Genetic Control of High Density Lipoprotein (HDL)-Cholesterol in AcB/BcA Recombinant Congenic Strains of Mice

Sean A. Wiltshire^{1,2}, Eduardo Diez^{1,3}, Qianqian Miao⁴, Marie-Pierre Dube⁵, Mireille Gagné³, Olivier Paquette³, Ronald G. Lafrenière⁶, Momar Ndao⁴, Lawrence W. Castellani⁷, Emil Skamene^{1,4}, Silvia M. Vidal^{1,2}, and Anny Fortin^{3,8}

¹Department of Human Genetics, McGill University, Montreal, Quebec, H3G 0B1, Canada

²Complex Traits Group, McGill University, Montreal, Quebec, H3G 0B1, Canada

³Emerillon Therapeutics Inc., Suite 1000, 416 de Maisonneuve Blvd. W., Montréal QC, H3A 1L2

⁴The Research Institute of the McGill University Health Centre, 1650 Cedar Avenue, Montreal QC, H3G 1A4

⁵Faculty of Medicine, University of Montreal, Montreal Heart Institute, 5000 Bélanger Ave. E., Montreal QC, H1T 1C8

⁶Centre of Excellence in Neurosciences of the Université de Montréal (CENUM), Ste Justine Hospital Research Centre, Room A-937B, 3175 Côte-Sainte-Catherine, Montreal, QC, H3T 1C5

⁷Department of Medicine/Cardiology University of California, Los Angeles, California 90095

⁸Department of Biochemistry, McGill University, Montreal QC, H3G1Y6

Abstract

Epidemiological studies show that high HDL-cholesterol (HDLc) decreases the risk of cardiovascular disease. To map genes controlling lipid metabolism, particularly HDLc levels, we screened the plasma lipids of 36 AcB/BcA RC mouse strains subjected to either a normal or a high-fat/cholesterol diet. Strains BcA68 and AcB65 showed deviant HDLc plasma levels compared to the parental A/J and C57BL/6J strains; they were thus selected to generate informative F2 crosses. Linkage analyses in the AcB65 strain identified a locus on chromosome 4 (*Hdlq78*) responsible for high post-high-fat diet HDLc levels. This locus has been previously associated at genome wide significance to two regions in the human genome. A second linkage analysis in strain BcA68 identified linkage in the vicinity of a gene cluster known to control HDLc levels. Sequence analysis of these candidates identified a *de novo*, loss of function mutation in the *ApoA1* gene of BcA68, which prematurely truncates the ApoA1 protein. The possibility of dissecting the specific effects of this new *ApoA1* deficiency in the context of isogenic controls makes the BcA68 mouse a valuable new tool.

Supplementary key words

mouse model; lipid profiles; genome-wide linkage analysis; *de novo* mutation in *ApoA1*

Introduction

Several epidemiological studies have shown that the concentration of HDL cholesterol (HDLc) inversely correlates with the risk of cardiovascular disease (CVD)(8). A number of mechanisms have been proposed to explain how HDL particles may mediate protection against atherosclerosis (reviewed in (1)). These include the stimulation of reverse cholesterol transport; inhibition of LDL oxidation; inhibition of inflammation by suppressing expression of adhesion molecules and macrophage chemotactic proteins; reduction of lipoprotein retention, and attenuation of endothelial dysfunction.

Twin and family studies have established that the level of plasma HDL is in part controlled by genetic factors (9). Over the past decade, the study of HDL metabolic pathways and the identification of naturally occurring rare mutations have established a list of more than 19 loci and 50 candidate genes possibly involved in HDL modulation (reviewed in (13, 24)). These candidate genes encode several different types of proteins: structural apolipoproteins; cellular receptors, adaptors, and transporters; transcription factors; as well as plasma- and cell-associated enzymes and transfer proteins. Rare variants of the ABCA1 transporter gene have been associated with decreased HDLc (4); genome wide association studies have revealed that common variants associated with blood lipids are also associated with CVD (35, 40). Mouse models generate novel targets for the control of lipid metabolism; they also confirm existing associations, as an overwhelming majority of these are conserved between mice and humans(17). Inbred strains of mice diverge widely in their response to high-fat, high-cholesterol diets, as measured by plasma lipid levels and susceptibility to fatty streak lesions and gallstone formation(32). The phenotypic variation observed between inbred strains in response to high-fat diet has provided the basis for quantitative trait locus (QTL) studies that aim to identify genes controlling atherosclerosis and blood lipid levels(38, 42, 43). For HDLc in particular, over 20 crosses involving at least 20 inbred strains have been used to map about 40 unique putative HDLc QTLs scattered on every somatic chromosome(43). The genetic control of HDLc is therefore acknowledged to be extremely complex.

The AcB/BcA set of recombinant congenic mouse strains (RCS) is a series of 36 inbred strains produced from the second backcross generation of the two progenitor strains A/J and C57BL/6J(7). Each individual RCS carries a small amount (about 12.5%) of DNA from one “donor” parental strain fixed as a set of discrete congenic segments on the background (87.5%) of the other parent. The AcB/BcA set has been genetically characterized using a panel of 625 SSLP markers and the position of all congenic fragments has been established(7). The AcB/BcA recombinant congenic strains have proven to be highly useful to study the genetic basis of the differential disease susceptibility reported between the A/J and C57BL/6J strains. This is exemplified by their recent use to study a series of multigenic phenotypes, such as susceptibility to malaria and *Salmonella* infections, pre-pulse inhibition

of acoustic startle, lung fibrosis, and several others (6, 7, 12, 18, 25, 26, 34). Individual gene effects contributing to a complex trait can segregate in a single RCS and can thus be studied in isolation. In some cases, the relatively small size of the congenic segments fixed in individual RCS facilitates the search and testing of candidate genes(25). In addition, secondary genetic effects can be detected in strains fixed for certain alleles at major mapped loci, yet showing deviations from expected phenotypes (6). Finally, reassortment of parental haplotypes or appearance of novel mutations during the breeding of individual strains may generate “hyper-phenotypes” that segregate as simple traits and that can quickly be cloned (26).

In order to map genes involved in lipid metabolism in general and HDL formation in particular, we undertook a systematic screening of plasma lipid levels in the 36 AcB/BcA RC strains before and after 4 weeks of a pro-atherogenic high-fat, high-cholesterol diet. From this initial screen, we focused on two strains showing phenotypes which deviated substantially from the parental A/J and C57BL/6J strains. Linkage analyses in secondary crosses, fine mapping, and sequence analyses identified the phenotype-causing genetic defect in one instance and has revealed novel and previously associated candidate genes in the second instance.

Materials and Methods

Animals

Inbred C57BL/6J (B6), AKR, and A/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The set of 36 AcB/BcA recombinant congenic strains (RCS), derived from A/J and B6 parents, were generated according to a previously described breeding scheme and genotyping protocol(7). (AcB65 X AKR) and (BcA68 X AKR) F1 crosses were generated; F2 crosses were then performed by standard (F1brother X F1sister) mating. All mice were handled according to the guidelines and regulations of the Canadian Council on Animal Care; all protocols were approved by the Montreal General Hospital Research Institute Animal Care Committee.

Diet and Lipid Measurements

For each RC strain, three to seven mice (5.5 on average) per sex, between 8 and 12 weeks of age, were fed a pro-atherogenic, high-fat, high-cholesterol diet (ID 90221, Teklad Research Diets, Madison, WI) for 4 consecutive weeks. The diet contained (by weight) 75% Purina Mouse Chow, 7.5% cocoa butter, 1.25% cholesterol, and 0.5% sodium cholate. Mice were weighed weekly. Blood collection was performed both pre- and post-diet following 4–6 hours of fasting. For pre-diet collection, blood was drawn from the lateral saphenous vein; cardiac puncture under anaesthesia was performed for exsanguination at the end of the diet period. Plasma lipid related variables, including HDL cholesterol (HDLc) and total cholesterol (TC), were measured by enzymatic assays employing colorimetric end points(3, 10). Each assay was performed in triplicates. The UCLA Lipid/Lipoprotein Laboratory, which performed the lipid analyses, participates in the Centers for Disease Control and Prevention National Heart, Lung, and Blood Institute Lipid Standardization Program

(Laboratory ID# LSP-251); it also undergoes quarterly certification by the Centers for Disease Control.

Genotyping

Tail biopsies were obtained from all animals and genomic DNA was isolated by a standard procedure involving proteinase K treatment. Primer pairs defining dinucleotide repeat markers informative for the A/J, C57BL/6J, and AKR mouse strains were purchased from Research Genetics (Huntsville, AL) and Biocorp Inc. (Montreal, QC). A complete list of microsatellite markers used for genotyping is included in Supplemental Table 1.

Statistical and Bioinformatics Analyses

Statistical analyses were conducted with the freely available program R; linkage was performed with the package 'R/qtl' version 1.23–16. The scanone function of the 'R/qtl' library was used to perform maximum likelihood interval mapping (EM) of the phenotype on genetic markers using sex as an additive covariate term. Significance values were evaluated with 10 000 permutations. The LOD support interval of QTL peaks was calculated using a 1.5 LOD drop by 'R/qtl' on the interval map. Sequence polymorphisms were identified in the candidate genes using the Sanger1 high density SNP set (65.2+ Million) from the mouse phenome database (<http://phenome.jax.org/SNP>)(14). P-values shown in the figures are a result of two-tailed T-Tests with *P < 0.05, **P < 0.01, and ***P < 0.001.

ApoA1 Sequencing

Regional transcript maps of chromosome 9 were obtained from the UCSC Genome Browser (<http://genome.ucsc.edu>). Exons from the *ApoA1* gene were amplified from genomic DNA of the parental C57BL/6J strain, as well as from BcA68. The following oligonucleotides were used for PCR amplification of 3 fragments containing the 4 exons of *ApoA1*: Exon 1, 5'-TTGTTCCAGGCTCAGAGGGCACTA-3' and 5'-ATCAACTGGGAGGCGCTATGGAG-3'; Exons 2–3, 5'-ATATCTCGCACCTTTAGCCATTCT-3' and 5'-CTCAGTTCCAGCATCTTCTCA-3'; Exon 4, 5'-TTCGGGGAACTAGGACCATAGCA-3' and 5'-AGTGCGGCACCATGTTCTCACG-3'.

The PCR products were visualized by agarose gel electrophoresis to confirm size. Cycle sequencing of individual *ApoA1* exons was performed on both DNA strands at the McGill University/Genome Quebec Innovation Centre (Montreal, QC). Reactions were done in the presence of fluorescently labeled BigDye™ Terminator (Applied Biosystems, Foster CA); the products were analyzed using an automated ABI 3700 instrument (Applied Biosystems, Foster CA). Genotyping of the QW22-23CStop mutation was done in 104 (BcA68XAKR)F2 mice by digesting the exon 2–3 fragment amplified with PCR using the *Mwo* I restriction enzyme according to manufacturer's directions. Digested fragments were visualized by electrophoresis through 3% agarose gels.

Western Blot Analysis

Mice sera were separated in 4–12% Novex NuPAGE Bis-Tris gradient gels (Invitrogen) under reducing conditions. Separated proteins were transferred to nitrocellulose membranes.

Nonspecific sites were blocked with 5% skim milk in TBS for 1h at room temperature. Membranes were incubated overnight with rabbit anti-Apo A1 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:500 dilution, followed by incubation with HRP-conjugated anti-rabbit IgG at a 1:200,000 dilution (Amersham Biosciences Co., Piscataway, NJ). The membranes were incubated in SuperSignal West Pico detection solution (Pierce, Rockford, IL) and exposed to X-ray film. The loading control gel was stained with Biosafe Coomassie Stain (BioRad).

Results

Screening of the AcB/BcA RCS

In order to identify genes involved in lipid metabolism in mice, 36 AcB/BcA recombinant congenic strains (RCS), along with parental controls (A/J and C57BL/6J), were fed a pro-atherogenic, high-fat diet (7.5% cocoa butter diet, 1.25% cholesterol, 0.5% cholate) for a period of 4 weeks. Lipid related fractions, including HDL cholesterol (HDLc) and total cholesterol (TC), were measured before and after the diet period; the strain distribution patterns were then analyzed. Figure 1 presents the amounts (mg/dL) of HDLc in the plasma of the AcB/BcA RC strains, along with that of the A/J and C57BL/6J (B6) parental strains, according to diet: normal diet (Figure 1A); and following four weeks of a pro-atherogenic, high-fat diet (Figure 1B). Figure 1C presents the difference between the two conditions (normal vs. high-fat diet) in plasma taken from the same animal (Figure 1C). Total serum cholesterol (TC) was also measured in the plasma of these animals under the same conditions (Figure 2).

When the B6 and A/J parental strains were fed a normal diet, HDL was the primary cholesterol transport particle found in their plasma, corresponding to about 85% and 90% of their TC respectively. Conversely, following four weeks of the high-fat diet, HDLc decreased to about 20% of the TC in both parental strains, paralleled with a 1.6- and 1.8-fold increase in TC, respectively (Figures 1C and 2C). A similar increase was observed in both sexes, although females had significantly lower levels of plasma cholesterol than males ($P < 0.001$). Strains of the AcB/BcA RC set presented a continuous phenotypic distribution of cholesterol levels (TC and HDLc) when fed either a normal or a high-fat diet, indicating the presence of multigenic determinants controlling plasma cholesterol. The diet-induced change in plasma cholesterol composition was similarly heterogeneous across RC strains (Figure 3). Changes in HDLc and TC levels were significantly correlated, although weakly so ($R^2=0.12$, $P < 5e12$); this suggests that HDLc and TC serum levels have some genetic controls in common, but that they also have some that are specific to each of them. Two notable outliers were identified, BcA68 and AcB65. BcA68 plasma contained extremely low levels of HDLc and TC, independently of diet (Figure 1, 2). When fed a high fat diet, TC levels in the plasma of BcA68 mice rose by 68.0 mg/dL, an increase comparable to that of other RC strains, increases in which ranged between 49.2 mg/dL and 158 mg/dL; this was not the case with HDLc levels. A second strain of interest, AcB65, also demonstrated a lack of change in HDLc levels following a high-fat diet (Figure 3). On a normal diet, the proportion of HDLc with respect to TC in this strain was similar to that of A/J (83.0 mg/dL of HDLc to 92.4 mg/dL of TC in AcB65 vs. 89.3 mg/dL of HDLc to 93.8 mg/dL of TC in

A/J), but it remained relatively high when the mice were fed a high-fat/cholesterol diet (80.2 mg/dL of HDLc to 211.6 mg/dL of TC in AcB65 vs. 35.3 mg/dL of HDLc to 154.0 mg/dL of TC in A/J). Unlike in AcB65, B6 serum post-high-fat diet is relatively HDLc poor (31.6 mg/dL of HDLc to 202.1 mg/dL of TC). Thus, AcB65 mice exhibit elevated post-high-fat diet HDLc levels compared to both parental mouse strains. The phenotypes seen in BcA68 and AcB65 transcend both parental strains, suggesting specific parental allele assortments or a spontaneously occurring mutation during the derivation of these strains.

Phenotype of outcrosses between strains of interest

In order to further investigate the mode of inheritance of the extremely low HDLc phenotype seen in BcA68 and the high post-diet HDLc phenotype seen in AcB65, informative outcrosses with the AKR strain were generated (Figure 4). When fed a normal diet, mice of the [BcA68xAKR]F1 generation cross showed intermediate HDLc levels as compared to those observed in the AKR and BcA68 parents, consistent with a co-dominant transmission of the trait. Segregation analyses showed that 26 of the 106 (BcA68 X AKR)F2 (24.6%) displayed the extremely low HDLc phenotype, suggesting that a single autosomal mutation is responsible for this phenotype in BcA68 mice. Similarly, an intermediate HDLc phenotype was observed in [AcB65xAKR]F1 mice, as compared to both parental strains. However, HDLc levels in the [AcB65xAKR]F2 mice were continuously distributed. Significant differences in HDLc levels were noted between [AcB65xAKR]F2 males and females, as shown in Figure 4B (74 mg/dL vs. 46 mg/dL, respectively).

Mapping *Hdlq78*, a locus conferring high post-diet HDL cholesterol

In order to map the locus (loci) controlling the high HDLc phenotype in AcB65 mice post-high-fat diet, we performed a genome scan using 106 SSLP markers on 186 [AcB65 X AKR]F2 animals. Linkage analysis performed on HDLc and non-HDLc (TC - HDLc) identified a single locus on chromosome 4 that controls HDLc levels (Figure 5). No significant linkage was found between TC (or non-HDLc) in the [AcB65xAKR]F2 cross. The peak of linkage occurred at the marker D4Mit178, with a LOD score of 5.97 and a P-value of 0.0003; the 1.5 LOD interval of the locus spanned between D4Mit288 and D4Mit16, covering 71 Mb on chromosome 4. This locus, which we will refer to as *Hdlq78*, controls approximately 8% of the observed phenotypic variance. The effect size of *Hdlq78* was 16.7 mg/dL in females (57.0±19.1 for KK (K for AKR alleles)) homozygotes vs. 40.3±11.2 for AA (A for A/J alleles) and 16.3 mg/dL in males (KK=83.2±18.8, AA=66.9±9.5mg/dL). It has been hypothesized that when human genome wide associations and mouse QTL analysis identify the same region for the same phenotype, it is because variation in the same orthologous segment is responsible (17). Therefore, the *Hdlq78* interval was compared to human association data as indicated in Figure 5D (40, 43). There appear to be two sub-peaks within the *Hdlq78* locus, suggesting the possibility that two loci underly the effect. The trough separating the two sub-peaks corresponds to where the two orthologous human regions on chromosomes 9p and 1p join. Both of these have been significantly associated with HDLc levels (Figure 5B, D). The more proximal peak observed corresponds to an association with the *TTC39B* gene, located at 82.86 Mb in the mouse, and the distal peak corresponds to an association with a cluster of genes from *MACF1-PABPC4*, located at 123.3 Mb in the mouse.

Mapping low HDLc levels in BcA68 mice

The extreme and bimodal phenotypic distributions in [BcA68XAKR]F2 mice suggested the possibility of a *de novo* mutation unrelated to its congenic segments. As such, a relatively low-coverage genotyping panel of 21 markers was typed on 41 mice from both extremes of the F2 distribution. From this analysis, the low HDLc phenotype was linked to a locus on chromosome 9 with a LOD score of 24.25, as shown in Figure 6A. After initial linkage, 8 additional markers were added to chromosome 9, which refined the peak of linkage to a region centered on D9Mit130. This peak of linkage coincided with a cluster of genes known to be involved in lipid metabolism (*ApoA1*, *Apoa4*, *Apoc3*, *Apoa5*, *Pafah1b2*). Sequence analyses in this cluster identified two consecutive point mutations in exon 3 of the *ApoA1* gene of BcA68: a G>T transition at the third position of codon 22 (TGG), producing a tryptophan to cysteine missense (W22C); and a C>T transition at position 1 of codon 23 (CAG), which replaces a glutamine by a stop (Q23Stop) (Figure 6C). The presence of these mutations predicts the synthesis of a truncated 22aa *ApoA1* protein in the BcA68 RC strain in place of the wild-type 264aa protein; however, no *ApoA1* protein was detected in this strain (Figure 6D). The 66-67GC>TT mutation in *ApoA1* destroys a *Mwo* I restriction site in the genomic DNA. Using this diagnostic restriction assay, the remainder of the BcA68-derived (BcA68 X AKR)F2 mice were genotyped in order to correlate the presence of the *ApoA1* mutant allele with the low HDLc phenotype. As shown in Figure 6E, this analysis clearly demonstrated the dosage effect of *ApoA1* on HDLc levels. Homozygous *ApoA1*^{66-67TT} F2 mice showed an average HDLc level of 5 ± 3 mg/dL (n=25) compared to 60 ± 12 mg/dL (n=23) in the homozygous wild type. Heterozygous *ApoA1*^{66-67GC/}*ApoA1*^{66-67TT} mice displayed an intermediate HDLc level of 38 ± 8 mg/dL (n=56). These data are similar to the ones previously reported for homozygous *ApoA1* (44) and *ApoA1/Apoc3/Apoa4* (22) knockout (KO) models, as well as the ones for wild-type littermates. The 22-23WQ>CStop mutation is not found in the A/J and C57BL/6J parental strains, or in any other strains of the AcB/BcA RCS set. It is a *de novo* mutation that emerged on a C57BL/6J genomic segment and that became fixed during the breeding of BcA68. When fed a normal diet, BcA68 animals appear healthy; they do not show any gross abnormality and present normal longevity. These *ApoA1*^{-/-} mice may be useful as an *in vivo* model of *ApoA1* deficiency.

Discussion

HDL cholesterol metabolism is acknowledged to be highly complex. In human populations, Teslovich et al. recently identified 95 loci associated with the control of lipid metabolism. Taken together, these loci accounted for 10–12% of the observed phenotypic variance (40). This complexity is mirrored in mice, where over one hundred quantitative trait loci have been reported to control natural variation in HDLc levels (reviewed in (43)). Mouse models are used to identify novel targets for the control of lipid metabolism, as well as to confirm existing associations, since an overwhelming majority of them are conserved between mice and humans (17). In mice, the degree of experimental variation is minimized, such that each linkage peak explains $10 \pm 8\%$ of phenotypic variation (43). The interrogation of the AcB/BcA RCS for plasma lipids on a normal and a high-fat, high-cholesterol diet allowed us to rapidly identify at least two strains (AcB65 and BcA68) that showed a highly deviant

HDLc-related phenotype compared to both the A/J and C57BL/6J parental strains. Following a change in diet, these RC strains responded less than other strains with respect to HDLc levels, but their TC response was normal. These “hyperphenotypes” may arise from unique re-assortment of parental alleles or may be due to novel mutations arising during derivation of the strains, which become fixed during the inbreeding of one or more strains (22).

Genetic analysis of the AcB65 strain, whose HDLc levels are high following a high-fat diet, revealed a novel, AKR-derived locus on chromosome 4, *Hdlq78*. The *Hdlq78* locus controlled 8% of observed phenotypic variance; and increased mean post-high-fat diet level of HDLc by 16.5 mg/dL on average. The 1.5 LOD interval of *Hdlq78* spans between D4Mit288 and D4Mit16; however, a trough separates two possible sub-peaks within the interval. These two peaks each contain interesting candidate genes known to be implicated in important pathways, as well as genes whose human orthologues have been strongly associated with the control of HDLc levels.

The proximal portion of *Hdlq78* is highly associated with HDLc levels in humans, especially the Tetratricopeptide repeat domain 39B (*TTC39B*) gene. While the function and mechanism of action of *Ttc39b* are not entirely clear, there is an association between its expression levels and HDLc levels (40). As a proof of concept, when *Ttc39b* is knocked down by shRNA in the mouse, significantly higher HDLc levels are observed(40); this makes *Ttc39b* a very strong candidate gene for HDLc regulation. Of the 336 known SNPs between A/J and AKR at this locus (14), none are predicted to alter the amino acid sequence of the full-length gene product (transcript ENSMUST00000102823, 617 amino acids). However, the *Ttc39b* gene has four additional putative protein coding transcripts (ENSMUST00000048274, ENSMUST00000030205, ENSMUST00000148811, and ENSMUST00000150522), which respectively encode 518, 209, and two 179 amino acid long proteins. A SNP located at position 82,905,359 of chromosome 4 (rs28317504) is predicted to result in the presence of a stop codon in the 209 and 179 amino acid long proteins, leading to a 39 amino acid truncation from the C-terminus. The function of these transcripts should be studied further to determine which of them, if any, contribute to HDL regulation. Other potential candidates within the proximal portion of *Hdlq78* include a family of 21 Major Urinary proteins (*Mup1-21*) with lipid binding ability (lipocalin domains), which are excreted into the urine.

The distal sub-peak of *Hdlq78* overlaps almost identically with the previously described *Hdlcho* locus (peak at 49cM ~105Mb) identified in a [B6xA/J]F2 cross (5). In addition to its association with HDLc control in the mouse, this region has also been strongly associated with HDLc regulation in humans, particularly the *MACF1*, *BMP8A*, *PPIEL*, and *PABPC4* genes (40). In the AcB65 strain, a non-synonymous coding change is present in the *Macf1* gene (rs2755370), leading to a V1756A substitution; another non-synonymous mutation is present in *Pabpc4* (rs32502744), which leads to an A502T substitution. In both cases, AKR and B6 strains share a common genotype different from the A/J or AcB65 genotype. Other notable genes within the distal portion of *Hdlq78* include the receptor for leptin satiety hormone (*Lepr*), a sterol transport protein (*Scp2*), and *Pcsk9*, which is involved in LDL receptor turnover(16), as well as *Angptl3*. Indeed, *Angptl3* deficiency has been reported to decrease HDLc levels via *Lipg* or *Lpl* (29, 36, 37). In this intercross, HDLc levels were

influenced in the same direction by both proximal and distal regions of *Hdlq78*. Linkage to two strongly associated human genes under these two regions raises interesting possibilities for furthering the study of human association data in a more controlled model. At this point, however, it is impossible to determine whether one or more distinct genes in the *Hdlq78* locus are actually responsible for the observed HDLc phenotype. The presence of several strong candidates, including those from human association studies, suggests future work should begin with the generation of congenic and sub-congenic mice in order to assess the contribution of individual genes.

A similar genetic analysis using the BcA68 RCS led to the identification of a nonsense mutation in the *ApoA1* gene, which resulted in extremely low levels of HDLc as a single Mendelian-type effect. This genetic effect was unlinked to donor-derived congenic segments in the AcB/BcA RCS set, but rather resulted from the presence of a *de novo* mutation in BcA68. Interestingly, this is the second *de novo* mutation controlling a phenotype of interest in the AcB/BcA RCS set(27).

The GC>TT substitution in exon 3 of the *ApoA1* gene of BcA68 introduces a premature termination codon at a position corresponding to amino acid 23 in the normal 243-amino acid, mature *ApoA1*. Considering the position of the mutation, this 22 amino acid truncated *ApoA1* protein is predicted to be functionally inert and was undetectable, leading to HDLc deficiency in BcA68. To our knowledge, this is the first naturally occurring *ApoA1* null mutation reported in mice. The fact that the 22-23WQ>CStop mutation is not present in any other strain of the AcB/BcA RC set suggests that it occurred relatively late in the generation of the BcA68 strain. In humans, homozygosity for several nonsense mutations in the *ApoA1* gene have been reported (15, 21, 28, 30, 31, 33, 39, 45), as well as compound heterozygosity where one of the mutations was a nonsense mutation (20, 23). These mutations were all associated with extremely low levels or absence of HDLc. This is in agreement with our observation that the extremely low HDLc phenotype inherited from BcA68 segregates as a recessive single gene effect in two different F2 crosses (data only shown for BcA68xAKR), corresponding to a high penetrance of the mutation. At this point, the size and composition of HDL particles from BcA68 mice have not been investigated, though it seems they contain no residual ApoA1 protein.

Despite the clear link between ApoA1 and HDL deficiency, a clear relationship between ApoA1 and the development of atherosclerotic lesions remains to be demonstrated (19, 41). According to previous calculations, about 91% of the genetic material of BcA68 mice is derived from the parental C57BL/6J inbred strain (7). Since C57BL/6J are susceptible to diet-induced atherosclerosis (32), it could be of interest to study the effect of a long-term atherogenic diet on the BcA68 strain, either on its natural genetic background or in crosses with sensitized strains (*ApoE*^{-/-}, human CETP transgenic model, etc.). Similarly, in humans, clinical heterogeneity is observed in patients with APOA1 deficiency; several pathologies have been reported, including CVD (21, 30), but also corneal opacity (15, 39), xanthoma (15, 21, 30), neuropathy (30, 31), and hepatomegaly (15, 39). Possible explanations for this pleiotropic effect of APOA1 deficiency may include the influence of environmental risk factors, such as age, diet, alcohol consumption, and smoking, but also of interactions with unknown additional genetic defects of various expressivities.

One important technical feature of the targeted *ApoA1* KO animals is to be taken into consideration. The *ApoA1* null mutation is introduced using targeted ES cells from a 129 background(44). Within the *ApoA1^{tm1Unc}* mouse, even after extensive backcrossing efforts, this mutation therefore remains surrounded by 129-derived genetic material. Unfortunately, *ApoA1* is part of a very tight cluster of genes involved in lipid metabolism: *ApoA1*, *Apoc3*, *Apoa4*, and *Apoa5*. Among the few genes with modulated expressions described in *ApoA1* KO mice (2), there was *Apoc3*, which sits immediately distal to *ApoA1* on mouse Chromosome 9 (less than 3 kb apart) (11). It remains to be seen whether *Apoc3* expression is modulated by the *ApoA1* null-mutation, or whether differences in expression are due to *Apoc3* alleles of C57BL/6 and 129 origin, or even if other genes within that 129 congenic segment indirectly affect its expression. Callow and colleagues elegantly showed evidence supporting the latter two possibilities in their paper (2). Differential expression of the *Apoc3* gene in the targeted *ApoA1* KO mouse could thus be responsible for some of the other detected differences in gene expression. In the BcA68 strain, the null mutation occurred within a large C57BL/6J-derived genomic segment. Therefore, transfer of the *ApoA1*-null mutation from the BcA68 mouse into a pure C57BL/6 genetic background through backcrossing will constitute a much more accurate *ApoA1*-null model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. HDLc levels in AcB/BcA recombinant congenic strains of mice. A) High-density lipoprotein cholesterol (HDLc) in AcB/BcA mice when fed a normal chow diet (A) or a high-fat diet (B). The difference in HDLc levels between the two diets per mouse (C). HDLc fractions were determined in 3–7 females and males prior to and following a four-week high-fat diet. Each point represents one mouse and is overlaid on a standard box and whisker plot.

Figure 2.

Total Cholesterol levels (TC) in AcB/BcA recombinant congenic strains of mice. A) Total plasma cholesterol in AcB/BcA mice when fed a normal chow diet (A) or a high-fat diet (B). The difference in TC levels between the two diets per mouse (C). TC fractions were determined in 3–7 females and males prior to and following a four-week high-fat diet. Each point represents one mouse and is overlaid on a standard box and whisker plot.

Figure 4. Segregation analyses of HDLc levels in AcB65 and BcA68-derived crosses. A) BcA68, AKR, F1, and F2 mice were fed a normal diet prior to HDLc measurements. B) AcB65, AKR, F1, and F2 mice were fed a pro-atherogenic, high-fat diet prior to HDLc measurements. Males and females are shown separately. Each point represents one mouse, with the mean indicated by horizontal bars.

Figure 5. Linkage in [AcB65xAKR]F2 and orthologous human candidate genes associated with HDLc regulation

A) LOD plot for [AcB65xAKR]F2 intercross (black), with males (grey) and females (light grey) plotted separately, shows significant linkage on chromosome 4 to HDL cholesterol levels.

B) LOD plot of peak linkage of HDLc on chromosome 4 defines the *Hdlq78* interval; peak linkage occurs at D4Mit178 (66.8Mb), with LOD=5.97, $P=0.0003$, and 8% variance explained. Bars below the X-axis represent human orthologous haplotype regions (upper bar, expanded in Figure 3D), as well as A/J (light grey) and B6 (black) chromosomal contributions to AcB65 (lower bar).

C) HDLc vs. genotype at D4Mit178 shows that the AA(AcB65) genotype leads to lower HDLc than KK (AKR) in both females (40.3 ± 11.2 vs. 57.0 ± 19.1) and males (66.9 ± 9.5 vs. 88.2 ± 18.8). D) Mouse chromosome 4 is roughly orthologous to human 9p31–32 and 1p31–36. Two regions (shown as diamonds in 3B and 3D) were highly significantly associated to HDLc by Teslovich et al. previously (40). Association is plotted against genome position as $-\log(P\text{-value})$ and genome wide significance ($P < 5 \times 10^{-8}$) as a dashed line.

Figure 6. Linkage of low HDLc levels in BcA68 identifies a novel mutation in the *ApoA1* gene
 A) Low density linkage mapping in the [BcA68xAKR]F2 intercross revealed a single peak with LOD of 24.25 on chromosome 9 at D9Mit130 (47.4 Mb).
 B) HDLc vs. genotype at D9Mit130 shows that mice with the KK genotype had normal levels of HDLc, while those with the BB (BcA68) genotype all had extremely reduced levels. C) Proximity to the *ApoA1*/*CIII*/*AIV*/*AV* gene cluster prompted the sequencing of *ApoA1*, which revealed the presence of a 66-67GC>TT mutation. D) Western blot analysis shows that no *ApoA1* protein is detectable in BcA68 mice (below). A Coomassie-stained sample of full serum is shown as a loading control (above), where the absence of a band at 28kd is also evident. E) The 66-67GC>TT mutation in *ApoA1* (resulting in 22-23WQ>CStop) destroys a *MwoI* restriction site in the genomic DNA, which is used as a marker here. The effect of homozygosity or heterozygosity with respect to either wild type (+; *ApoA1*66-67GC) or mutated (-; *ApoA1*66-67TT) *ApoA1* alleles on plasma HDLc levels is shown.