Common variants of APOA5 have consistently shown association with differences in plasma triglyceride (TG) levels. These single nucleotide polymorphisms (SNPs) fall into three common haplotypes: APOA5*1, with common alleles at all sites; APOA5*2, with rare alleles of −1131T→C, −3A→G, 751G→T, and 1891T→C; and APOA5*3, distinguished by the c56C→G (S19W). Molecular modeling of the apoAV signal peptide (SP) showed an increased angle of insertion (65°) at the lipid/water interface of Trp-19 SP compared with Ser-19 SP (40°), predicting reduced translocation. This was confirmed by 50% reduction of Trp-19-encoded SP-secretary alkaline phosphatase (SEAP) fusion protein secreted into the medium from HepG2 cells compared with the Ser-19-SEAP fusion protein (p < 0.002). Considering APOA5*2 SNPs, there was no significant difference in the relative luciferase expression in Huh7 cells transiently transfected with a −1131T construct compared with the −1131C (fragments −1177 to −516 or −1177 to −3). Similarly, for the −3A→G in the Kozak sequence, in vitro transcription/translation assays and primer extension inhibition assays showed no alternate AUG initiation codon usage, demonstrating that −3A→G did not influence translation efficiency. Although 1891T→C in the 3′-untranslated region disrupts a putative Oct-1 transcription factor binding site, when inserted 3′ of the luciferase gene the T→C change demonstrated no significant difference in luciferase expression. Thus, association of APOA5*2 SNPs with TG levels is not due to the individual effects of any of these SNPs, although cooperativity between the SNPs cannot be excluded. Alternatively, the effect on TG levels may reflect the strong linkage disequilibrium with the functional APOC3 SNPs.

There is strong evidence from clinical studies, epidemiological studies, and animal models (reviewed in Ref. 1) that apoAV plays a key role in triglyceride (TG) metabolism. Transgenic and knock-out mouse models identified an inverse relationship between apoAV and plasma TG levels (2). APOA5 variants determine between individual differences in plasma TG in all ethnic groups studied to date (3, 4). The role of apoAV in TG metabolism is further supported by the recently identified rare mutation Q145X, introducing a premature termination of apoAV and resulting in apoAV deficiency and severe hypertriglyceridemia (5). However the exact function(s) of apoAV remain to be clarified. APOA5 expression is up-regulated in liver regeneration after rat hepatectomy (6), suggesting that it acts as a break on very low density lipoprotein assembly (7). ApoAV is found preferentially on high density lipoprotein but is thought to transfer to very low density lipoprotein in the postprandial state (8) and has been shown to activate lipoprotein lipase in vitro and in vivo (9).

The APOA5 gene itself is relatively polymorphic. The five most common APOA5 haplotypes can be defined by seven SNPs (10). Two haplotypes, APOA5*2 and APOA5*3, are of particular interest as they show association with raised plasma TG levels (4). APOA5*1 is the wild type haplotype defined by the common alleles at the seven sites. This is found at a frequency of 69% in Caucasian populations (10). Haplotype APOA5*2 is defined by the rare alleles at four sites (10), −1131T→C (SNP3 (2)), −3A→G, 751G→T (SNP 2, previously referred to as IVS3 + 476G→T (2)), and 1891T→C (SNP 1, previously referred to as c1259T→C (2)) in the 3′-UTR of the gene. The third haplotype, APOA5*3, is distinguished from APOA5*1 by the rare allele of the cSNP 56C→G, which results in the substitution of Trp for Ser at residue 19 (S19W; SNP5 (2)). Haplotypes APOA5*2 and APOA5*3 occur at frequencies of 4% in a Caucasian study (11). The rare alleles of 1764C→T and V153M, the two remaining SNPs, both on chromosomes defined by the other common alleles, represent the other two APOA5 haplotypes. Family studies suggest that V153M is nonfunctional because it shows no co-segregation with hypertriglyceridemia in a carrier family (4), whereas there is no information about the association of 1764C→T with plasma lipids. We have therefore concentrated our functional studies on SNPs that define haplotypes APOA5*2 and *3.

The proximity of the APOA5 to APOA4/APOC3/APOA1 in the same gene cluster on chromosome 11q23 raised the question of whether the TG-raising effects of APOA5 SNPs simply reflected linkage disequilibrium (LD) with functional variants in the other TG-raising gene, APOC3, or whether these were independent functional effects (3). Haplotype analysis of the APOA5/A4/C3 gene cluster identified that whereas the S19W showed association with plasma TG levels, independent of APOC3, the −1131T→C is in strong LD with the APOC3 −482G→T, and thus associations of haplotype APOA5*2 may indeed be due to the effects of −482G→T, which disrupts the normal insulin responsiveness of apoCIII. No detailed functional studies have been reported on these two TG-raising APOA5 haplotypes, and thus the purpose of this study was to investigate the basis for their effects and to determine which of these variant sites are functional.

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1 The abbreviations used are: TG, triglyceride(s); SNP, single nucleotide polymorphism; UTR, untranslated region; LD, linkage disequilibrium; SP, signal peptide; SEAP, secretory alkaline phosphatase; nt, nucleotide(s).
Functional Studies of Common APOA5 Polymorphisms

MATERIALS AND METHODS

Molecular Modeling of apoaV Signal Peptide Variant

The Ser-19 (wild type) and variant Trp-19 23-amino acid signal peptides (SP) of apoaV were three-dimensionally constructed as α-helices using Hyperchem 5.0 (Hypercube, Inc.). Their conformations were minimized by the Polak-Ribiere algorithm in an AMBER force field. The two peptides were analyzed by the IMPALA algorithm, described elsewhere (12). This enables analysis of the insertion of a peptide into a modeled membrane using simple restraint functions designed to mimic the membrane properties. The position of the peptide in the modeled membrane is minimized by a Monte Carlo procedure.

Functional Studies of S19W SNP Defining haplotype APOA5*3 Replacement of the Secreted Alkaline Phosphatase Signal Peptide with the apoaV Signal Peptide Variants, Ser-19 and Trp-19

To test the effect of the apoaV signal peptide variant S19W, APOA5 signal peptide sequences with either a C or G at position 56 were used to replace the endogenous signal peptide sequence of the secreted alkaline phosphatase (SEAP) in the Great EscAPe SEAP Reporter System 3 vector (BD Biosciences) by PCR concatenation following the method of Tuohy and Groden (13). The forward primer consisted of a SEAP sequence 5′ to its signal peptide followed by the APOA5 signal peptide sequence: internal forward (IntF) 5′-ATCGCACCACATGACAGATATGGCAAGATTGCTGCTGCCTACCTGGCTGCTGCTTGGTCTGGCG-3′. The reverse primer contained overlap with a post-signal peptide sequence and the 3′ end of the APOA5 signal sequence: internal reverse (IntR) 5′-GGGAATCTTGCTCACCTGGGATGATTGCCTGGGTGGC-3′. The primer pair was used with 10% fetal bovine serum. SEAP was assayed after transfection of HepG2 cells, which were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. SEAP was assayed after transfection of HepG2 cells, which were maintained in RPMI 1640 supplemented with 10% fetal bovine serum.

Results represent the mean from nine repeat experiments, each performed in sets of eight repeat wells.

Kozak Sequence – 3A→G

Two different assays were performed to test the functionality of the 3A→G variant.

In Vitro Translation and Translation Assay—Two APOA5 cDNA clones (5′-UTR and coding sequence only), a kind gift from Len Pennacchio, were used: 3A/56C and 3G/56C. The cDNAs were subcloned into pGEM 7Zf driven from a T7 promoter (Promega) and confirmed by sequencing. The transcription/translation experiments were performed by using the TnT quick-coupled transcription/translation system (Promega). The plasmids were transfected into vitro and translated by using the T7-coupled reticulocyte lysate system (Promega TnT Quick coupled transcription/translation and using the manufacturer’s protocol and using FluoroTect Green (Promega FluoroTect™ Green LYS tRNA) to label newly synthesized protein. Size and quantification of the proteins were checked by SDS-PAGE, run on a Typhoon 8600 (Molecular Dynamics) at 532 nm excitation, and analyzed with ImageQuant software. Three separate DNA preparations of each clone were used, and samples were duplicated for each experiment.

 Primer Extension Inhibition (Toe Printing) Assay—To study the initiation AUG of protein synthesis, a primer extension inhibition assay (14) in rabbit reticulocyte lysates was used. The same constructs were used as in the transcription/translation assay. Plasmid DNA, linearized by digestion with EcoRI, provided the template for transcription by T7 RNA polymerase using the Riboprobe System-T7 (Promega) and Ribo mG cap analog (Promega). Capped mRNAs were purified by extraction with phenol, concentrated by ethanol precipitation, resuspended in water, and stored at −70 °C. An oligonucleotide, AGAGGCTTCCAGTGTTTTCGCCA, labeled at the 5′ end with 6-carboxyfluorescein (6-FAM), was used to prime the reverse transcriptase step. The fluorescently labeled primer was pre-annealed to the DNA by heating for 1 min at 65 °C followed by incubation at 37 °C for 5 min in 40 mM Tris-HCl, pH 7.5, and 0.2 mM EDTA. The primer-RNA complexes were then held on ice for ~15 min, whereas the reticulocyte reaction mixtures were assembled. Ribosome binding reactions were carried out using a micrococcal nuclease-treated rabbit reticulocyte lysate (Promega). The reaction mixtures contained 45% reticulocyte lysate, 90 μg/ml cycloheximide, 200 μM sparsomycin, 2 mM magnesium (CH3COO)2, and 100 μM sodium acetate for the assay, 25-μl aliquots of this mixture containing 2 μl of mRNA/primer (0.1–0.2 μg of mRNA) were incubated at 25 °C for 6 min and then diluted with 20 volumes of cold buffer, 50 mM Tris-HCl, pH 7.5, 40 mM KC1, 6 mM MgCl2, 5 mM dithiothreitol, 110 μg/ml cycloheximide, and each of the four dNTPs at 575 μM. Primer extension was initiated by adding 2 units/μl Superscript II reverse transcriptase to the mix and incubated at 25 °C for 10 min. Reactions were stopped by extraction with phenol and precipitation with ethanol.
before resuspension in 10 μl of water. 2-μl aliquots, together with GeneScan-500 ROX (dichlororhodamine acceptor dye) size standards (Applera), were run on an ABI 377 automated sequencer and analyzed using GeneScan software. Experiments were repeated twice, each time in triplicate.

3′-UTR 1891T→C

To test the function of the 1891T→C variant in the 3′-UTR, an 816-bp fragment was amplified by PCR from the DNA sample from an individual with haplotype APOA5*3 from +1752 to +2568 with a T at position 1891. Oligonucleotides were designed with XbaI sites at the 5′ end of the forward oligo and HpaI at the 5′ end of the reverse oligo. In addition, GG and CG tails were introduced at the 5′ ends of forward and reverse oligos. Oligonucleotides for the short fragments were as follows: forward primer, 5′-GCTCTAGA GGCCCATTCCCAGCTCCTTGT-3′; reverse primer, 5′-GGGTAAACTGTGCTTTGGGGATAGTGGTGAGG-3′.

PCR products were TA-cloned into pGEM-T vector system (TM042) following the manufacturer’s conditions (Promega). Site-directed mutagenesis was used to introduce the T→C at position 1891 using the Stratagene QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by sequencing. Subcloning into the XbaI/HpaI polylinker site of either the pG3L control or promoter Luciferase reporter vector (TM033) followed. Minipreps were purified using the QIAprep Spin miniprep kit (Qiagen) and sequenced with Applera BigDye Terminator (TM033) followed. Minipreps were purified using the GenElute HP plasmid Maxiprep kit (Sigma).

Huh7 cell transfection and luciferase activity measures were carried out as described above for the −1131T→C variant.

RESULTS

Functional Predictions and Effects of Haplotype APOA5*3

Molecular Modeling of the apoaV Signal Peptide S19W Variant—The first 23 N-terminal residues of apoaV form a signal peptide. We had previously shown that signal peptides insert obliquely into the lipid membrane (15, 16). This tilted orientation was important to the translocation activity of the signal peptide, with an angle of insertion of 45° giving the highest activity (15). The most stable position in a modeled membrane for the Ser-19 SP and the Trp-19 SP are shown in Fig. 1. The Ser-19 SP assumes an angle of insertion relative to the phospholipids bilayer of 40°, with its mass center at 10 Å from the bilayer center. For the Trp-19 SP, the best position relative to the bilayer is at an angle of 65°, with its mass center at 5 Å. For comparison, modeling of the endogenous secretory alkaline phosphatase SP shows an angle of insertion of 40° relative to the bilayer plane with a mass center at 9 Å from the bilayer center (data not shown). This increase of the angle of insertion from 40° to 65° for Ser-19 compared with Trp-19 is predicted to lead to reduced “activity,” i.e. translocation across the endoplasmic reticulum. These predictions also suggest that the apoaV Ser-19 SP should work as efficiently as the endogenous SP when fused to SEAP.

Influence of the APOA5 Signal Peptide Variant S19W on Secretory Alkaline Phosphatase Secretion—The effect of the substitution of the SEAP signal sequence by the APOA5 signal sequence, encoding either Ser or Trp at residue 19 and creating two different apoaV-SEAP fusion proteins, was examined. The secretion of alkaline phosphatase from HepG2 cells into the medium was then assayed quantitatively. As presented in Fig. 2, compared with control SEAP with its endogenous signal peptide, the apoAV wild type Ser-19 signal peptide acted effectively as a signal sequence, as predicted by the molecular modeling. However, substitution of Ser-19 by Trp-19 reduced the amount of secreted SEAP by 49% (p = 0.002), proving that this SNP is indeed functional. According to the manufacturer’s protocol, the SEAP in the medium is directly proportional to the intracellular mRNA and protein. For this reason the intracellular concentration of the fusion proteins was not measured.

FIG. 1. Molecular modeling of Ser-19 and Trp-19 signal peptides inserted in a simplified lipid bilayer. Best position for the Ser-19 (a) and Trp-19 (b) signal peptides after IMPALA minimization (12) is shown. The dashed line represents the phospholipid head group/water interface (placed at 18 Å from the bilayer center); the dotted line represents the phospholipid head group/phospholipid acyl chain interface (at 13 Å from the bilayer center); and the solid line represents the bilayer center (at the origin (z = 0)). The bilayer is symmetric. The peptides are represented as ribbons.

FIG. 2. Quantitative estimation of secretion of the apoaV signal peptide-SEAP fusion proteins from HepG2 cells. HepG2 cells were transiently transfected with the wild type SEAP construct with its endogenous signal peptide, the APOA5 Ser-19-SEAP or APOA5 Trp-19-SEAP constructs. Co-transfection with β-galactosidase acted as a control for transfection normalization, and the SEAP basic vector acted as the negative control. Wild type SEAP was set at 100%. Comparison of the amount of secreted apoaV Ser-19-SEAP and apoaV Trp-19-SEAP into the medium shows a statistically significant difference (p = 0.002). Results represent five separate transfections, and each sample was replicated five times. Bars = S.D.

Functional Effects of Haplotype APOA5*2

Haplotype APOA5*2 is defined by four variant sites all in complete LD, namely −1131T→C, −3A→G, 715G→T, and 1891T→C. Of these variants, 715G→T, 476 bp into intron 3, is unlikely to be functional and was not examined further. The potential functional effects of the other three variants were tested.

Promoter −1131T→C—This variant within the promoter of APOA5 could alter transcription, and in order to study this further, its effect on the expression of luciferase, acting as a reporter gene, was examined. To test whether −1131T→C was acting independently or could be influenced by the −3A→G
change (with which it shows complete LD), four different constructs were made, two shorter fragments (from −1177 to −516) that included only the −3 site (from −1177 to −3) in the naturally occurring combinations (−1131T/−3A and −1131C/−3G). The effects of these four constructs on luciferase expression in the luciferase assay are shown in Fig. 3. Results from nine separate experiments on two different DNA preparations (each with eight repeats) showed no significant difference between the −1131C or −1131T short constructs on luciferase expression (p = 0.53), although these constructs showed more than double the effect on luciferase activity compared with the pGL3 Basic vector. Similarly there was no statistically significant difference between the −1131T/−3A and −1131C/−3G constructs (p = 0.34), although both constructs showed significantly lower luciferase activity compared with the short constructs (p < 0.0001).

Kozak Sequence −3A→G—Two different assays were used to test the function of this variant. The in vitro transcription/translation assay was used to investigate whether the −3A→G in the Kozak sequence affected the translation efficiency of −3G and −3A cDNAs. Evaluation was carried out by lysine fluorescence (a representative gel is shown in Fig. 4a), with the major band representing the translated apoAV proteins. Quantification of these results from three repeat experiments (Fig. 4b) shows that there was no significant difference (p = 0.59) in fluorescence emission between the two constructs, establishing that the site at −3 was not influencing translation efficiency.

This was confirmed by the complementary primer extension inhibition “toe printing” assay, which examines codon usage. The premise is that the −3A→G change in the Kozak sequence could result in leaky translation, such that translation might start at an alternative AUG initiation site, because of poor discrimination. ApoAV has AUG codons at residues 1 and 4. The essence of this assay is that the interaction of ribosomes with mRNA can be limited to the initiation step by including antibiotics that inhibit elongation (14, 17). A 5′-fluorescently labeled oligo, 230 nt 3′ of the residue 1 start site and 221 nt 3′ of residue 4, was used to prime reverse transcriptase to the position of the bound ribosome. Representative electropherograms from the two constructs −3A or −3G are shown in Fig. 5. As expected, the full-length 5′ end product of 285 bp was visible. A 253-bp fragment was present for both constructs and is likely to represent an echo band (14). As reported by Kozak (14), the 80 S ribosome bound to the AUG will protect 15–16 nt 3′ of the codon, and thus the expected bands, if both AUGs are used, should be 214 or 205 nt long. The peak at 209 nt probably represents the 80 S ribosome blocked codon 1 fragment, which is slightly shorter than expected, representing 21 nt blocked by the 80 S ribosome. This might be because of distortion by the fluorescent label. A small peak at 230 nt was seen with both constructs, which would be the expected size fragment corresponding to codon 1 usage. No peak at 221 nt (which would represent codon 4 usage) was seen. Importantly, no second additional peak representing the 9-nt shorter fragment was evident in the −3G construct. These results thus confirm results from the transcription/translation assay by demonstrating that only the AUG at residue 1 is used and the −3A→G change does not result in leaky translation.

3′-UTR 1891T→C—The function of the T→C change in the 3′-UTR of APOA5, acting as a 3′-UTR for the luciferase gene, was tested in two different vectors, pGL3 control vector (which in addition to the SV40 promoter has the SV40 enhancer) and pGL3 promoter vector (which lacks the SV40 enhancer). There was no significant difference in the effect of the T or C construct on luciferase expression in either vector system (Fig. 5).

DISCUSSION

The novel aspect of this study is the resolution of the functionality of APOA5 SNPs by demonstrating the altered signal peptide function of the APOA5*2 S19W and the lack of functional effects of any of the three APOA5*2 SNPs tested individually. Haplotype APOA5*2 3A/56C→G, resulting in S19W, introduces an aromatic amino acid, Trp, in exchange for a polar Ser at position 5 from the cleavage site of the apoAV signal peptide. Although the sequences of signal peptides are not highly conserved, they do observe certain constraints, with positively charged residues at the N terminus, a hydrophobic core, and neutral but polar residues at the C terminus (18). Amino acids at −3 and −1 relative to the cleavage site must be small and neutral for cleavage to occur (19). The rigidity of this requirement is borne out by the amino acid substitutions of Ala by Thr at the −1 cleavage site of vasopressin-neurophysin II (20), a Gly to Ala change at −1 in collagen X (21), and a Pro to Ser substitution at −3 in the preproparathyroid hormone (22), which cause severe genetic disorders. However, amino acid
changes at less critical residues within the signal sequence are not associated with such extreme phenotypes. Alteration to the hydrophobic core of the ApoB signal peptide, due to deletion of a Leu-Ala-Leu triplet, reduces the total hydrophobic potential of the signal peptide (15) and is predicted to decrease translocation across the endoplasmic reticulum. This has been confirmed in vitro in both heterologous and homologous systems (23, 24). The biological consequence is that carriers of the APOB deletion allele have modestly lower plasma TG than those homozygous for the insertion allele (25). The APOA5 S19W change has a similar effect on SP function as the ApoB SP deletion and alters the predicted angle of insertion of the modeled signal peptide to the water/lipid interface from 40° to 65°. The prediction of reduced translocation and therefore secretion of Trp-19 apoAV compared with the Ser-19 apoAV was fully supported by the apoAV SP-SEAP fusion experiments. This fusion protein showed almost 50% lower SEAP in the medium of HepG2 cells compared with the Ser-19-SEAP fusion protein. One caveat is that the intracellular SEAP concentration was not measured; however, a difference between intracellular Ser-19-SEAP fusion protein and Trp-19-SEAP fusion protein concentrations would be difficult to interpret because it might not reflect true intracellular levels because of potential differences in degradation of the two proteins. This reduced secretion would be predicted to result lower plasma apoAV in Trp-19 subjects, although this has not been measured. Because mouse models show an inverse relationship between plasma apoAV and plasma TG levels (2), reduced apoAV should thus result in lower plasma apoAV in Trp-19 homozygotes (3), which correlates well with the prediction from the functional studies.

The Kozak sequence, from −6 to −1 preceding the initiating AUG codon, represents a highly conserved motif in eukaryotes which regulates ribosomal translation (14, 17). The transcription/translation assay used to examine the change within the Kozak sequence, −3A→G, had previously been used to differentiate between the translation efficiency of a C→T change at −1 of the Kozak sequence in annexin V (28). However, the APOA5 −3A→G change did not affect the translation efficiency of apoAV. The primer extension inhibition assay was used to validate these results and demonstrated that leaky translation, which would have resulted in translation initiation from the +4 AUG rather than +1 AUG of APOA5, did not occur. 97% of vertebrates have a purine at position −3 (29), and thus the change from A→G is conservative and unlikely to affect translation efficiency. These two experiments therefore confirmed the lack of functionality of this change within the Kozak sequence.

Initial analysis using TRANSPLORER™ to identify potential transcription factor binding sites in the APOA5 3′-UTR identified a putative Oct-1 site (upper case shows the expected consensus sequence for Oct-1; lower-case letters do not fit the consensus sequence).
consensus sequence), CtCtgtCaTatCCG (+1883 to +1897), which would be disrupted by the 1891T→C change. However, using this APOA5 sequence as a 3′-UTR for luciferase showed that the 1891T→C change did not alter luciferase expression, confirming that this SNP is also not functional. Thus, functional studies of the three APOA5*2 SNPs (the intron 3 SNP is unlikely to be functional), showed that none of these sites significantly altered transcriptional strength, translation efficiency, or 3′-UTR function.

There are three possible explanations for the association of this haplotype with raised plasma TG levels. First, there could be an additional functional site in APOA5 that is in LD with these three variant sites. However, extensive resequencing (10, 30) has not identified any other common, potentially functional SNPs that could account for the APOA5*2 haplotype association. Second, these three SNPs of APOA5*2, although individually not functional, might act cooperatively and thus affect expression levels. An example of such interaction is seen with the α1-antitrypsin gene, where the 11478G→A (TaqI) SNP in the 3′-UTR disrupts an Oct-1 site and acts in cooperation with a promoter NF-IL6 site as part of the acute phase response (31). Finally, the strong LD between the −1131T→C and the APOC3 −482C→T variant, identified in our haplotype analysis (3) and subsequently confirmed by O’Muircheartaigh et al. (10), suggests that this APOC3 SNP, which disrupts the insulin responsive −UTR function. There are three possible explanations for the association of this SNP, which disrupts the insulin responsive −UTR function. There are three possible explanations for the association of this SNP, which disrupts the insulin responsive −UTR function.

In conclusion, these studies highlight the complex genotype-phenotype relationship that can occur between genotypes (in this difference in genotypes) that show strong LD.

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