Hyplip2, a New Gene for Combined Hyperlipidemia and Increased Atherosclerosis

Xuping Wang, Peter Gargalovic, Jack Wong, Jennifer L. Gu, Xiaohui Wu, Hongxiu Qi, Pingzi Wen, Li Xi, Bing Tan, Rocky Gogliotti, Lawrence W. Castellani, Aurobindo Chatterjee, Aldons J. Lusis

Objective—We previously reported the mapping of a quantitative trait locus (QTL) on chromosome 15 contributing to hyperlipidemia in a cross between inbred strains MRL/MpJ (MRL) and BALB/cJ (BALB). Using marker-assisted breeding, we constructed a congenic strain in which chromosome 15 interval from MRL is placed on the genetic background of BALB. The congenic allowed us to confirm the QTL result and to further characterize the properties and location of the underlying gene.

Methods and Results—On chow and high-fat (atherogenic) diets, the congenic mice exhibited higher levels of plasma triglycerides and cholesterol than BALB mice. In response to the atherogenic diet, the congenic mice but not BALB mice exhibited a dramatic ≈30-fold increase in atherogenic lesions accompanied by ≈2-fold decrease in high-density lipoprotein cholesterol levels. With respect to atherosclerotic lesions and some lipid parameters, this chromosome 15 gene, designated Hyplip2, exhibited dominant inheritance. Expression array analyses suggested that Hyplip2 may influence inflammatory and bile acid synthesis pathways. Finally, we demonstrated the usefulness of subcongenic strains to narrow the locus (50 Mbp) with the goal of positionally cloning Hyplip2.

Conclusions—Our data demonstrate that the Hyplip2 gene significantly contributes to combined hyperlipidemia and increased atherosclerosis in mice. (Arterioscler Thromb Vasc Biol. 2004;24:1928-1934.)

Key Words: hyperlipidemia • atherosclerosis • QTL • chromosome 15 • FCHL

Atherosclerosis is a disease of the large arteries that is the major cause of coronary heart disease and stroke. The disease is extremely complex, and a multitude of important genetic and environmental risk factors have been identified.1–3 Although there has been dramatic progress in identifying genes contributing to Mendelian disorders associated with atherosclerosis, such as familial hypercholesterolemia and Mendelian forms of hypertension, progress in dissecting the common, complex forms of the disease has been disappointing.

One approach to these difficulties is to study animal models exhibiting genetic differences relevant to atherosclerosis. Among inbred strains of mice and rats are numerous variations contributing to traits such as lipoprotein metabolism, blood pressure, diabetes, and atherosclerotic lesion formation. Over the past 2 decades, we and others have examined such traits in genetic crosses and have found that, as in humans, the traits are generally inherited in a complex fashion, with multiple contributing loci.4–7 With the development of dense maps of genetic markers, it became feasible to perform quantitative trait locus (QTL) mapping of the major loci, and dozens of loci relevant to atherosclerosis have been mapped during the past decade. An important step in the characterization and eventual isolation of genes underlying QTLs is the isolation of the individual loci of interest as congenic strains, in which a locus from one strain has been introgressed by a series of back-crosses onto the background of a second strain. The congenic strain thus serves as a means of confirming the QTL mapping and further narrowing the location of the gene by the use of crossovers or analysis of subcongenic mice.8 Congenic strains, first developed by Snell in the 1940s9 to examine the genetic basis of histocompatibility, have classically been constructed using a series of back-crosses, requiring 10 or more generations of breeding. With the availability of genetic markers, the process can be accelerated by constructing maps of the back-cross progeny and selecting animals with the least donor contribution at several successive generations, a procedure termed the “marker-assisted selection protocol.”10

One particularly interesting QTL for lipoprotein metabolism resides in the central part of mouse chromosome 15. Genetic variation of this locus occurs among multiple mouse...
strains, because we and others have observed linkage at this locus in 5 separate genetic crosses.5,6,11–13 The most striking linkage at this locus was observed in a cross between the inbred strains MRL/MpJ (MRL) and BALB/cJ (BALB).6 In this cross, the chromosome 15 locus exhibited a lod score of 11.1 for total cholesterol levels, 6.7 for very-low-density lipoprotein (VLDL)/low-density lipoprotein (LDL) cholesterol levels, and 2.7 for triglyceride levels. The phenotype suggested that it might be in some aspects relevant to familial combined hyperlipidemia, the most common discreet dyslipidemia, with a population frequency of 1% to 2%.14 We now report the construction and characterization of a congenic strain for the chromosome 15 locus using the marker-assisted selection protocol. The congenic mice contain a region of chromosome 15 encompassing the QTL from strain MRL on the genetic background of BALB. Analysis of the congenic mice showed the expected genetic effects of the locus, including increased total cholesterol and triglyceride levels as well as high-density lipoprotein (HDL) cholesterol alterations on chow and high-fat diets. The locus also had a striking effect on diet-induced atherosclerosis. Whereas BALB mice are very resistant to atherogenesis, exhibiting few or no lesions when maintained on a high-fat diet for 8 weeks, the congenic mice developed significant fatty streak lesions characteristic of susceptible strains such as C57BL/6J. This appears to be caused, at least in part, by the dramatic increase in LDL/VLDL cholesterol and decrease in HDL cholesterol levels in the congenic mice in response to the diet. The congenic strain should facilitate the positional cloning of the underlying gene and its biochemical characterization.

Methods

Animals and Diets

Strain MRL/MpJ and BALB/cJ mice were obtained from the Jackson Laboratory (Bar Harbor, Me). The housing and care of mice were performed according to the approved institutional guidelines. Genetic crosses were conducted in our laboratory and housed as previously described.15 The mice were fed a standard rodent chow diet containing 4% fat (Ralston-Purina Co) or an atherogenic high-fat, high-cholesterol diet containing 15% fat, 1.25% cholesterol and 0.5% cholic acid (TD 90221; Food-Tek Inc).

Plasma Lipids

Mice were bled under isoflurane anesthesia after overnight fasting. Blood was collected through the retro-orbital vein into EDTA anticoagulant as described.15 For measurement of chase plasma lipids, mice were bled at 8 to 10 weeks of age; for measurement of lipids on the atherogenic diet, mice were bled before euthanization at ~5 months of age. Plasma total cholesterol, HDL cholesterol, and triglyceride levels were measured with enzymatic assays. Gel filtration chromatography using fast protein liquid chromatography (FPLC) equipped with Superose 8 columns (Pharmacia) was used to examine the classes and sizes of lipoprotein particles, as previously described.16

Aortic Lesion Analysis

Methods for the quantification of atherosclerotic lesions in the aortic root were the same as previously reported.7 In brief, the heart and proximal aorta were dissected, washed with phosphate-buffered saline, embedded in optimal cutting temperature compound, and then frozen on dry ice. Serial 10-μm-thick cryosections from the middle portion of the ventricle to the aortic arch were collected on superfrost/plus microscope slides (Fisher number 12 to 550-15). In the region beginning at the aortic valves, every other section was collected. In all other regions, every fifth section was collected. Then sections were stained with oil red O and hematoxylin and counterstained with fast green. Lesion areas were quantitated by light microscopy.

Statistical Analysis

Data are presented as the mean±SEM. The ANOVA t test was performed using Statview (Abacus Concepts, Inc) to compare differences between groups in lipid levels and atherosclerotic lesions. Differences were considered statistically significant at P<0.05.

Results

Construction of Chromosome 15 Congenic Strain

A previous study involving an MRL × BALB cross localized a QTL for elevated plasma lipids, including LDL/VLDL cholesterol and triglycerides, on mouse chromosome 15, between genetic markers D15Mit152 and D15Mit28.6 To generate a congenic strain for the region, we used the “marker-assisted selection protocol” in which back-cross progeny with the least donor genetic contribution, but heterozygous for the region of interest, were selected at each generation. The chromosome 15 QTL region was identified by typing 5 microsatellite markers (D15Mit152, D15Mit26, D15Mit17, D15Mit101, D15Mit28) within a 16-centimorgan (cM) interval.17 A total of 69 genetic markers, with an average spacing of ~15 cM, were typed in the first (N2) back-cross generation (see online Methods section, available online at http://atvb.ahajournals.org). The single male exhibiting the lowest level of heterozygosity (42%) was harem-bred to generate mice for the second (N3) back-cross generation, from which a male with 15% heterozygosity was selected for further breeding. A similar protocol was used until the fourth (N5) back-cross generation, when mice heterozygous at only the chromosome 15 locus were identified and intercrossed to generate the homozygous congenic strain, which we designate BALB.MRL chr.15 or CON15. The details of the marker-assisted selection protocol are summarized in Table I (available online at http://atvb.ahajournals.org). The CON15 mice and BALB mice exhibited similar growth curves and body weight. The size of the chromosome 15 congenic region was determined by typing additional flanking markers. The border on the proximal side was between D15Mit176 (7cM) and D15Mit180 (11 cM), and the border on the distal side was between D15Mit76 (54 cM) and D15Mit16 (61 cM) (Figure 4).

Plasma Lipid Levels

Plasma lipid levels were examined at the N4 and N5 generations and were found to be consistent with the QTL mapping studies. Thus, mice containing the chromosome 15 region from MRL tended to have elevated total cholesterol and triglycerides levels as compared with the background BALB strain. The levels and distributions of plasma lipids in chase-fed BALB mice, congenic chromosome 15 (CON15) mice, and F1 hybrid mice are shown in Table I and Figure 1. Significant increases in the levels of triglyceride and total cholesterol were observed in the CON15 mice of both sexes. The increased triglyceride in CON15 mice was present in
VLDL/LDL fractions after FPLC fractionation (Figure 1). The elevated cholesterol in CON15 mice was primarily caused by increased HDL cholesterol levels (Table 1 and Figure 1). In general, F1 hybrid mice exhibited lipoprotein profiles either intermediate between the parental strains or similar to the CON15 mice (Figure 1). As judged by gel filtration chromatography using FPLC, the sizes of HDL particles in the CON15 were somewhat smaller than those of BALB mice, and F1 mice had intermediate-size HDL (Figure 1). Unesterified cholesterol levels and free fatty acid levels were increased in CON15 mice of both sexes (Table 1).

### Increased Atherosclerosis in Chromosome 15 Congenic Mice

The effect of the chromosome 15 locus on atherogenesis was examined using a diet-induced model of fatty streak formation. A total of 14 BALB mice, 23 CON15 mice, and 7 (BALB x CON15) F1 heterozygous mice were fed the high-fat, high-cholesterol diet for 8 weeks and examined for lesions in the aortic sinus. Only female mice were used because they are more susceptible to diet-induced atherosclerosis than are males. Lesion areas were quantitated after oil red O staining of serial frozen sections, as described in Methods. Figure 2 shows the observed lesion sizes after log transformation. Only 2 of the 14 BALB mice had significant lesions, with an average area of $34 \pm 24 \mu m^2/section$. All but 1 of the 23 CON15 mice had significant lesions, with an average area of $904 \pm 221 \mu m^2/section$. Thus, the increase in the size of lesions was $30\times$ fold. Heterozygous mice exhibited lesions that were approximately the same size as the CON15 parent (Figure 2), suggesting dominant inheritance. There were no apparent effects of the locus on lesion composition, although the small number of significant lesions in BALB mice prevented detailed comparisons (data not shown).

### Genetic–Dietary Interactions Mediated by Chromosome 15 Locus

The levels of plasma lipids in the atherosclerosis study were determined after 8 weeks of feeding the atherogenic diet. As compared with the chow diet, large increases in total cholesterol levels occurred in BALB, CON15, and F1 mice. Striking differences, however, were observed in HDL cholesterol and

<table>
<thead>
<tr>
<th></th>
<th>Male BALB</th>
<th>Male CON15</th>
<th>Female BALB</th>
<th>Female CON15</th>
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<td>Total cholesterol (mg/dL)</td>
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<td>71±2</td>
<td>80±1*</td>
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<td>Unesterified cholesterol (mg/dL)</td>
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<td>Free fatty acids (mg/dL)</td>
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<td>53±1.5*</td>
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<tr>
<td>HDL (mg/dL)</td>
<td>31±3</td>
<td>46±4*</td>
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<td>VLDL+LDL (mg/dL)</td>
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Results are based on a total of 25 male BALB mice, 52 male CON15 mice, 16 female BALB mice, and 57 CON15 female mice. Values are expressed as mean±SEM.

*Significantly different from BALB within the same sex group ($P<0.05$).

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**TABLE 1. Effect of the Chromosome 15 Locus on Plasma Lipid Levels in Male and Female Mice on a Chow Diet**

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**Figure 1.** Gel filtration (FPLC) analysis of plasma lipoproteins in BALB, CON15, and F1 mice maintained on a chow diet. Plasma samples (400 μL) were applied to Superose 8 columns and the collected fractions were assayed for cholesterol and triglyceride levels as previously described. In these studies, VLDL/IDL eluted in fractions 9 to 20, LDL in fractions 20 to 32, and HDL in fractions 32 to 43.

**Figure 2.** CON15 locus has a major impact on atherosclerotic susceptibility. Female mice (BALB/c=13, CON15=23, F1=8) were placed for 8 weeks on high-fat diet containing 15% fat, 1.25% cholesterol, and 0.5% cholic acid, and lesions in the aortic root were examined in serial frozen sections as previously reported.
LDL/VLDL cholesterol levels. Whereas HDL cholesterol levels in BALB mice remained relatively constant after challenge with the atherogenic diet, they decreased strikingly in CON15 mice. In contrast, the CON15 mice exhibited much higher LDL/VLDL cholesterol levels in response to the atherogenic diet than BALB mice, and the F1 mice were intermediate (Table 2). Additional FPLC analysis in selected pools of animals showed similar results, with the exception of the HDL cholesterol levels (Figure 3). We determined that this was caused by high variation in the HDL concentration within the selected 4 animals as compared with the HDL concentration for the group of animals used in the lipid analysis (Table 2). Consistent with previous observations, the atherogenic diet resulted in decreased triglyceride levels in all of the strains.

These changes in plasma lipids are likely to underlie, at least in part, the differences in atherogenesis. Because HDL protects against atherogenesis whereas LDL/VLDL promotes atherogenesis, one measure of the atherogenic profile is the ratio of HDL cholesterol to LDL/VLDL cholesterol on the atherogenic diet. This was >3-times lower for CON15 mice (0.14) than BALB mice (0.55), and F1 mice were intermediate (0.37).

TABLE 2. The Chromosome 15 Locus Determines Response to the Atherogenic Diet

<table>
<thead>
<tr>
<th></th>
<th>Chow Diet</th>
<th>Atherogenic Diet</th>
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<tr>
<td></td>
<td>BALB</td>
<td>CON15</td>
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<tr>
<td>Triglycerides (mg/dL)</td>
<td>31±5</td>
<td>55±5*</td>
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<td>Total cholesterol (mg/dL)</td>
<td>82±4</td>
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<td>HDL (mg/dL)</td>
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<td>LDL/VLDL (mg/dL)</td>
<td>22±4</td>
<td>32±4</td>
</tr>
</tbody>
</table>

Plasma lipid levels in BALB, CON15, and F1 female mice used in the lesion study on chow and atherogenic diets. Results are based on a total of 13 BALB mice, 23 CON15 mice, and 8 heterozygous F1 mice. Values are expressed as mean±SEM.

*Significantly different from BALB on the same diet (P<0.05).

Use of Subcongenics for Fine Mapping

It should be feasible to subdivide the chromosome 15 congenic region to more narrowly map the gene or genes underlying the effects on lipid metabolism and atherosclerosis. To demonstrate the feasibility of this, we have subdivided the congenic region into proximal (closer to the centromere) and distal segments (Figure 4). The proximal congenic mice carried MRL alleles at markers D15Mit180 (11 cM from centromere) and D15Mit152 (18 cM), and BALB alleles at markers D15Mit26 (27 cM), D15Mit101 (29 cM), D15Mit28 (34 cM), D15Mit96 (44 cM), D15Mit76 (54 cM), and D15Mit16 (61 cM). The distal congenic carried BALB alleles at D15Mit152, D15Mit176, D15Mit180, and D15Mit26, and MRL alleles at D15Mit101, D15Mit28, D15Mit96, and D15Mit76. A comparison of lipid levels in the parental (BALB and CON15) and subcongenic lines clearly shows that the MRL gene determining elevated lipids resides in the distal subcongenic rather than the proximal subcongenic region. Thus, for total cholesterol levels in males, the proximal subcongenic was not significantly different from BALB (P=0.21) but was significantly different from CON15 (P<0.001), whereas the distal subcongenic was not significantly different from CON15 (P=0.47) but was significantly different from BALB (P=0.001) and CON15 (P<0.001).
different from BALB (P<0.001). Similar trends, although not all statistically significant, were observed for all the other lipid parameters (Figure 4). The isolated distal subcongenic 2 region encompasses ≈50 million base pairs or 30 cM.

Metabolic Alterations
To help identify the biochemical pathways affected by the chromosome 15 locus, we performed expression array analysis on multiple liver samples from the BALB and CON15 male mice on chow and atherogenic diets. Based on the selected criteria (please see online Methods), a number of significant changes in gene expression were observed in response to the atherogenic diet and between the BALB and CON15 strains (Tables II, III, and IV, available online at http://atvb.ahajournals.org). Striking decreases in expression of several key enzymes involved in bile acid synthesis, cholesterol-7α-hydroxylase, sterol-12α-hydroxylase, and oxysterol-7α-hydroxylase were observed in both strains in response to the atherogenic diet (Table III). A number of genes that showed significant decreases in mRNA expression in response to atherogenic diet (squalene epoxidase, fatty acid binding protein 5, and sterol-C4-methyl oxidase) are direct SREBP targets or were shown to be upregulated in SREBP transgenic mice.18 Feeding mice the atherogenic diet also resulted in increased expression of a number of genes involved in inflammation and detoxification, indicating the pro-inflammatory effect of atherogenic diet. As indicated in the data tables, quantitative real-time polymerase chain reaction was used in selected cases to confirm array data and gave similar results (data not shown).

Direct comparison of gene expression profiles between CON15 and BALB livers from mice fed chow or atherogenic diets revealed changes in a number of lipid metabolism-related genes (Tables II and IV). A large subset of differentially expressed genes belongs to pathways of fatty acid metabolism, bile acid biosynthesis, and extracellular matrix remodeling. Interestingly, several genes that belong to the bile acid metabolism pathway, eg, oxysterol-7α-hydroxylase (CYP39a1) are expressed at significantly lower levels in CON15 than BALB livers with both diets. Two of the differentially expressed genes (lymphocyte antigen 6 complex-locus D and atrogin 1) mapped near the peak of the QTL locus and represent potential Hyplip2 candidates that will be further evaluated.

Discussion
Our results confirm the QTL mapping of a chromosome 15 locus contributing to plasma lipid levels in a cross between inbred strains BALB and MRL, and they have also revealed that the locus has a major impact on atherosclerosis susceptibility. The availability of the congenic strain should enable fine mapping and positional cloning of the underlying gene. These points are discussed.

The lipid phenotype of the chromosome 15 congenic strain is consistent with expectations from the original mapping study.6 Both LDL/VLDL cholesterol levels and HDL cholesterol levels, as well as triglyceride levels, were affected by the locus. The locus also exhibited a marked effect in response to an atherogenic diet challenge. Particularly striking was the effect of the locus on the levels of HDL cholesterol in response to the high-fat diet. When challenged with the high-fat diet, HDL cholesterol levels in BALB mice increased slightly, but in the CON15 mice, HDL cholesterol levels decreased ≈2-fold (Table 2). This difference in dietary responsiveness has previously been observed in surveys of inbred strains of mice.12,19 Thus, most strains of mice, including BALB and C3H/HeJ, exhibit either an increase or no change in HDL cholesterol in response to the high-fat diet, whereas other strains, including members of the C57BL family of strains, exhibit a marked decrease in HDL cholesterol levels. We show in this study that the chromosome 15 locus can independently mediate the response of HDL cholesterol to a high-fat diet. Previous studies have suggested that in the C57BL family of strains, the decrease in HDL cholesterol is related to a decrease in the expression of cholesterol-7α-hydroxylase (Cyp7a1), the rate-limiting enzyme in bile acid synthesis.12,20,21 The direct causal relationship between plasma HDL cholesterol levels and the expression levels of Cyp7a1 has not been demonstrated. Clearly, the correlation is strain-specific and appears to be controlled by multiple genetic loci, regulating hepatic apoa-I mRNA levels as well as enzymes affecting HDL turnover.12,21 It is noteworthy that in this study both strains exhibited large decreases in Cyp7a1 and 2 other key enzymes (Cyp8b1 and Cyp7b1) of the bile acid biosynthesis pathway when fed the atherogenic diet (Table III). Thus, unlike the C3H/HeJ mice, BALB mice do exhibit decreases in Cyp7a1 when placed on atherogenic diet, despite having no significant change in HDL cholesterol levels. Although both strains exhibited decrease in a number of bile acid metabolism enzymes when placed on atherogenic diet, using the selection criteria (see online Methods) there were also differences between CON15 and BALB strains on a particular diet. CON15 mice had significantly lower mRNA levels of oxysterol-7α-hydroxylase (Cyp39a1) on chow and high-fat diets, cholesterol-7α-hydroxylase (CYP7a1) on a chow diet, and sterol-12α-hydroxylase (CYP8b1) on a high-fat diet (Table II and IV). Whether these differences contribute to the observed decrease in HDL levels is not clear at this point. Any effect of chromosome 15 locus on the expression of these enzymes would have to occur in trans, because none of these genes reside on chromosome 15.12 Particularly noteworthy in this respect is a QTL study by Schwarz et al,13 indicating that a gene influencing lipid levels in liver also resides in the region, at position 42 cM, only 10 cM away from the peak of Hyplip2 locus. Future studies will evaluate the influence of Hyplip2 locus on liver cholesterol metabolism.

The phenotype of the chromosome 15 congenic mice includes elevated levels of triglycerides and cholesterol, suggesting that it may be relevant to the common, complex human disorder familial combined hyperlipidemia (FCH).14 FCH is a mixed hyperlipidemia that is characterized by elevated levels of triglycerides and cholesterol and, frequently, by decreased HDL levels. Although FCH was recognized ≈30 years ago, the genetic cause of the disease remains poorly understood. Recently, Pajukanta et al identified the upstream transcription factor 1 as a strong candidate for FCH in the Finish population.22 In addition, some modi-
fiers such as the apolipoprotein AI-CIII-AIV-AV gene cluster and the hepatic lipase gene have also been identified.\textsuperscript{12,24} Clearly, FCH involves multiple genetic and environmental factors as well as genetic heterogeneity.\textsuperscript{25} One powerful approach to these problems is to use animal models to identify genes contributing to FCH traits and then to test these genes in human populations. The \textit{Hyplip1} gene in mouse exhibits a hypertriglyceridemia phenotype, and it was recently positionally cloned and shown to correspond to the thio-redoxin interacting protein \textit{Txnip}.\textsuperscript{8} Subsequent studies, however, showed that \textit{Txnip} is unlikely to represent a major FCH gene in human population.\textsuperscript{22,26} The chromosome 15 congenic strain provides a second possible mouse model of human mixed hyperlipidemias. The peak of the chromosome 15 QTL maps in close proximity to mouse marker D15Mit17.\textsuperscript{6} This locus corresponds very closely (within 1Mbp) to a syntenic region on human chromosome 8 centered around marker D8S1128, previously identified as a susceptibility locus in genome scan studies of Finish FCHL population.\textsuperscript{27}

The isolated distal subcongenic 2 region contains several possible candidate genes, including the sterol regulatory element binding protein, squalene epoxidase, apolipoprotein \textit{L}, carnitine \textit{o-palmitoyl} transferase 1, and a number of enzymes involved in lipid metabolism (www.informatics.jax.org). Rather than performing extensive sequencing and expression studies of these candidates, we are focusing on the narrowing the congenic interval to a few centimorgans or less by the subcongenic strategy.

The chromosome 15 locus has a dramatic effect on diet-induced atherosclerosis. Although mapping studies in mice have provided suggestive evidence for the locations of a number of genes contributing to atherogenesis, this is only the third report in which a congenic strain has been shown to affect lesion formation. One congenic strain, B6.C \textit{H-25}\textsuperscript{t}, on mouse chromosome 1, led to the identification of apolipoprotein \textit{AII} as a contributing factor in crosses between C57BL/6J and BALB/cJo or C3H/HeJ.\textsuperscript{14} Recently, Mehrabian et al reported the mapping of a locus for atherosclerosis on mouse chromosome 6 in a cross between the resistant strain CAST and the susceptible strain C57BL/6J.\textsuperscript{6} By testing candidate genes in the limited region, they provided strong evidence that the chromosome 6 gene corresponds to 5-lipoxygenase, a gene known to be involved in inflammation but not previously considered likely to contribute importantly to atherogenesis. A number of putative loci contributing to atherosclerosis susceptibility in various crosses have been reported.\textsuperscript{19,28–32} It will be important to examine the effect of the chromosome 15 locus on atherogenesis in a model of advanced atherosclerosis, such as apoE-null mice or LDL receptor-null mice.

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References


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