance derives from a spontaneous single-locus modification. This is supported by the distribution of phenotypes in the cross between B6By and B6J (Fig. 5B). Of course, there is the concern that the responsible gene or genes may have been introduced inadvertently by genetic contamination of B6By mice by an unrelated and resistant strain. Our results in gene mapping, however, do not support this alternative hypothesis. In testing of more than 85 microsatellite markers

TABLE 2. Effect of *diet1* locus on fasting plasma lipid levels in [(B6By × A/J) × B6By] N_2 mice

		Total Cholesterol		HDL Cholesterol		LDL/VLDL Cholesterol		ΔLDL/VLDL Cholesterol	
	n	Chow	Ath	Chow	Ath	Chow	Ath	Chow vs. 5-Wk Ath Diet	
		mg/dL		*mg/dL*		*mg/dL*		*mg/dL*	
Male									
By/A	100	102 ± 14	219 ± 63	90 ± 10	64 ± 18	11 ± 7	155 ± 62	144 ± 64	
By/By	92	100 ± 14	159 ± 74	88 ± 10	41 ± 16	12 ± 6	118 ± 66	105 ± 67	
P		0.3715	<0.0001	0.0990	<0.0001	0.5107	<0.0001	<0.0001	*P* = 0.34
Female									
By/A	105	77 ± 12	202 ± 83	66 ± 10	26 ± 9	11 ± 5	176 ± 82	165 ± 82	
By/By	100	73 ± 12	141 ± 82	64 ± 10	21 ± 7	9 ± 5	120 ± 80	111 ± 79	
P		0.0036	<0.0001	0.0630	<0.0001	0.0013	<0.0001	<0.0001	

Plasma lipids were measured in backcross mice before and after 5 weeks on the atherogenic diet. The data are separated depending on sex and genotype of each mouse at D2Mit117. Backcross mice are either homozygous for the By allele (By/By) or heterozygous (By/A). Lipid values are expressed as mg/dL and represent the mean ± SD of determinations. For chow determinations (Chow), plasma was obtained from 8-week-old mice fed the chow diet. Mice were then fed the atherogenic diet (Ath) for 5 weeks and plasma was obtained for the Ath determinations. The change or "delta" in LDL/VLDL (ΔLDL/VLDL Cholesterol) was calculated as $(TC\text{-}HDL)_{Ath}$ minus $(TC\text{-}HDL)_{Chow}$ for each animal. The number of animals in each group (*n*) is indicated. *P* values for group differences were evaluated by ANOVA (Statview 5.0).

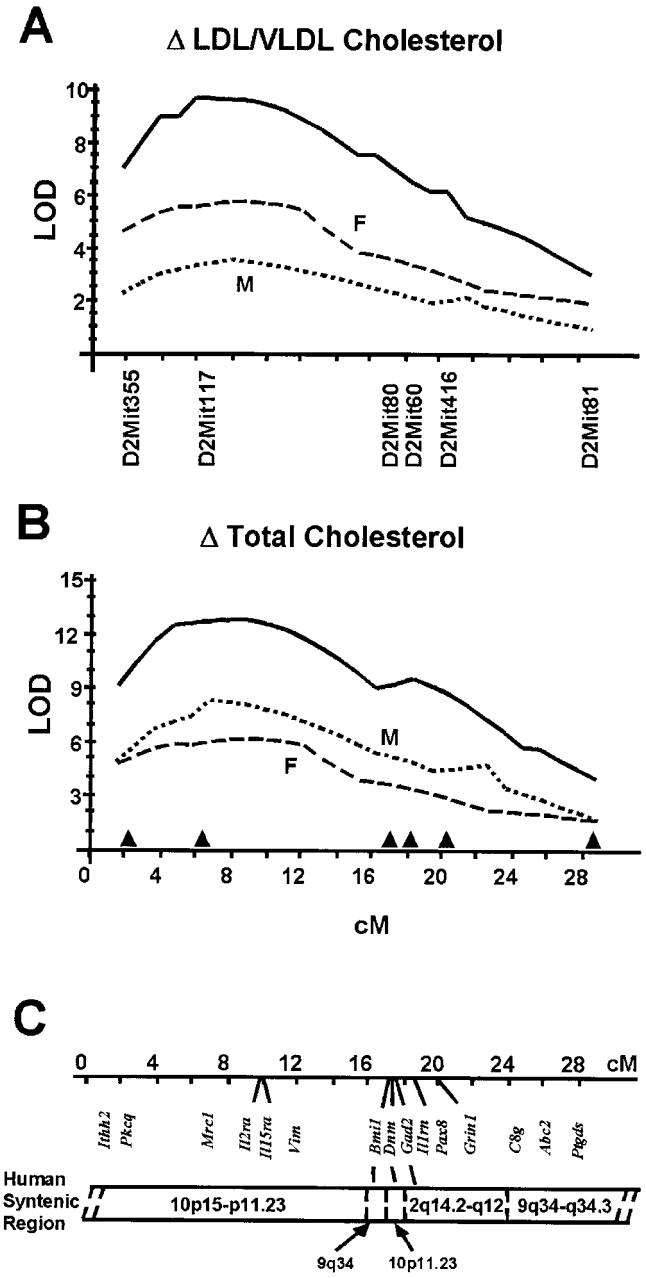

Fig. 7. Linkage analysis. To determine the linkage of the hypo-response to the diet (*Diet1* locus), 400 randomly picked [(B6By × A/J) × B6By] N_2 mice were typed for microsatellite markers shown on proximal chromosome 2. The data were analyzed using Map Manager QT and the resulting lod scores for LDL/VLDL levels (A) and ΔTC (B) in response to 5 weeks Ath diet are shown. Map positions are indicated in recombination units (cM) calculated from observed recombination frequencies in the N_2 cross. Separate lod score plots for male (M) and female (F) mice are shown by dashed lines. (C) Known genes mapping to proximal mouse Chr.2 are shown above the human syntenic regions. Map positions for the genes, relative to microsatellite markers, were obtained from the Mouse Genome Database (MGD) (37).

widely distributed over the mouse genome, we saw no evidence of allelic differences between B6By and B6J mice. This continues to be the case as we fine map the region surrounding *Diet1* (data not shown). These results are most consistent with the hypo-responsive phenotype having arisen as a single de novo mutation on the genetic background of B6J mice.

In Table 1, we noted evidence for both "level" and "variability" differences between B6By and B6J plasma cholesterol levels. In contrast, we see mostly evidence for a variability effect of the *Diet1* locus in data from the [(B6By × A/J) × B6By] N_2 mice (Table 2). On a chow diet, there are no large differences in absolute levels of either total cholesterol or LDL/VLDL cholesterol between mice heterozygous or homozygous for Bailey alleles near D2Mit117. Perhaps the impact of *Diet1* on basal cholesterol levels is obscured by effects of other genes introduced in the backcross with A/J. Alternatively, a separate locus may be responsible for the differences in basal levels seen between B6By and B6J. Definitive evidence regarding the "level" effects of *Diet1* awaits development of appropriate congenic strains. Finally, it is possible that the depletion of A/J alleles from the hypo-responsive pool may reflect absence of a dominant hyper-responsive A/J gene allele rather than presence of a recessive hypo-responsive B6By gene allele. This seems unlikely as no other B6By locus was observed to account for the original observation. Again, the appropriate congenic strains will provide final proof.

In general, atherosclerosis in humans is multifactorial, being the result of numerous genetic and environmental factors. The effects of gene–environment interactions are almost impossible to measure except in clonal populations such as in twin studies or in inbred mice where responses may be monitored in a constant genetic background. For instance, Kåre Berg (20) compared plasma lipid variations in pairs of monozygotic twins to search for polymorphisms near candidate genes that might contribute to differences in dietary response. While feasible, this approach is limited in its ability to finely localize the responsible allelic variations. Thus further characterization of *Diet1* represents a rare opportunity to explore an environmental response gene.

The finding that B6By mice are largely resistant to atherosclerosis is notable. Given that the genetic background is nearly identical to B6J, which is among the most susceptible inbred strains, these results suggest that the *Diet1* locus exerts a major effect on aortic fatty lesion development. The high degree of susceptibility of B6J mice compared to other inbred strains has been attributed, in part, to the reduced HDL cholesterol levels in this strain (12, 14). Similarly, the expression of an apoA-I transgene in B6J mice leads to increased HDL and reduced aortic lesion scores (32). Interestingly, HDL cholesterol levels in B6By mice are lower on both chow and Ath diets (Table 1), suggesting that HDL levels are not the determinant of the reduced lesions in this strain. The results instead direct the focus to the difference between B6J and B6By in LDL/VLDL levels. Mechanisms that have been proposed for the pro-atherogenic effect of elevated LDL cholesterol include increased abundance and uptake by macrophages and smooth muscle cells in the artery wall of products resulting from modification of LDL, such as oxidation and

aggregation (reviewed in 33). Modified LDL also appears to be refractory to removal by reverse cholesterol transport mechanisms, further contributing to its accumulation in lesions. The nature and extent to which LDL modification plays a role in the difference in susceptibility to atherosclerosis between B6J and B6By mice requires further study.

The physiological mechanism leading to *Diet1* hyporesponse remains to be elucidated. Our present results show that the phenotype does not arise simply from altered food consumption or major variations in intestinal absorption of cholesterol. Instead, the *Diet1* phenotype appears to result from an alteration in cholesterol processing, storage, or clearance. The nature of this alteration and identity of the affected tissues are under investigation.

Large differences in response to dietary cholesterol have been observed in humans and likely play a role in genetic susceptibility to atherosclerosis. The Masai of East Africa, with a diet of milk, blood, and meat, consume much higher levels of fat and cholesterol than the average Westerner and yet have mean serum cholesterol levels of only 135 ± 34 mg/dl and minimal atherosclerosis (2). While Alaskan arctic Eskimos are similar to the general US population both in terms of dietary lipids (cholesterol and fat) and serum cholesterol levels, they show markedly less ischemic heart disease and atherosclerosis (1). To some extent, these racial differences may be attributed to differences in the suppression of total body cholesterol synthesis in response to diet. For instance, it is estimated that the Masai suppress cholesterol synthesis by 50% while US whites show suppression of only 25% and Eskimos show intermediate suppression (1, 2). The Ath diet given to B6By and B6J mice in the experiments presented here is, of course, much higher in cholesterol than most human diets. Thus, an attractive hypothesis is that differences in suppression of de novo cholesterol synthesis might act to produce the observed differences in plasma cholesterol and atherosclerosis. However, in preliminary experiments, we find that the high levels of cholesterol and cholate in the Ath diet completely suppress cholesterol synthesis in both B6J and B6By animals (data not shown). Thus, the higher plasma cholesterol levels seen in B6J mice are unlikely to be the result of higher levels of residual cholesterol synthesis.

Striking differences in plasma lipid metabolism and atherosclerosis susceptibility in response to diet have been observed among various inbred mouse strains (13, 17, 34). Further, quantitative trait locus (QTL) mapping studies have identified numerous genetic loci associated with these genetic–dietary interactions. In some cases, the QTL corresponds to a candidate gene with obvious relevance to current understanding of lipid physiology and atherogenesis (35). More commonly, the underlying genes are not apparent and, moreover, the loci represent one gene in a multigenic trait so that it is difficult to identify its specific physiologic impact. By contrast, while the *Diet1* gene is unknown, it is likely monogenic and therefore amenable to direct physiologic characterization.

Several of the previously identified diet/lipid–metabolism QTLs localize to mouse chromosome 2. In particular, QTLs for obesity (34, 36) and lipoprotein and insulin metabolism (34) have been observed on distal and central regions of the chromosome. However, the *Diet1* locus is a broad peak localized between 2 and 17 cM at the proximal terminus of mouse chromosome 2 (Fig. 7) with no overlap with previously identified QTLs. The human syntenic region corresponding to *Diet1* (Fig. 7C) lies on chromosome 10 (p11 to p15) with nearby syntenic regions on chromosome 2 (q12–14.2) and chromosome 9 (q33–34.3). Within these regions, there are no genes with obvious impact on lipid metabolism. The known genes in or near the locus include those with neural (*Dnm*, *Gad2*, *Grin1*), immunological (*Il15ra*, *Il2ra*, *Il1rn*, *C8g*, *Mrc1*), homeotic (*Pax8*, *Bmi1*), and signal transduction (*Pkcq2*) functions. Thus, it is likely that the *Diet1* locus represents a novel gene that impacts response to dietary lipids. Characterization of the responsible mutation will be significant to our understanding of energy homeostasis and atherosclerosis.

The original observation of reduced diet responsiveness in C57BL/6ByJ mice was the result of a strain survey studying genetic–dietary interactions carried out by C. Warden, L. W. Castellani, J. Fisler, and A. J. Lusis. In addition, we wish to acknowledge the indispensable assistance of Sharda Charugundla in plasma lipid determinations, and Mark Cuevas and Robert Cohen for preliminary phenotypic characterization. This work was supported, in part, by NIH grants HL58627 and HL28481. Finally, we would like to thank the reviewers for their thoughtful and thorough reviews and for their very helpful suggestions.

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