

Intestinal Transcription and Synthesis of Apolipoprotein AI Is Regulated by Five Natural Polymorphisms Upstream of the Apolipoprotein CIII Gene

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Abstract

To understand the factors contributing to the synthesis of human apolipoprotein AI (apoAI), relative apoAI synthesis was measured from endoscopic biopsy samples obtained from 18 healthy volunteers. The relative amount of apoAI synthesis was directly correlated with steady state intestinal apoAI mRNA levels and a 10-fold within-group variability was observed. Analysis of genomic DNA from the subjects revealed five polymorphic sites which defined two haplotypes in the intestinal enhancer region of the apoAI gene located upstream of the apolipoprotein CIII gene transcriptional start site (+1): (−641 C to A, −630 G to A, −625 T to deletion, −482 C to T, and −455 T to C). The population frequencies of the wild-type and mutant alleles were 0.53 and 0.44, respectively. Mean steady state apoAI mRNA levels and mean relative apoAI synthesis were 49 and 37% lower, respectively, in homozygotes for the mutant allele and 28 and 41% lower, respectively, in heterozygotes than in homozygotes for the wild-type allele ($P < 0.05$ for both). Site-directed mutants of apoAI gene promoter/reporter constructs containing the above mutations were transfected into Caco-2 cells and showed a 46% decrease in transcriptional activity compared with the wild type ($P < 0.001$); however, no significant differences were observed in HepG2 cells. Electrophoretic mobility shift assays showed that the mutated sequences from −655 to −610 bound Caco-2 cell nuclear protein(s) while the wild type did not. These results indicate that intestinal apoAI gene transcription and protein synthesis are genetically determined and are reduced in the presence of common mutations which induced binding of nuclear protein(s), possibly a transcriptional repressor. (*J. Clin. Invest.* 1997; 99: 1958–1965.) Key words: mutation • transcription factor • enhancer • high density lipoprotein • intestine • apolipoproteins

Introduction

Apolipoprotein AI (apoAI), a lipid binding protein involved in the transport of cholesterol and other lipids in plasma, is an

important constituent of chylomicrons produced in the intestine and is the major structural protein of plasma high density lipoprotein (HDL), which has been implicated in the pathogenesis of atherosclerosis and coronary heart disease (1, 2). Therefore, factors contributing to the synthesis of apoAI are of potential importance. The gene encoding human apoAI is located on chromosome 11q and is part of a 15-kb gene cluster with the apolipoprotein CIII (apoCIII) and apolipoprotein AIV genes. The apoCIII gene is separated ~2.9 kb from the apoAI gene and is transcribed convergently from the opposite strand of DNA (3).

apoAI is synthesized mainly in the liver and small intestine of mammals, and while significant progress has been made in understanding the factors that regulate hepatic expression of this gene, the mechanisms governing its expression in the intestine are less well defined. We have shown previously that the DNA regions from nucleotides −595 to −192 and −222 to −110 bp immediately upstream of the apoAI gene are necessary and sufficient for expression of the apoAI gene in human colon carcinoma (Caco-2) and human hepatoma (HepG2) cells, respectively (4–6). A third 0.6-kb DNA fragment located between nucleotides −770 and −194 upstream of the apoCIII gene enhances transcription mediated by either of these two tissue-specific apoAI gene promoters (4). Data from transgenic mice had confirmed the importance of the region upstream of apoCIII in intestinal apoAI gene expression and it may constitute an intestinal control element (ICE)¹ for apoAI gene transcription in vivo (7).

In this study we describe a frequent, naturally occurring series of polymorphisms in the intestinal transcriptional control region for the apoAI gene that is associated with expression of apoAI mRNA and relative synthetic rates of apoAI from the human intestine. Reduced intestinal specific repression of the apoAI gene transcription was observed in vitro in mutated versus wild-type apoAI gene promoter/chloramphenicol acetyl transferase (CAT) reporter constructs, a result which directly correlated with reductions in intestinal apoAI synthesis and mRNA levels observed in vivo in the presence of the mutant allele. Electrophoretic mobility shift assays (EMSAs) have identified a nuclear protein binding activity which is markedly enhanced in the presence of these mutations. Taken together, these results suggest that intestinal apoAI gene transcription and protein synthesis are genetically determined and are reduced in the presence of common mutations which induced binding of nuclear protein(s), possibly a transcriptional repressor, to the apoAI ICE.

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1. *Abbreviations used in this paper:* CAT, chloramphenicol acetyl transferase; EMSA, electrophoretic mobility shift assay; ICE, intestinal control element; oligo, oligonucleotide; TK, thymidine kinase.

Methods

Subjects. These studies were conducted on 18 healthy student volunteers of Columbia University, College of Physicians and Surgeons. The subjects were males with a mean age of 23.8 ± 3.2 yr, BMI 24.0 ± 2.9 kg/m², and none were using any medications. Subjects were on a carefully controlled American Heart Association Step I diet (30% calories from fat, 55% from carbohydrates, 15% from protein) for 6 wk before duodenal biopsy. Subjects were supplied two meals at a special feeding center and had all other food provided as well. In some cases, subjects were rebiopsied after an additional 6 wk yielding a total of 32 biopsy samples. Data from the total number of biopsies were used for the data presented in Fig. 3 to increase sample size and statistical power. All studies were approved by the Human Studies Committee at that institution. Each volunteer had a sample of peripheral blood taken for protein determination as well as extraction of genomic DNA as described below. Duodenal biopsy was carried out using topical pharyngeal anesthesia and a pediatric endoscope which was passed into the first portion of the duodenum and 200–400 mg of mucosal tissue was collected.

Determination of plasma apoAI levels and relative apoAI synthesis. Plasma apoAI levels were determined using radioimmunoassay as reported previously (8). Relative apoAI synthesis was determined as follows. Tissue samples obtained by biopsy were pulse labeled with L-[4,5-³H]leucine (specific activity 120 Ci/mmol; Amersham, Arlington Heights, IL). After incubation for 20 min at 37°C, biopsies were homogenized and subjected to immunoprecipitation using polyclonal rabbit anti-human apoAI serum as described previously (8–10). Synthetic rates for apoAI were expressed as counts per minute incorporated into immunoprecipitable supernatant apoAI divided by counts per minute incorporated into trichloroacetic acid-precipitable protein that was determined on an aliquot of starting homogenate. apoAI synthesis is expressed as a percentage of total protein synthesis in each sample and is thus denoted "relative apoAI synthesis."

apoAI mRNA determination. Immediately after duodenal biopsy, tissue was submerged in ice-cold 4 M guanidine isothiocyanate solution. Total RNA was extracted from tissues as previously described (11). 10 µg of total RNA was slot-blotted on a nitrocellulose membrane and was hybridized with a ³²P-labeled apoAI cDNA probe or an actin probe for normalization. The filters were washed twice with 0.5% SDS, 150 mM NaCl, and 15 mM Na-citrate (pH 7.0) for 10 min at room temperature, and with 0.5% SDS, 75 mM NaCl, and 7.5 mM Na-citrate (pH 7.0) for 15 min at 55°C, subjected to autoradiography, and the mRNA level was determined by densitometry.

Genomic DNA extraction, amplification, and sequencing. 5 ml of a peripheral blood sample was immediately centrifuged to separate white blood cells and its genomic DNA was extracted (12). Two regions, the apoAI gene promoter (–595 to +397) and the apoCIII gene upstream region (–825 to –277), were amplified by PCR. The sense and antisense strand oligonucleotide (oligo) primers used for amplification of apoCIII upstream region were 5'-AAC CCA GGA AGG TGA ACG AGA GAA TCA GTC-3' and 5'-GCT GGT GAG AGG GGA AAT GGC TTG GCA TA-3' (13). These oligos were synthesized by an Applied Biosystems 394 DNA/RNA synthesizer™

(Applied Biosystems, Foster City, CA). 25–100 ng of genomic DNA was amplified at 94°C for 1 min, 65°C for 2 min, 72°C for 2 min, and repeated for 30 cycles in 0.8 mM MgCl₂, 0.25 mM dNTP, 25 pmol of each primer, and 2.5 U of *taq* polymerase with manufacturer's buffer (Promega, Madison, WI). The amplicons from both regions were excised after 1% agarose gel electrophoresis and sequenced in both directions (14).

Plasmid constructs and transfection of cells for CAT assays. Based on information obtained from sequencing (see Results), using site-directed mutagenesis, mutated constructs were derived from a construct containing sequences necessary for maximal intestinal apoAI gene transcription denoted –2500 AI.CAT [CIII: –770/–194] (4). This construct consisted of the apoAI gene promoter (–2500 to +397) fused to the CAT gene, and the wild-type apoCIII upstream region (–770 to –194) ligated into the BamHI site 3' to the CAT gene (4). Constructs were named as follows: Wt (wild-type), 5M (containing point mutations at nucleotide positions –641, –630, –625, –482, and –455 upstream of the apoCIII gene), 3M (only nucleotides –641, –630, and –625 mutated), and 2M (only nucleotides –482 and –455 mutated). The BamHI fragments from the Wt and 5M constructs were ligated into the BamHI site of the thymidine kinase (TK) promoter in the TK-CAT reporter (15). The resulting constructs, Wt-TK-CAT and 5M-TK-CAT, were isolated in both orientations and used for transfections.

Human colon carcinoma (Caco-2) and human hepatoma (HepG2) cells were cultured in DME (GIBCO-BRL, Gaithersburg, MD) with 10% fetal calf serum, penicillin, and streptomycin at 37°C in 5% CO₂. Cells were seeded at 2×10^6 cells per 100 mm dish before transfection. To correct for variation in DNA uptake by the cells, 2 µg of plasmid pRSV-β-gal (16) was cotransfected with 10 µg of either Wt, 5M, 3M, 2M, Wt-TK-CAT, or 5M-TK-CAT plasmids by the calcium phosphate coprecipitation method as described (17, 18). 24 h after transfection, cells were treated with 15% glycerol, and an additional 48 h later, cells were harvested and protein was extracted as described previously (6) and CAT activities were determined.

Oligonucleotide preparation and EMSAs. Pairs of sense and antisense oligonucleotides for EMSA (Table I) were synthesized, purified by polyacrylamide gel electrophoresis, and appropriate sense and antisense strand pairs were annealed (17). Nuclear extracts from Caco-2 cells were prepared from 20 confluent 150 mm dishes as described (19) and the protein concentration of the extract was 7 mg/ml (20). Oligos were labeled using 100 µCi of [³²P]ATP using T4 polynucleotide kinase (New England Biolabs, Boston, MA) according to the manufacturer's instruction. For EMSAs, 0.2 mg/ml poly(dI-dC) and ~0.7 mg/ml of nuclear protein were incubated for 15 min at room temperature in 2.5 mM Hepes (pH 7.5), 10 mM KCl, 2% glycerol, 0.01% Nonidet P-40™, 1 mM ZnSO₄, and 0.1 mM DTT. 0.2 ng of labeled probe was added to the mixture and incubated for an additional 30 min before loading on a 6% polyacrylamide (39:1 bisacrylamide) gel (5). For competition experiments 100-fold molar excess of specific or nonspecific competitor oligos were added together with the probe. After running the gel in 25 mM Tris, 25 mM boric acid, and 0.5 mM EDTA buffer at 25 mA for 1.5 h, the gel was dried and exposed to x-ray film.

Table I. Sequences of Oligonucleotides for EMSA

WtU	5' - CCCCTCCCTACACTCAGGGGGAGGCGGCGGTGGGGCACACAGGGT - 3'
MU	5' - _____ A _____ A _____ de1 _____ - 3'
WtD	5' - GGAGCCACTGATGCCCGGTCTTCTGTGCCTTTACTCCAAACATCCCCCAGCCCAAGC - 3'
MD	5' - _____ T _____ C _____ - 3'

Only sense strands of complementary double-stranded oligos are shown. Wt, wild-type sequence; M, mutated sequence; U, upstream three polymorphic sites (–641, –630, and –625); D, downstream two polymorphic sites (–482 and –455). Polymorphic sites are underlined in oligos WtU and WtD.

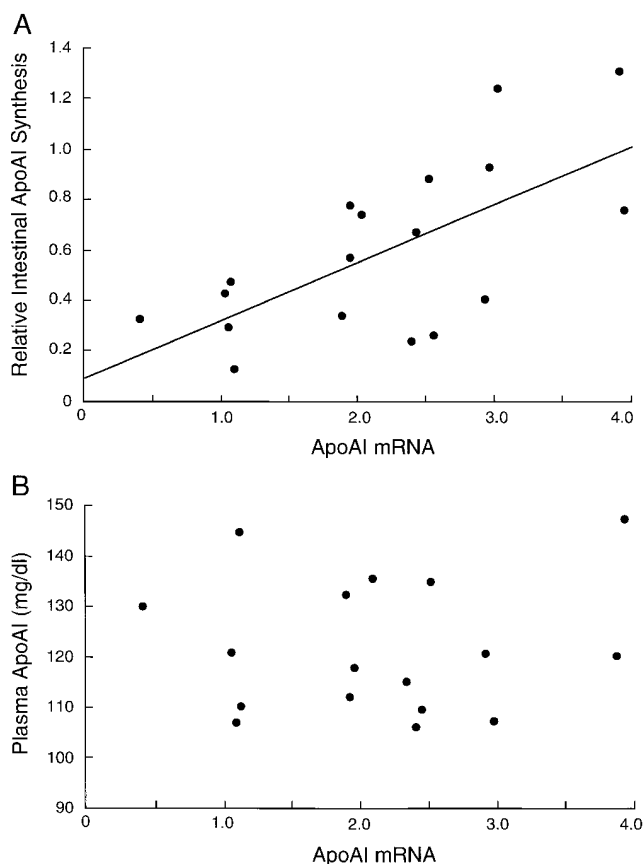


Figure 1. Relationship between relative intestinal apoAI synthesis (A) or plasma apoAI levels (B), and intestinal mRNA levels. $y = 0.11 + 0.23x$, $r = 0.66$, where r is correlation coefficient, for the association seen in A. There was no significant association seen in B. The units for apoAI mRNA levels and relative synthesis are arbitrary.

Results

Correlation between human intestinal apoAI mRNA levels and intestinal apoAI synthesis. Samples of intestinal tissue were obtained from healthy volunteers who underwent peroral endoscopic biopsy of the duodenum and had mRNA and relative apoAI synthesis measured from the biopsy specimens as well as simultaneous plasma measurements of apoAI. As shown in Fig. 1 A, intestinal apoAI mRNA levels displayed a high degree of interindividual variability as exemplified by the 10-fold difference between the lowest and the highest and steady state

apoAI mRNA (0.40 vs. 3.98) and relative synthesis of apoAI (0.11 vs. 1.39) values across the cohort (units arbitrary). In addition, a significant correlation was found between mRNA levels and intestinal apoAI protein synthesis ($r = 0.66$, $P < 0.05$; Fig. 1 A), but no association was found between intestinal apoAI mRNA levels and apoAI levels in the plasma (Fig. 1 B). Importantly, the interindividual variability could be reproduced when some of the subjects were biopsied a second, and in some cases, a third time. These results indicate that relative synthesis of intestinal apoAI is highly variable from individual to individual, suggesting that these are genetically determined. Similar variation in intestinal apoAI mRNA levels raised the possibility that intestinal apoAI synthesis might be determined at the level of transcription.

Five polymorphic sites in strong linkage disequilibrium are found in the intestinal control element for apoAI gene transcription. To determine whether the regions responsible for transcription of the apoAI gene could explain the observations above, we analyzed the DNA sequences from the genomic DNA of each subject. Previously, we have defined two transcriptional control regions which are necessary for intestinal apoAI gene expression (Fig. 2). Region I contains an intestinal specific promoter element between nucleotides (-595 and -192, relative to the transcriptional start site of the apoAI gene (+1), and region II, between nucleotides -770 and -194, relative to the transcriptional start site of the apoCIII gene (+1), contains an enhancer element required for proper intestinal expression of the human apoAI gene in transgenic mice (4, 7). No sequence polymorphisms were observed in region I for any of the subjects. However, as shown in Table II, five distinct polymorphic sites at nucleotide positions -641, -630, -625, -482, and -455 were found within region II. These polymorphisms have been described independently and were shown to be associated with protection against or susceptibility to severe hypertriglyceridemia and are in strong linkage disequilibrium with each other, thus defining two haplotypes (21). The allele frequency in our study population was 0.53 for allele 1 and 0.44 for allele 2 (Table II). Thus, a highly prevalent series of mutations is present in the intestinal transcriptional control element for the apoAI gene.

The genotypes in the intestinal control element for apoAI gene transcription are associated with reduced relative intestinal apoAI synthesis in vivo. We examined the relationship of genotype to intestinal apoAI mRNA levels and relative apoAI synthesis. To perform this analysis, 32 biopsy samples obtained from the 18 study subjects were used. Second and sometimes third biopsy samples were obtained under dietary control (see Methods) for 6 wk before the biopsy. Genotypes are denoted

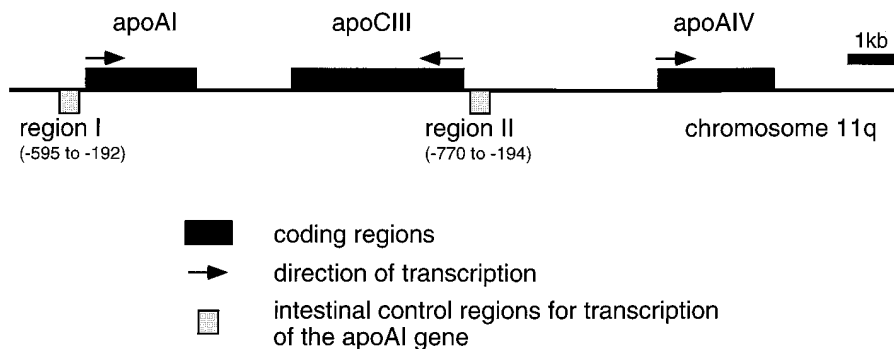


Figure 2. Map of apoAI, apoCIII, and apolipoprotein AIV gene cluster. Region I defines an intestinal specific promoter element between nucleotides -595 and -192 relative to the transcriptional start site of the apoAI gene (+1) which we have reported previously (4). Region II, between nucleotides -770 and -194, relative to the transcriptional start site of the apoCIII gene (+1), is an ICE that enhances transcription mediated by the presence of region I. Regions I and II are not drawn to scale.

Table II. Haplotypes and Allele Frequencies in Our Study Population

Allele	Polymorphic sites upstream of apoCIII gene					Allele frequency*
	-641	-630	-625	-482	-455	
1	C	G	T	C	T	0.53
2	A	A	del	T	C	0.44

*A single hybrid allele which may represent a rare recombination event (frequency = 0.03) was excluded from this analysis. Allele 1, wild-type; allele 2, mutated.

11 for homozygotes for allele 1, 22 for homozygotes for allele 2, and 12 for heterozygotes. The association of relative apoAI synthesis with genotypes is shown in Fig. 3. Mean apoAI mRNA levels and relative protein synthesis for each genotype are shown in Table III. Both genotypes 12 and 22 had significantly lower mRNA levels and relative apoAI synthesis compared with genotype 11 ($P < 0.05$ for 11 vs. 12 and 11 vs. 22). No significant association was observed between genotype and plasma apoAI levels (Fig. 3B). These data suggest that a series of mutations in the intestinal control element for the apoAI gene located upstream of the apoCIII gene is correlated with and may be directly responsible for variation in measured apoAI gene expression and protein synthesis in the intestine.

Intestinal apoAI gene transcription is significantly reduced in vitro by five polymorphisms in the apoAI intestinal transcriptional control element. Using site-directed mutagenesis, sequence changes were introduced into the ICE in the construct -2500 ALCAT [CIII: -770/-194] to generate the following constructs containing the mutations described above (see Methods): 5M (all five sites mutated), 3M (only nucleotides -641, -630, and -625 mutated), and 2M (only nucleotides -482 and -455 mutated) (Fig. 4A). These plasmids were transfected into Caco-2 and HepG2 cells and the resulting CAT reporter activity was determined and compared with that of the wild type as shown in Fig. 4B. In Caco-2 cells, the relative CAT ac-

Table III. Mean Intestinal apoAI mRNA Levels and Relative Synthesis as a Function of Genotype

Genotype*	apoAI mRNA [‡]	Relative apoAI synthesis [§]
11	380 ± 198 [§]	1.18 ± 0.48
12	275 ± 89	0.70 ± 0.31
22	204 ± 61	0.74 ± 0.28

*See Table II. [‡]Mean ± SD (units arbitrary); mRNA levels are reported on relative intensity by densitometry; apoAI levels are expressed as percentage of total protein synthesis (see Methods). [§] $P < 0.05$ 11 vs. 12, 12 vs. 22 (mRNA only); $P < 0.02$ 11 vs. 22.

tivity of the construct with all five mutations (5M) was reduced by 54% ($P < 0.001$) compared with the activity of the wild-type construct. The transcriptional activities of the 3M construct as well as the 2M construct were also reduced by 26% ($P < 0.001$) compared with that of the wild-type construct. In contrast, when this series of constructs was transfected into HepG2 cells, transcriptional activity was 90, 123, and 124%, respectively, of that of the wild type, but only the 2M construct had a transcriptional activity that differed significantly from the wild type. Thus, these mutations appear to have a direct and potentially intestinal cell-specific effect on transcription.

Next, we determined whether the ICE can activate transcription from a heterologous promoter and whether the mutations described above could affect transcription in this context. The ICE from nucleotides -770 and -194 was cloned into the TK-CAT reporter as was the same fragment containing all five mutations. The resultant plasmids (in both orientations) WT-TK-CAT and 5M-TK-CAT were transfected into Caco-2 and HepG2 cells and their activities were compared with that of the TK-CAT plasmid as a control. The results in Fig. 5 show marked activation (7–10-fold) of the transcriptional activity of the wild-type construct in both cell types, compared with the control, a result that was independent of orientation (data not shown). These results indicate that the ICE behaves as a classical enhancer. However, when the five

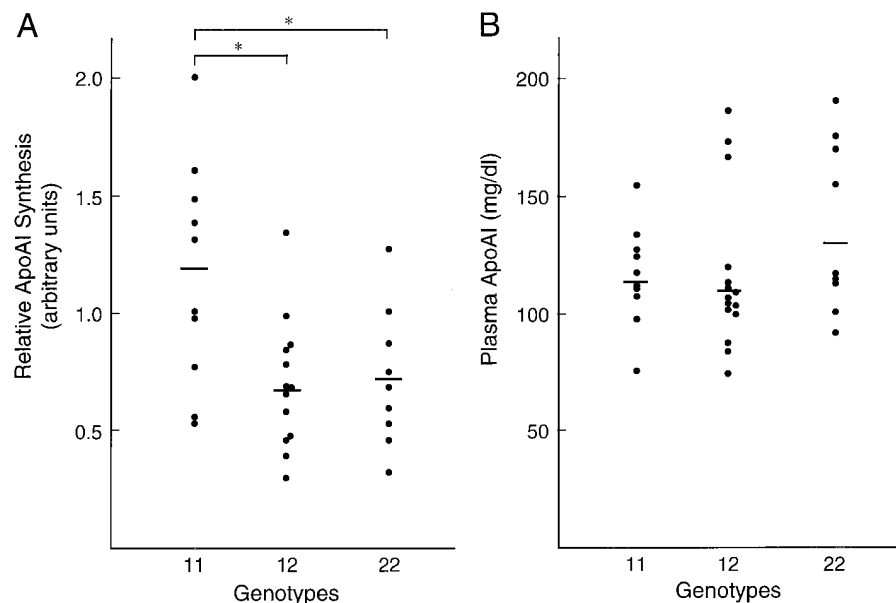


Figure 3. Association of relative intestinal apoAI synthesis (A) or plasma apoAI levels (B) with genotype. The association of apoAI synthetic rates with the three possible genotypes based on alleles shown in Table II. Horizontal bars show means in both A and B. * $P < 0.05$ by Student's *t* test.

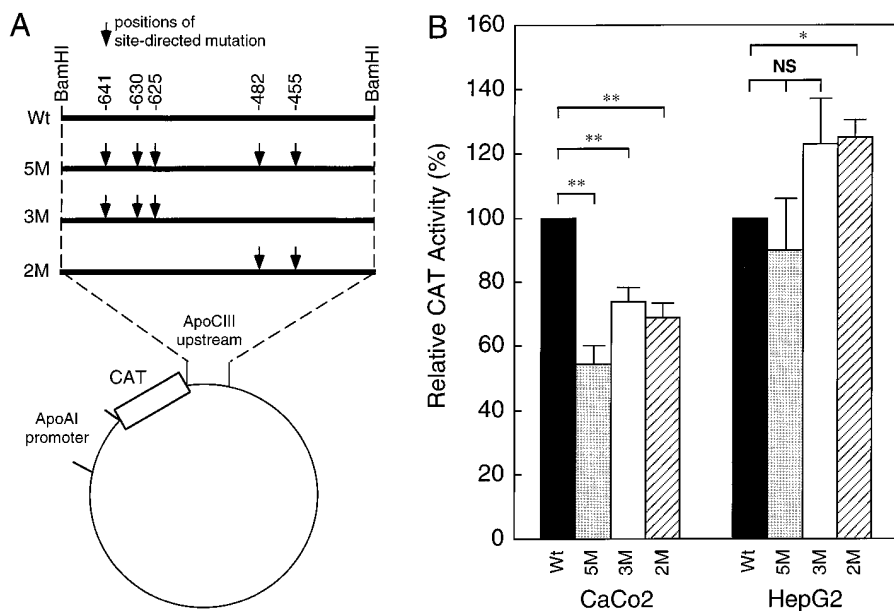


Figure 4. (A) Constructs used to test the effect of genotype on transcription. Map of constructs used in transient transcription assays which include the apoAI gene promoter region (-2500 to +1 of apoAI gene transcription starting site) fused to the CAT gene, and the apoCIII upstream region (-770 to -194) flanked by BamHI sites 3' to the CAT gene (4). The upper four horizontal bars are the enlargement of apoCIII gene upstream portion in the constructs for Wt (wild-type), 5M (mutations at nucleotides -641, -630, -625, -482, and -455), 3M (nucleotide -641, -630, and -625 mutated), and 2M (nucleotides -482 and -455 mutated). (B) Intestinal specific transcriptional repression by mutations in the intestinal control region of the apoAI gene promoter. Relative CAT activity for construct Wt, 5M, 3M, and 2M transiently transfected into CaCo-2 and HepG2 cells. Each experiment was repeated and carried out in triplicate. Transcriptional activity is expressed relative to that of Wt construct. $**P < 0.001$ by Student's *t* test.

mutations were present (5M-TK-CAT) enhancer activity was reduced 48% in HepG2 cells and 49% in CaCo-2 cells ($P < 0.01$, Wt vs. 5M). Thus, five point mutations are sufficient to reduce enhancer activity in a non-cell type restricted fashion and to an extent similar to that observed in the natural promoter context in CaCo-2 cells (Fig. 4B).

Binding of nuclear proteins is induced by mutations in the intestinal control element of the apoAI gene. To examine whether nuclear protein binding to these transcriptional regulatory regions is affected by the presence or absence of these mutations, we performed EMSAs using CaCo-2 nuclear extracts. To facilitate this analysis the polymorphisms were divided into two groups: the three upstream mutations (-641, -630, and -625) and the downstream pair (-482 and -455). The upstream wild-type (WtU), upstream mutant (MU), downstream

wild-type (WtD), and downstream mutant (MD) oligonucleotides were used as probes (Table I). Weak binding activity was seen using probe WtU (Fig. 6, lane 1). Sequence specificity was demonstrated by competition with unlabeled specific (Fig. 6, lane 2) and nonspecific (Fig. 6, lane 3) oligos in 100-fold molar excess. When oligo MU was used as a probe, markedly enhanced binding was observed (Fig. 6, lane 4) that was competed by the unlabeled MU oligo (Fig. 6, lane 5) but not an unrelated oligo (Fig. 6, lane 6). Specific binding of nuclear proteins could not be demonstrated with probes WtD or MD using a variety of different binding conditions (Fig. 6, lanes 7-12).

To examine the binding requirements for nuclear proteins to the upstream series of mutations we prepared probes from various oligos shown in Fig. 7 and performed EMSA. Compared with probe WT (Fig. 7, lane 1) probe MU (Fig. 7, lane 2) showed enhanced binding. The substitution at position -630 did not significantly affect binding (Fig. 7, lane 3); however, truncation of the probe (Fig. 7, lanes 4 and 5) showed no binding, indicating the importance of sequences including positions -625 and -641 for binding. Finally, the absence of complexes in Fig. 7, lanes 9-12, indicated that sequences from -640 to -620 were necessary for DNA-protein complex formation.

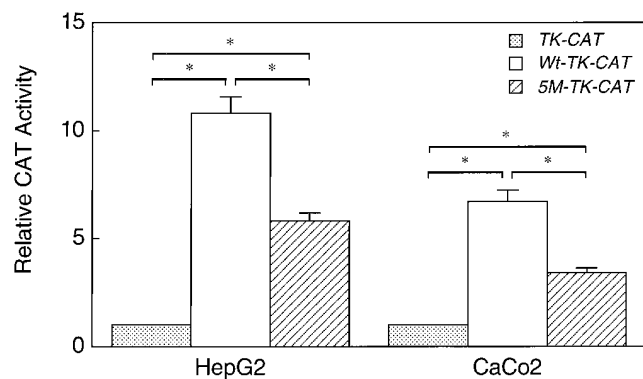


Figure 5. Five mutations in the ICE affect enhancer activity. Sequences from -770 to -194 upstream of the apoCIII gene without (WT) or with (5M) variants were ligated into TK-CAT reporter and transfected into CaCo-2 and HepG2 cells. Results are expressed as relative activities to that of TK-CAT. $*P < 0.01$ by Student's *t* test.

Discussion

In mammals, it has been estimated that as much as 50% of daily synthesis of apoAI occurs in the intestine (22-25). Despite the major contribution of the intestine to the apoAI of circulating HDL and chylomicrons (26), the factors regulating intestinal apoAI expression and synthesis, or even whether intestinal apoAI expression is constitutive or regulated, are not well understood. Progress has been made in defining the role of the liver in apoAI synthesis and the regulatory mechanisms governing hepatic apoAI expression and HDL synthesis (7, 27-31); however, few studies have addressed the regulation of

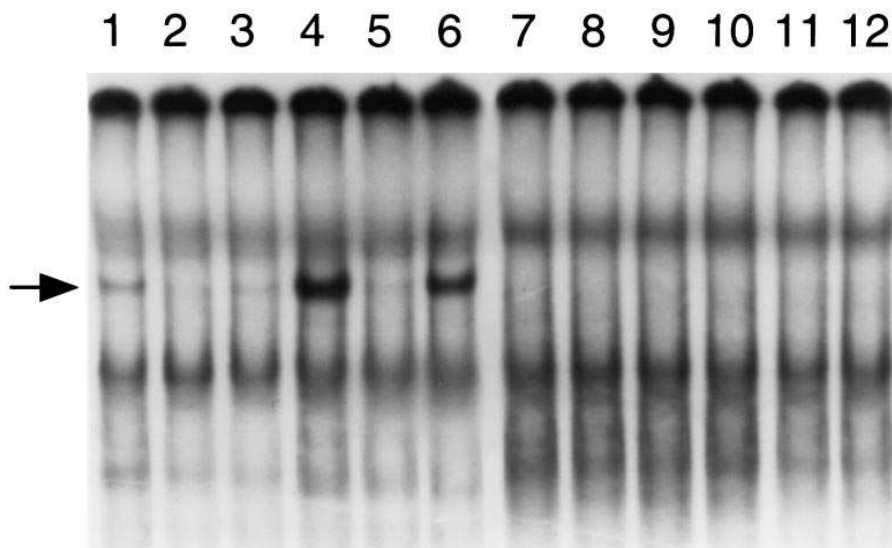


Figure 6. Binding of nuclear proteins is induced by mutations. EMSA using Caco-2 nuclear extracts and double-stranded oligo probes indicated in Table I. Probes WtU (lanes 1-3), Mu (lanes 4-6), WtD (lanes 7-9), and MD (lanes 10-12). Data are shown for the labeled oligo alone (lanes 1, 4, 7, and 10), in the presence of 100 M excess of specific competitor (lanes 2, 5, 8, and 11), and 100 M excess of nonspecific competitor (lanes 3, 6, 9, and 12). The arrow indicates the specific complex.

intestine-specific expression of apoAI (4, 6, 32, 33). In this study, using human intestinal tissue samples, we found evidence that intestinal apoAI synthesis is highly variable and at least partly determined by a series of polymorphisms that modify the binding of nuclear proteins to a sequence in the

ICE for apoAI gene transcription. The ICE behaves as a classical transcriptional enhancer whose activity is reduced significantly in the presence of these polymorphic nucleotides. These data provide strong evidence for transcriptional control of apoAI gene expression in the intestine and its dependence on a frequently occurring series of linked polymorphisms in an enhancer region responsible for apoAI gene transcription.

Our findings of marked interindividual variation of apoAI mRNA expression and protein synthesis suggested the possibility of genetic variation among individuals. The strong correlation between these two values certainly suggested that apoAI expression in the intestine is not constitutive but may be regulated. However, the possibility of posttranscriptional modification of apoAI mRNA or enhanced mRNA stability could not be excluded. The intestinal biopsy mRNA samples were not sufficient enough to explore this possibility nor to perform nuclear run-on assays. However, the 10-fold variation across our sample cohort strongly suggested a transcriptional basis for this effect. Several studies in rodents and nonhuman primates have suggested that diet and hormones affect hepatic synthesis of apoAI at the level of transcription (34-38). Additionally it has been shown that differences in hepatic apoAI production between two species of nonhuman primates are the result of differences in the apoAI gene promoter (34, 35). Recently a single polymorphism in the human apoAI gene promoter has been shown to be associated with altered transcription of apoAI in the liver (39). Thus, there is precedence for transcriptional control of apoAI synthesis and our data provide the first evidence for transcriptional control of apoAI synthesis in the intestine.

The lack of association between apoAI plasma levels and intestinal mRNA (Fig. 1 B) may be indicative of additional levels of regulation for apoAI levels in plasma such as in posttranslational processing, assembly, secretion, modification, and catabolism of apoAI in the peripheral circulation, as well as the contribution of hepatic apoAI synthesis to plasma levels. There is evidence for posttranscriptional regulation of apoAI in regenerating rat hepatocytes and in transgenic mice expressing human apoAI (27, 40-42). However, the mechanisms regulating gene expression may be different in states of

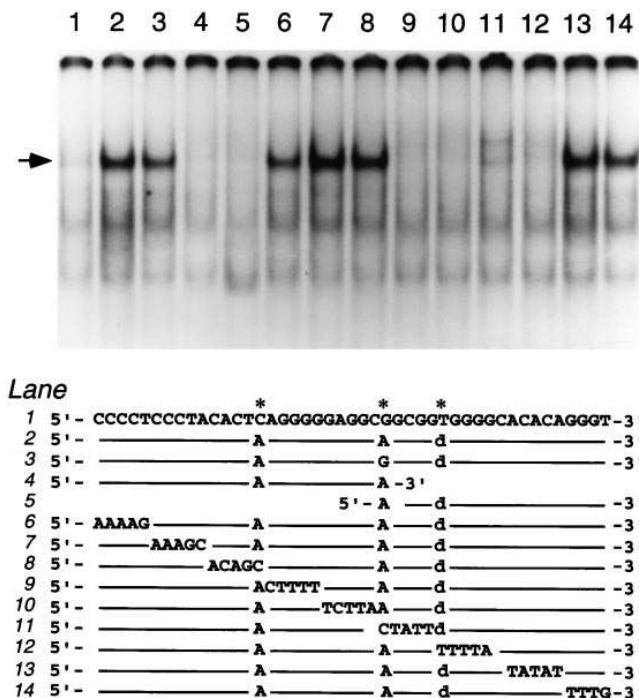


Figure 7. Scanning mutagenesis to localize the sequences required for binding. The sequences of the probes are indicated below the autoradiogram. Binding is compared with that of probe Wt (lane 1), and mutated (lanes 2 and 3) and truncated (lanes 4 and 5) versions of the probe are shown. Lanes 6-14 show the results of scanning mutagenesis (every five base pairs) of the probe MU and binding by EMSA using Caco-2 cell nuclear extracts. An arrow indicates the specific complex. Asterisks indicate the three variants at positions -641, -630, and -625.

cellular growth and organ regeneration or in other species. Certainly pre- and posttranscriptional regulation could be present concurrently. Multiple levels of regulation of expression may be responsible for the differences in the degree of in vivo variability we observed between intestinal apoAI mRNA from the subjects (10-fold, Fig. 1) and that observed in transcription assays in vitro (2-fold, Fig. 4). Thus, our data provide three important concepts: intestinal apoAI synthesis is not constitutive and thus may potentially be manipulated; individual intestinal synthesis rates may be genetically determined; and intestinal apoAI production is likely under the influence of transcriptional signals.

In this report we investigated whether a transcriptional mechanism could explain variation in human intestinal apoAI synthesis. Sequence analysis revealed two alleles in the ICE that have been reported to be associated with hypertriglyceridemia (21). In our study, the rare allele (frequency = 0.44) was associated with reduced intestinal apoAI synthesis as well as decreased apoAI gene transcription in the intestinal cells (Figs. 3 and 5). In the study of Dammerman et al. (21), allele 2 was in strong linkage disequilibrium with an *Sst* I S2 allele in the 3' untranslated region of the apoCIII gene. Further, the combination of the promoter polymorphisms and the *Sst* I RFLP were the strongest predictors of susceptibility or resistance to hypertriglyceridemia. The authors suggested that perhaps a combination of transcription effects by the former and RNA stability by the latter were potential mechanisms that explained this observation. In contrast, Surguchov et al. have suggested more recently that hypertriglyceridemia was solely attributable to the *Sst* I allele (43). Our data show that promoter polymorphisms in the ICE alone, both in the homologous and heterologous context, are sufficient to confer effects on apoAI gene transcription, an effect completely independent of the *Sst* I RFLP.

It is interesting that in the context of the TK promoter, reduced transcription is observed in both Caco-2 and HepG2 cells in the presence of the ICE variants (Fig. 5). However, in the natural promoter context, transcription is reduced only in Caco-2 cells (Fig. 4). This is not completely surprising since this element has been defined as an intestinal cell-specific transcriptional element in both cell-based assays and in mouse models of apoAI gene regulation (4, 7). Our data would argue that the factor(s) that reduce transcription are not cell specific, but they act in a promoter-specific context. In previous studies we have shown that the ICE acts synergistically with elements in the proximal apoAI promoter to confer intestinal cell-specific transcriptional activity (4). Thus, these factors reduce the activity of the ICE in the context of active promoters: the TK promoter which is active in HepG2 and Caco-2 cells, and the natural promoter that is active only in intestinal cells. In the later case, reduced transcriptional activity presumably results from disruption of the synergistic interactions of transcription proteins that confer intestine-specific activity on this promoter.

DNA-protein interactions in the ICE region have been described previously (13, 33). Using rat liver nuclear extracts (13), DNase I protection has shown footprints that contain the two downstream mutations (-482, -455). Footprints upstream of -680 have been reported with Caco-2 nuclear extracts (33). However, none of these directly involve the polymorphic nucleotides at position -641, -630, and -625. It should be noted that in both of these studies the wild-type se-

quence was used as a probe. Our EMSA indicate that the minimum sequence required for DNA-protein binding is (-641)-5'-AAG GGG GAG GCA GCG GGG GGC-3'(-620) (Fig. 7). In functional studies of the apoCIII promoter, deletion of the region from -686 to -583 resulted in a twofold increase in intestinal transcription of apoCIII, suggesting that a transrepressor activity for the apoCIII gene might also be located in this region (13).

The DNA region from -461 to -453 upstream of the apoCIII gene contains recently described insulin response element, and allele 2 results in enhanced apoCIII gene transcription in cultured HepG2 cells (44). While binding to this region was not observed in our EMSAs, transcriptional activity of the apoAI gene in intestinal cells was significantly reduced in the presence of these mutations. It is thus interesting to speculate that a common mutation results in decreased apoAI transcription and enhanced apoCIII gene transcription, and that apoAI gene transcription might be regulated, in part, by insulin. Syndromes associated with insulin responsiveness/sensitivity are characterized by increased very-low-density lipoprotein triglyceride levels and low HDL-cholesterol (45). In fact, allele 2 has been associated with an increased relative risk of hypertriglyceridemia (21), a phenotype associated with overexpression of apoCIII (46).

In summary, we report that a common genetic variant in the ICE for apoAI gene transcription located within the apoCIII gene promoter is associated with apoAI gene expression and protein synthesis in the intestine. The presence of mutations in this enhancer results in both reduced apoAI protein synthesis and gene transcription. Binding of nuclear protein(s) to the mutated allele is enhanced compared with the wild-type allele. While numerous studies have reported that mutations in the promoter of a gene modify DNA-protein binding, few studies have reported the effect of naturally occurring gene promoter polymorphisms (47-55). This report provides clear evidence of a frequent promoter mutation that alters DNA binding of nuclear proteins. The identity of this factor(s) may aid us in understanding the mechanism underlying variations in intestine-specific expression of apoAI and plasma HDL levels.

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