Identification of Promoter Variants in Baboon Endothelial Lipase That Regulate High-Density Lipoprotein Cholesterol Levels

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Background—High-density lipoprotein cholesterol (HDL) levels are a major risk factor for cardiovascular disease. Previously we identified a quantitative trait locus on baboon chromosome 18 that regulates HDL. From positional cloning studies and expression studies, we identified the endothelial lipase gene (LIPG) as the primary candidate gene for the quantitative trait locus. The mechanism by which LIPG variation influences HDL levels has not been determined.

Methods and Results—We identified 164 LIPG polymorphisms in a panel of sibling baboons discordant for HDL1 and genotyped putative regulatory polymorphisms in a population of 951 pedigreed baboons. With the use of quantitative trait nucleotide analysis we identified 3 polymorphisms in the LIPG promoter associated with variation in serum HDL1 levels. In addition, we demonstrated that these 3 polymorphisms affect LIPG promoter activity in vitro. In silico analysis was used to identify putative transcription factors that differentially bind the functional promoter polymorphisms.

Conclusions—These results reveal LIPG variants that specifically contribute to HDL1 levels and demonstrate mechanisms by which these polymorphisms may regulate LIPG promoter activity. Results from the present study provide a mechanism, namely variation in LIPG promoter activity possibly caused by altered transcription factor binding, by which LIPG variation affects HDL levels. (Circulation. 2007;116:1185-1195.)

Key Words: cardiovascular diseases ■ cholesterol ■ gene expression ■ genetic polymorphisms ■ genetics ■ genomics

High serum levels of high-density lipoprotein cholesterol (HDL) are associated with a lower risk of cardiovascular disease and with a lower extent of atherosclerotic lesions in humans and experimental animal models. However, HDL actually comprises a heterogeneous mixture of apolipoprotein (apo) AI–containing lipoproteins that differ in size and composition and, potentially, in protective capability. HDL patterns vary greatly among individuals and within individuals in different environments, yet little is known about the precise HDL subpopulations that may help protect against cardiovascular disease. Humans accumulate appreciable amounts of large apoE-rich HDL1 particles (ie, larger than HDL2) under circumstances of impaired lecithin:cholesterol acyltransferase or cholesteryl ester transfer protein activities and low-density lipoprotein receptor deficiency. When fed a high-fat diet, some baboons also accumulate HDL1 particles heterogeneous in size (12.5 to 22.0 nm in diameter) and density (1.028 to 1.080 g/mL), that are rich in apoE and apoAI; compositional analyses of these particles indicate they are ~30% protein and rich in cholesteryl esters and phospholipids (both ~30% on a weight-percent basis). Concentrations of HDL1 appear to be largely independent of the concentrations of HDL2 (r=0.15) and HDL3 (r=−0.51). Our previous studies have demonstrated that a substantial proportion of variation in HDL1 in these animals is under the control of a single major gene. Prior linkage analyses have localized a quantitative trait locus (QTL) for HDL1 to chromosome 18, and positional cloning of candidate genes in the QTL interval suggested that endothelial lipase (LIPG) was the candidate gene for the HDL1 QTL. In addition, LIPG liver expression studies in a panel of baboons discordant for HDL1 showed an inverse correlation between LIPG liver expression and HDL1 serum concentrations consistent with LIPG studies in mice and LIPG association studies in humans. Furthermore, we found higher LIPG mRNA expression in the low HDL1 responders than the high responders as well as decreased baboon liver LIPG expression in response to increased dietary fat and cholesterol (Table 1).

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Endothelial lipase (EL, the protein encoded by LIPG), the most recently identified member of the triglyceride lipase family, is expressed mainly in liver and vasculature where it hydrolyzes triglycerides from lipoproteins. Specifically, EL reduces triglycerides in VLDL and HDL and augments apolipoprotein A-I (apoA-I) in HDL. This study identified 3 promoter polymorphisms that regulate HDL1 and suggests an inverse association between HDL1 and EL gene activity.

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family,\textsuperscript{18} was reported to have phospholipase activity and triglyceride lipase activity. EL is more active as a phospholipase than as a triglyceride lipase (phospholipase to triglyceride lipase ratio, 1.6).\textsuperscript{25} Studies in mouse models suggest that \textit{LIPG} variation influences HDL metabolism. McCoy et al demonstrated with an in vitro system of \textit{LIPG} cloned into an adenoviral vector and transfected into COS cells that, for all lipoprotein fractions, HDL particles are the preferred source of EL substrate.\textsuperscript{25} Studies in mouse models show that a decrease in EL expression and activity, by gene deletion of \textit{LIPG} in knockout mice\textsuperscript{17,20} and by antibody inhibition,\textsuperscript{19} resulted in significant increases in plasma HDL in mice. Furthermore, overexpression of \textit{LIPG} in transgenic mice resulted in decreased plasma HDL.\textsuperscript{18} The present findings suggest that EL lipolytic activity plays an important role in HDL metabolism in mice.

In studies in humans also suggest that \textit{LIPG} plays a role in regulation of HDL levels. De Lemos et al identified 17 variants in human \textit{LIPG}.\textsuperscript{22} The authors state that 6 of these variants are likely to be functional because 4 encode amino acid changes and 2 are located in the promoter. Statistical analyses did not show a significant association of these variants with HDL, which suggests that genetic variation in \textit{LIPG} is linked to HDL levels\textsuperscript{32}; however, the mechanism(s) by which these intronic variants might influence HDL levels was not determined. A third study examined an \textit{LIPG} promoter single-nucleotide polymorphism (SNP) and a nonsynonymous SNP in exon 3 as a haplotype and found a marginal association with HDL and strong association with HDL3 and apoAI serum concentrations.\textsuperscript{23}

In a fourth association study, EL plasma concentration was found to be inversely correlated with a number of metabolic syndrome phenotypes, which included HDL.\textsuperscript{21} Taken together, these data suggest that genetic variation in \textit{LIPG} influences HDL levels. However, these studies do not define the mechanisms by which \textit{LIPG} variation impacts HDL.

In the present paper, we identify baboon \textit{LIPG} promoter, coding sequence, intron and 3′UTR polymorphisms, and promoter polymorphisms that influence HDL serum concentrations. Furthermore, we demonstrate that the polymorphisms in the \textit{LIPG} promoter that affect \textit{LIPG} promoter activity also influence HDL1. The present results indicate that variation in \textit{LIPG} expression affects HDL1 serum concentrations. On the basis of sequence data, the promoter variants that influence HDL1 are predicted to alter transcription factor binding to the \textit{LIPG} promoter, which suggests a mechanistic link between \textit{LIPG} variation and HDL variation.

**Methods**

### Pedigreed Baboons and Blood Samples

Samples from 951 pedigreed baboons were analyzed in the present study. These animals have been genotyped\textsuperscript{26} and are organized into eleven 3- to 4-generation pedigrees that have a rich diversity of relative pairs. Blood samples were obtained from the femoral artery of animals fed a high-fat diet (ie, 40% of calories from lard). Baboons were immobilized with ketamine, blood was taken from the femoral artery, and serum was prepared by low-speed centrifugation. Samples were frozen at −80°C until use. Animals were maintained at Southwest Foundation for Biomedical Research, a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

### Measurement of HDL Cholesterol in Serum

Serum HDL concentrations were quantified after precipitation of apoB–containing lipoproteins with heparin-Mn\textsuperscript{27} on a Ciba-Corning Express Plus Clinical Chemistry Analyzer (Medfield, Mass.) and reagents supplied by F. Hoffmann-La Roche (Basel, Switzerland).\textsuperscript{27} HDL particles were resolved on the basis of size with nondenaturing gradient gel electrophoresis as described previously.\textsuperscript{24} The proportion of cholesterol in large (ie, >13 nm in diameter) HDL1 particles was estimated with use of Sudan black B staining, and cholesterol concentrations in HDL1 were calculated as described.\textsuperscript{28} Previous studies have indicated that approximately half the variation in HDL1 levels on the high-fat diet is under genetic control.\textsuperscript{13}

### Sibling Pair Baboon Panel for \textit{LIPG} Resequencing

On the basis of phenotypic and genotypic analysis of the pedigreed baboon population, 5 sibling pairs with contrasting phenotypes for HDL1 were chosen. One high-HDL responder was a sibling to 3 low-HDL responders, which resulted in a total of 8 baboons in the panel for resequencing. The sibling pairs differed by at least 1 SD for HDL1 serum concentrations. In addition, members of each selected sibling pair were discordant for at least 1 of the marker loci within the support interval for an HDL1 QTL on chromosome 18. For details of sibling pair HDL phenotype data, see Cox et al.\textsuperscript{15}

### Preparation of Lymphocyte Genomic DNA for Sequencing and Genotyping

DNA extraction from lymphocytes were performed as described by Cox et al.\textsuperscript{26}

### Identification of Sequence Polymorphisms in Baboon \textit{LIPG}

A baboon BAC clone that contained \textit{LIPG} was isolated from a BAC library (BACPAC Resources, Children’s Hospital Oakland Research Institute, Oakland, Calif.) with use of a baboon \textit{LIPG} gene fragment generated by polymerase chain reaction (PCR) with primers based on human \textit{LIPG} sequence. Baboon \textit{LIPG} were first sequenced in the BAC clone and then \textit{LIPG} were resequenced for the 8-baboon panel of low- and high-HDL1 sibling pairs to identify sequence polymorphisms. Briefly, genomic DNA (50 ng) was amplified with \textit{LIPG} primers (10 mmol/L each) (online Supplement Table 1), 10×1.5 mmol/L Mg\textsuperscript{2+} buffer, 10 mmol/L dNTP mix, and TaKaRa Taq DNA Pol (Fisher, Pittsburgh, Pa) in a 50 μL reaction. PCR products were subcloned into pTOPO with the TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.) and transfected into One Shot compe-
Genotyping SNPs

SNPs were genotyped by direct sequencing of genomic DNA from 951 pedigreed HDL1-phenotyped baboons. Genomic DNA was amplified with use of primers listed in online Data Supplement Table I and subjected to cycle sequencing with use of primers listed in online Data Supplement Table II and automated sequencer analysis as described above.

Genotyping the Indel −3609 and the Intron 9 Microsatellite Marker

Microsatellite markers were genotyped as described in Cox et al.26 The forward and reverse primers for the indel were (−3195) 5′-[(FAM)TTCCAGTCTCAGTTGAGTTGA-3′] and (−2775) 5′-AGAAGCTTGCAGTCTTATT-3′, respectively. The forward and reverse primers for the intron 9 microsatellite marker were (22 592) 5′-[FAM]CTGCTGGAGATGCTCTG-3′ and (22 877) 5′-CTTCAAGGCTTCCCAAT-3′. Data cleaning was performed by Cox et al.26

Human and Rhesus Macaque LIPG

Sequence Data

Rhesus LIPG is not yet annotated in the rhesus genome sequence; therefore, rhesus LIPG sequence data were based on alignment of the rhesus genome sequence with human LIPG7 in the March 2006 human genome build at UCSC genome browser.30

Analysis of Effects of Polymorphic Amino Acids on EL 3-Dimensional Structure

Human EL (Q9Y5X9) was used as the reference protein for analysis. Human EL was imported into Deepview Swiss Pdb Viewer software (Swiss Institute of Bioinformatics, Basel, Switzerland).33 Baboon EL sequence for each of the 3 polymorphic amino acids was imported into Deepview Swiss Pdb Viewer and aligned with the human EL model. The Deepview mutation function was used to evaluate structural impact of amino acid substitutions, only the most probable rotamer variants were considered. The predicted functional consequences of each mutation were evaluated with use of the second-generation baboon linkage map.35 Briefly, we estimated the genetic variance attributable to the QTL (σ²T) by specifying the expected genetic covariances between arbitrary relatives as a function of the identity-by-descent relationships at a given marker locus assumed to be tightly linked to a locus that influences the quantitative trait.34 We used the Markov Chain Monte Carlo routines implemented in the computer package Loki (Centre National de Génopépigen, Evry, France)35 to estimate multipoint identity-by-descent probabilities for all relative pairs, tested linkage hypotheses at 1-cM intervals along each chromosome with likelihood ratio tests, and converted the resultant likelihood test statistics to the logarithm of the odds (LOD) score of classic linkage analysis.36 We estimated that genome-wide probability values were estimated with use of modifications to a method suggested by Feingold et al37 to account for both the finite marker density in the baboon linkage map and the size and complexity of the baboon pedigrees.

Genetic Association Analyses

Pedigree-based measured genotype analyses were conducted within the variance components framework as part of the quantitative trait linkage disequilibrium (QTLD) procedures38 in SOLAR. This approach is described in detail elsewhere.39 Briefly, like the transmission disequilibrium test, the QTLD procedures model the genetic association as a fixed effect on a quantitative trait mean and decompose the genotype scores into between- and within-pedigree components, b and w, respectively (like the quantitative transmission disequilibrium test).39,40 However, it also tests for population stratification and, in the absence thereof, exploits more of the genetic information in the large extended baboon pedigree than does the quantitative transmission disequilibrium test alone.24

Of the 594 phenotyped baboons for which we had genotype data at a minimum of 20 (of the 24) LIPG promoter SNPs, all had genotype data at SNP −2117, and 543 (91.5%) had genotype data at SNP −1990. Imputation of SNP genotypes was exact (integers, not probabilities, based on complete concordance with Mendelian expectations) and only relevant to the Bayesian quantitative trait nucleotide (BQTN) analyses. That is, imputed SNP genotypes were not used in the QTLD suite of analyses (the results of which are reported in Table 2) that identified the same 2 LIPG promoter SNPs as associated with HDL1 levels in these baboons.

Bayesian Quantitative Trait Nucleotide Model

We also assessed the contribution of the LIPG polymorphisms to the previously detected HDL1 QTl on PHA18 with BQTN analysis. BQTN analysis41 is a novel statistical genetic method that uses a Bayesian model averaging approach with either SNP genotypes or haplotypes to identify sequence variants that are either functional or exhibit the highest disequilibria with the true functional sites. The BQTN model that we employed simultaneously uses information from all possible biological relationships to disentangle the genetic architecture of a quantitative trait.34,42 The technique is described in detail elsewhere.43–44

Conditional Linkage/QTN Analysis

To examine whether the associated LIPG promoter SNP(s) found by the BQTN analysis can account for our reported QTL for serum HDL1 concentrations on PHA18q, we conducted analyses that combined the BQTN analysis with identity-by-descent–based variance component linkage analysis. Although a detailed description of this approach has been presented elsewhere,45 its basic rationale is described here. If a variant or set of variants in LIPG is entirely responsible for the observed linkage signal, a linkage analysis conditional on a fixed-effect of the polymorphism would reduce the LOD score to near zero. Alternatively, if the associated polymorphism is in less than complete linkage disequilibrium with the true functional site or other unmeasured functional polymorphisms are present, linkage analysis generally will give a non-zero LOD score.

Statistical Genetic Analysis of Indel Polymorphisms

We used the standard fixed-effects regression approach implemented in QTLD procedures (above) to perform a measured genotype analysis to test for association between the indel polymorphism and serum HDL1 levels. We used likelihood ratio tests to compare the likelihoods of a model in which the mean effect of the indel
model, the residual phenotypic variance (that which remains after the mean effects of age, age², sex, sex×sex, age² sex, and body weight are accounted for) was partitioned into components that represent the additive genetic effects of the QTL and genes other than the QTL plus unmeasured nonadditive genetic effects (often referred to as the random environmental component). Because they had not been required to detect and localize genetic effects on this trait, other components of the genetic variance (e.g., dominance or epistasis) were not partitioned/estimated for these analyses.

### Genotype Data Preparation

#### Genotype Error Detection and Elimination

For both the SNP and indel polymorphisms, we used the Sim-Walk2 Markov Chain Monte Carlo routines to detect and eliminate any genotypes that were inconsistent with Mendelian segregation in the large single-pedigree configuration that contains the animals from which data were obtained for the present study. A more detailed description of the basic genotype data processing methods is provided elsewhere.

#### SNP Processing

All further SNP processing was conducted with use of SOLAR routines as described in detail (with examples) in Blangero et al. Because it evaluates all possible combinations of SNPs, the BQTN procedure limits the sample to those individuals for whom no data are missing. To maximize our sample size and statistical power, we used SimWalk2 SNP haplotype estimation algorithms implemented in SOLAR to impute as many missing genotypes as possible. For missing genotypes that could not be assigned exactly from the haplotypes, SOLAR extends this method to impute missing genotypes as the weighted average of all haplotypes that are consistent with an individual’s haplotype and genotype data, where the weights are the estimated haplotype frequencies. We limited imputation in preparation for BQTN analyses to those individuals with genotypes at a minimum of 20 SNP sites in the LIPG promoter.

It is common to observe variable patterns of linkage disequilibrium (LD) correlations (ρd) among SNPs within genes such that pairs or sets of SNPs exist for which the estimated value of ρd is at or near 1.0. It is not possible to discriminate statistically between the members of such sets (referred to as isocorrelated redundant SNP sets) in an association analysis. We used a method advanced by Cheverud to estimate the effective number of independent SNPs within the LIPG promoter. This estimate provides a correction for the multiple testing in the association analyses that is more reasonable than a Bonferroni correction based on the assumption of independence among the LIPG promoter SNPs. Furthermore, it provides a means to determine a threshold for identifying isocorrelated redundant SNP sets based on the absolute value of the LD correlations (ie, |ρd|) between all pairs of SNPs within the LIPG promoter. From each isocorrelated redundant SNP set, we selected 1 SNP to represent the others in the BQTN analysis.

### Baboon LIPG Promoter Analysis—Cell Culture Assays

Reporter constructs for LIPG promoter variants were made by amplifying the LIPG promoter (−3721 to 86) from animals with 2 of the 3 polymorphisms shown by QTN analysis to regulate HDL1 levels (−1990 SNP and −3069 indel); the same nucleotides were present in each construct for all other 5′ flanking region polymorphisms. PCR was performed with LIPG promoter primers with restriction sites at the 5′ end with an extra 4 nucleotides to facilitate restriction endonuclease digestion (forward primer that included a Kpn I site: 5′-GGCCG-GGTACC-3′) among SNPs within genes such that pairs

### Table 2: Summary of QTLD Analyses of LIPG 5′ Flank Genotyped Polymorphisms for HDL1 Levels in Baboons Fed a High-Fat Diet

<table>
<thead>
<tr>
<th>SNP</th>
<th>Stratification</th>
<th>Measured Genotype</th>
<th>QTDT</th>
<th>QTLD</th>
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<tbody>
<tr>
<td>416</td>
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<tr>
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<tr>
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<td>0.107329</td>
<td>0.516896</td>
<td>0.537130</td>
<td>0.393789</td>
</tr>
<tr>
<td>555</td>
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<td>0.155361</td>
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<tr>
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<tr>
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</table>

Outliers (mean±4 SD) removed prior to analysis. QTDT indicates quantitative transmission disequilibrium test.

*P* values are from 4 In likelihood ratio tests performed as part of the QTLD procedure. First, the stratification test: model in which *b* and *w* are estimated vs one in which *b*=*w*. A significantly better likelihood when *b*≠*w* indicates presence of population stratification. Second, the measured genotype test: a model in which *b*=*w* vs one in which *b*=0 and *w*=0. A significantly better likelihood when *b*=0 indicates association (regardless of stratification). Third, the QTLD: a model in which *b* and *w* are estimated vs one in which *b* is estimated and *w*=0. Significantly better likelihood when *w*=0 indicates association (in the absence of population stratification).

polymorphism on HDL1 was estimated to that of a model in which its mean effect was constrained to zero. We also conducted a conditional multilocus linkage analysis to assess whether the indel polymorphism accounted for a significant proportion of the genetic variance attributable to the HDL1 QTL on PHA18.

The above series of statistical genetic analyses were conducted to address the following objectives: to confirm our earlier detection and localization of an HDL1 QTL to baboon chromosome 18q in pedigreed baboons from this same population; to prioritize polymorphisms in the 5′ flanking region of LIPG, a gene within the support interval for this QTL, for further experimental consideration; and to assess the contribution of these polymorphisms to this QTL. To improve our chances of detection of polymorphisms relevant to the detected genetic effects on serum HDL1 in this population, all analyses utilized variance component models based on the linkage model originally used to localize the QTL for the trait. In the present...
ng) (Promega) in serum-free media. The pGL4.74 [hLuc/TK] is a secondary reporter vector that contains the Renilla-luciferase gene used to normalize for transfection efficiency. After incubation, cells were grown for 24 hours, at which time the cells were lysed. An aliquot of lysate was used to determine transfection efficiency by Renilla quantification and to determine promoter activity by luciferase quantification with the Dual-Luciferase Reporter Assay System (Promega). The pGL4.13 [Luc2/SV40] was included as a positive control. The pGL4.10 [Luc2] vector without a promoter was included as a negative control for expression and a no-DNA negative control was included. Data were normalized against the Renilla luciferase activity. All assays were performed in triplicate and each experiment was performed 3 times. Data were analyzed with use of single-factor ANOVA analyses with α=0.05 followed by all pairwise multiple comparison procedures with the Student-Newman-Keuls method.

In Silico LIPG Promoter Analysis for Transcription Factor Binding
SiteSeer\(^\text{48}\) was used to determine predicted transcription factor binding to the LIPG promoter binding for ~3069 indel variants and ~2117 and ~1900 variants. SiteSeer settings were: GC content for background probability=35%; species=human, mouse and rat; minimum number of occurrences=1; expectation value=1; and minimum expectation ratio=1.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Multipoint Linkage Analysis
We performed linkage analysis of HDL for the 951 baboons in the present study and obtained significant evidence for a QTL on chromosome 18. Figure 1 presents a plot of the multipoint LOD scores on chromosome 18 from the present analysis. The peak multipoint LOD score was 4.25 and the genome-wide probability value was 0.0012 (locus-specific P=0.0000049). The 1-LOD support interval for this QTL was an 18-cM region between 49 cM and 67 cM from the pter-most marker locus. LIPG, marked by the locus D18Spha1 (which maps to intron 9), falls within this 18-cM interval. The mean effects of age, sex, age×sex, age\(^2\)×sex, and body weight (kg) accounted for ≈6.2% of the total phenotypic variance in serum HDL1 levels in these pedigreed baboons. The additive effects of genes accounted for ≈53% of the remaining 93.8% of the variance, with 15% attributable to variation at the QTL and 38% to genes other than those at the QTL. The QTL accounted for ≈28% of the total additive genetic effects on variation in serum HDL1 levels.

Identification of Polymorphisms in Baboon LIPG
The entire baboon LIPG gene was sequenced with the exception of ~1700 nucleotides in intron 1, 1200 nucleotides in intron 2, 1910 nucleotides in intron 8, and ~2600 nucleotides in intron 9. These estimates are based on baboon genomic DNA PCR product sizes. Baboon LIPG was resequenced for a sibling pair panel (n=8) discordant for HDL1 serum concentrations. Baboon LIPG DNA sequence are available at the National Center for Biotechnology Information website (accession number EU00687).

Analysis of baboon LIPG sequence data from the sibling pair panel showed 9 indels (Supplementary Table III) and 3 repetitive elements (Supplementary Table IV). In addition, 2 insertions were found in baboon LIPG compared with human LIPG (Supplementary Table V). We identified 150 SNPs in these animals (Supplementary Table VI): 6 polymorphisms were in LIPG coding region exons and 3 of these encode amino acid changes; 25 polymorphisms were in the 5’ flanking region; 100 polymorphisms were in LIPG introns; and 19 polymorphisms were in the 3’ UTR. Identification of 162 polymorphisms in ~30,000 bases of sequence data reveals variation in 1 of 185 nucleotides for baboon LIPG. A microsatellite marker was identified in baboon LIPG intron 9. This marker was genotyped for 951 pedigreed HDL1-phenotyped baboons. Inclusion of this microsatellite marker in the baboon chromosome 18 linkage map is reported in Cox et al.\(^\text{26}\)
Comparison of Baboon EL Predicted Amino Acid Sequence with Human and Rhesus Macaque

Baboon EL predicted amino acid sequence is aligned with human and rhesus macaque amino acid sequence (Figure 2). Baboon versus human EL sequence alignment showed 11 amino acid differences (97.8% amino acid identity). Baboon versus rhesus macaque EL sequence alignment showed 3 amino acid differences (99.4% amino acid identity). Primate species comparison showed 6 nonconserved amino acid differences between baboon and human, no nonconserved amino acid difference between baboon and rhesus, and 1 nonconserved amino acid difference between rhesus and human; 5 conserved amino acid differences existed between baboon and human and 1 conserved amino acid difference between baboon and rhesus. Human and baboon polymorphic amino acids are denoted in Figure 2.

In Silico Analysis of Baboon EL Polymorphic Amino Acids

The effects of amino acid polymorphisms for Asn212Asp, Asn292Ser, and Ile446Val were analyzed. The results showed that, for polymorphic amino acid Asn212Asp, the predicted conformation indicates that these amino acids are buried inside the EL protein. In this location, both of these amino acids are usually involved in the formation of salt bridges that stabilize hydrogen bonds. Consequently, both amino acids are predicted to function similarly in this EL location. For polymorphic amino acid Asn292Ser, both of...
these amino acids typically play a role in overall protein structure. Predictions based on the EL Deepview Swiss Pdb Viewer protein model indicate that exchange of these 2 amino acids at this position is unlikely to influence overall conformation or effect enzyme activity. The polymorphic amino acid Ile446Val is positioned on the protein’s surface, and both of the amino acids at this position are hydrophobic. In this location, hydrophobic amino acids are usually involved in binding/recognition of hydrophobic ligands such as lipids. Because this is a conserved polymorphic amino acid, this polymorphism is not predicted to effect protein structure or activity.

Statistical Genetic Analyses of Baboon LIPG 5’ Flanking Region Polymorphisms

Linkage Disequilibrium and Effective Number of SNPs

The 24 SNPs and the indel polymorphism in 5’ flanking region of LIPG were genotyped in DNA from 951 pedigree baboons for which serum lipoprotein levels had been measured on the high-fat diet. Figure 3 shows the variable general pattern of disequilibria among the 24 markers in this region. LD between SNP pairs was estimated with use of the absolute value of the correlation coefficient (ie, $|\rho|$). Mean $|\rho|$ in this region was 0.318 (SD=0.2798). With use of the method of Cheverud,47 we used the $|\rho|$ values to estimate the effective number of independent SNPs to be 18.39 (76.6%). This corresponded to the number of SNPs that exhibit pair-wise LD correlations below a threshold of 0.93. We used this threshold to exclude 6 of 24 SNPs from 3 isocorrelated redundant SNP sets (Figure 3), which left 18 for the BQTN analyses. We also used effective number to obtain the multiple test correction for standard association tests. To maintain a type I error rate of 0.05, we required $P$=0.00272 in our series of QTLD association analyses.

QTLD Analyses of LIPG Promoter SNPs

In the analyses of the effects on HDL1 levels of the 24 SNPs in the promoter of LIPG in pedigree baboons fed the high-fat diet, 6 of the 24 SNPs were eliminated because they were likely redundant because of complete LD with others in 1 of 3 haplotype blocks. The measured genotype analysis, the quantitative transmission disequilibrium test, and the QTLD analysis (Table 2) all indicated that SNP −1990 had a significant mean effect and that SNP −2117 had a marginally significant effect on HDL1 levels in baboons fed the high-fat diet.

BQTN Analyses of LIPG Promoter SNPs

After imputation of missing SNP genotypes for pedigree baboons typed at 20 or more sites, we conducted BQTN analyses with data from 594 animals. BQTN analyses of all possible combinations of the 18 relatively more independent LIPG promoter SNPs (ie, 262 144 models of gene action) identified the 2-SNP combination of −1990 and −2117 as most important in the LIPG promoter for serum HDL1 levels on the high-fat challenge diet. With a Bayesian information criterion of −15.16, positive evidence supports this 2-SNP model over the next best model with approximate posterior probability of 93%.
Inclusion of these 2 SNPs, −2117 and −1990, in the linkage model for HDL1 results in a significant (P=0.00131) improvement in the likelihood of the model compared with that of the alternative restricted model in which the effects of the 2 SNPs are constrained to equal zero. Concordant with this result, the peak multipoint LOD score on baboon chromosome 18 (at 68 to 69 cM from pter-most marker) is improved rather than diminished by the addition of the SNPs to the linkage model (ie, with βSNPs=0, LOD=5.01; with βSNPs estimated, LOD=5.41). To better understand this result, we reparameterized the linkage models for HDL1 to estimate the effects, if any, of the 2 SNPs on the QTL-specific genetic variance. Covariate effects accounted for ≈7% of the total phenotypic variance in serum HDL1 levels in the BQTN models that we maximized on data from these 594 pedigreed baboons. Of the 93% remaining (residual) phenotypic variance in this trait, 56% was attributable to the additive effects of genes and 44% to unmeasured nonadditive genetic factors. Whereas the 2 SNPs identified by the BQTN analysis accounted for 2.8% of the residual phenotypic variance (and 2.6% of the total variance) in this trait, they accounted for 4.5% of the total additive effects of genes on variation in HDL1 levels. However, modeling of the additive genetic effects of these 2 promoter SNPs resulted in a concomitant reduction of the proportion of the residual variance that was caused by genes other than those at the QTL.

**Statistical Genetic Analysis of the Indel Polymorphism**

We conducted a measured genotype analysis to detect an effect of the indel polymorphism (−3069) on HDL1 levels in the group of pedigreed baboons in which we detected the baboon chromosome 18 QTL that regulated HDL1. We scored deletion homozygotes as zero and any other genotypes at this locus as 1 to test for the mean effect of 1 or more insertions (variation in indel genotype) on HDL1 levels by comparing the likelihood of a model in which the coefficient for 1 or more insertions was estimated to that of a model in which it was constrained to equal zero. Presence of at least 1 insertion results in a significant (P=0.027) decrease (24%) in mean serum HDL1 levels. Our multipoint linkage analyses of serum HDL1 levels, conditional on the presence of 1 or more insertions, did not account for a significant proportion of the additive genetic variance attributable to the HDL1 QTL on baboon chromosome 18.

**Statistical Genetic Analysis of Significant HDL1 LIPG Promoter Polymorphism Effects on HDL**

We compared the maximum likelihood of a quantitative genetic model in which we estimated only the mean effects of age, sex, age×sex, age², age³×sex, and body weight, plus the additive contributions of genes and unmeasured environmental factors on variation in total serum HDL with those of models in which we also estimated the mean effects of each of the 2 previously nominated SNPs and the deletion-insertion polymorphism on total serum HDL levels. These measured genotype analyses, conducted with data from the same pedigreed baboons fed the same high-fat diet, found that both SNP −1990 and the indel exerted significant effects on mean total serum HDL levels, accounting for 2.4% (P=0.00001) and 2.0% (P=0.005) of the total phenotypic variance in this trait, respectively.

**In Vitro Analysis of LIPG Promoter Polymorphisms Shown by QTN Analysis to Influence HDL1 Cholesterol Variation**

Activity for LIPG promoter variants for the indel at −3069 and the SNP at −1990 were analyzed with a dual reporter vector system and HepG2 cells. Results of these assays showed significant differences between groups (P=0.0001) and showed the greatest activity in the −3069 deletion with “T” at −1990. A change of the −1990 SNP to “C” decreased promoter activity. A change of the −3069 indel to the 216 nucleotide insertion and keeping “T” at the −1990 SNP also decreased activity. Substitution of the −3069 216 insertion for the 228 insertion further decreased promoter activity. All promoters showed activity greater than the no-promoter negative control (Figure 4).

**Predicted Transcription Factor Binding Sites in Polymorphic Regions**

We performed in silico analysis of the baboon LIPG promoter to determine the putative effects of polymorphisms −3069, −2117, and −1990 on transcription factor binding. Seven transcription factors, TH2B (AGCTCA, R01934), RAS1 (GCTCCGGTCT, R01305), CFOS (GATGTCC, R00465; GTGACGTGTT, R00471), ALBU (AAACATA, R00087), DPOLB (GCCCC, R00290), WAP (CCAAAGT, R01597), and TERT (CACCCTG, R09480), are all predicted to bind either insertion (216 or 228) but not the deletion. CFOS has 2 predicted binding sites in the insertion region. One transcription factor, ALBU (AACATA, R00087), is predicted to bind the C but not the T at SNP −1990. No transcription factors are predicted to bind either nucleotide at SNP −2117.

**Discussion**

Previously, we reported a QTL for HDL1 levels located on chromosome 18, and the present study has replicated that finding in 951 pedigreed baboons. Studies in our laboratory identified LIPG as the primary candidate gene that accounts for the QTL on chromosome 18, which regulates HDL1 in a population of pedigreed baboons. Furthermore, these studies showed an inverse correlation between HDL1 serum concentrations and LIPG expression in baboon siblings discordant for HDL1. As a first step to identify variation in LIPG that influences HDL1, we resequenced baboon LIPG from −3721 in the 5′ flanking region to the 3′ end of the 3′UTR for the panel of 8 baboon siblings discordant for HDL1 serum concentrations and identified 164 polymorphisms, which included 25 promoter polymorphisms and 3 nonsynonymous exon polymorphisms. The baboon LIPG coding region SNPs identified in the present study did not associate with HDL1 phenotypes in the discordant sibling pair panel. In addition, the baboon EL polymorphic amino acids do not encode predicted conformational changes. Therefore, in the present study, we focused on identification of variation in putative regulatory regions that could influence HDL1 serum concentrations.
Comparison of baboon and human LIPG gene sequence shows 2 insertions and 2 microsatellite markers not present in human. In addition, human LIPG contains 55 validated polymorphisms from LIPG resequencing for polymorphism identification22,44 and from submissions to the National Center for Biotechnology Information SNP database (build 126, http://www.ncbi.nlm.nih.gov/projects/SNP). Comparison of 55 human validated polymorphisms with 164 validated polymorphisms in baboon LIPG suggest that baboon LIPG has ≈3 times more variation than human LIPG.

Comparison of baboon, human, and rhesus EL protein sequence showed that baboon EL is 97.8% identical to human EL and 99.4% identical to rhesus EL. Both baboon and rhesus differ from human EL at amino acid 15 in the putative signal domain from amino acids 1 to 20. Baboon is identical to human and rhesus in the heparin binding domain, the poly valine region from 118 to 121, the charge relay system amino acids at 169, 193, and 274, and the potential N-linked glycosylation sites at amino acids 80, 136, 393, 469, and 491. In addition, the putative pairs of residues linked by intrachain disulfide bonds at amino acids pairs 64 and 77, 252 and 272, 297 and 316, 308 and 311, and 463 and 483 are conserved. These data indicate that baboon EL function compared with human EL is conserved.

As mentioned above, previous studies in our laboratory showed an inverse correlation between HDL1 serum concentrations and LIPG expression in baboon sibs discordant for HDL1. Therefore in the present study, we focused on identification of variation in putative regulatory regions that influence HDL1 serum concentrations. As a first step, we genotyped all polymorphisms identified in the 5′ flanking region for 951 pedigreed HDL1-phenotyped baboons. QTN Analyses of the 25 5′ flanking region polymorphisms showed that genetic variation in the LIPG promoter contributes to variation in HDL1 levels. Furthermore, the effect of the promoter on HDL1 serum concentrations in these pedigreed baboons can be associated with variation at 2 SNP, (−2117, −1990) and 1 indel (−3069). These results conclusively demonstrate that LIPG promoter variation impacts HDL1 serum concentrations. In addition, results that show that 2 of the 3 polymorphisms contribute to variation in total HDL suggest that LIPG promoter variants may also play a role in general HDL metabolism.

On the basis of these results, we performed in vitro assays to show that the variants identified by QTN analysis influenced LIPG promoter activity. In these experiments, LIPG promoter activity differed for all indel alleles at −3069. We showed that promoter activity differed between the 2 alleles at −1990. Therefore, LIPG promoter variants that were identified by statistical analysis to influence HDL1 levels encode variation in LIPG promoter activity. In silico analysis of these polymorphisms predicts transcription factor binding to both insertions; these factors are not predicted to bind to the deletion because of the absence of the target sequences. The analysis did not predict differential binding of transcription factors when the 216-nucleotide insertion is compared to the 228-nucleotide insertion nor did the analysis predict differential binding of transcription factors when the −1990 nucleotide variants are compared.

The promoter polymorphisms that influence HDL1 in baboons are not found in human LIPG. As with other studies that compared genetic mechanisms that regulate cardiovascular disease quantitative trait variation in baboon and human, it is likely that the same mechanism is used in humans as baboons but via a different regulatory polymorphism. For example, a SNP was discovered in 1 splice site of apo(a) that

Figure 4. Variation in LIPG promoter activity with the −3069 indel and the −1990 SNP. LIPG promoter variants for an indel and a SNP, shown to influence HDL, were subcloned into the pLuc vector. A, the negative control pLUC vector with no promoter; B, the 228-nucleotide deletion at −3069 with SNP variant C at −1990; C, the 228-nucleotide deletion at −3069 with SNP variant T at −1990; D, the 12-nucleotide deletion (216-nucleotide insertion) at −3069 with SNP variant T at −1990; and E, the 228-nucleotide insertion at −3069 with SNP variant T at −1990. Promoter constructs were transfected into HepG2 cells, and promoter activity was quantified after 24 hours. Assays were run in triplicate and repeated 3 times. Expression values are shown as a ratio of luciferase to Renilla activities. Error bars indicate SEM. Promoter activity was different from all other promoters (*P<0.05).
caused deletion of 1 exon and encoded a transcript positive apo(a)-null allele.\textsuperscript{50} Although this same SNP was not found in human apo(a), a different SNP was discovered in an apo(a) splice site that created a truncated version of apo(a) and encoded a transcript-positive apo(a)-null allele.\textsuperscript{51} These studies demonstrated that the same basic mechanism created a transcript positive null allele that used different polymorphic nucleotides. Similarly, we predict that promoter polymorphisms in human \textit{LIPG} will affect circulating endothelial lipase and ultimately HDL metabolism. Future studies to define the proteins that differentially bind the \textit{LIPG} promoter as a result of these polymorphisms will be required to determine the factors that mediate \textit{LIPG} variant effects on HDL1 levels. Results from the present study suggest that HDL levels may be modified therapeutically via modulation of \textit{LIPG} expression; definition of the factors that differentially bind the \textit{LIPG} promoter will provide the means to test this.

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\textbf{Disclosures}

None.

\textbf{References}


High-density lipoprotein cholesterol (HDL) levels are a major risk factor for cardiovascular disease. In light of the recent cancellation of Torcetrapib clinical trials, it has become clear that new approaches to raise HDL must be developed. Studies in humans and mice indicate that endothelial lipase plays a major role in regulation of HDL levels by catalyzing the hydrolysis of HDL phospholipids and facilitating clearance of HDL from the circulation. The central question is whether genetic variation in the endothelial lipase gene (LIPG) influences variation in HDL. Association studies in humans and functional studies in mice support the hypothesis that genetic variation in LIPG significantly influences HDL. However, few if any LIPG polymorphisms have been rigorously demonstrated to affect endothelial lipase activity and function. Furthermore, the mechanism(s) by which LIPG variation influences HDL levels has not been determined. Results from our study demonstrate that variation of specific nucleotides in the LIPG promoter regulates LIPG expression and influences variation in HDL (specifically large HDL particles). On the basis of previous studies that compared functional variants in baboon and human genes, it is unlikely that the same polymorphisms in human LIPG gene promoters regulate HDL. However, it is likely that the same mechanism (ie, LIPG promoter variation regulation of LIPG expression) will be found to regulate human LIPG expression and influence HDL levels. Therefore, our findings suggest that HDL levels may be modified therapeutically via modulation of LIPG gene expression. Future studies will focus on identification of human LIPG functional variants that could serve as therapeutic targets to increase HDL levels.
Identification of Promoter Variants in Baboon Endothelial Lipase That Regulate High-Density Lipoprotein Cholesterol Levels
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