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Complex Inheritance of the 5-Lipoxygenase Locus Influencing Atherosclerosis in Mice

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ABSTRACT

We previously mapped a locus on chromosome 6 with a large effect (LOD > 6) on aortic lesion size in a (C57BL/6J × CAST/Ei) F_2 cross and identified arachidonate 5-lipoxygenase (5LO) as a candidate gene in this region. Subsequent studies with the 5LO knockout model showed effects on atherosclerosis and aortic aneurysms. We now report detailed genetic analysis of the chromosome 6 locus. We created a panel of overlapping and reciprocal subcongenic lines from the B6.CAST Ldlr$^{-/-}$ chromosome 6 congenic strain (CON6.Ldlr$^{-/-}$) and analyzed aortic lesion size in different subcongenic lines. Our results revealed that there are at least two subregions, designated as *Ath37* and *Ath38* that affect the size of aortic lesions independently of 5LO. We also showed that homozygote 5LO null mice develop smaller atherosclerotic lesions. We conclude that the relation between the mouse chromosome 6 locus and atherosclerosis is complex and is due to at least two genes with large effects within this region. This complexity should be considered when interpreting results of knockout studies.

ATHEROSCLEROSIS is a chronic inflammatory disease, which is influenced by a large number of environmental and genetic factors (Lusis 2000). Roberts and Thompson (1976) originally showed that inbred mouse strains differ in susceptibility to atherosclerosis, and subsequently Paigen *et al.* (1987, 1989) mapped the first genomic regions linked to atherosclerosis development in mice (*Ath1, Ath2, and Ath3*). At present, >20 loci contributing to atherosclerosis susceptibility have been identified in crosses between various strains of mice (Allayee *et al.* 2003; Wang *et al.* 2005a). Recently, *Tnfsf4* was identified as the gene underlying the *Ath1* locus and polymorphisms of this gene were found to be associated with myocardial infarction in humans (Wang *et al.* 2005b).

Several years ago, we reported the presence of linkage between the middle/distal segment of chromosome 6 and the size of atheroma (LOD = 6.7) in 250 F_2 mice generated from the athero-susceptible C57BL/6J (B6) and athero-resistant CAST/Ei (CAST) parental strains (Mehrabian *et al.* 2001). In this cross, the CAST allele was associated with decreased aortic lesions. The genetic effect of the chromosome 6 locus on lesion size was subsequently confirmed by introgressing this locus from CAST onto the B6 background and analyzing the lesion area in the congenic strain (CON6). Similarly, when CON6 mice on the low-density receptor null background (Ldlr$^{-/-}$) were fed with a high-fat diet, they had reduced lesion size compared with the B6.Ldlr$^{-/-}$ mice despite hypercholesterolemia, suggesting that this locus influences lesion development by a mechanism distinct from lipid metabolism (Mehrabian *et al.* 2001).

Arachidonate 5-lipoxygenase (5LO) is an enzyme that converts arachidonic acid to various leukotrienes, which are potent proinflammatory mediators (Mehrabian and Allayee 2003). The 5LO gene is located within the CON6 locus, and our results showed that the CAST allele of 5LO exhibits decreased levels of both 5LO mRNA and protein (Mehrabian *et al.* 2002). Furthermore, 5LO$^{+/-}$ Ldlr$^{-/-}$ mice fed with a Western-type diet developed significantly smaller lesions compared to the B6 Ldlr$^{-/-}$ mice. Moreover, resistance to atherosclerosis by the chromosome 6 congenic region or the 5LO knockout was mediated, in part, by bone-marrow-derived cells as judged by bone marrow transplantation experiments, consistent with a role for 5LO (Mehrabian *et al.* 2001, 2002). From this work, we concluded that 5LO is a primary candidate gene in the CON6 locus (Mehrabian *et al.* 2002).

Other studies have confirmed that the 5LO pathway plays a critical role in atherosclerosis. In two separate mouse studies with 5LO inhibitors, it was shown that blocking the 5LO pathway results in reduced atherosclerosis (Aiello *et al.* 2002), especially during early lesion development (Subbarao *et al.* 2004). In humans, there is an abundance of cells expressing 5LO in the atherosclerotic lesions (Spanbroek *et al.* 2003). We also found significant association between promoter variants of 5LO and intima-media thickness of the carotid artery (Dwyer *et al.* 2004). Similarly, variants of the 5LO

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activating protein (FLAP) and LTA4 hydrolase (LTA4H) genes were found to be associated with the risk of stroke and myocardial infarction (Helgadottir *et al.* 2004, 2005, 2006). Recently, Hakonarson *et al.* 2005 showed that short-term treatment of coronary artery disease patients with a FLAP inhibitor significantly reduced the levels of biomarkers associated with the risk of myocardial infarction, and Cipollone *et al.* (2005) found that the expression level of 5LO is elevated in symptomatic compared to asymptomatic plaques and is associated with acute ischemic syndromes.

Despite the mounting evidence favoring the hypothesis that 5LO pathway plays a role in atherosclerosis, a recent study by Zhao *et al.* (2004) observed little or no effect of a 5LO knockout on atherosclerosis when studied on either Ldlr$^{-/-}$ or apolipoprotein E null (Apoe$^{-/-}$) backgrounds. However, they observed that 5LO$^{-/-}$apoE$^{-/-}$ mice developed fewer aneurysms than Apoe$^{-/-}$ mice. In light of the supporting evidence favoring the role of the 5LO pathway in atherosclerosis from both mouse and human studies, we reasoned that the differing conclusions drawn from this study may be due to the complexity of the 5LO locus. If the chromosome 6 region flanking the 5LO gene contains other genes that influence atherosclerosis or aneurysm development, then studies of the 5LO knockout mice could be complicated by variations between the knockout and the control mice in the flanking region of 5LO, unrelated to the 5LO gene itself. That is, since the knockout was created on the strain 129 genetic background and then backcrossed to a B6 background by a series of crosses, the region around 5LO will retain genes derived from strain 129 rather than from B6.

In addition to 5LO, there are other genes in the chromosome 6 locus, such as PPAR-gamma (PPARγ), CD163, and α-2-macroglobulin (A2m), which have been previously associated with atherosclerosis and other known risk factors for cardiovascular disease in humans or animals (Li *et al.* 2000; Chawla *et al.* 2001; Chen *et al.* 2001; Krimbou *et al.* 2001; Schaer 2002). This, coupled with the growing evidence that some of the QTL for complex traits consist of multiple closely linked causal genes (Legare and Frankel 2000; Rogner *et al.* 2001; Liu *et al.* 2002; Diament and Warden 2004; Yalcin *et al.* 2004; Flint *et al.* 2005), lead us to hypothesize that the chromosome 6 locus may also represent such a multigenic region. To examine this, we created a panel of overlapping and reciprocal subcongenic lines from CON6.Ldlr$^{-/-}$ mice and characterized each line for development of atheroma in the proximal aorta. Our results showed that the chromosome 6 locus contains at least two genes influencing atherosclerosis.

MATERIALS AND METHODS

Animals, diets, and markers: Strain C57BL/6J Ldlr$^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The housing and care of mice were performed according to the approved institutional guidelines. All mice were housed in groups of five or fewer animals per cage and maintained on a 12-hr light–dark cycle at an ambient temperature of 23°. The subcongenic mice were fed on a standard rodent chow containing 4% fat (Ralston-Purina) until 12 weeks of age. At 12 weeks of age, female mice on the Ldlr$^{-/-}$ background were started on an adjusted, Western-type diet containing 42% fat, 0.15% cholesterol, and 19.5% casein without sodium cholate (TD 88137; Food-Tek) and maintained on this diet for 8 weeks. 5LO null mice on the B6 background were fed a standard rodent chow containing 4% fat (Ralston-Purina) until 8 weeks of age. At 8 weeks of age, female mice were placed on a cholic-acid-containing atherogenic diet (HFA) (TD 90221; Food-Tek) and maintained on this diet for 18 weeks or for other times indicated.

To generate chromosome 6 subcongenic Ldlr$^{-/-}$ mice, B6.CAST chromosome 6 congenic mice containing the low-density-lipoprotein-receptor null mutation (referred to here as "CON6.Ldlr$^{-/-}$") (Mehrabian *et al.* 2001) were crossed to C57BL/6J.Ldlr$^{-/-}$ mice. The resulting (B6.Ldlr$^{-/-}$ × CON6.Ldlr$^{-/-}$) F_1 animals were then either backcrossed or intercrossed to B6 Ldlr$^{-/-}$. The resulting (B6.Ldlr$^{-/-}$ × CON6.Ldlr$^{-/-}$) F_2 and [(B6.Ldlr$^{-/-}$ × CON6 Ldlr$^{-/-}$)F_1 × B6.Ldlr$^{-/-}$] N2 mice were then genotyped for 10 microsatellite markers (*D6Mit152*, *D6Mit123*, *D6Mit102*, *D6Mit104*, *D6Mit44*, *D6Mit193*, *D6Mit61*, *D6Mit111*, *D6Mit198*, and *D6Mit14*) in the CON6 region. F_2 mice exhibiting recombination within the interval were then bred to produce homozygous subcongenic strains.

The primer sequences for microsatellite markers used in this study were obtained from the Broad Institute at http://www.broad.mit.edu/resources. The physical location of each marker was obtained from the Ensemble mouse genome database (build 33) at http://www.ensembl.org/Mus_musculus with the exception of the D6Mit14 marker. The approximate physical location of D6Mit14 was estimated by first finding the location of this marker relative to D6Mit198, the most distal marker on our map, using T31 radiation hybrid panel, followed by converting the centiray distance obtained from radiation hybrid mapping to a megabase distance using information available on The Jackson Laboratory T31 Mouse Radiation Hybrid Database.

For each subcongenic mouse, the genotype of two candidate genes, 5LO and PPARγ, were determined using primers designed to amplify the nearby microsatellite markers located at 115.7 and 116.7 Mb, respectively. The primer located at 115.7 is named *D6UCLA3* and the sequences of the forward and reverse primers are GAATCCAGCACCCTCTTCTG and TGTACTGTGCTGGGCAAGTT. The primer located at 116.7 Mb is named *D6UCLA5* and the sequences of the forward and reverse primers are GTTTGCATGTGTGTGTGTGC and GAA CAAAGGAAGAAGATTCCCTAA. For *D6UCLA3*, the C57BL/6J and CAST/Ei PCR-amplified product sizes are 149 and 161 bp, respectively. For *D6UCLA5*, the C57BL/6J and CAST/Ei PCR-amplified product sizes are 156 and 160 bp, respectively.

Aortic lesion and aneurysm analysis: Methods for the quantification of atherosclerotic lesions in the aortic root have previously been reported (Mehrabian *et al.* 2002). In brief, the heart and proximal aorta were dissected, washed with phosphate-buffered saline, embedded in optimal cutting temperature compound (Tissue-Tek), 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 no. 12–550-15). In the region beginning at the aortic valves, every other section was collected. In all other regions, every fifth section was collected. Sections were then stained with Oil red-O and hematoxylin

and counterstained with Fast Green. Lesion areas were quantified by light microscopy.

To determine the percentage of incidence and degree of severity of aneurysms in aortic sections, we used a semiquantitative method that took into account the extent of medial destruction as well as the number of sections in which aneurysms occurred (Shi *et al.* 2003). With this method, each section examined received a score between 0 and 6, with 0 being no aneurysm (no disruption of the media) and 6 being complete destruction of the media with the elastin layer being disrupted for >2 μm and the aortic lesion being protruded into the adventitia. More details on quantitation and representative figure of each score (1–6) is provided in supplemental Figure 1 at http://www.genetics.org/supplemental/. The "relative score" for aneurysms was calculated by summing over all the scores of the aortic sections examined for each mouse.

Statistical analysis: The lesion data are presented as a scattergram with the mean for each group indicated on the graph. The results mentioned in the text are the means and the standard errors. The ANOVA *t*-test was performed using Statview (version 5.0) (Abacus Concepts, Berkeley, CA) to compare differences among groups in atherosclerotic lesions. The chi-square test was implemented to compare the difference in the frequency of occurrence of aneurysms between the subcongenics with B6 5LO and the subcongenics with CAST 5LO. In all the tests applied, differences were considered statistically significant at $P < 0.05$.

RESULTS

Construction of CON6 subcongenic lines: To analyze the effect of chromosome 6 locus on lesion size and refine this genetic region, we created a panel of overlapping and reciprocal subcongenic lines. Overall, a total of 16 lines were constructed (Figure 1A). This was achieved by intercrossing (B6.Ldlr$^{-/-}$ × CON6 Ldlr$^{-/-}$) F_1 mice and genotyping the F_2 progeny for nine microsatellite markers across the CON6 locus (for details of markers and their corresponding physical location, see MATERIALS AND METHODS). These markers span ~43 cM covering the middle and distal section of mouse chromosome 6. Recombinant mice were then selected and intercrossed to produce a series of homozygous subcongenic lines. Each subcongenic line is genetically distinct from the others in terms of the length and the boundaries of the CAST introgressed region.

The chromosome 6 locus contains more than one gene affecting atherosclerosis: To investigate if the genetic effect of the CON6 locus on atherosclerosis is due to one or multiple genes, we compared the size of aortic lesions in two subcongenics that divided the CON6 locus into two nonoverlapping halves (lines 3 and 10). In subcongenic line 3, the proximal region is derived from CAST strain, with the proximal recombination breakpoint located between *D6Mit152* and *D6Mit123* and the distal recombination breakpoint located between *D6Mit44* and *D6Mit193* (Figure 1A). In subcongenic line 10, the distal region of the CON6 locus is derived from the CAST strain. In this line, the proximal recombination breakpoint is located between *D6Mit193* and *D6Mit61* and the distal recombination breakpoint is located between *D6Mit14* and the end of the chromosome 6 (Figure 1A). Since these two subcongenic lines retained no overlapping CAST region, we were able to study the effect of each region on atherosclerosis independently of the other.

After feeding each line and the parental strains with a high-fat Western-type diet for 8 weeks, the size of the atherosclerotic lesions was quantified in Oil red-O-stained ascending aortic sections (see MATERIALS AND METHODS). In subcongenic line 3, the size of the lesions was significantly smaller than that in the B6.Ldlr$^{-/-}$ mice ($P < 0.0001$) (Figure 1B). Likewise, line 10 developed smaller atheroma compared to the B6.Ldlr$^{-/-}$ mice ($P < 0.0001$) (Figure 1B). There was no significant difference between the size of the lesions in lines 3 and 10. When compared to the lesion size of the CON6. Ldlr$^{-/-}$ mice, both subcongenic lines had slightly higher average lesion size values (CON6.Ldlr$^{-/-}$ = 57,000 μm^2/section ± 39,000 *vs.* line 3 = 81,000 μm^2/section ± 46,000 and line 10 = 78,000 μm^2/section ± 36,000). However, these differences were not statistically significant for either line 3 ($P = 0.16$) or line 10 ($P = 0.19$). These results show that the effect of the CON6 locus on atherosclerosis could be attributed to at least two subregions. The first subregion (referred to here as the "proximal region") is located between *D6Mit152* and *D6Mit193* and the second subregion (referred to here as the "distal region") is located distal to the *D6Mit193* marker.

Fine mapping of the distal region: The distal region spans ~25 Mb and contains 277 annotated genes. To find the smallest interval associated with lesion formation in the distal region, we compared the phenotype of subcongenics 11, 12, 13, 14, and 15, which share overlapping CAST segments in the distal region. Lines 11, 14, and 15 all had significantly smaller atherosclerotic lesions compared to B6.Ldlr$^{-/-}$ mice. The genotyping information of these three lines revealed that the resistant lines 11, 14, and 15 all had the CAST allele of the *D6Mit111* marker. This analysis indicated that the region associated with smaller lesion size can be confined to the 10-Mb interval surrounding the *D6Mit111* marker between markers *D6Mit61* and the *D6Mit198*. In contrast, line 12 had the same phenotype as that of the B6.Ldlr$^{-/-}$ mice and line 13 had an intermediate phenotype when compared to that of the B6.Ldlr$^{-/-}$ ($P = 0.037$) and CON6.Ldlr$^{-/-}$ mice ($P < 0.0001$). The results for lines 12 and 13 suggested that at the telomeric end of chromosome 6, outside the 10-Mb interval surrounding the D6Mit111 marker, there are at least two genes linked in repulsion with small but significant effects on lesion formation (Figure 2B).

From the five subcongenics used to analyze the distal region, lines 12 and 14 had recombination breakpoints within the 10-Mb fine-mapped interval. These two lines were selected for further genotyping to increase the resolution of the fine mapping. Each line was genotyped

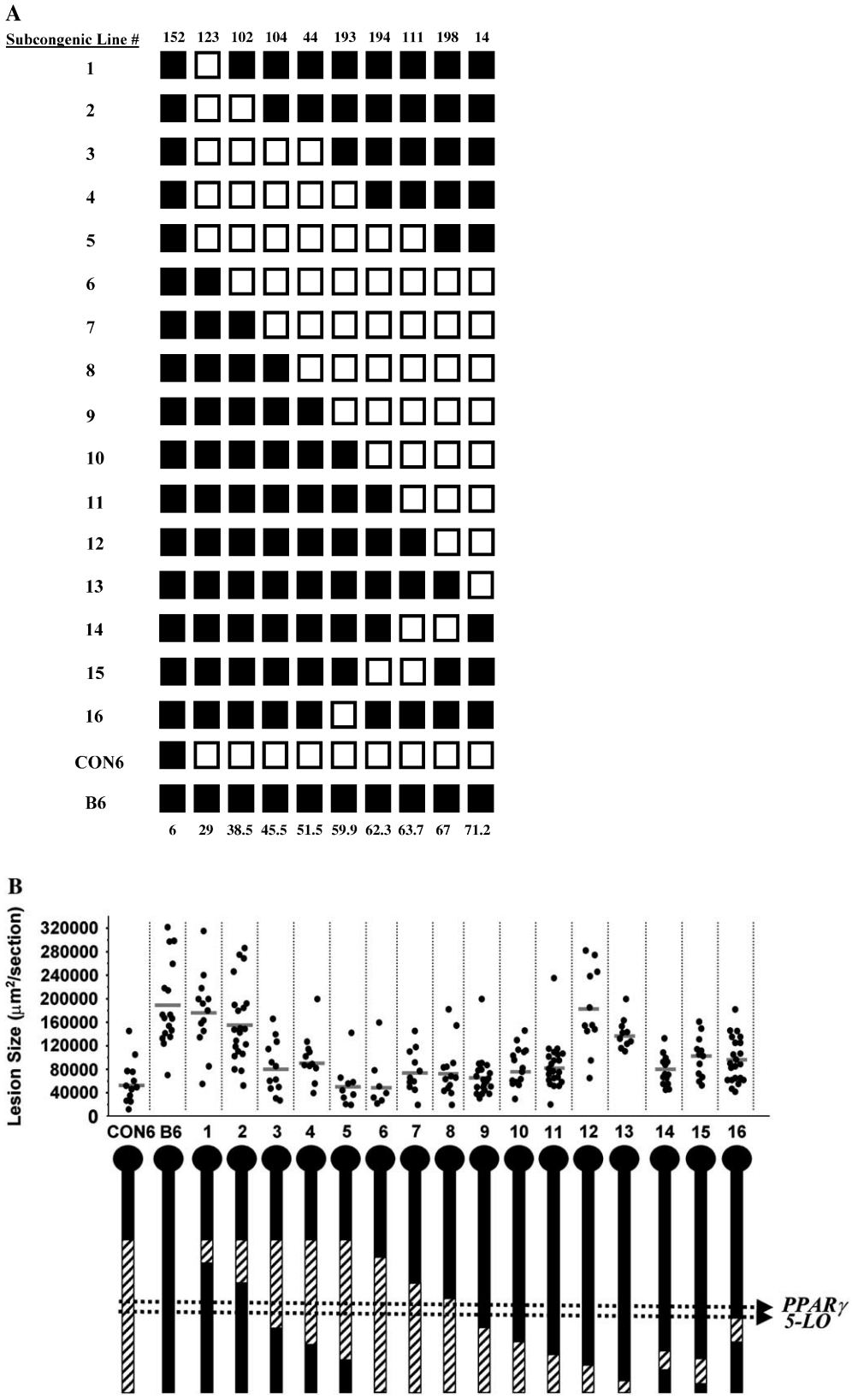

FIGURE 1.—The genotype and phenotype of the subcongenic lines. (A) The genotype of the 16 CON6 subcongenic lines for 10 microsatellite markers originally used to derive the congenic strain. The solid and open boxes represent the B6 and the CAST genotypes, respectively. The short name of the markers is given at the top, where 152 indicates *D6Mit152*; 123, *D6Mit123*; 102, *D6Mit102*; 104, *D6Mit104*; 44, *D6Mit44*; 193, *D6Mit193*; 61, *D6Mit61*; 111, *D6Mit111*; 198, *D6Mit198*; and 14, *D6Mit14*. B6 refers to C57BL/6J Ldlr$^{-/-}$, and CON6 refers to B6.CAST chromosome 6 congenic on an Ldlr$^{-/-}$ background. The numbers at the bottom are the centimorgan positions of each marker obtained from the Mouse Genome Informatics database. (B) An overview of the aortic lesion size in each subcongenic line. (Top) The scatter plot of lesion size measured for each subcongenic mouse. The shaded line corresponds to the mean of each group. The *x*-axis is the line number for each subcongenic. (Bottom) The schematic of each subcongenic. Hatched boxes depict the CAST congenic region. The location of the 5LO and PPARγ genes are shown with broken arrows across the subcongenic lines. Mice used for these analyses were all female and were fed a Western diet beginning at 3 months of age for 8 weeks. All mice are on an Ldlr$^{-/-}$ background.

for four additional markers: *D6Mit220*, *D6Mit301*, *D6Mit258*, and *D6Mit291*. The marker pattern along with the phenotype of each strain is shown in Figure 2A. As shown in Figure 2A, the resistant line 14 has retained the CAST alleles for markers *D6Mit220* and *D6Mit301*, indicating that the resistant gene(s) in the distal region is located proximally to the *D6Mit258* marker. Consistent with this analysis is the marker pattern for the

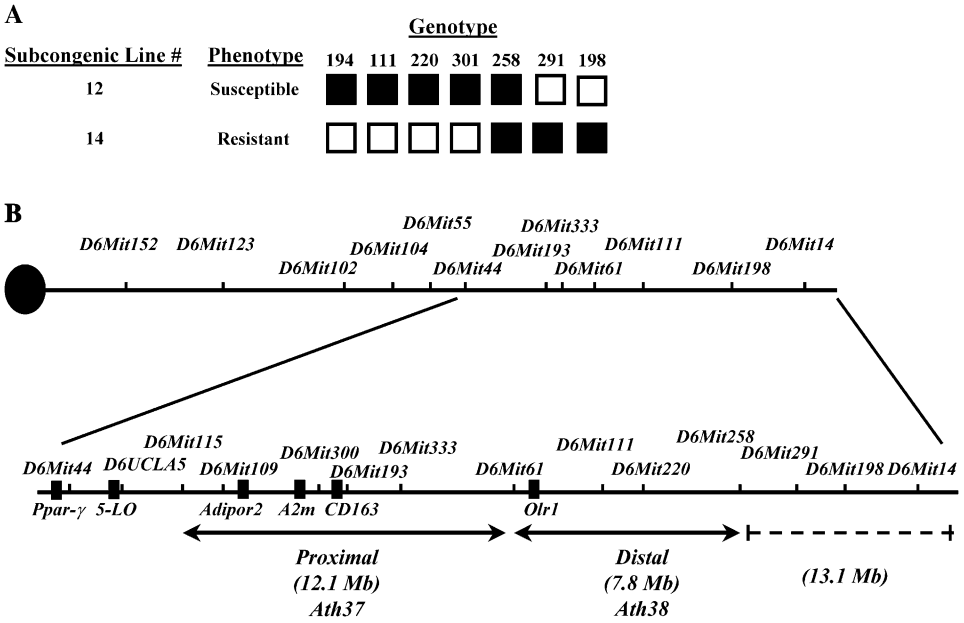

FIGURE 2.—Fine mapping of the CON6 distal locus. (A) The phenotype classification and genotype of the subcongenic lines 12 and 14 used to narrow the boundaries of the fine-map distal locus. Shown are the subcongenic line number, the phenotype of each line, and the genotype information for each line for seven microsatellite markers located in the fine-mapped region. The number 61 indicates *D6Mit61*; 111, *D6Mit111*; 220, *D6Mit220*; 301, *D6Mit301*; 258, *D6Mit258*; 291, *D6Mit291*; and 198, *D6Mit198*. The solid and open boxes represent the B6 and the CAST genotypes, respectively. (B) The CON6 locus has at least two genes affecting atherosclerosis. The map of the CON6 locus along with the location of the two major loci affecting atherosclerosis are shown. The size of the fine-mapped proximal and distal novel loci for atherosclerosis is indicated below their respective intervals. The dashed line indicates the interval where genes with small effect are located. As mentioned in the text there may be at least two genes with opposing effects in this interval. The relative locations of the six candidate genes (PPARγ, 5LO, Adipor2, A2m, CD163, and Olr1) are shown with solid squares on the map.

susceptible line 12. This line exhibits a B6.Ldlr$^{-/-}$ phenotype and has B6 alleles for *D6Mit220*, *D6Mit301*, and *D6Mit258*, which again indicates that the protective gene(s) is located proximally to the *D6Mit258* marker. In summary, these data allowed us to narrow the distal region to the 7.8-Mb interval between markers *D6Mit61* and *D6Mit258* for the QTL with the major effect. We designate the gene in the distal region *Ath38* (Figure 2B).

Complex inheritance of the proximal region: We next focused on the proximal region. To carry the genetic analysis of this region, we created and characterized subcongenic line 16 (Figure 1, A and B). The marker pattern (Figure 1A) shows that the CAST region retained in this line is the region surrounding marker *D6Mit193* but excluding the 5LO gene. The phenotypic characterization of this line revealed that the size of aortic lesions is significantly smaller than that of B6.Ldlr$^{-/-}$ ($P < 0.0001$) but larger than that of the CON6.Ldlr$^{-/-}$ mice (line 16 average lesion size = 95,000 μm^2/section ± 38,000 *vs.* CON6.Ldlr$^{-/-}$ average lesion size = 57,000 μm^2/section ± 39,000, $P = 0.011$). These data suggested that line 16 has a protective phenotype.

To determine the size of the proximal region, we genotyped line 16 with four additional markers surrounding *D6Mit193*. The results showed that the proximal recombination breakpoint is located between *D6Mit115* and *D6Mit109* and the distal recombination breakpoint is between *D6Mit218* and *D6Mit61*. Since this line had the B6 alleles of both the 5LO and the distal region, we concluded that, within the proximal region, the 12.1-Mb interval between *D6Mit115* and *D6Mit61* contains at least one athero-protective gene. We designate this gene *Ath37* (Figure 2B).

5LO null mice develop smaller lesions during the initial stages of atherosclerosis: As discussed above, multiple studies involving inhibitors of the 5LO pathway and disruption of leukotriene receptors have suggested that 5LO deficiency protects against atherosclerosis and that the effect of 5LO is strongest during early stages of atherosclerosis (AIELLO *et al.* 2002; SUBBARAO *et al.* 2004). To test if the effect of 5LO is strongest during the initial stages of lesion development, we examined the effect of the 5LO deficiency on atherosclerosis in a diet-induced model that results primarily from the development of fatty-streak lesions (NISHINA *et al.* 1993). As shown in Figure 3, 5LO$^{-/-}$ mice exhibited considerably reduced atherosclerosis compared to C57BL/6J mice in the diet-induced system (~10-fold). These data are consistent with other studies suggesting that reduced 5LO activity has a larger effect on the development of early lesions as compared to its effect on more advanced lesions. However, it is important to note that the 5LO$^{-/-}$ mice used here and in other studies (ZHAO *et al.* 2004) carry flanking genes derived from strain 129 rather than from the B6 strain. Genotyping of the 5LO$^{-/-}$ mice used in our study revealed that the 129-derived region extends from at least markers *D6Mit55* to *D6Mit333* and thus overlaps with the *Ath37* locus that we mapped in this study.

The CAST 5LO locus does not influence the development of aneurysms in the proximal aorta: ZHAO *et al.* (2004) reported that 5LO deficiency decreases the frequency and severity of both abdominal and aortic medial elastic lamina destruction (aneurysm) in a hyperlipidemic

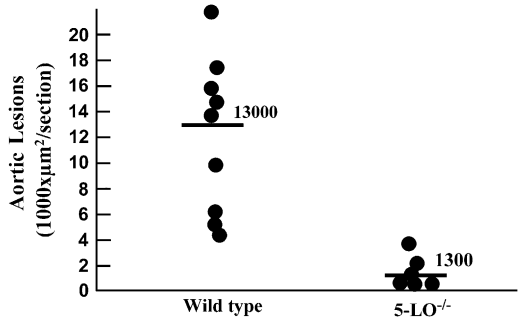

FIGURE 3.—5LO null mice have reduced atherosclerosis. Scattergram of aortic lesions in $5LO^{-/-}$ ($n = 6$) and C57BL/6J ($n = 9$) mice. Two-month-old, chow-fed female mice of each group were fed with the high-fat, atherogenic diet for 18 weeks prior to lesion assessment. Numbers within each plot indicate the mean leasion size for each group. B6 $5LO^{-/-}$ mice developed ~10-fold smaller aortic lesions as compared to B6 wild-type mice.

background. Therefore, we asked if the 5LO CAST allele, with dramatically decreased 5LO expression as compared to the B6 allele, has a similar effect in subcongenic lines. To determine this, we divided the subcongenics into two groups on the basis of the homozygosity for the parental 5LO genotype and scored aortic arch sections for the presence or absence of aneurysms. In cases where aneurysms were present, they were scored on a scale of 0–6 using predetermined criteria based on the morphology of the aortic section (see MATERIALS AND METHODS and supplemental Figure 1 at http://www.genetics.org/supplemental/). The results of this analysis showed no significant differences in the frequency of aneurysms between homozygous subcongenic lines bearing the CAST 5LO allele and those containing the B6 5LO allele. In addition, among the mice having aneurysms, there were no significant differences in regard to the severity of the aneurysms in these two groups (Figure 4).

DISCUSSION

We performed fine mapping of the CON6 locus using a panel of 16 reciprocal and overlapping subcongenic lines. This panel offers several advantages for studying the genetic effects of different subregions within a QTL. First, similarly to the recombinant inbred mice, each line is genetically homogeneous, allowing a large number of mice of each genotype to be tested. The ability to examine multiple mice from each line is especially important in studying complex traits such as atherosclerosis in which environmental and other nongenetic factors cause large variations in the phenotype. Moreover, these subcongenic lines can now be studied for other traits that may be associated with the atherosclerotic phenotype. Also, such a panel allows for the simultaneous detection of multiple genes contributing to the phenotype and their fine mapping. For our study, the combined analyses of the CON6.$Ldlr^{-/-}$ subcongenic panel allowed us to identify and fine map two major genes that conferred aortic lesion resistance (Figure 2B). Although 7.8 Mb was the best resolution that we achieved in our study, one could achieve a higher fine-mapping resolution using this strategy by developing more overlapping subcongenics within the locus of interest. Another advantage of using subcongenic lines is that it allows one to test the presence of gene–gene interactions between genes located in separate subregions represented in the panel (LEGARE and FRANKEL 2000; ROGNER *et al.* 2001). For example, in our data, *Ath37* and *Ath38* reduced the lesion size by 43 and 41%, respectively. If these two loci acted independently, one might expect to see an approximately additive protective effect on lesion size when the two are combined. However, the CON6.$Ldlr^{-/-}$ mice, which contain both the proximal and the distal loci, exhibit only a slightly increased protective phenotype, suggesting that there may be an epistatic interaction between these two loci.

Although we observed that there are at least two other genes at the chromosome 6 locus that contribute to lesion development, our results are consistent with previous evidence that 5LO affects atherosclerosis (MEHRABIAN *et al.* 2001; DWYER *et al.* 2004; HELGADOTTIR *et al.* 2004; HUANG *et al.* 2004), at least in earlier stages. Recently, however, ZHAO *et al.* (2004) observed inconsistent effects of 5LO deficiency on the background

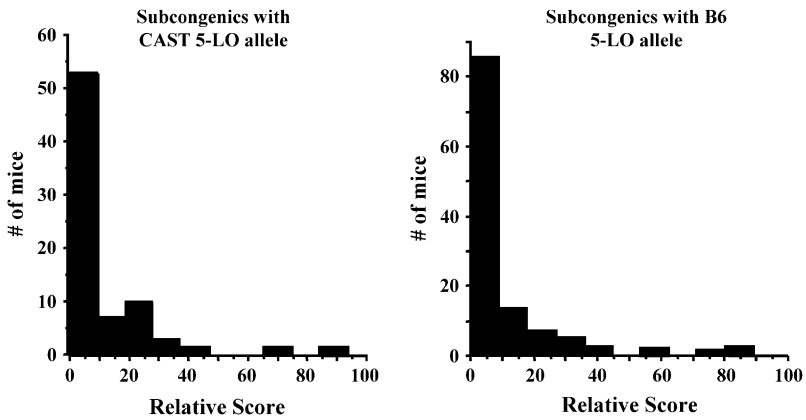

FIGURE 4.—Aneurysms in subcongenic mice. The frequency plot for the incidence and severity of aneurysms in female subcongenic $Ldlr^{-/-}$ mice homozygous for the CAST 5LO allele (left) and the B6 5LO allele (right). The number of mice analyzed for the CAST and the B6 group are 76 and 116, respectively. The severity of aneurysms for each mouse was estimated using a semiquantitative method (see MATERIALS AND METHODS) and represented as a "relative score" on the plot.

of the Apoe null mutation in two separately maintained colonies. In this study, one colony showed a 25% decrease in lesions and the other showed no difference. These authors also failed to observe significant differences in lesion development between the $5LO^{-/-}$ $Ldlr^{-/-}$ double knockout and the $Ldlr^{-/-}$ mice (Zhao *et al.* 2004). They, however, did observe an effect of 5LO deficiency on the frequency and severity of aneurysms in $Apoe^{-/-}$ mice (Zhao *et al.* 2004). In contrast, we observed a significant reduction in lesion size in 5LO null homozygous mice compared to wild-type B6 mice and no evidence for aneurysms in the subcongenic lines with CAST 5LO hypomorph allele. These differing findings may be due to differences in experimental design such as the methodology for lesion and aneurysm quantitation, the age of the animals, and/or type and length of the diet, all of which differed in some respects. One significant difference in experimental design was that Zhao *et al.* (2004) assessed aneurysms in the distal aorta whereas we studied aneurysms in the proximal aorta. However, in light of the study presented here, a plausible explanation would be that there are genetic factors differing between the two studies that confound the results. In particular, it could be that the presence of 129 genomic DNA flanking the targeted allele of 5LO could influence lesion or aneurysm development. Here we provide evidence that two regions distal to 5LO contain genes that affect atherosclerosis. Genotyping of the $5LO^{-/-}$ mice obtained from the Jackson Laboratory and used in our study indicates that in this colony the 14-Mb region surrounding the targeted 5LO gene consists of the 129 genomic sequence. How much of the 129 genomic DNA remained in the $5LO^{-/-}$ mouse used by Zhao *et al.* (2004) is not known. But clearly, given that multiple genes at the chromosome 6 locus affect atherosclerosis, the extent of the 129 flanking region could influence the results if strain 129 differed from strain B6 with respect to alleles of these genes. We acknowledge that in our report the evidence for the antiatherogenic effects of loss of 5LO in the 5LO null mice may be confounded by the presence of the *Ath37* 129 allele surrounding the targeted 5LO gene. In addition, we were unable to genetically isolate the 5LO CAST allele from the *Ath37* locus to directly investigate the effect of the CAST 5LO on atherosclerosis. However, the results obtained from the 5LO null mice are consistent with other mouse (Aiello *et al.* 2002; Subbarao *et al.* 2004) and human studies (Dwyer *et al.* 2004; Helgadottir *et al.* 2004, 2005; Cipollone *et al.* 2005), which collectively point toward the importance of the 5LO pathway in pathogenesis of atherosclerosis. It is noteworthy that recent studies have shown that 5LO deficiency results in complex metabolic effects, including adiposity and insulin resistance (Mehrabian *et al.* 2005). These effects predict that 5LO deficiency would result in increased atherosclerosis, but inhibition of the 5LO pathway, both genetically and pharmacologically, results in reduced atherosclerosis. Combined, these results suggest that 5LO deficiency both promotes atherogenesis (through its metabolic effects) and blocks atherosclerosis (perhaps due to its local effects in the subendothelial space in arteries) with the latter being dominant, at least in the early stages of atherosclerosis.

Our studies have revealed two novel major atherosclerosis genes, neither of which significantly impact lipoprotein levels. The more proximal locus (designated *Ath37*) spans 12.1 Mb between markers *D6Mit115* and *D6Mit61*. This interval contains 175 genes and includes several candidate genes. One of the candidate genes located in this interval is Adiponectin receptor 2 (Adipor2) (Shimada *et al.* 2004; Kadowaki and Yamauchi 2005). The natural ligand of Adipor2 is adiponectin, which in both animal and human studies has been shown to have antiatherogenic effects (Kadowaki and Yamauchi 2005). In summary, in humans, plasma adiponectin levels are inversely correlated with the marker for coronary artery disease, the circulating C-reactive protein (Ouchi *et al.* 2003). In mice, adenovirus-mediated overexpression of adiponectin resulted in reduced progression of atherosclerosis in $Apoe^{-/-}$ mice (Okamoto *et al.* 2002). In addition, adiponectin stimulates endothelial cells to produce nitric oxide (Chen *et al.* 2003a) and decreases lipid accumulation in human monocyte-derived macrophages by suppressing the expression of class A scavenger receptor (Ouchi *et al.* 2001). Since adiponectin has many antiatherogenic effects and because it is known to bind and act only through Adipor1 or Adipor2, it would be plausible to hypothesize that some of the antiatherogenic effects of adiponectin are mediated through Adipor2. CD163, the macrophage-specific scavenger receptor for the hemoglobin–haptoglobin complex, is another candidate gene located in the *Ath37* locus (Schaer 2002). A recent study by Aristoteli *et al.* (2006) showed that the plasma levels of CD163, which previously were found to be present in human atherosclerotic lesions (Ratcliffe *et al.* 2001), are significant predictors of coronary artery disease in humans. Although the exact role of CD163 in the pathogenesis of atherosclerosis is not known, the ability of IL-6 to increase expression of cell-surface CD163 protein (Buechler *et al.* 2000) and to induce hemeoxygenase-1 (HO-1) mRNA expression has led to the speculation that internalization of hemoglobin–haptoglobin complex by CD163 is an initial event in production of antiinflammatory heme metabolites by HO-1 (Moestrup and Moller 2004). The third candidate gene located in the *Ath37* region is A2m. A2m is known to interact with lecithin:cholesterol acyltransferase (LCAT) and enhance its clearance (Krimbou *et al.* 2001). Since LCAT plays a major role in reverse cholesterol transport and regulation of the plasma high-density lipoprotein (HDL) levels, it is plausible to hypothesize that A2m-enhanced clearance of LCAT can lead to lower plasma HDL levels and increase atherosclerosis.

The more distal locus (designated *Ath38*) spans 7.8 Mb between markers *D6Mit61* and *D6Mit258*. This locus contains 92 annotated genes, including a candidate gene, Olr1. Olr1 is known to be expressed in macrophages and vascular endothelial cells and is inducible by a variety of proinflammatory cytokines (Nagase *et al.* 1998; Morawietz *et al.* 1999, 2001). Moreover, this gene has been found to be abundantly present in human (Kataoka *et al.* 1999) and rabbit atherosclerotic lesions (Chen *et al.* 2000). Several independent studies have also found evidence for association between OLR1 and acute myocardial infarction (Mango *et al.* 2003; Tatsuguchi *et al.* 2003) and coronary artery disease (Chen *et al.* 2003b; Ohmori *et al.* 2004). Perhaps the most direct evidence suggesting that Olr1 plays a role in the pathogenesis of atherosclerosis comes from the transgenic experiments. Recently, Inoue *et al.* (2005) overexpressed the bovine Olr1 cDNA under endothelial-specific promoter in mice, which resulted in a 10-fold increase in the size of atheroma-like lesions in intramyocardial vessels of transgenic animals fed a high-fat diet. They, however, saw no statistical difference in lesions in the aorta of these animals, which they attribute to lack of Olr1 overexpression in the aortic endothelial cells compared to myocardial vessel endothelial cells.

In addition to *Ath37* and *Ath38*, which have strong effects on lesion formation, we were able to find evidence for the presence of at least two other genes with smaller effects at the telomeric end of the chromosome 6. These genes, however, seem to be linked in repulsion, meaning that the CAST allele of one of these genes is athero-protective and the CAST allele of the other gene is atherogenic. There are no apparent candidate genes that we could identify in this 13.1-Mb region.

In summary, our data emphasize the complications that can arise in the genetic dissection of polygenic diseases such as atherosclerosis. Clearly, the chromosome 6 locus has a dramatic impact on atherosclerosis because it contains multiple genes contributing to the trait. The presence of closely linked genes affecting the same trait complicates not only the genetic analysis of the locus but also the interpretation of the results obtained from experiments with knockout mice.

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