Haplotype Analysis in Multiple Crosses to Identify a QTL Gene

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Identifying quantitative trait locus (QTL) genes is a challenging task. Herein, we report using a two-step process to identify *Apoa2* as the gene underlying *Hdlq5*, a QTL for plasma high-density lipoprotein cholesterol (HDL) levels on mouse chromosome 1. First, we performed a sequence analysis of the *Apoa2* coding region in 46 genetically diverse mouse strains and found five different APOA2 protein variants, which we named APOA2^a to APOA2^e. Second, we conducted a haplotype analysis of the strains in 21 crosses that have so far detected HDL QTLs; we found that *Hdlq5* was detected only in the nine crosses where one parent had the APOA2^b protein variant characterized by an Ala^{61}-to-Val^{61} substitution. We then found that strains with the APOA2^b variant had significantly higher ($P \leq 0.002$) plasma HDL levels than those with either the APOA2^a or the APOA2^c variant. These findings support *Apoa2* as the underlying *Hdlq5* gene and suggest the *Apoa2* polymorphisms responsible for the *Hdlq5* phenotype. Therefore, haplotype analysis in multiple crosses can be used to support a candidate QTL gene.

[Supplemental material is available online at www.genome.org. The following individuals kindly provided reagents, samples, or unpublished information as indicated in the paper: D. Puppione.]

Most common human diseases, such as atherosclerosis, diabetes, and obesity, are complex traits determined by many genetic and environmental factors. The genetic factors are usually studied in animal models, most commonly mice, and frequently through a process known as quantitative trait locus (QTL) analysis, which has the advantage of finding novel key genes in a metabolic pathway. To date, more than 1800 mouse QTLs have been found (Mouse Genome Informatics, http://www.informatics.jax.org); however, identifying the genes underlying these QTLs has been an extremely challenging task (Nadeau and Frankel 2000; Korstanje and Paigen 2002).

The level of plasma high-density lipoprotein cholesterol (HDL), although not a disease, is also a complex trait. It has been intensely studied because, in humans, it is inversely correlated with the risks of coronary artery disease, and therapies that raise HDL levels may significantly reduce these risks (Boden and Pearson 2000). QTL analysis has identified many genomic regions that regulate HDL levels, both in mice and in humans—to date, 37 mouse and 29 human HDL QTLs have been identified (update of review by Wang and Paigen 2002). One of the mouse HDL QTLs, *Hdlq5* (Wang et al. 2003), on distal chromosome 1 (cM 92), has been repeatedly identified in nine of those crosses (update of review by Wang and Paigen 2002). An obvious candidate for the *Hdlq5* gene was *Apoa2* (cM 92.6), because its encoded protein, apolipoprotein A-II (APOA2), helps maintain plasma HDL levels in mice (for review, see Blanco-Vaca et al. 2001).

To determine whether *Apoa2* was the *Hdlq5* gene, we took the approach of haplotype analysis. Recent single nucleotide polymorphism (SNP) maps indicate that the genome of common inbred mouse strains is defined by 1–2 Mb haplotype blocks (Wade et al. 2002; Wiltshire et al. 2003), which can be used to narrow a QTL, because its underlying gene should be in subregions where the parental strains have different haplotypes (Park et al. 2003; Manenti et al. 2004). We extended this haplotype analysis further by using it to identify a QTL gene. In doing so, we performed a sequence analysis of the coding region of *Apoa2* in 46 genetically diverse mouse strains, and a haplotype analysis of strains both in the nine crosses that detected *Hdlq5* and in the 12 crosses that failed to detect *Hdlq5*. We found that not only did the haplotype analysis support *Apoa2* as the *Hdlq5* gene, but it also suggested that the *Apoa2* mutation is responsible for the *Hdlq5* phenotype. Thus, haplotype analysis in multiple crosses can be used to support a QTL gene.

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RESULTS

Inbred Mouse Strains Have Five APOA2 Protein Types

Polymorphisms of mouse *Apoa2* coding sequence have been reported before; a total of 15 SNPs cause eight amino acid changes in APOA2 (Doolittle et al. 1990; Higuchi et al. 1991; Purcell-Huynh et al. 1995; Suto et al. 1999; Kitagawa et al. 2003). For our purpose of testing *Apoa2* as the candidate gene for *Hdlq5*, we sequenced all four *Apoa2* exons in 46 genetically diverse and widely used inbred mouse strains, including 43 whose plasma HDL concentrations were known (Mouse Phenome Database, http://www.jax.org/phenome) and three that were parents in crosses to map HDL QTLs (CASA, MRL, and NZO). We found that the four exons of *Apoa2* had a total of 16 SNPs, resulting in nine amino acid changes and producing five APOA2 protein variants, which we named type "a" (APOA2^a) to "e" (APOA2^e; see Table 1 and Supplemental Fig. 1). The "b" strains were distinct with regard to two features: Their APOA2 sequence had an Ala^{61}-to-Val^{61} substitution, and none of them were wild-derived strains (all were inbred laboratory mice). The other APOA2 types each had at least one wild-derived strain: "a" type contains *M. m. castaneus*, *M. m. musculus*, and *M. m. molossinus* (a mixture of the previous two); "c" type contains *M. m. musculus*; "d" type contains *M. m. domesticus*; and "e" type contains *M. spretus*.

Ala^{61} Is Conserved in Eight Mammalian Species

By comparing the APOA2 peptide sequences of eight mammal species, we found that APOA2 Ala^{61} was conserved in mice (except those having APOA2^b), humans, chimpanzees, monkeys, horses, cattle, pigs, and rats (Fig. 1), suggesting that Val^{61} is a mutation in mice with APOA2^b.

Table 1. Inbred Mouse Strains Can Be Separated into Five Groups According to Their APOA2 Amino Acid Sequences

APOA2	AA (28–32–39–43–49–61–77–85–99)	n	Strains
a	P S Q D M A R N P	25	AKR, BTBR, BUB, C57BL/6, C57BL/10, C57BLKS, C57BR, C57L, C58, CASA,[1] CAST,[1] CE, CZECHII,[2] DBA/1, DBA/2, I, JF1,[3] MA, MOLF,[3] MSM,[3] NOD, PL, RIII, SEA, SWR
b	P S Q E V V R N P	13	129, BALB/cJ, BALB/cByJ, C3H, CBA, FVB, KK, LP, MRL, NON, NZB, NZW, RF
c	Q S Q E V A R N P	5	A, NZO, PWK,[2] SJL, SM
d	Q S Q E V A K N P	2	PERA,[4] WSB[4]
e	Q N H E V A K K L	1	SPRET[5]

(AA) Amino acid.
[1]*M. m. castaneus*; [2]*M. m. musculus*; [3]*M. m. molossinus* (a mixture of *M. m. castaneus* and *M. m. musculus*); [4]*M. m. domesticus*; [5]*M. spretus.* [1–5]All are wild-derived inbred mice, and all the others are inbred laboratory mice, each being a mosaic of *M. m. domesticus* and either *M. m. musculus* or *M. m. castaneus* (Wade et al. 2002).

Hdlq5 Was Only Detected in Crosses Whose Parental Strains Differed at APOA2 Amino Acid 61

Of the 21 different mouse crosses used to identify HDL QTLs, *Hdlq5* was found only in the nine where one parent carried APOA2^b and the second parent carried other APOA2 types (Table 2). Because mice with APOA2^b have a unique Val^{61}, our results clearly showed that *Hdlq5* was found only when the two parental strains differed at amino acid 61, that is, one should carry Ala^{61} and the other should carry Val^{61}. *Hdlq5* may not be detected even though the two parental strains have different APOA2 types; for example, *Hdlq5* was not found when C57BL/6 (having APOA2^a) and SPRET (having APOA2^e) were crossed (Table 2).

Haplotype analysis of the strains carrying APOA2^b excluded the possibility that an unknown gene in linkage disequilibrium with *Apoa2* was underlying *Hdlq5* in these nine crosses: The APOA2^b strains do not share a common haplotype block near *Apoa2* (Table 3). The nearby SNPs reduced the region the strains shared to one containing only two genes, *Apoa2* and *Fcer1g* (Fc receptor, IgE, high affinity I, γ polypeptide). There is no evidence that *Fcer1g* is involved in lipoprotein metabolism.

Mice with APOA2^b Had Higher Plasma HDL Concentrations Than Did Those With Either APOA2^a or APOA2^c

To determine whether any of the APOA2 variants were associated with variant plasma HDL levels, we analyzed the plasma HDL concentrations of the 43 inbred mouse strains from the Mouse Phenome Database. In male mice, HDL levels of strains having APOA2^b (103 ± 6 mg/dL, mean ± SEM, 12 strains) were significantly higher than those of strains having either APOA2^a (67 ± 3 mg/dL, $n = 24$) or APOA2^c (62 ± 4 mg/dL, $n = 4$) ($P = 9 \times 10^{-7}$ and $P = 0.002$, respectively; Fig. 2A). Similar results were found in female mice: HDL levels of strains having APOA2^b (81 ± 8 mg/dL, n = 12) were significantly higher than those of strains having either APOA2^a (52 ± 2 mg/dL, n = 24) or APOA2^c (46 ± 2 mg/dL, n = 4) ($P = 4 \times 10^{-5}$ and $P = 0.02$, respectively; Fig. 2B).

The distinguishing feature of the APOA2^b is the presence of the amino acid substitution of alanine to valine at position 61. HDL levels of strains having Val^{61} were significantly higher than those having Ala^{61} in both male and female mice (103 ± 6 vs. 69 ± 3 mg/dL, $P = 3 \times 10^{-6}$, and 81 ± 8 vs. 53 ± 3 mg/dL, $P = 6 \times 10^{-5}$, respectively; Fig. 2C,D).

DISCUSSION

Identifying the gene underlying a QTL is a challenging task. Whereas considerable success has been achieved in identifying genes responsible for Mendelian traits—more than 1400 genes for them have been found (Page et al. 2003), fewer than 50 have been identified for polygenic or quantitative traits (Glazier et al. 2002; Korstanje and Paigen 2002).

Although there is no "gold standard" for positively identifying a QTL gene, the Complex Trait Consortium recently suggested that an identified gene should meet more than one of the following eight criteria (Biola et al. 2003): (1) polymorphisms in either its coding or regulatory regions have been found; its function has been (2) linked to the quantitative trait being analyzed,

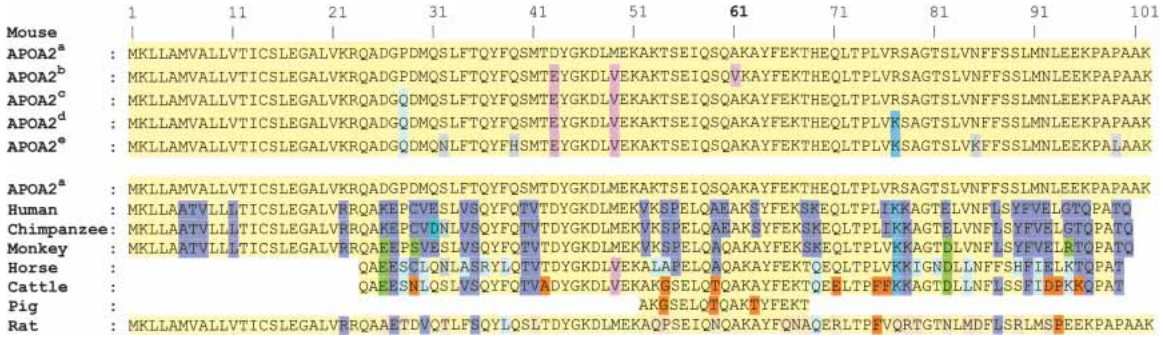

Figure 1 APOA2 protein sequence comparison in eight mammal species. Amino acids that are identical to those in mice having APOA2^a, APOA2^b, APOA2^c, APOA2^d, and APOA2^e are highlighted yellow, rose, light green, pale blue, and gray, respectively; those that are identical to APOA2 from human (lavender), chimpanzee (sky blue), monkey (green), horse (light turquoise), cattle (orange), and rat (tan) are also highlighted. Sources, with GenBank accession no. or reference: Human (*Homo sapiens*), NP_037244; Chimpanzee (*Pan troglodyte*), AAM49808; Cynomolgus monkey (*Macaca fascicularis*), P18656; Horse (*Equus caballus*), D. Puppione (pers. comm.); Cattle (*Bos taurus*), P81644; Pig (*Sus scrofa*), CAD91908; Rat (*Rattus norvegicus*), NP_037244.

Table 2. *Hdlq5* Was Only Detected When the Two Parental Strains in a Cross Differed at AA[61] of APOA2

Cross	APOA2 AA	Ala[61] × Val[61]		Ala[61] × Ala[61]			Val[61] × Val[61]
	APOA2 type	a × b	b × c	a × a	a × e	c × c	b × b
Hdlq5 detected		Yes	Yes	No	No	No	No
Examples		B6 × 129,[1] B6 × C3H,[2] B6 × FVB,[3] B6 × KK,[4] B6 × NZB,[5] CAST × 129,[6] RIIIS × 129[7]	NZB × SM,[8] SJL × 129[9]	AKR × DBA/2,[10] B6 × CASA,[11] B6 × CAST,[12] B6 × DBA,[13] CAST × DBA,[14] MOLF × B6[15]	B6 × SPRET[16]	SJL × NZO,[17] SM × A[18]	BALB × KK,[19] KK × RR,[20] MRL/*pr* × BALB[21]

(AA) Amino acid. The strains with the "b" allele (Val[61]) are underlined.
[1]Ishimori et al. 2004; [2]Machleder et al. 1997; Mehrabian et al. 1993; [3]Dansky et al. 1999; [4]Suto et al. 1999; [5]Wang et al. 2003; [6]Lyons et al. 2004b; [7]Lyons et al. 2004a; Korstanje et al. 2004; Mehrabian et al. 1993; Purcell-Huynh et al. 1995; [9]Schwarz et al. 2001; [10]Schwarz et al.; [11]Sehayek et al. 2003; [12]Mehrabian et al. 2000; [13]Colinayo et al. 2003; [14]Lyons et al. 2003; [15]Welch et al. 2001; [16]Warden et al. 1993; [17]Giesen et al. 2003; [18]Anunciado et al. 2000; [19]Shike et al. 2001; [20]Suto and Sekikawa 2003; [21]Gu et al. 1999.

Table 3. Haplotype Analysis of *Apoa2* Region in Mice with APOA2[b]

Marker[1]	Gene[2]	Start (bp)[3]	End (bp)[3]	Strains with APOA2[b]										
				129	BALB	C3H	CBA	FVB	KK	LP	NON	NZB	NZW	RF
Wrn3		175048841	175048962	2	2	2	1	2	3	3	2	2	1	1
	Nr1i3	175071723	175076568											
Wrn1		175081943	175082139	2	1	2	2	2	2	1	1	1	1	1
	Apoa2	175082812	175084093											
	Fcer1g	175087300	175092009											
Wrs1		175092624	175092806	2	2	1	1	1	1	2	1	1	1	2
	Ndufs2	175092583	175104835											
Wrs3[1]		175106847	175107095	3	3	3	3	2	1	3	1	2	1	3

[1]Microsatellite markers that we designed. We used PCR to amplify them. PCR products were scored according their sizes, with 1 < 2 < 3 in size. The sequences of the PCR primers for these markers are: *Wrn3:* forward: 5′-CCCAAAGGATTTACACATGC-3′, reverse: 5′-TACATACACCTGCCACATGC-3′; *Wrn1:* forward: 5′-TGCAGCATTTTCTCTGTGTG-3′, reverse: 5′-AAGGAATGGGGGTTATGAAG-3′; *Wrs1:* forward: 5′-CTAGTTCATGCACAGAAAGCC-3′, reverse: 5′-CCAGGATATTGTGTTCGGAG-3′; *Wrs3:* forward: 5′-AGTTCCCTCCTCTAACACCC-3′, reverse: 5′-CACCCAGAAGTCATCTCTGC-3′.
[2]Genes that are closest to *Apoa2,* according to Ensembl Mouse Genome Server (http://www.ensembl.org/Mus_musculus/), Build 32.
[3]Positions were retrieved from Ensembl Mouse Genome Server (http://www.ensembl.org/Mus_musculus), Build 32. The distance from the neighboring genes to *Apoa2: Nr1i3:* 6244 bp; *Fcer1g:* 3207 bp; *Nudfs2:* 8490 bp.

(3) tested in vitro, (4) tested in transgenic animals, (5) tested in knock-in animals, (6) assessed in deficiency-complementation tests, or (7) tested by mutational analysis; and (8) it has an homologous QTL for the same phenotype in another species. The most conclusive evidence comes from knock-in studies by replacing one allele with another and testing for function. We herein report using haplotype analysis in multiple crosses as a way of testing polymorphisms (criterion 1) to provide genetic evidence that a gene underlies a QTL.

By using this genetic approach, we provided two strong lines of evidence that *Apoa2* underlies *Hdlq5*. First, haplotypes of *Apoa2* were associated with plasma HDL concentrations: Mice with APOA2[b] had significantly higher plasma HDL levels than those with either APOA2[a] or APOA2[c]. Second, among the 21 crosses used to map HDL QTL, *Hdlq5* was only detected in the nine where the parental strains had different amino acid at position 61 in APOA2 protein. We excluded the possibility that a gene in linkage disequilibrium with *Apoa2* was underlying *Hdlq5* because *Fcer1g*, the only gene that was in linkage disequilibrium with *Apoa2*, has no known functions in lipoprotein metabolism.

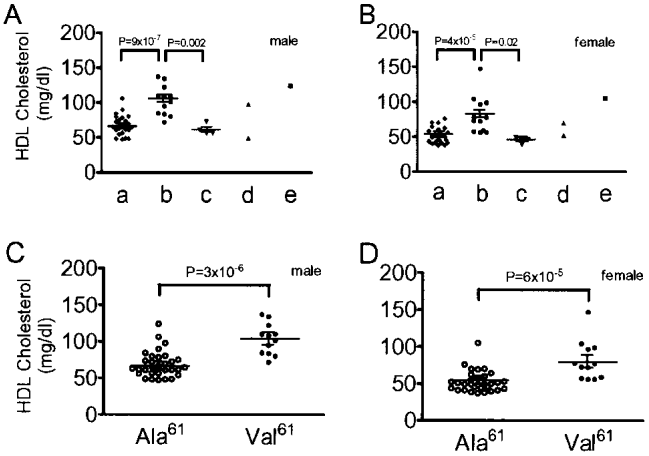

Figure 2 HDL concentrations in inbred mouse strains with five types of APOA2. The 43 strains were separated into five types (24 "a," 12 "b," 4 "c," 2 "d," and 1 "e") according to amino acids located at nine different APOA2 positions (see Table 1). Plasma HDL concentrations (mean ± SEM, mg/dL) were obtained from groups of 7- to 10-week-old males (*A*) and females (*B*) fasted for 4 h (all the strains in Table 1 except CASA, MRL, and NZO; Mouse Phenome Database). Each group consisted of between 10 and 40 mice (24 ± 7 males and 22 ± 6 females, mean ± SD). Plasma concentrations of HDL in male (*C*) and female (*D*) mice with Ala[61] (all the strains except APOA2[b] mice) and those with Val[61] (APOA2[b] mice) were also compared.

Haplotype analysis enabled us not only to identify *Apoa2* as the gene for *Hdlq5* but also to pinpoint the Ala[61]-to-Val[61] substitution as the possible causal change for this QTL. First, mice with Val[61] had significantly higher HDL levels than did those with Ala[61]. Second, *Hdlq5* was only found in the crosses in which one parental strain had Val[61] and the other had Ala[61] in APOA2. Third, in the nine crosses that detected *Hdlq5*, the strain with Val[61] always had the high allele of this QTL. This method may not be limited to finding a QTL gene with amino acid differences among the parental strains; it may also be used to find the key SNPs in the regulatory elements of a candidate gene.

Although *Apoa2* is mostly likely the gene underlying *Hdlq5*, and Ala[61]-to-Val[61] substitution in APOA2 is most probably the causal polymorphism, other possibilities exist. First, polymorphisms in the promoter region of *Apoa2* may lead to mRNA difference and thereby difference in plasma HDL levels. The following results, however, suggest that there is no correlation between *Apoa2* mRNA and plasma HDL levels and that *Apoa2* promoter polymorphisms are unlikely to affect plasma HDL levels. In the B6xNZB cross in which *Hdlq5* was found, B6 mice (having APOA2[a]) have higher levels of *Apoa2* mRNA but lower levels of plasma HDL levels, compared with NZB mice (having APOA2[b]) ($P < 0.05$) (Wang et al. 2003). In the B6xC3H cross in which *Hdlq5* was found, B6 mice had lower levels of both *Apoa2* mRNA and HDL levels, compared with C3H mice (having APOA2[b]; $P < 0.05$; X. Wang and B. Paigen, unpubl.). On the other hand, B6 mice have similar levels of *Apoa2* mRNA but lower levels of plasma HDL levels, compared with BALB/c mice (Doolittle et al. 1990). Second, a polymorphic enhancer in the same haplotype as *Apoa2* coding sequence may regulate another gene, and this gene could regulate HDL levels. Although unlikely, we cannot exclude this possibility.

It has been reported that compared with C57BL/6 mice (having APOA2[a]), BALB/c mice (having APOA2[b]) have similar liver *Apoa2* mRNA levels, but higher APOA2 protein synthesis rate in hepatocytes (Doolittle et al. 1990). This suggests that a change in messenger sequence, presumably the nucleotide change that causes Ala[61]-to-Val[61] substitution, increases the efficiency of *Apoa2* messenger translation, leading to more rapid

production of APOA2 protein and therefore larger HDL particle and higher plasma HDL concentrations. We found no sequence changes in the 3′- or 5′-UTR regions between C57BL/6 and BALB/c mice (Supplemental Fig. 1).

Our findings are important for future HDL research for two reasons. First, to avoid *Hdlq5*, whose strength may mask other HDL QTLs (and hence their discovery), future HDL QTL mapping efforts in mice should cross parental strains with the same amino acid 61 in APOA2. Second, a human QTL for plasma HDL levels was identified in the *APOA2* region (Elbein and Hasstedt 2002). To determine whether *APOA2* regulates human HDL concentrations, APOA2 SNPs should be analyzed in human populations.

Obviously, the analysis we conducted will be more powerful when the same QTL is detected in multiple crosses. Now that about 1500 QTLs, many of which were detected in multiple mouse crosses, have been mapped in the mouse genome (http://informatics.jax.org) and a wide variety of phenotypic data from many commonly used and genetically diverse mouse strains are easily accessible in the Mouse Phenome Database (hppt://www.jax.org/phenome), many QTL genes can be discovered quickly by analyzing their SNPs and haplotypes in multiple crosses, as exemplified in this study.

METHODS

Obtaining and Genotyping Genomic DNA

We obtained genomic DNA from Mouse DNA Resource at the Jackson Laboratory (http://www.jax.org/dnares/index.html). PCR genotyping of the four microsatellite markers (*Wrn3*, *Wrn1*, *Wrs1*, and *Wrs3*) in the *Apoa2* region was carried out for 35 cycles under the following conditions: 94°C, 30 sec; 55°C, 30 sec; 72°C, 1 min. Polymorphisms were detected by electrophoresing the PCR products on 4% Nusieve 3:1 agarose gels in 1× Tris-borate–EDTA running buffer for 2 h at 190 volts. Gels were then stained with ethidium bromide and photographed under ultraviolet light.

Sequencing *Apoa2*

To define *Apoa2* haplotypes, we sequenced the four exons of mouse *Apoa2*. We amplified each of the four exons with PCR with the same protocol as shown above except using an annealing temperature of 59°C instead of 55°C, and checked the product sizes on 4% Nusieve 3:1 agarose gels. The PCR products were sequenced using Big Dye Terminator Cycle Sequencing Chemistry and the ABI 3700 Sequence Detection System.

Retrieving Data

We retrieved plasma HDL concentrations of the 43 inbred mouse strains from Mouse Phenome Database. Mice had been fed LabDiet 5K52 (6% fat) and were fasted for four hours before their blood was sampled. After precipitating non-HDL cholesterol with polyethylene glycol (20% PEG 8000 in 0.2 M glycine), plasma HDL concentrations were measured with Beckman Coulter Synchron CX5 chemistry analyzer.

APOA2 protein sequences of human, chimpanzee, monkey, cattle, pig, and rat were obtained from GenBank, and D. Puppione (UCLA, Los Angeles, CA) kindly provided horse APOA2 sequence.

Analyzing Statistical Difference

Student's *t*-test was used to compare the plasma HDL concentrations among the different groups.

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