Phenotypic characterization of the *Ath-1* gene controlling high density lipoprotein levels and susceptibility to atherosclerosis

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Abstract The *Ath-1* gene determines the levels of high density lipoprotein (HDL) lipid in response to a high fat diet challenge as well as susceptibility to diet-induced atherosclerosis in mice (Paigen et al. 1987. *Proc. Natl. Acad. Sci. USA.* **84:** 3763–3767). As yet, the identity of the *Ath-1* gene and how it acts to affect HDL levels are completely unknown. In an effort to clarify the nature of the gene, we have examined HDL phenotypes in strains carrying either the susceptible or resistant alleles. When challenged with a high fat diet, the susceptible strain C57BL/6 exhibited a marked decrease in the levels of HDL cholesterol and apolipoprotein A-I (apoA-I), the major protein of HDL, whereas the resistant strains C3H and BALB/c maintained high levels of both. Separation of HDL subfractions by polyacrylamide gradient gel electrophoresis revealed that the decrease was particularly striking among the larger HDL species. The rates of synthesis of apoA-I in liver and intestine were similar in the strains and were unaffected by the high fat diet. Although the rates of synthesis of apoA-II and the levels of apoA-II mRNA were decreased in response to the high fat diet, similar decreases were observed in both the susceptible and resistant strains. We conclude that the *Ath-1* gene results in a rapid decrease in both HDL lipid and HDL apolipoprotein levels in the susceptible strain in response to the high fat diet and that this is mediated primarily at the level of HDL catabolism. — **LeBoeuf, R. C., M. H. Doolittle, A. Montcalm, D. C. Martin, K. Reue, and A. J. Lusis.** Phenotypic characterization the *Ath-1* gene controlling high density lipoprotein levels and susceptibility to atherosclerosis. *J. Lipid Res.* 1990. **31:** 91–101.

Supplementary key words apoA-I • apoA-II • HDL subfraction • high fat diet

Epidemiological studies have revealed an inverse correlation between plasma high density lipoprotein (HDL) cholesterol levels and the incidence of heart disease (1–3). Precisely how HDL protects against heart disease is unknown, although it has been speculated that high HDL levels may facilitate the transport of cholesterol from peripheral tissues to liver, a process known as reverse cholesterol transport. To address the problem, and also to identify genetic factors contributing to HDL metabolism, we and others are developing a mouse animal model (4–6). Various inbred strains of mice exhibit large differences in susceptibility to diet-induced atherosclerosis (reviewed in ref. 6) as well as in HDL metabolism (7, 8). Preliminary studies revealed an association between the levels of HDL lipid and atherosclerosis among inbred strains of mice, and subsequently it was shown in two genetic crosses that the levels of HDL lipid in mice maintained on a high fat diet cosegregate with susceptibility to diet-induced atherosclerosis (4). These results led to the identification of a gene, designated *Ath-1*, that controls the levels of HDL as well as susceptibility to atherosclerosis (4, 9). This gene is located on mouse chromosome 1, in a linkage group that is conserved between mice and humans (10, 11). As yet, the identity of *Ath-1* and how it acts to affect HDL levels in response to a dietary challenge are completely unknown. We now report the results of biochemical studies involving the characterization of phenotypes associated with different *Ath-1* alleles.

EXPERIMENTAL PROCEDURES

Animals and diets

Mice were obtained from Jackson Laboratory, Bar Harbor, ME. The strains used were BALB/cJ (BALB), C3H/HeJ (C3H), and C57BL/6J (C57BL/6). Female mice, 2–4 months of age, were used. Normal chow was

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

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Purina chow containing 4% fat. The high fat diet was prepared by mixing the Thomas-Hartcroft diet purchased from Teklad Test Diets, Madison, WI, with Purina breeder chow (diet 5015) in a ratio of 1 part to 3 parts, respectively. The resulting diet contained, by weight, 15% cocoa butter, 1.25% cholesterol, and 0.5% cholic acid (12). The fatty acid composition has been reported earlier (12). All mice were fasted for 8–12 h prior to collection of blood and tissue.

Nondenaturing gradient polyacrylamide gel electrophoresis

Nondenaturing polyacrylamide gradient gel electrophoresis was performed essentially as described by Nichols, Krauss, and Musliner (13). Gels containing a 2–16% linear polyacrylamide gradient were electrophoresed in 90 mM Tris, 80 mM boric acid, and 3 mM EDTA, pH 8.35. Prior to electrophoresis, plasma samples (10 μl) were stained for lipid by incubation at 4°C overnight with 8 μl freshly prepared Sudan Black B dye solution (5 parts 1% Sudan Black B in ethylene glycol–3 parts 40% sucrose). Electrophoresis was carried out at 25 volts for 20 min, followed by 50 volts for 16 h.

Lipid and protein determinations

Plasma cholesterol concentrations were determined using a hexane extraction procedure (14) and triglycerides using a colorimetric micromethod (15). Proteins were determined by a modification (16) of the method of Lowry et al. (17). HDL cholesterol and VLDL + LDL cholesterol values were determined following the selective precipitation of VLDL + LDL by phosphotungstate (18). ApoA-I was not detected in selected precipitated fractions by immunoblot analysis.

Apolipoprotein quantitation

The levels of apoA-I and apoA-II were determined by a quantitative immunoblotting method. ApoE was quantitated on separate gels, also by immunoblotting. Plasma aliquots (0.5 μl) were subjected to SDS-polyacrylamide gel electrophoresis as described (19). After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose filters (0.2-μm pore size, Sartorius) using 150 mA for 15–20 h as described (20). Apolipoproteins were detected after incubation of filters with monospecific antisera followed by incubation with iodinated protein A. Antisera dilutions of 1:1000 (apoA-I and E) or 1:500 (apoA-II) were used. Increasing amounts of antisera in the incubation step did not result in increasing signal intensity. Signal intensities for apoA-II and E were linear for plasma volumes ranging from 0.3 to 2.5 μl, and for apoA-I, from 0.1 to 1.5 μl. Antisera prepared in rabbits to mouse apoA-I and rat apoE were as previously described (21). Monospecific antiserum to mouse apoA-II was prepared in rabbits using apoA-II that had been purified by gel filtration and ion exchange chromatography from mouse HDL (19). Apolipoprotein bands were visualized by autoradiography and quantitated by excision and counting of radioactivity in appropriate areas of the filters. Absolute concentrations of apolipoproteins were determined by comparison with standard curves of isolated mouse apoA-I, apoA-II, and apoE.

Messenger RNA quantitation

A mouse cDNA probe for apoA-II was isolated by screening a C57BL/6 mouse liver cDNA library with a rat apoA-II cDNA probe and its identity was confirmed by sequencing (M. Lucero and A. Lusis, unpublished results). A partial mouse apoA-I cDNA probe (22) was a gift from R. Elliot, Roswell Park Memorial Institute, Buffalo, NY. RNA was isolated from mouse livers and quantitated by Northern blotting as previously described (23, 24) using tubulin mRNA as an internal standard (24).

Rates of synthesis measurements

For most rates of synthesis measurements, whole livers were perfused for 15 min with [^{35}S]methionine (ICN) as described previously (25). Briefly, following laparotomy, the portal vein was cannulated and the liver was excised; after a 5–10-min perfusion with gassed O_2–CO_2 95:5 KRB-HEPES (Kreb's Ringer Bicarbonate supplemented with 10 mM HEPES) at 37°C, the pulse was initiated by adding isotope at a concentration of 250 μCi/ml. After 15 min, cycloheximide was added to the perfusion medium to a final concentration of 10 μg/ml, and the liver was perfused for an additional 5 min. The liver was then immediately homogenized in 10 vol (by liver weight) of lysis buffer (3% Triton X-100, 0.1% N-lauryl sarcosine, 1 mM phenyl methyl sulfonate fluoride, 0.15 M NaCl, 0.1 M Tris, pH 7.5). Nuclei and tissue debris were removed by centrifugation (12,000 *g*, 20 min) and the supernates were stored at −80°C until used for immunoprecipitation.

To establish linearity of [^{35}S]methionine incorporation (see Fig. 6A), freshly isolated hepatocytes were used (25). Briefly, the liver was first perfused with chelator (1 mM EGTA) followed by collagenase (0.05% of type V collagase, Sigma). The hepatocytes were washed 3 times by low-speed centrifugation (50 *g*, 2 min) to remove debris and most nonparenchymal cells (26). The hepatocytes were then incubated in a Dubnoff metabolic shaker at a concentration of about 1×10^7 cells/ml KRB-HEPES for 5 min before initiating the pulse (200 μCi/ml [^{35}S]methionine). After 1, 5, 10, 15, and 20 min, equal aliquots of 1×10^6 cells were quickly spun out (less than 10 sec) and cell pellets were immediately lysed with 0.5 ml of ice-cold lysis buffer. The lysates were treated as above.

For rates of synthesis measurements in jejunum and ileum, the entire small intestine was excised and the lumen was rinsed with ice-cold normal saline to remove intestinal contents. Two 1-cm longitudinal rings were removed from the mid-jejunum and the region adjacent to the ileocecal junction. The rings were opened up into sheets by cutting longitudinally and pulsed for 15 min in KRB-HEPES as described above. The pulse was terminated by adding cycloheximide (10 μg/ml). The luminal enterocytes were lysed in hot (80°C) 1% SDS by pipetting the intestinal sheets several times through a 3-mm aperature. The unlysed portion of the intestine was removed, and the DNA released by SDS lysis of the enterocyte was sheared by repeated passage of the lysate through a 20-gauge needle. We found that lysis in the presence of hot SDS prevented the degradation of apoA-I seen after Triton X-100 homogenization, presumably by instantly denaturing lumenal proteases. Particulates were removed from the intestinal lysates by centrifugation and the supernates were stored as described above.

ApoA-I and apoA-II were immunoprecipitated from the lysates with rabbit antisera directed against the mouse apolipoproteins (see Fig. 6). A concentration of antiserum of 10 μl/mg liver protein was used throughout; this amount of antiserum immunoprecipitated quantitatively both apoA-I and A-II in liver (see Fig. 6B). For intestine, an antiserum concentration of 20 μl/mg protein was used. For both liver and intestine, we routinely immunoprecipitated the lysates a second time to assure quantitative removal of antigen. In all rates of synthesis measurements, the incorporation of [^{35}S]methionine into apoA-I and A-II used to calculated relative rates of synthesis was the summed value of the first and second immunoprecipitates. The first immunoprecipitates always accounted for 85–95% of the summed values. In Fig. 7A, cpm incorporated/g protein was expressed as pg [^{35}S]methionine incorporated/g protein using the specific activity of the radiolabeled methionine added (1000 Ci/mmol).

Immune complexes of apoA-I and A-II were allowed to form overnight at 4°C, and, generally, apoA-I and A-II were immunoprecipitated from 1 mg of liver protein and 2 mg intestinal protein. Ten μl of a 10% insoluble Protein A (lyophilized cell powder; Sigma) was used to quantitatively bind IgG from a 1 μl antiserum (27). The pellet was washed once in 0.5 M LiCl, 0.1 M Tris, pH 7.5, and twice in 0.15 M NaCl, 0.1% N-lauryl sarcosine, 0.1 M Tris, pH 7.5. The immune complexes were dissociated from the staph A pellet by boiling in 2% SDS, 2% β-mercaptoethanol, 25% glycerol, 0.1 M Tris, pH 6.8.

All immunoprecipitates were subjected to SDS-PAGE utilizing a 14% polyacrylamide gel and a cross-linkage concentration of acrylamide-bis-acrylamide 40:1. SDS-PAGE and fluorography were performed as described elsewhere (25). To quantitate counts incorporated into apoA-I or A-II, appropriate regions of the dried gel were excised using the fluorogram as a template. These gel slices were rehydrated in 1 ml 80% Protosol (New England Nuclear) and counted by liquid scintillation spectrometry after addition of 10 ml scintillant (Econofluor (NEN)–Protosol–glacial acetic acid 20:1:0.04).

Total protein synthesis was estimated by determining the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material. Five μl of pulse-labeled tissue lysate was added to 1 ml 0.01% BSA. After the addition of 1 ml 20% trichloracetic acid, the precipitate was allowed to form for 20 min at room temp. The precipitates were collected on GF-C glass fiber filters (Whatman), washed with several ml of 10% trichloroacetic acid, 5 ml absolute ether, and then air dried. The precipitates were dissolved in 1 ml 80% Protosol and counted as described above for the gel slices.

RESULTS

HDL lipid

Our previous genetic studies utilized crosses between the susceptible strain C57BL/6 and the resistant strains BALB and C3H (4). In these studies, HDL levels were estimated by the intensity of lipid straining of plasma lipoproteins after agarose gel electrophoresis. Levels were found to cosegregate with susceptibility to diet-induced atherosclerosis in two sets of recombinant inbred strains. Susceptible recombinant inbred strains, which developed numerous lipid-containing lesions in the aorta, showed about 50% reduction in HDL lipid staining in mice on the high fat diet, in common with the C57BL/6 parental strain. Resistant recombinant inbred strains, which developed few if any lesions, showed little or no reduction in HDL levels on the high fat diet, in common with the BALB or C3H parental strains. Thus, both phenotypes cosegregated as a single major Mendelian gene, a finding that has now been confirmed by Paigen and coworkers (9) using additional backcrosses. This gene, designated *Ath-1*, was found to map near the structural locus for apoA-II (designated *Apoa2*) on mouse chromosome 1, but studies with congenic and recombinant inbred strains indicate that it is distinct from the *Apoa2* gene (4).

As shown previously (4), when maintained on a high fat diet, the susceptible strain C57BL/6 exhibited substantially lower levels of HDL lipid, as judged by Sudan Black staining, than the resistant strains C3H or BALB. This results from both a lower basal HDL lipid level (in mice maintained on chow) as well as a substantial decrease in the levels of HDL lipid when animals were placed on the high fat diet (**Fig 1**). In contrast to C57BL/6, the resistant strains BALB and C3H did not exhibit a significant change in HDL lipid when placed on

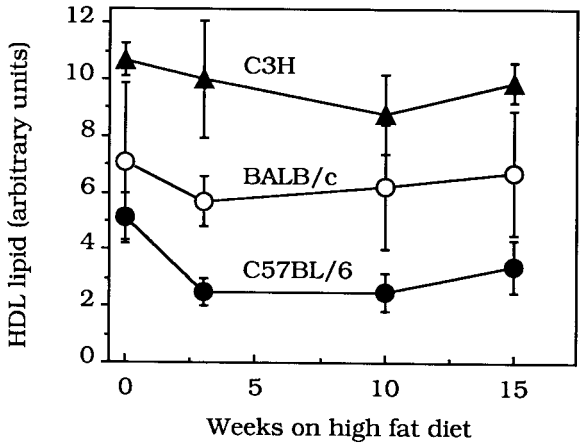

Fig. 1. Genetic control of HDL lipid levels in response to a high fat diet challenge. Mice were maintained on a high fat diet as described in Methods. HDL lipid concentrations in plasma were determined by lipid staining and agarose gel electrophoresis of lipoproteins as described previously (4). Relative intensities of the alpha-migrating band were determined by quantitative scanning densitometry (23). Each point represents the mean value (expressed in arbitrary units) ± the standard error using 4 to 6 animals of each strain per time point. Values for C57BL/6 at 3 and 10 weeks were significantly different than at 0 weeks ($P = 0.05$).

the high fat diet (Fig. 1). The response of HDL lipid to the high fat diet challenge in strain C57BL/6 was relatively rapid, as plateau levels were achieved by 3 weeks (Fig. 1). It is noteworthy that the levels of HDL lipid of strain C3H are higher, on both the chow and high fat diets, than those of strain BALB. Although the difference in HDL lipid levels between C3H and BALB mice in the experiment shown in Fig. 1 reached statistical significance ($P<0.05$) only at the 3-week point of the high fat diet, we have consistently observed the difference in several studies (data not shown). Previous studies have shown that C3H mice develop fewer and smaller lesions than BALB mice (6), and it is interesting to speculate that this may be due to the higher levels of HDL in C3H mice. As previously noted (23), there was no obvious association between the levels of total plasma cholesterol or of LDL + VLDL lipid and resistance to atherosclerosis (**Fig. 2**). In contrast to the response of HDL lipid, the levels of total plasma cholesterol continued to increase during the 15-week time course of the experiment (**Table 1**).

Studies involving the isolation of lipoprotein fractions followed by cholesterol determinations yielded results that were similar to, but less marked than, those obtained by staining for total lipid (Fig. 2). The level of HDL cholesterol in strain C57BL/6 decreased about twofold (from 64.7 to 30.5 mg/dl) following the high fat diet challenge ($P<0.0001$), while that of strain BALB was not significantly affected ($P=0.5$) and that of strain C3H showed a small decrease ($P=0.02$). On the chow diet, strain C3H had a significantly higher HDL cholesterol than either strains C57BL/6 or BALB ($P<0.01$), whereas on the high fat diet strain C57BL/6 had a significantly lower HDL cholesterol than either strains C3H or BALB ($P<0.0001$), and strains C3H and BALB did not differ significantly ($P=0.01$).

HDL size heterogeneity

To ascertain whether *Ath-1* affects the size distribution of HDL, nondenaturing polyacrylamide gradient gel electrophoresis was used (13). Plasma samples from BALB, C3H, and C57BL/6 mice maintained on chow or high fat diets for 3 weeks were prestained with Sudan Black and electrophoresed for 24 h through 2–16% nondenaturing gels. In contrast to the single broad HDL band observed after agarose gel electrophoresis, three distinct HDL size classes, referred to from largest to smallest as M-HDL_1, M-HDL_2, and M-HDL_3, were seen on gradient gels. Typical patterns are shown in **Fig. 3A**. All three forms were immunoblotted and shown to contain both apolipoproteins A-I and A-II (data not shown). The predominant form observed in all three strains was M-HDL_3, whereas M-HDL_1 was present at very low levels.

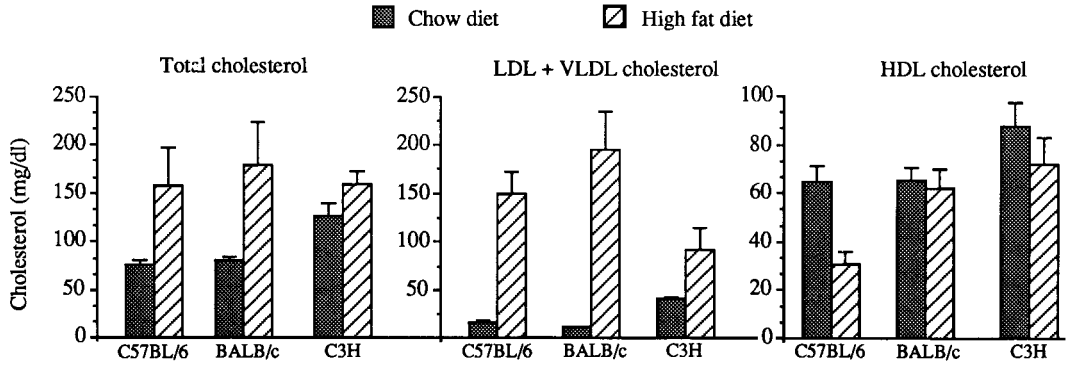

Fig. 2. Genetic control of HDL cholesterol concentrations in response to a high fat diet challenge. Plasma lipoproteins were isolated from mice maintained on chow (solid bars) or high fat diets (diagonal bars) for 10 weeks; cholesterol was determined as described under Methods. Each experiment is the mean value (± standard error) obtained for 4 to 6 animals.

TABLE 1. Plasma cholesterol concentrations as a function of time on the atherogenic diet

Strain	Week[a]	Cholesterol Exp. 1[b]	Cholesterol Exp. 2
		mg/dl	
C57BL/6	0	69.2 (4.4)[c]	76.5 (2.0)
C57BL/6	3	145.8 (56.8)	155.2 (33.3)
C57BL/6	10	140.8 (32.7)	157.2 (19.6)
C57BL/6	15	176.1 (66.1)	190.7 (15.7)
BALB	0	73.0 (4.7)	80.1 (1.8)
BALB	3	110.6 (31.6)	117.3 (17.8)
BALB	10	168.7 (39.1)	179.4 (17.9)
BALB	15	255.5 (114)	274.1 (33.7)
C3H	0	112.6 (11.8)	128.7 (6.2)
C3H	3	118.7 (9.4)	126.1 (2.1)
C3H	10	142.6 (11.0)	159.3 (5.1)
C3H	15	153.2 (45.9)	163.2 (5.6)

[a]Zero week values are those of chow-fed animals, which exhibited no significant changes in lipoprotein concentrations with time.
[b]Experiments 1 and 2 were done in separate laboratories and at separate times.
[c]Numbers in parentheses are standard errors for four to six animals.

Densitometric gel scanning and integration of the peaks representing the two major species of HDL yielded the results shown in Fig. 3B. As observed with agarose gel electrophoresis, strain C57BL/6 showed an approximately twofold decrease in HDL lipid stain intensity in response to the high fat diet as compared to the intensity observed for chow-fed mice. In contrast, strains BALB and C3H showed little or no change in total lipid staining. Moreover, strain C3H was found to have significantly higher levels of HDL lipid staining than either strains BALB or C57BL/6 (Fig. 3). On the other hand, there were clear differences between the strains in the effects of the high fat diet on the different HDL species. In strain C57BL/6, the HDL_1 and HDL_2 species were entirely eliminated by the high fat diet challenge. Strain BALB, on the other hand, exhibited either no decrease or an increase in the levels of HDL_1 and HDL_2 in response to the high fat diet (Fig. 3). These results in mice are in apparent concordance with human studies, which suggest that the HDL_2 species are of primary importance in terms of risk of atherosclerosis. On the other hand, the resistant strain C3H showed a substantial decrease in M-HDL_2 in response to the high fat diet (Fig. 3). We conclude that mice do indeed exhibit discreet HDL subclasses and that the levels of these classes can be selectively altered by a high fat diet in a genetically determined fashion.

Levels of HDL apolipoproteins

The major apolipoproteins of mouse HDL are apoA-I and apoA-II. To examine the effects of *Ath-1* on these proteins, we purified both from mouse plasma (19) and raised monospecific antibodies to the proteins in rabbits (data not shown). **Fig. 4** shows a typical Western blot using these antibodies. Quantitative immunoassays for both proteins were developed using isolated, homogenous mouse apoA-I and apoA-II as protein standards (see Methods). As shown in **Fig. 5**, the levels of apoA-I in strain C57BL/6 decreased significantly (from 120 to 75 mg/dl; $P<0.01$) in response to the high fat diet challenge, while they were not significantly affected by diet in strains BALB and C3H (Fig. 5). The time course for the reduction of apoA-I was similar to that observed for HDL lipid.

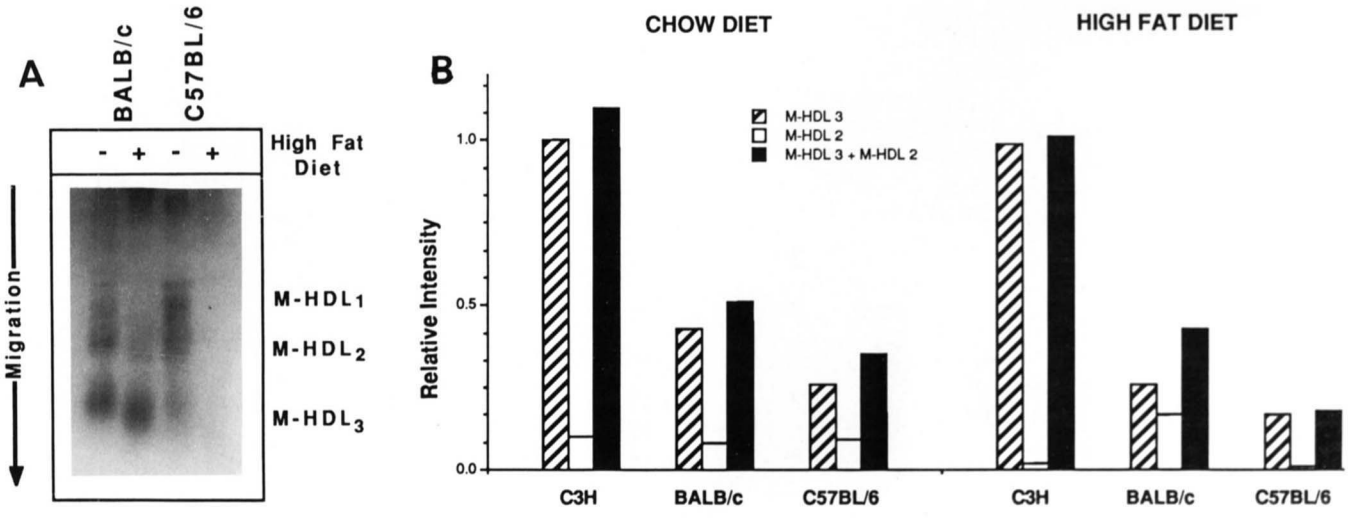

Fig. 3. HDL size heterogeneity and its response to a high fat diet. Mice were maintained on chow or high fat diets for 3 weeks. Ten μl of plasma was prestained for neutral lipids using Sudan Black (4) and electrophoresed to equilibrium in a 2–16% nondenaturing polyacrylamide gel. A. Representative migration pattern of HDL observed with BALB and C57BL/6 mice: (−) chow; (+) high fat diet. B. Integration of the M-HDL_2 and M-HDL_3 peaks in densitometic scans of gradient gels, which is an estimate of the neutral lipid comprising each HDL size class. These studies were performed in duplicate with very similar trends.

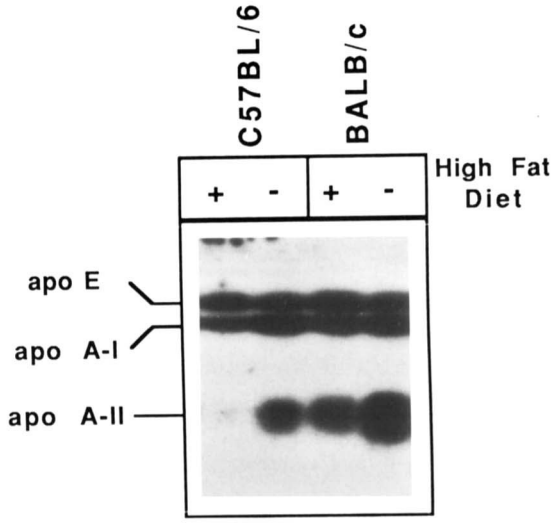

Fig. 4. Immunoblotting of apoA-I and apoA-II. Plasma was subjected to SDS-polyacrylamide gel electrophoresis and Western transfer was performed essentially as previously described (19, 20). The resulting filters were incubated with monospecific antibodies followed by iodinated protein A and then autoradiographed: (+) high fat diet challenge; (−) normal chow diet.

In C57BL/6 the levels of apoA-I reached plateau levels by 3 weeks, while in the resistant strains no changes were observed even after 15 weeks. Interestingly, the levels of apoA-I in mice on the chow diet were similar for all three strains, despite the difference in HDL cholesterol levels which were clearly higher in C3H than C57BL/6 mice (compare Figs. 2 and 5). As discussed below, this is likely to reflect HDL structural differences between the strains.

The strain C57BL/6 had considerably reduced levels of apoA-II as compared to strains BALB and C3H on the chow diet, with levels of 15.7, 31.7, and 34.7 mg/dl, respectively (Fig. 5). When challenged with the high fat diet, all three strains exhibited significant (about twofold; $P < 0.01$) decreases in apoA-II levels (Fig. 5). Thus, in contrast to apoA-I and HDL cholesterol, there was no indication of a specific effect of the *Ath-1* gene on apoA-II concentrations in response to a high fat diet challenge. The difference in basal apoA-II levels between the strains is also not controlled by the *Ath-1* gene. Rather, it appears to be due to a mutation of the apoA-II structural gene, since the levels of apoA-II segregate in genetic crosses with the apoA-II structural locus rather than with the *Ath-1* gene (M. Doolittle, R. LeBoeuf, and A. Lusis, unpublished results). This is clearly demonstrated in the congenic strain B6.C.*H-25*, which contains the BALB apoA-II structural gene on a C57BL/6 background. This strain resembles BALB in terms of apoA-II levels (about 30 mg/dl) but C57BL/6 in terms of both susceptibility to atherosclerosis and decreased HDL lipid in response to a high fat diet challenge (4).

ApoE is a relatively minor component of mouse HDL. Total apoE, which occurs primarily in very low density lipoprotein (VLDL) fractions in mice maintained on both chow and fat diets (19), did not differ significantly between the three strains (Fig. 5). There was a slight but significant increase in apoE levels in all three strains in response to the high fat diet.

Genetic and dietary control of HDL structure

While the above results indicate that there are major structural differences in the HDL between the resistant strain C57BL/6 and the susceptible strains BALB and C3H, these appear to be due largely to the effects of the apoA-II structural gene variation. Thus, the ratio of HDL cholesterol to apoA-I remained relatively constant upon challenge with the high fat diet (**Table 2**). Clearly, however, the structure of HDL, most notably the ratio of apoA-I to apoA-II, was significantly altered by the high fat diet in each of the strains examined (Table 2). In terms of atherosclerosis, the importance of these structural differences as compared to quantitative differences in HDL is unknown, although studies of genetic recombinants be-

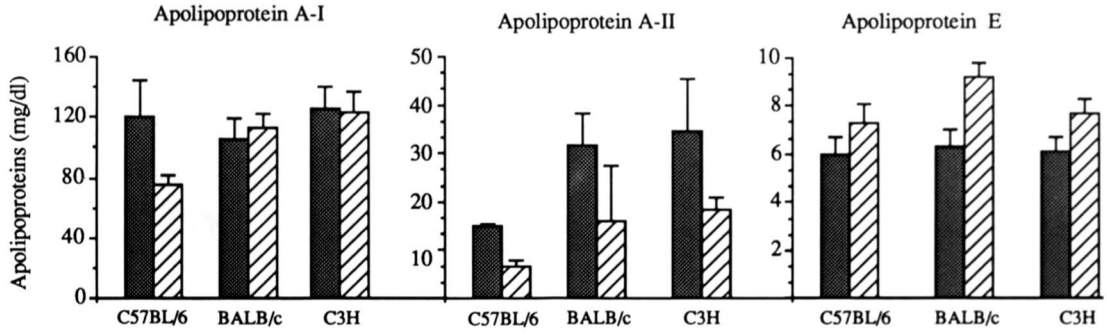

Fig. 5. Levels of apolipoproteins associated with HDL: Genetic and dietary regulation. The levels of apoA-I, apoA-II, and apoE in three strains of mice maintained on chow (solid bars) or a high fat diet (diagonal bars) for ten weeks are indicated. Values represent the mean values for 4 to 6 animals along with the standard errors. Apolipoprotein levels were determined by quantitative immunoblotting as described under Methods.

TABLE 2. Structural differences between HDL of strains C57BL/6, BALB and C3H

Strain	Diet	ApoA-I HDL-CHOL	ApoA-II HDL-CHOL	ApoA-I ApoA-II
C57BL/6	Chow	1.9	0.23	7.9
C57BL/6	High fat	2.5	0.21	11.6
BALB	Chow	1.7	0.49	3.4
BALB	High fat	1.8	0.26	7.0
C3H	Chow	1.4	0.41	3.6
C3H	High fat	1.7	0.25	6.8

Values are expressed as weight/weight ratio for mice maintained on a chow diet or, for 10 weeks, on a high fat diet.

tween *Ath-1* and *Apoa2* (discussed above) suggest that apoA-II levels are not of primary importance with respect to susceptibility. Finally, it should be noted that mouse plasma contains little, if any, free apoA-I or apoA-II (data not shown); thus, the differences in apolipoprotein to HDL cholesterol ratios discussed above cannot be attributed to the presence of free apolipoproteins under certain conditions.

Ath-1 does not control the synthesis of apoA-I

As discussed above, the most notable molecular effect of *Ath-1* is a reduction of both apoA-I and HDL cholesterol in the susceptible but not resistant strains upon high fat diet challenge. This could result from either decreased synthesis or from increased catabolism of HDL. To test this, we have examined the synthesis of the major apolipoproteins of HDL in different strains maintained on either chow or high fat diets.

Fig. 6A shows that the antiserum used for immunoblotting (Fig. 4) precipitates apoA-I and A-II monospecifically from BALB and C57BL/6 livers pulse-labeled for 15 min with [^{35}S]methionine. Intestinal ring segments from jejunum and ileum were also pulse-labeled and shown to synthesize apoA-I but not A-II; likewise, apoA-II mRNA was not detected in intestine (data not shown). An appropriate amount of antiserum was used to quantitatively immunoprecipitate all antigen present in liver and intestinal lysates; the concentration of 10–20 μl antiserum per mg tissue protein used was well within the linear portion of the titer curves for both apolipoproteins (Fig. 6B; see Experimental Procedures). **Fig. 7A** shows that [^{35}S]methion-

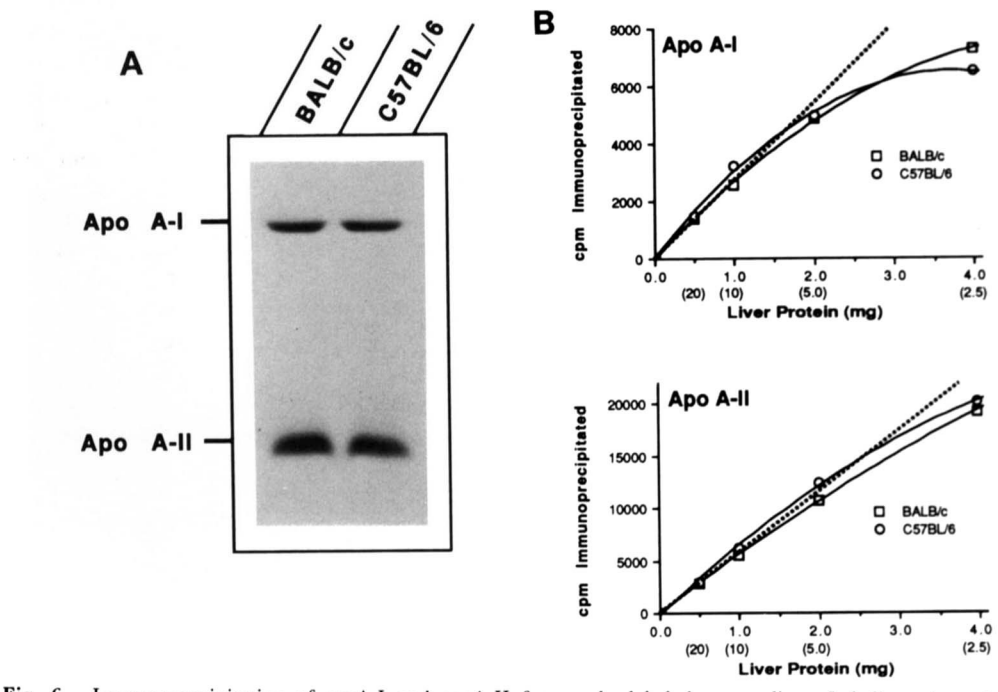

Fig. 6. Immunoprecipitation of apoA-I and apoA-II from pulse-labeled mouse liver. Labeling, tissue lysis, and immunoprecipitation of apoA-I and A-II are described in Methods. A. Immunoprecipitates of apoA-I and A-II, from BALB and C57BL/6 livers from mice maintained on normal chow diet, perfused for 15 min with [^{35}S]methionine and electrophoresed in a 14% SDS polyacrylamide gel. ApoA-I (mol wt 27,000) and A-II (mol wt 8,400) are indicated. A combination of two monospecific antisera for apoA-I and apoA-II was used at a volume to volume ratio of 1:3. B. ApoA-I and A-II were immunoprecipitated from increasing amounts of tissue lysate (indicated as liver protein on the abcissa) holding antiserum volume constant (10 μl). Numbers in parentheses below the abcissa represent μl antiserum per mg protein; values of 10–20 μl/mg were used throughout to determine rates of synthesis, well within the linear portion of the curves (dashed line). Each point represents the cpm immunoprecipitated as determined by excising the appropriate region of the SDS gel using the fluorogram as a template (see Methods).

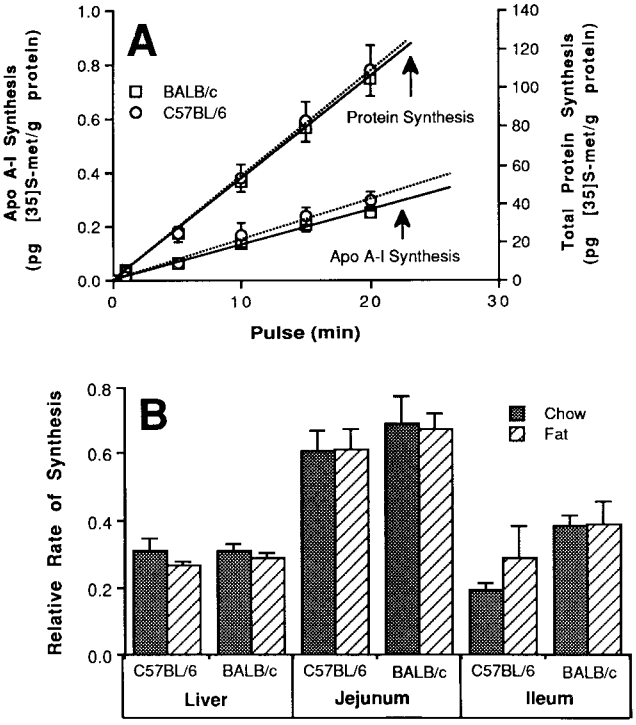

Fig. 7. Rate of apoA-I synthesis in liver and intestine. The determination of [^{35}S]methionine incorporation rates into total proteins and apoA-I is described under Methods and in the legend to Fig. 6 A. Linear incorporation rate of [^{35}S]methionine into total proteins and apoA-I. Isolated hepatocytes from chow-fed BALB and C57BL/6 mice were pulse-labeled as described in Methods. Each point represents the mean ± the standard error of eight (protein synthesis) or six (apoA-I synthesis) animals. B. Relative rates of synthesis of apoA-I determined as a percentage of the [^{35}S]methionine incorporated into total proteins. This precludes any effects on apoA-I synthesis due to altered methionine pool sizes. Each histogram represents the mean ± one standard error of 4 to 6 animals.

ine is incorporated linearly into both total proteins and apoA-I through at least 20 min of pulse time in BALB and C57BL/6 hepatocytes. Using a 15-min pulse time in liver, jejunum, and ileum of BALB and C57BL/6 mice maintained on chow or high fat diets for 3 weeks showed that relative rates of apoA-I synthesis were not influenced by diet (Fig. 7B). Quantitation of hepatic apoA-I mRNA also failed to reveal differences between the strains BALB, C3H, or C57BL/6 when maintained on either chow or high fat diets, supporting the above biosynthetic data (data not shown). Thus, the decreased levels of apoA-I, and presumably HDL, in strain C57BL/6 appear to be the result of increased catabolism. Attempts to examine this directly will be complicated by the complex and poorly understood mechanisms involved in HDL turnover.

Rates of synthesis of apoA-II

As mentioned previously, the difference in the basal level of apoA-II is controlled by the A-II structural gene locus and not *Ath-1*. However, as shown in Fig. 5, apoA-II

TABLE 3. ApoA-II synthesis in chow-fed and fat-fed mice

Strain	Diet	N[a]	Plasma Level	Relative Rate of Synthesis[b]
			mg/dl ± SE	*% of total synthesis*
BALB/c	Chow	4	46.7 ± 2.20	0.936 ± 0.055
BALB/c	High fat	5	30.5 ± 2.87[c]	0.661 ± 0.043[c]
C57BL/6	Chow	4	18.9 ± 1.50	0.589 ± 0.041
C57BL/6	High fat	5	11.8 ± 0.66[c]	0.500 ± 0.039

[a]N = number of animals. Animals used for determinations of plasma apoA-II plasma levels and relative rate of synthesis were identical.
[b]The relative rate of apoA-II synthesis was determined as described in the legend to Fig. 7 and under Methods. Relative rate of apoA-II synthesis in the strains has been corrected to take into account a difference in methionine content of the apoA-II; strain C57BL/6 contains 4 moles of methionine per mole apoA-II whereas strain BALB contains 3 moles of methionine per mole apoA-II (A. Lusis and M. Lucero, unpublished results).
[c]Significant decrease compared to chow diet at the $P < 0.005$ level.

plasma levels in both the susceptible and resistant strains decline about 50% in response to high fat feeding. This decrease may be due to decreased rates of apoA-II synthesis, particularly in the BALB strain (**Table 3**). Although the decrease in apoA-II synthesis in strain C57BL/6 did not reach statistical significance, a significant decrease in apoA-II mRNA levels was observed (see below). Synthesis measurements were not performed for strain C3H. The decrease in apoA-II synthesis could be accounted for by decreased levels of apoA-II mRNA. As shown in **Table 4**, the levels of apoA-II mRNA did not differ significantly among the three strains for a given diet, suggesting that the rates of synthesis differences result from differences in translational efficiency (M. H. Doolittle, R. C. LeBoeuf, and A. J. Lusis, unpublished results). However, the high

TABLE 4. High fat diet results in decreased hepatic apoA-II messenger RNA levels

Strain	Diet	N[a]	Hepatic ApoA-II mRNA Levels[b] (arbitrary units)
C3H	Chow	4	1.59 ± 0.13
C3H	High fat	4	1.18 ± 0.07[c]
BALB/c	Chow	5	1.63 ± 0.07
BALB/c	High fat	5	1.06 ± 0.05[c]
C57BL/6	Chow	5	1.72 ± 0.04
C57BL/6	High fat	4	1.19 ± 0.12[c]

The levels of hepatic apoA-II mRNA were determined by densitometric scanning of Northern blots using mRNA from mice maintained on chow or high fat diets (for 3 weeks). Values are expressed in arbitrary densitometric units. In these studies, we also examined levels of α-tubulin (24) mRNA, which did not differ between strains or vary with diet. Relationships for apoA-II mRNA levels remained similar if normalized against α-tubulin mRNA (data not shown).
[a]N = number of animals used.
[b]Numbers represent the mean values ± the standard errors.
[c]Significant decrease compared to chow diet at the $P < 0.005$ level.

fat diet challenge resulted in a 25–35% decrease in apoA-II mRNA levels in each strain (Table 4). It is interesting to note that only in the resistant strains, BALB and C3H, does the ratio of A-I/A-II protein increase about twofold with fat feeding; the ratio in the susceptible strain C57BL/6 is only slightly increased (Table 2). Although it is unknown whether this higher apoA-I ratio could play a role in resistance to diet-induced atherosclerosis, it is conceivable that such changes would influence lecithin: cholesterol acyltransferase and HDL receptor activity, both thought to be important in reverse cholesterol transport.

DISCUSSION

Several conclusions have emerged from these results. First, the HDL from the susceptible C57BL/6 strain undergoes both qualitative as well as quantitative changes in response to a high fat diet. Gradient gel electrophoresis has shown that multiple size classes of HDL exist in the mouse, as is reported in humans (13). In humans, heart disease is associated with low concentrations of HDL_2 and HDL_3 (reviewed in ref. 27). In accordance with this concept, both the M-HDL_2 and M-HDL_3 size classes were reduced in the susceptible C57BL/6 strain with fat feeding. Although the resistant strain C3H also demonstrated a decrease in M-HDL_2, the M-HDL_3 levels remained the highest among the strains, and overall HDL cholesterol concentration did not change. We also confirmed the previous observation based on semi-quantitative agarose gel determinations that *Ath-1* controls HDL lipid levels as judged by measurement of lipid and apolipoprotein concentrations from isolated HDL. Thus, the susceptible strain C57BL/6 exhibited about a 50% reduction in HDL cholesterol and a 35% reduction in apoA-I when maintained on a high fat diet, while the resistant strains exhibited no significant changes in either HDL cholesterol or apoA-I. In C57BL/6 mice, both HDL cholesterol and apoA-I levels reached a plateau by 3 weeks on the high fat diet and remained unchanged thereafter. The more marked differences seen in the reduction of HDL lipid using the gradient gel electrophoretic approach compared to isolation of HDL by selective phosphotungstate precipitation may be due to the semi-quantitative nature of HDL neutral lipid estimations by Sudan Black stainability.

Second, these results clarify the nature of structural variations of HDL among the strains. We previously noted substantial differences in the size of HDL from strains expressing different alleles of the apoA-II gene, and we showed that these differences segregated with the apoA-II gene in genetic crosses (28). Genetic studies have shown that the apoA-II gene variations are distinct from the *Ath-1* phenotype (4). Although the *Ath-1* gene appears to influence HDL structure to some extent, the ratio of apoA-I to HDL cholesterol remained relatively constant in the susceptible strain when challenged with the high fat diet. Thus, *Ath-1* appears to act, at least in part, by reducing the number of HDL particles in C57BL/6 mice.

Third, our studies indicate that the decreased levels of HDL cholesterol and apolipoproteins in the susceptible strain C57BL/6 are not due to decreased synthesis of apoA-I. Both mRNA levels and rates of synthesis of apoA-I were unaffected by diet in the strains examined. Thus, it appears likely that the *Ath-1* gene acts by altering the catabolism of HDL particles in susceptible but not resistant strains. HDL turnover has been studied in humans in relation to nutritional change and disorders of HDL metabolism (reviewed in ref. 29). These studies demonstrate a link between metabolism of HDL and triglyceride-rich lipoproteins, with an inverse association between HDL and VLDL removal rates (30). This leads to low HDL levels in hypertriglyceridemia, a combination that may promote development of atherosclerosis. Kinetic analysis of human VLDL and HDL flux in vivo shows that production of these lipoproteins may be positively correlated, with increased formation of VLDL particles leading to the generation of more HDL. When VLDL removal is high, the fraction of the HDL pool that is removed each day is lowered. Conversely, when VLDL catabolism is low, the fractional catabolic rate of HDL is high. This appears to occur in cases of lipoprotein lipase deficiency, resulting in low HDL levels in this disorder (31). In the fat-fed mouse, levels of VLDL and LDL cholesterol increase in all strains, but this increase is accompanied by a reduction in HDL cholesterol only in the susceptible C57BL/6 strain. Furthermore, the increase in VLDL + LDL cholesterol is of a higher magnitude in C57BL/6 (see Fig. 2). Consequently, a much lower proportion of total cholesterol occurs in the HDL fraction in C57BL/6 mice (17%) as compared to BALB/c mice (61%) and C3H mice (45%). It is possible that the *Ath-1* gene has a role in regulating the rate of interconversion or removal of VLDL and HDL.

Our overall goal is to identify the *Ath-1* gene and determine how it acts to affect HDL levels and atherosclerosis. This would have considerable importance for understanding the genetic control of HDL metabolism and would allow studies of the homologous gene in humans. One approach toward this end is to define in detail the biochemical effects of the gene. With some luck, this could lead to the identification of the *Ath-1* gene product. For example, the present studies suggest that the *Ath-1* gene affects HDL catabolism raising the possibility that proteins involved in HDL processing (such as hepatic lipase or lecithin:cholesterol acyltransferase) or HDL turnover may be responsible. Unfortunately, the processes involved in HDL assembly and turnover are as yet poorly understood, making detailed biochemical studies difficult. On

the basis of the chromosomal location of the *Ath-1* gene, it is possible to rule out various candidate genes which map elsewhere (32). An alternative to the biochemical approach is to attempt to isolate the *Ath-1* gene on the basis of its chromosomal location. Although such reverse genetic strategies have been applied successfully to the identification of several human disease genes, most of these human studies have been aided by the presence of readily detectable gene deletions. The *Ath-1* gene polymorphism, on the other hand, affects HDL metabolism in a relatively subtle fashion and is unlikely to involve a deletion.

Recent studies indicate that additional genetic loci distinct from *Ath-1*, conferring resistance or susceptibility to diet-induced atherosclerosis, occur among other inbred strains of mice (33, 34). Analysis of the one large set of recombinant inbred strains, derived from the resistant A/J strain and the susceptible C57BL/6 strain, by two different groups produced very different results and conclusions. Although the diets used differed slightly, the explanation for the differing results is unclear (33, 34).

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