A common *Hpa* I RFLP of apolipoprotein C-I increases gene transcription and exhibits an ethnically distinct pattern of linkage disequilibrium with the alleles of apolipoprotein E

Yan Xu,* Lars Berglund,* Rajasekhar Ramakrishnan,§ Richard Mayeux,† Colleen Ngai,* Steven Holleran,§ Benjamin Tycko, Todd Leff,†† and Neil S. Shachter[1,*]**

Division of Preventive Medicine and Nutrition, Department of Medicine,* Gertrude H. Sergievsky Center and Department of Neurology,† Department of Pediatrics,§ and Department of Pathology,** College of Physicians and Surgeons of Columbia University, 630 W. 168th Street, New York, NY 10032; and Department of Biological Chemistry, University of Michigan and Department of Cell Biology,†† Parke-Davis Pharmaceuticals, 2800 Plymouth Road, Ann Arbor, MI 48106

Abstract Apolipoprotein (apo) C-I is a constituent of triglyceride-rich lipoproteins (TGRL) that interferes with their hepatic clearance. Functional polymorphism in the apoC-I gene has not been established. We determined that an *Hpa* I site variably present at −317 relative to the apoC-I gene is produced by a 4-bp CGTT insertion. The apoC-I *Hpa* I alleles showed an ethnically distinct pattern of linkage disequilibrium with the alleles of the adjacent apoE gene. The frequency of apoC-I *Hpa* I-positive (H2) with apoE ε2 was 0.98, without significant ethnic difference. In contrast, the frequency of H2 with apoE ε4 was 0.85 in European-Americans but only 0.55 in African-Americans ($P < 0.001$). The frequency of H2 with apoE ε3 was 0.02 in European-Americans and 0.08 in African-Americans ($P < 0.001$). African-American apoE ε3/ε3 carriers of apoC-I H2 had 19% lower fasting triglyceride levels than H1 homozygotes ($P = 0.03$) along with 18% higher HDL-cholesterol levels ($P = 0.02$). ApoB levels were 21% lower ($P = 0.002$). H2-allelic reporter-gene constructions showed 50% higher expression in transient transfection studies. We localized the source of this difference in expression to the CGTT insertion itself. Deletion studies of the H1 allele showed a negative transcriptional effect of the polymorphic region. An H1 oligodeoxynucleotide showed specific binding of a hepatoma-cell nuclear protein not evident with an H2 oligodeoxynucleotide. The H2 sequence may decrease the binding of a negatively acting transcription factor, leading to overexpression of apoC-I. This may produce a functional effect on lipoprotein levels but confirmation is needed in other populations.—Xu, Y., L. Berglund, R. Ramakrishnan, R. Mayeux, C. Ngai, S. Holleran, B. Tycko, T. Leff, and N. S. Shachter. **A common *Hpa* I RFLP of apolipoprotein C-I increases gene transcription and exhibits an ethnically distinct pattern of linkage disequilibrium with the alleles of apolipoprotein E.** ***J. Lipid Res.*** **1999.** 40: **50–58.**

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Apolipoprotein (apo) C-I is a constituent of triglyceride-rich lipoproteins (TGRL) and high density lipoproteins (HDL) whose importance in plasma lipoprotein metabolism is increasingly evident. ApoC-I displaces apoE from triglyceride-rich emulsions and lipoproteins and interferes with their hepatic clearance (1, 2). ApoC-I decreases binding of β-very low density lipoproteins (VLDL), a model of post-lipolysis lipoprotein remnants, to a remnant receptor, the low density lipoprotein (LDL) receptor-related protein (LRP) (3, 4). ApoC-I has also been shown to decrease the apoE-mediated binding of human VLDL and intermediate density lipoproteins (IDL) to the LDL receptor, an effect that may be independent of apoE displacement (5, 6).

Elevated plasma cholesterol and triglyceride levels in human apoC-I transgenic (HuCI Tg) mice were initially reported by Simonet et al. (7) in a study establishing the role of DNA sequences 5′ to the apoC-I′ pseudogene in the liver-specific expression of apoC-I and apoE. We performed a detailed physiological characterization of HuCI Tg mice generated using a DNA construction of the apoC-I gene and the core 154 bp liver-specific enhancer (8, 9). Similar mice have been reported by others (10). HuCI Tg mice exhibit combined hyperlipidemia with cholesterol enrichment of VLDL and IDL + LDL, raising the question of whether variation in apoC-I expression might have a clinically important effect on lipoprotein metabolism.

Abbreviations: apo, apolipoprotein; β-VLDL, β-migrating very low density lipoproteins; LRP, low density lipoprotein receptor-related protein; HuC-I, human apoC-I; Tg, transgenic; TG, triglycerides; TGRL, TG-rich lipoproteins; H1, *Hpa* I-site negative apoC-I gene allele; H2, *Hpa* I-site positive apoC-I gene allele.

[1]To whom correspondence should be addressed.

Very little information is available on apoC-I genetic variation in humans. An *Hpa* I polymorphism was localized to a site 317-bp 5′ to the apoC-I transcription initiation site (11). However, the *Hpa* I-negative sequence was not reported. In one population-based genetic association study, this polymorphism was associated with disordered lipoprotein metabolism. Homozygosity for the minor allele (H2) of the *Hpa* I RFLP was reported in 97% of apoE $\varepsilon 2/\varepsilon 2$ allelic individuals with familial dysbetalipoproteinemia, a disorder characterized by decreased lipoprotein remnant clearance. In contrast, 17% of normal controls and 22% of $\varepsilon 2/\varepsilon 2$ individuals without hyperlipidemia were reportedly homozygous for H2 (12). However, this association with familial dysbetalipoproteinemia was not confirmed in subsequent work by the same investigators, leaving its role uncertain (13). In the current study, we have determined the DNA sequence basis of the *Hpa* I polymorphism and defined its association with apoE allele status and lipoprotein phenotype in both European-Americans and African-Americans. In addition, we have characterized the effect of *Hpa* I allele status on apoC-I gene expression using a reporter-gene assay. We have localized the source of the observed difference in gene expression and have observed allele-specific differences in the binding of nuclear proteins, as seen in an electrophoretic mobility shift assay (EMSA). We have also determined the transcriptional effect of this DNA region by successive deletion and have identified the molecular weight of a nuclear factor that binds the region specifically.

METHODS

Study population

Data were analyzed from healthy individuals without dementia who were part of a random sample of Medicare recipients (14). Subjects lived within three contiguous ZIP codes in the community of Washington Heights in northern New York City. The Columbia University institutional review board reviewed and approved this project.

DNA samples were extracted from buffy coat and frozen at −20°C. Only individuals that could be unambiguously assigned as African-American or European-American were analyzed. All apoE $\varepsilon 2/\varepsilon 2$, $\varepsilon 2/\varepsilon 4$, and $\varepsilon 4/\varepsilon 4$ individuals were genotyped for apoC-I, along with a randomized selection from the other genotypes based on a statistical power analysis. Altogether 414 samples were genotyped for apoC-I. All lipid profiles were obtained on fresh plasma at the time of initial phlebotomy in the same CDC-certified Lipid Research Clinic Core Laboratory. Plasma levels of triglycerides and cholesterol were determined using Boehringer Mannheim enzymatic reagent kits on a Hitachi autoanalyzer model 705. HDL cholesterol levels were analyzed after precipitation of apoB-containing lipoproteins with phosphotungstic acid. LDL cholesterol levels were calculated using the Friedewald formula. ApoA-I and apoB levels were determined on simultaneously drawn serum samples frozen at −70°C, using commercially available immunoturbidometric reagents on a Beckman Array 360 nephelometer.

ApoE and apoC-I genotyping

Genotype for the $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$ alleles of apoE was determined using a described technique (15). Genotypic status for the *Hpa* I apoC-I promoter polymorphism was determined by two-step nested polymerase chain reaction (PCR) followed by restriction digestion with *Hpa* I and electrophoresis on 1.5% agarose (ultraPure Agarose, Life Technologies, Gaithersburg, MD). The sequence of the upstream external primer was GCATGCAGCC CCCAGTCACGCATCCCCTGC and began at −492 relative to the apoC-I gene start site (16). The downstream external primer was GAATCCTTGCTGGAGGGCTTGGTTGGGAGGT and corresponded to the first exon. The upstream internal primer was TTGTTCAATCGATCACGACCCTCTCACGT. The downstream internal primer was GCAGGACCTTTATCAGGCTGGGGAGC.

Statistical analysis

The results of transfection experiments were compared by *t*-test. The issue of admixture in the population study was addressed by stratification (European-American and African-American). There was a strong association, in both racial backgrounds, of the $\varepsilon 2$ allele with H2 and the $\varepsilon 3$ allele with H1 that was evident in the apoE allelic homozygotes. In light of this, the allelic assignment of the apoC-I *Hpa* I genotype was necessarily highly accurate in the apoE heterozygotes ($\varepsilon 2/\varepsilon 3$, $\varepsilon 2/\varepsilon 4$, and $\varepsilon 3/\varepsilon 4$). Misclassification would require that the rarer apoC-I allele be present in association with both of the apoE alleles, an event too infrequent to affect our conclusions. Genotype data from apoE heterozygotes were therefore combined with the data from homozygotes and presented as haplotypes. Ethnic differences in the association of apoE and apoC-I alleles were studied by constructing frequency tables with ethnicity as one dimension and the number of H2 alleles as the other dimension. The tables were constructed separately for each of the three apoE alleles. Two-tailed statistical significance was determined by the Fisher exact test or a chi-squared test, as appropriate. Because of the different frequencies of the H2 allele in the different ethnic groups and in different apoE genotypes, lipids and lipoproteins were analyzed separately for each ethnic group/apoE genotype combination. Comparisons were made between apoC-I genotypes in each combination by a two-tailed *t*-test. Sufficient numbers and apoC-I allelic variation to permit analysis were present only in apoE $\varepsilon 3/\varepsilon 3$ and apoE $\varepsilon 3/\varepsilon 4$. Due to skewing, the triglyceride data were log-transformed prior to statistical analysis. Means and standard deviations are based on the antilog of the log transformed values and are reported as means ± coefficients of variation expressed as percentages of the means.

Gene expression test constructions

ApoE $\varepsilon 2$ and $\varepsilon 3$ allelic cosmids with and without, respectively, an *Hpa* I site 5′ to the apoC-I gene were the generous gift from Marten H. Hofker (University of Leiden, The Netherlands). Both cosmids contained the complete apoC-I gene and downstream regulatory sequences. A series of plasmids was generated from these cosmids (see Fig. 1). These, at first, utilized DNA elements specific to each cosmid to regulate the expression of an inserted luciferase reporter gene and subsequently involved exchanges of genetic material from the two cosmids.

A 719 bp *Sac* I fragment within both cosmids extends from −380 relative to the start of apoC-I gene transcription to a site within the second intron of the apoC-I gene (11). PCR primers were generated to the area of the 5′ *Sac* I site and to the second exon of the apoC-I gene. Sequences were introduced into the downstream primer that created a *Not* I site and a *Sac* I site immediately prior to the ATG translation initiation codon. Ten cycles of PCR were performed on both cosmids with these primers and both PCR products were cloned into the *Sac* I site of pUC 18. The modified firefly luciferase gene from pGL3 (Promega, Madison, WI) was then cloned into the *Not* I site of both plasmids. The apoC-I gene segments of both DNA constructions were sequenced

to verify the absence of PCR artifacts. The *Hpa* I-negative (apoE3 cosmid origin) and *Hpa* I-positive (apoE2 cosmid origin) DNA constructions were designated pH1 and pH2, respectively.

*Sal*I–*Bam*HI fragments bracketing the apoC-I gene (from 3 kb 5′ to 2 kb 3′ to the gene) were then cloned into a modified version of pUC 18 from which the *Sac* I site had been eliminated. These plasmids were digested with *Sac* I and the *Sac* I inserts from pH1 and pH2, containing the apoC-I promoter and inserted luciferase gene, were ligated, replacing the native *Sac* I fragments. These plasmids were designated pH1long and pH2long. Still larger DNA constructions were made via the insertion into the *Bam*H I sites of pH1long and pH2long of the contiguous 3′ 5.5 kb *Bam*H I fragments that contained the apoC-I liver-specific enhancer. The latter constructions permitted the screening of a 16-kb region of DNA for allele-specific sequences affecting gene transcription. These plasmids were designated pH1long-enh and pH2long-enh. Plasmids pH1long-H2enh and pH2long-H1enh were produced by exchanging the *Bam*H I enhancer fragments from the two genetic backgrounds. Plasmids pH1-H1enh and pH2-H1enh utilized the initial reporter gene constructions, containing only 376 (380) bp of upstream sequence joined to the same (apoE3, apoC-I H1-allelic) enhancer sequence.

Successive 5′ deletions of plasmid pH1 were performed using Exonuclease III and Mung Bean Nuclease (Exo-Size™ Deletion Kit, New England Biolabs, Beverly, MA). Plasmids were sequenced to confirm the 5′ termini and the absence of extraneous deletions. The original −376 plasmid was compared with plasmids with 5′ termini at −324, −293, −234, −134, and +1 relative to the apoC-I gene transcriptional start site.

Cell culture

HepG2 cells were purchased from American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Life Cell Culture Technologies, Inc.) supplemented with 10% (v/v) fetal calf serum (FCS, Life Technologies, Inc.), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. They were incubated in 6-well tissue culture plates in a humidified air/CO_2 (19:1) incubator at 37°C.

Transient transfection and gene transcription assays

All transfections were performed at least nine times with at least three independently isolated plasmid preparations. Plasmids were prepared using Qiagen kits (Qiagen, Chatsworth, CA) and were transfected into HepG2 cells using Lipofectin (Life Technologies, Gaithersburg, MD). Twenty-four hours before transfection, cells were seeded at a density of 10^5 cells/well in 6-well plates and maintained in DMEM with 10% FCS and antibiotics. Transfection was performed 24 h later when the cells were approximately 50% confluent. One hundred μl of DMEM containing 0.54 pmol of apoC-I/luciferase DNA construction and 0.157 pmol (0.75 μg) of the pCMVβ β-galactosidase-expressing plasmid (Promega) was mixed with 100 μl of DMEM containing 10 μg of Lipofectin reagent (Life Technologies). The Lipofectin-DNA complex was incubated at room temperature for 15 min, during which time the cells were washed twice with serum-free medium. Eight hundred μl of DMEM was then added to the Lipofectin-DNA complex and the solution was pipetted into one well. At 5 h post-transfection, the Lipofectin-DNA complex was aspirated and replaced with DMEM containing FCS and antibiotics. Cell extract was prepared 48 h later and β-galactosidase and luciferase assays were performed using kits (β-Galactosidase Enzyme Assay with Reporter Lysis Buffer and Luciferase Assay System, Promega, Madison, WI). A Lumat LB 9501 luminometer was used (Wallac, Inc., Gaithersburg, MD). The results of the luciferase assay were normalized for β-galactosidase activity as an index of transfection efficiency.

Nuclear extract preparation

HepG2 cells were grown to confluence, and nuclear extracts were prepared as described (17). All buffers were supplemented with 2.5 μg/ml leupeptin and 1 mm DTT. Protein concentrations in the extracts were determined by the BCA protein assay (Pierce Chemical Co., Rockford IL).

Electrophoretic mobility shift assay

For the *Hpa* I-positive (H2) allele, a 34-bp double-stranded oligodeoxynucleotide was used that included 15 bases on either side of the 4-bp (CGTT) insertion, which we determined was specific to this allele. The top strand of this oligo was ATAAACCC CTTCCTTCGTTAACTCAGCGTCTGAG. For the *Hpa* I-negative (H1) allele a 30-bp double-stranded oligodeoxynucleotide was used that included the same bases minus the 4-bp (CGTT) insertion. The sequence of the top strand of this oligo was ATAAAC CCCTTCCTTAACTCAGCGTCTGAG. Probe labeling, association of extract with probe, non-denaturing polyacrylamide gel electrophoresis (PAGE) and autoradiography were performed essentially as described (18). The specific activities of the probes were essentially identical. One μl (2.5 μg) of nuclear extract was preincubated on ice for 10 min in a solution composed of 0.2 μl of 50 mm DTT, 1.5 μl of reaction buffer (0.1 m HEPES, pH 7.9, 20% Ficoll, 0.3 m KCl) and 2 μl of 1 mg/ml poly(dI-dC). A probe (10,000 counts/min), with or without unlabeled oligodeoxynucleotide competitors in 100-fold molar excess, was then added in a volume of 3.3 μl and incubated for 20 min at room temperature. The unbound free probe was separated from the DNA-protein complexes by electrophoresis for 2 h at 25 mA through a 7% nondenaturing polyacrylamide gel (acrylamide–bis-acrylamide 29:1) in 0.25× TBE buffer. The gels were then exposed to Dupont Reflection film at −70°C for 3 days.

Southwestern blot

We performed Southwestern blotting, as described, to provide further information on the molecular weight and binding specificity of the DNA binding protein(s) (19). Total nuclear proteins were separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane using standard Western blotting techniques. The blot was then incubated with the H1-sequence double-stranded oligodeoxynucleotide probe.

RESULTS

ApoE and apoC-I polymorphism in human subjects: ethnic differences in association of apoE and apoC-I alleles

In order to assess the presence of any independent functional effect of the polymorphism, we first determined the association of the apoC-I polymorphism with the variants of the linked apoE gene, which have known effects on plasma lipoprotein levels. We performed genotyping for *Hpa* I allele status in a multiethnic population of elderly individuals of known apoE genotype (14). The lipoprotein profile, apoB and apoA-I levels were also determined. The genetic diversity of the population was dealt with by stratification and only individuals who could be classified as African-American or European-American were analyzed. An association of apoC-I allele status with apoE allele status was observed in the entire sample and for both ethnic origins, as shown in **Table 1**. Examination

TABLE 1. ApoC-I allele status by apoE genotype and ethnicity

ApoE Ethnicity	ApoC-I Genotype H1/H1	H1/H2	H2/H2	Total
ε2/ε2				
European-American	0	2 (25%)	6 (75%)	8
African-American	0	0	2 (100%)	2
Total	0	2 (20%)	8 (80%)	10
ε2/ε4				
European-American	0	1 (50%)	1 (50%)	2
African-American	0	8 (40%)	12 (60%)	20
Total	0	9 (41%)	13 (59%)	22
ε2/ε3				
European-American	0	22 (100%)	0	22
African-American	0	15 (94%)	1 (6%)	16
Total	0	37 (97%)	1 (3%)	38
ε3/ε3				
European-American	110 (96%)	5 (4%)	0	115
African-American	70 (83%)	13 (15%)	1 (1%)	84
Total	180 (90%)	18 (9%)	1 (1%)	199
ε3/ε4				
European-American	8 (14%)	49 (84%)	1 (2%)	58
African-American	32 (47%)	32 (47%)	4 (6%)	68
Total	40 (32%)	81 (64%)	5 (4%)	126
ε4/ε4				
European-American	0	1 (33%)	2 (67%)	3
African-American	0	14 (87%)	2 (13%)	16
Total	0	15 (79%)	4 (21%)	19

of apoE allelic homozygotes revealed a strong, though not absolute, association of apoE ε3/ε3 with H1 and of apoE ε2/ε2 and, to a lesser extent, ε4/ε4 with H2. Breakdown by ethnic origin revealed that the associations, for both apoE ε3/ε3 and apoE ε4/ε4, were weaker in African-Americans than in European-Americans. Nevertheless, in all ethnic backgrounds, the extent of linkage disequilibrium between the apoE and apoC-I alleles was sufficient to allow us to calculate haplotypes, the relative chromosomal assignment of the alleles of apoE and apoC-I, with a high degree of certainty and thereby include the data from apoE allelic heterozygotes. These haplotypes are shown in **Table 2**, broken down by ethnic origin, and are presented along with statistical comparisons of the extent of association of the apoE and apoC-I alleles. In summary, apoE ε2 was strongly associated with apoC-I *Hpa* I-positive (H2). Of 78 apoE ε2 alleles evaluated (38 in European-Americans and 40 in African-Americans) only two were associated with apoC-I H1, both in European-Americans. The apoE ε4 allele was also associated with apoC-I H2, but to a lesser extent, and the strength of this association was strongly influenced by ethnic background. Of 186 apoE ε4 alleles, 122 (66%) were H2 and 64 (34%) were H1. However, in European-Americans, 10 of 66 alleles (15%) were H1 while in African-Americans 54 of 120 alleles (45%) were H1. The allele frequency in European-Americans versus African-Americans was significantly different at $P < 0.001$.

The apoE ε3 allele, in contrast, was strongly associated with apoC-I H1. A significant ethnic difference was present in the strength of this association as well. Only 6 of the 310 apoE ε3 alleles (2%) in European-Americans were of apoC-I H2 genotype, while 20 of 252 alleles (8%) in African-Americans were apoC-I H2. These differences were significant for European-Americans versus African-Americans at $P < 0.001$.

TABLE 2. ApoE/ApoC-I haplotypes by ethnicity

ApoE Ethnicity	ApoC-I H1	H2	Total	E vs. A[a]
ε2				
European-American	2 (5%)	38 (95%)	40	
African-American	0	40 (100%)	40	
Total	2 (3%)	78 (97%)	80	ns
ε3				
European-American	304 (98%)	6 (2%)	310	
African-American	232 (92%)	20 (8%)	252	$P < 0.001$
Total	536 (95%)	26 (5%)	562	
ε4				
European-American	10 (15%)	56 (85%)	66	
African-American	54 (45%)	66 (55%)	120	$P < 0.0001$
Total	64 (34%)	122 (66%)	186	

[a]E, European-American; A, African-American.

Effect of apoC-I polymorphism on lipoproteins

To attempt to evaluate the independent effects of the apoC-I polymorphism on clinical lipid parameters, the effect of apoC-I *Hpa* I allele status was assessed on a variety of lipoprotein and apolipoprotein measurements. Because of the small number of males (22% of the sample) the analysis was repeated with the exclusion of males and the conclusions remained unchanged. The number of individuals in the apoE ε2/ε2, ε2/ε4, and ε4/ε4 groups and the degree of variation in the apoE ε2/ε3 group (24/25 were H1/H2) were too limited to allow comparison (Table 1). No effect of apoC-I allele status on lipoproteins was evident in the apoE ε3/ε4 context (**Table 3**).

In the apoE ε3/ε3 genotype, the largest group, statistically significant effects of the apoC-I polymorphism on plasma lipoproteins were noted in African-Americans (**Table 4**). Mean triglycerides were 116 mg/dl ± 22% in African-American H2 carriers (n = 14) versus 144 mg/dl ± 36% in the H1 homozygotes (n = 70, $P = 0.03$). HDL-cholesterol measurements reflected the differences in triglycerides and were 62 ± 25 mg/dl in H2 carriers versus 51 ± 14 mg/dl in H1 homozygotes ($P = 0.02$). ApoA-I,

TABLE 3. Lipoprotein and apolipoprotein levels in apoE ε3/ε4 by ethnic background and apoC-I genotype

	European-American H1/H1 (n = 8)	H1/H2, H2/H2 (n = 50)	African-American H1/H1 (n = 31)	H1/H2, H2/H2 (n = 36)
	mg/dl ± %		*mg/dl ± %*	
Cholesterol	200 ± 39	207 ± 35	205 ± 45	207 ± 44
Triglycerides	194 ± 55	169 ± 44	136 ± 44	156 ± 36
HDL cholesterol	51 ± 15	45 ± 16	50 ± 14	49 ± 10
LDL cholesterol	111 ± 29	124 ± 30	125 ± 40	125 ± 41
	(n = 4)	(n = 17)	(n = 12)	(n = 20)
ApoB	113 ± 26	122 ± 29	114 ± 28	125 ± 44
ApoA-I	193 ± 27	150 ± 36	166 ± 28	155 ± 18

TABLE 4. Lipoprotein and apolipoprotein levels in apoE ε3/ε3 by ethnic background and apoC-I genotype

	European-American		African-American	
	H1/H1 (n = 110)	H1/H2, H2/H2 (n = 4)	H1/H1 (n = 70)	H1/H2, H2/H2 (n = 14)
	mg/dl ± %		*mg/dl ± %*	
Cholesterol	216 ± 35	198 ± 32	206 ± 34	199 ± 51
Triglycerides	174 ± 42	186 ± 27	144 ± 36	116 ± 22[a]
HDL cholesterol	47 ± 15	41 ± 9	51 ± 14	62 ± 25[a]
LDL cholesterol	131 ± 30	118 ± 36	125 ± 31	113 ± 36
	(n = 49)	(n = 4)	(n = 38)	(n = 10)
ApoB	125 ± 28	114 ± 20	119 ± 19	94 ± 26[b]
ApoA-I	147 ± 23	136 ± 15	149 ± 31	150 ± 17

[a] $P < 0.05$; [b] $P < 0.01$.

the principal structural protein of HDL, was unchanged. ApoB, the structural protein of LDL, was lower, 94 ± 26 mg/dl in H2 carriers versus 119 ± 19 mg/dl in H1 homozygotes ($P < 0.002$). The LDL cholesterol was 113 ± 36 mg/dl in the H2 carriers versus 125 ± 31 in H1 homozygotes ($P = 0.21$).

ApoC-I promoter reporter-gene assays

DNA constructions are shown in **Fig. 1**. ApoCI H1 (*Hpa* I-negative) and H2 (*Hpa* I-positive) allele-specific DNA constructions were made with 376 bp (380 bp) of promoter sequence driving a luciferase reporter gene inserted into exon 2. These were termed pH1 and pH2. Larger constructions (pH1long and pH2long) contained 3 kb of sequence 5′ and 2.5 kb 3′ to apoC-I. The largest (16-kb) DNA constructions (pH1long-enh and pH2long-enh) extended 5.5 kb farther 3′ and included the liver-specific enhancer. These allowed us to screen 16 kb of allele-specific DNA for differential effects on transcription. In **Table 5** we show the results of simultaneous transfections of constant molar amounts of all six DNA constructions, permitting the comparison of constructions of different length. The pHlong constructions expressed at slightly above the level of the pH constructions. The pHlong-enh constructions showed an over 3-fold increase in expression due to the effects of the enhancer. The effect of construction length on gene expression was highly statistically significant for both the H1 and the H2 constructions. Comparisons of gene expression by allele are shown in **Table 6**. The H2-allelic DNA constructions consistently showed significantly higher levels of gene expression. The relative increase was greater with the pH2long-enh construction (vs. pH1long-enh), which contained the liver-specific enhancer, than with the shorter DNA constructions. However, full-length DNA constructions with the 5.5-kb enhancer regions exchanged (pH1 long-H2enh and pH2long-H1enh) indicated that the effect did not segregate with the enhancer fragment (Table 6). Constructions made with the small (380 bp upstream) promoter fragments joined to the identical (H1-allelic) liver-specific enhancer (pH1-H1enh and pH2-H1enh) similarly showed increased expression of the H2-allele apoC-I promoter. The DNA sequence of the two 380-bp promoter fragments was determined and differed only by the four bp CGTT insertion which creates the *Hpa* I site, indicating that the presence of these four bp actually produced the gene expression change.

ApoC-I promoter deletion studies

Progressive deletion of the 5′ region of the pH1 construction was performed to yield DNA constructions terminating at −324, −293, −234, −134, and +1 relative to the apoC-I gene transcriptional start site (**Fig. 2**). The −324 construction, which deleted the *Hpa* I-negative allele to within a few bases of the *Hpa* I insertion site (−317), produced a significant increase in gene transcription. Complete deletion of the HpaI site (−293) produced an additional increase in gene transcription. No further change was observed with deletion to −234, while continued deletion produced decreasing gene transcription. These studies strongly support the presence of a negatively acting transcriptional regulatory sequence in the region of −324 to −293, the location of the polymorphic site.

Sequence-specific binding of nuclear proteins

To evaluate the mechanism of action of the polymorphism, we performed a DNA electrophoretic mobility shift assay (EMSA) and observed differences in the binding of proteins from HepG2 cell nuclear extract (NE) to the region of the four bp insertion (**Fig. 3**). Double-stranded DNA probes to the region produced several bands not competed by poly-dIdC. Two bands were consistently observed with the H1 probe. These were not competed by a 100-fold excess of nonspecific oligodeoxynucleotide competitor. The bands were progressively competed by increasing concentrations of specific competitor, unlabeled oligodeoxynucleotide of the same sequence as the probe. In contrast, no residual specific bands were seen with the H2 probe in the presence of excess nonspecific competitor. These results suggest that the 4-bp insertion that creates the H2 sequence may increase transcription by destroying a binding site for a negatively acting transcription factor, resulting in a net positive effect on gene transcription.

Southwestern blot

We performed Southwestern blotting to provide further information on the molecular weight and binding specificity of the DNA-binding protein(s). HepG2 cell nuclear protein extract was fractionated by SDS-PAGE and probed with the H1-sequence double-stranded oligodeoxynucleotide. This technique identified a single protein band (approximate molecular mass 80 kD) that exhibited specific binding to the H1 probe (**Fig. 4**). One hundred-fold excess of scrambled-sequence double-stranded oligo produced minimal diminution in band intensity. In contrast, the same amount of unlabeled specific competitor virtually eliminated the band. Of note, a fainter nonspecific band was unchanged.

DISCUSSION

We observed an ethnically distinct pattern of linkage disequilibrium between the alleles of apoE and the *Hpa* I

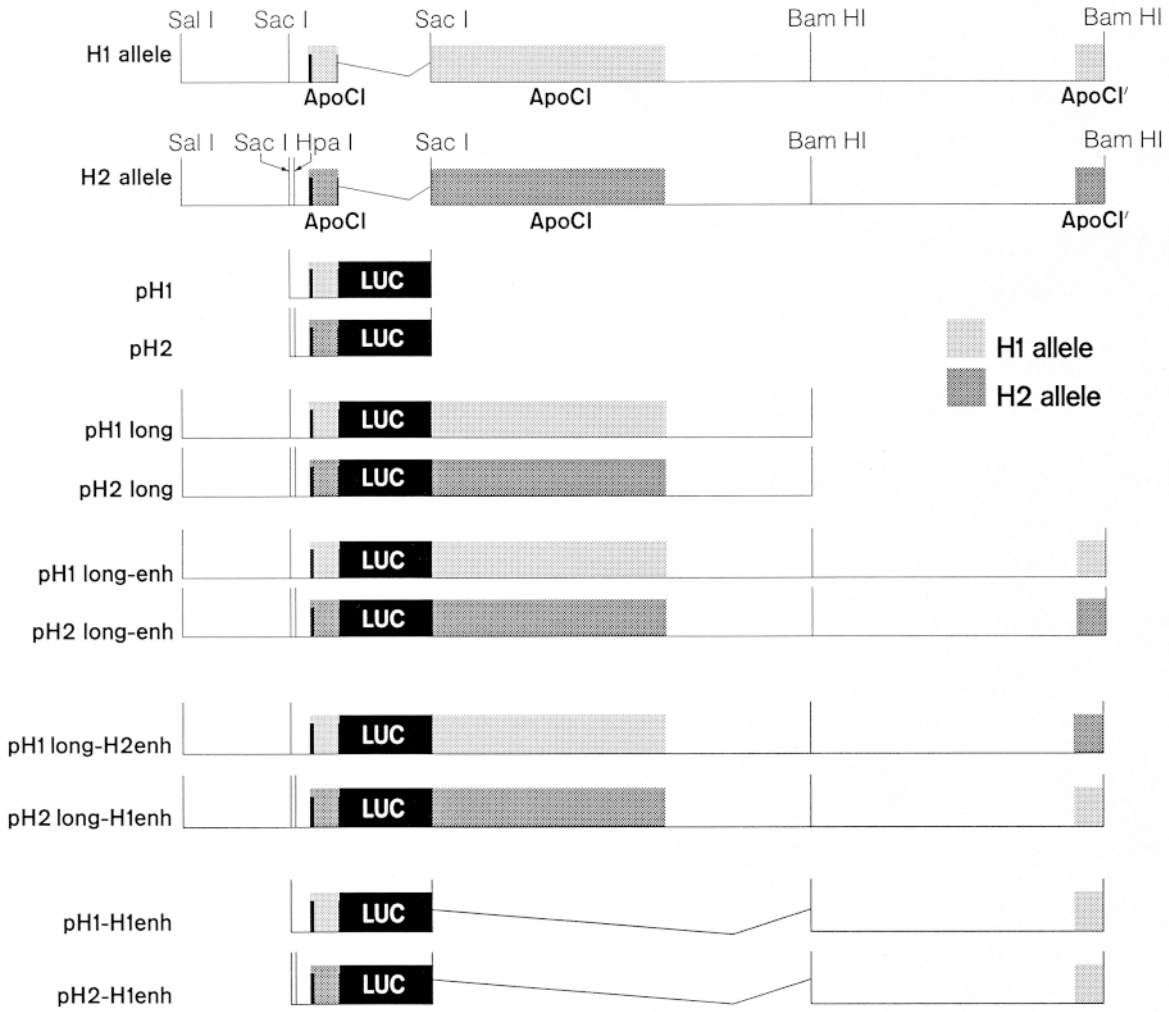

Fig. 1. DNA constructions of regulatory elements involved in apoC-I gene expression. The pH1 (*Hpa* I-negative) and pH2 (*Hpa* I-positive) allele-specific DNA constructions were made with 376 (380) bp of 5′ flanking sequence driving a luciferase reporter gene inserted into exon 2 prior to the apoC-I translation initiation codon (ATG). Larger constructions were based on the pH1 and pH2 constructions and contained the entire apoC-I gene along with 3 kb of 5′ flanking sequence and 2.5 kb of sequence 3′ to the apoC-I gene. These were termed PH1long and PH2long. These showed luciferase expression at levels only slightly above pH1 and pH2. A modest allele-specific increase in expression of the apoE2-background, H2 allele was present in both sets of constructions. Luciferase activity more than tripled in the largest DNA constructions. These extended 5.5 kb farther 3′ and included the liver-specific enhancer. They were termed pH1long-enh and pH2long-enh. The pH2long-enh construction showed a more marked increase and exhibited 1.5-fold more activity than the pH1long-enh construction ($P < 0.0005$). Data are shown in Table 5. Full-length (16 kb) DNA constructions with the 5.5-kb enhancer regions exchanged were termed pH1long-H2enh and pH2long-H1enh. Constructions were also made with the small pH1 and pH2 constructions joined to the same (H1-allelic) liver enhancer fragment. These were termed pH1-H1enh and pH2-H1enh. These, similarly, showed increased expression of the H2-allelic apoC-I 5′ flanking sequences. These expression data are shown at the bottom of Table 6.

TABLE 5. Promoter activity, comparisons by length

Construction	Activity	n	pH vs. pHlong	pH vs. pHlong-enh	pHlong vs. pHlong-enh
pH1	77.6 ± 28.4	15	$P < 0.01$		
pH2	99.6 ± 15.5	15	$P < 0.003$		
PH1long	100	15			$P < 0.0003$
PH2long	132 ± 37	15			$P < 0.001$
PH1long-enh	315.5 ± 181.8	15		$P < 0.0001$	
pH2long-enh	495.3 ± 322	15		$P < 0.0003$	

A description of the DNA constructions appears in the legend to Figure 1. A constant molar amount of each plasmid was transfected. Comparisons are of β-galactosidase-normalized luciferase activity relative to pH1long, which was normalized to 100 for each transfection; n, refers to the number of replicates of each transfection.

(H1 and H2) alleles of apoC-I. In European-Americans, allele frequencies of the apoC-I polymorphism were similar to those found in a Dutch population, with strong associations of apoE ε3 with H1 and of apoE ε2 and ε4 with H2 (13). In African-Americans, this association was significantly weaker for both apoE ε3 and apoE ε4.

The lesser degree of linkage disequilibrium between the alleles of apoE and apoC-I, which was found in African-Americans, permitted us to observe independent effects of the apoC-I polymorphism. The presence of the H2 allele was associated with lower fasting plasma triglyceride and apoB levels and higher HDL levels in apoE ε3/ε3-genotype individuals, though the number of such indi-

TABLE 6. Promoter activity, comparisons by allele

Construction	Activity	n	H1 vs. H2
pH1	100	18	
pH2	127.7 ± 29.1	18	$P < 0.001$
pH1long	100	18	
pH2long	131.0 ± 33.9	18	$P < 0.002$
pH1long-enh	100	48	
pH2long-enh	153.5 ± 43.0	48	$P < 0.000001$
pH1long-H2enh	100	12	
pH2long-H1enh	142.9 ± 15.7	12	$P < 0.000001$
pH1-H1enh	100	9	
pH2-H1enh	187.3 ± 45.4	9	$P < 0.0001$

DNA constructions are described in Fig. 1. A constant mass was transfected for each allelic pair. Comparisons are of β-galactosidase-normalized luciferase activity of the H2 apoC-I DNA construction relative to the corresponding H1 construction. The H1 allele activity was normalized to 100 for each transfection.

viduals was not large. The effect on apoB is consistent with the known effect of apoC-I to decrease remnant clearance and appears analogous to apoE2, which also decreases remnant clearance and produces lower LDL and apoB levels (20–23). The mechanism of the lowered LDL and apoB associated with apoE2 is controversial, but may reflect decreased partitioning of dietary triglycerides to the liver with subsequent decreased secretion of VLDL, the precursor of LDL. In contrast, the lowering of plasma triglycerides by the overexpressing H2 allele in the apoE ε3/ε3 context, which is not described in apoE ε2/ε2 individuals, was unanticipated and may be due to chance. However, a different analogy to apoE2 may be instructive. Modest degrees of expression of apoE2 in transgenic mice lead to markedly lowered plasma cholesterol and triglycerides, apparently due to decreased VLDL secretion (24). Higher levels of expression result in

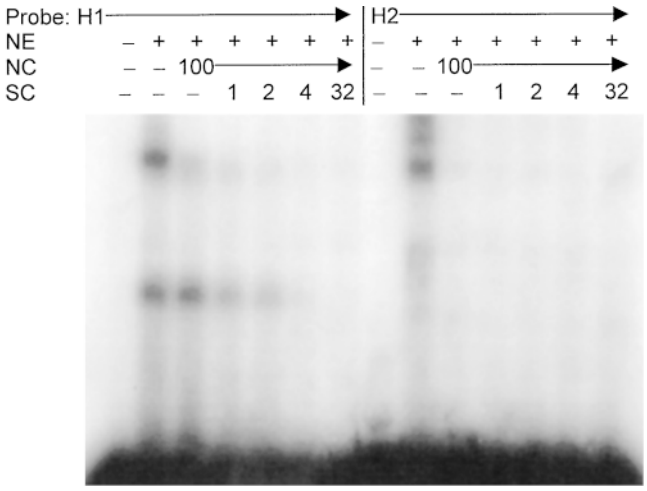

Fig. 3. Electrophoretic mobility shift assay using double-stranded oligodeoxynucleotides. A 34-bp oligo was used at the right of the figure and included 15 bp on either side of the 4-bp CGTT insertion found in the H2-allelic sequence. A 30-bp oligo lacking the 4-bp insertion was used at the left of the figure and corresponded to the H1-allelic sequence. NE indicates HepG2 cell nuclear extract. Two bands were consistently observed with the 30-bp Hpa I-negative probe (H1) in the presence of nuclear extract. These were not competed by a 100-fold excess of unlabeled scrambled sequence oligodeoxynucleotide (nonspecific competitor, NC) but were competed by unlabeled oligodeoxynucleotide of the same sequence as the probe (specific competitor, SC, in 1-, 2-, 4-, and 32-fold excess). In contrast, no bands were seen with the 34-bp Hpa I-positive probe (H2) in the presence of excess nonspecific competitor.

hypertriglyceridemia via the expected decrease in remnant clearance. Modest levels of expression of apoC-I may, similarly, lead to decreased hepatic secretion of VLDL triglycerides and apoB due to the decreased partitioning

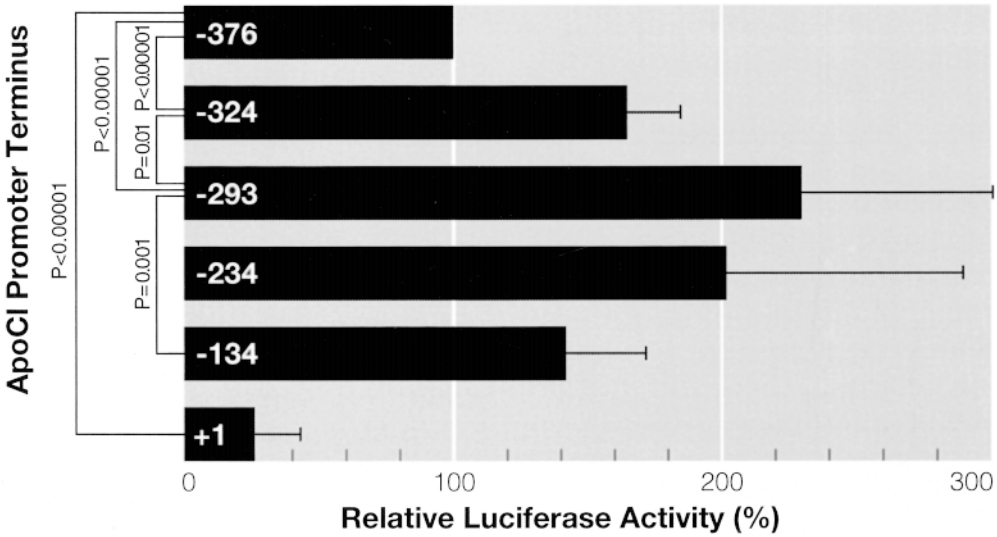

Fig. 2. ApoC-I promoter deletion studies. The original –376 H1-allelic plasmid was compared to deleted plasmids with 5′ termini at −324, −293, −234, −134, and +1 relative to the apoC-I gene transcriptional start site. Equal numbers of plates of each plasmid were transfected at a time. Results are presented as means ± SD normalized to the −376 plasmid (=100). *P* values comparing the promoter activities of the DNA constructions are indicated. There is evidence for a strong negatively acting element in the area of the polymorphic region (−317); deletions in this area produce a highly significant positive effect on transcription.

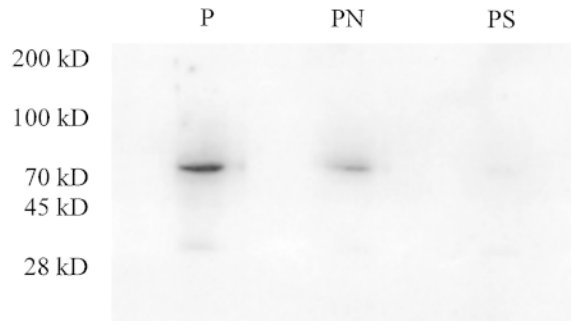

Fig. 4. Southwestern blot. Three identically loaded lanes of HepG2 cell nuclear extract, separated by SDS-PAGE and transferred to a nitrocellulose membrane, are shown. The blot was cut and each lane was separately incubated with either H1 probe alone (P), probe + 100× nonspecific DNA competitor (PN), or probe + 100× specific competitor (PS). While a lower MW nonspecific band appears unchanged in lane PS, the higher MW (specific) band is markedly diminished.

of dietary triglycerides to the liver related to delayed remnant clearance.

We did not see an effect of the H2 allele on plasma lipid levels in the apoE ε4 context, diminishing our confidence in this putative mechanism. However, this may be due to the biochemical differences between the apoE3 and apoE4 proteins. While apoE4 exhibits normal (similar to apoE3) binding to the LDL receptor (25), apoE4 exhibits increased affinity for triglyceride-rich lipoproteins (TGRL) versus HDL and is catabolized twice as fast as apoE3 when injected into apoE ε3/ε3 individuals (26). When apoE on TGRL is limiting, as in the apoE ε2 and apoE ε3 contexts, the effects of increased apoC-I on the partitioning of dietary triglycerides to the liver might be greater and lead to significantly decreased VLDL secretion that would be reflected in lower fasting lipid levels.

Due to the close linkage of the chromosome 19 apolipoprotein gene cluster, the actual DNA sequences responsible for the lipoprotein phenotype associated with the apoC-I *Hpa* I RFLP cannot be established through purely genetic means. However, the mechanism of the apoC-I *Hpa* I RFLP was strongly suggested by in vitro gene-expression studies. A highly significant 1.5-fold increase in apoC-I gene transcription was observed in a reporter-gene assay in association with the 4-bp insertion that creates the *Hpa* I site at −317 relative to the start of apoC-I gene transcription (the H2 allele). The use of recombinant DNA constructions containing DNA from both allelic backgrounds allowed us to localize the source of this difference in transcription to the actual polymorphism itself. The apoC-I promoter deletion studies have shown that this region of the H1-allelic sequence does have a negative effect on transcription. Based on mobility shift data, the H2-allelic sequence exhibits decreased binding of a nuclear protein that is specifically bound by the H1-allelic sequence. Therefore, the H2-allelic insertion may disrupt the binding of a negatively acting transcriptional factor to produce a positive effect on transcription. A Southwestern blot confirmed the specific binding of a single 80 kD nuclear protein to the H1 sequence. Further investigation of the molecular biology of this protein is warranted.

The ε4 allele of apolipoprotein E is one of very few common decreased-longevity alleles that have been described. Interestingly, the predictive value of apoE ε4 allele status is lower in African-Americans (27) who, we now show, also exhibit a lesser degree of linkage disequilibrium between the alleles of apoE and apoC-I. The effects of the polymorphism on lipoprotein parameters remain hypothetical and will require confirmation in larger populations. However, further definition of the genetics, regulation, and function of apoC-I may produce insights relevant to the pathogenesis of both coronary heart disease and Alzheimer's disease.

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