A functional polymorphism in the apolipoprotein B promoter that influences the level of plasma low density lipoprotein

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Abstract Apolipoprotein (apo) B is the structural protein moiety of plasma low density lipoprotein (LDL), an important risk factor for coronary heart disease (CHD). There is evidence that the rate of synthesis of apoB-containing lipoproteins may play an important role in the regulation of plasma LDL levels. However, it is generally thought that transcriptional regulation of the apoB gene is not a significant determinant of the synthesis of apoB-containing lipoproteins, and by inference, of the regulation of the plasma LDL concentration. Here we report the discovery of a common polymorphism in the promoter region of the apoB gene, a C to T substitution at position −516. The −516T allele is associated with an increase in the basal transcription of the apoB gene (+41%, \( P < 0.05 \)) in vitro in transfected HepG2 cells. Healthy middle-aged men who are homozygous for the −516T allele have 12% higher plasma LDL cholesterol levels than healthy homozygotes for the −516C allele \( (P < 0.05) \). The frequency of the −516T allele is significantly higher in young postinfarction patients \( (0.38) \) than in population-based controls \( (0.30) \) when the comparison is restricted to subjects without severe hypercholesterolemia who are homozygous for the apoE3 allele \( (P < 0.05) \). It is concluded that variation in the rate of transcription of the apoB gene can affect plasma LDL levels and influences the risk of CHD in middle-aged men.—van 't Hooft, F. M., S. Jormsjö, B. Lundahl, P. Tornvall, P. Eriksson, and A. Hamsten. A functional polymorphism in the apolipoprotein B promoter that influences the level of plasma low density lipoprotein. J. Lipid Res. 1999. 40: 1686–1694.

Supplementary key words DNA • cholesterol • restriction fragment length polymorphism • lipoproteins • coronary heart disease

The apolipoprotein (apo) B gene is a tissue-specific gene that is expressed mainly in liver and intestine (reviewed in refs. 1 and 2). In the human liver, transcription of the apoB gene gives rise to an mRNA product that is translated to yield a protein of 4536 amino acids, designated as apoB-100 \( (3) \). Triglyceride, cholesteryl ester, and phospholipid molecules associate with the protein both during and after synthesis of apoB-100 in the liver, ultimately leading to the assembly of triglyceride-rich very low density lipoprotein (VLDL) particles. After the secretion of VLDL by the liver, the triglyceride moiety of the lipoprotein is hydrolyzed in the circulation by the lipolytic enzyme lipoprotein lipase (LPL), a process which initially results in the formation of intermediate density lipoprotein (IDL) particles and ultimately leads to the generation of low density lipoprotein (LDL) particles \( (4) \). In the human intestine, however, the apoB mRNA is edited at position 6666, causing a C for U substitution that creates a stop codon \( (5, 6) \). Translation of the edited mRNA yields apoB-48, a protein consisting of the amino-terminal 2152 amino acids of apoB-100. ApoB-48 is assembled into lipoprotein particles by the addition of lipids, and secreted from the enterocytes as triglyceride-rich chylomicrons. Like VLDL, the triglyceride moiety of the chylomicrons is hydrolyzed by LPL, but unlike VLDL, this process does not lead to the generation of LDL, but instead to the formation of chylomicron remnants, lipoprotein particles which are effectively removed from the circulation by the liver. In short, the apoB-100 synthesized by the liver and secreted as part of the triglyceride-rich VLDL particle is the primary precursor of LDL apoB.

Plasma levels of LDL cholesterol and apoB (apoB-100) correlate directly with the incidence of coronary heart disease (CHD). Thus, an imbalance between production and degradation of apoB-containing lipoproteins has the profound consequences of hypercholesterolemia and, potentially, atherosclerosis. While genetic factors account for approximately one-half of the variation in plasma LDL levels, genetic variation in the apoB gene is an important determinant.

Abbreviations: apo, apolipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; CHD, coronary heart disease; LPL, lipoprotein lipase; bp, base pair; BMI, body mass index; EMSA, electromobility shift assay; DMEM, Dulbecco’s modified Eagle’s medium.

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cholesterol concentration in humans (7), considerable efforts have been made during the past decade to define mutations associated with hypercholesterolemia. Most research has focused on mutations that might affect the interaction of apoB-containing lipoproteins with cellular receptors (8, 9). However, a significant cause of hypercholesterolemia might also be overproduction of apoB-containing lipoproteins (10).

The processes regulating the synthesis of VLDL by hepatocytes have been studied extensively in cell culture systems (for review see ref. 11). Many groups have reported that the amount of apoB synthesized in the liver cells is in excess of the amount secreted (see for example ref. 12). It thus appears that a (substantial) proportion of the newly synthesized apoB molecules is degraded intracellularly (1). It is generally thought that this degradation process is an important regulatory mechanism and that lipid availability plays a major role in the posttranslational regulation of VLDL secretion. Transcriptional control is not believed to be a major modulator of apoB production by the liver, while several in vitro studies have shown that the steady state level of apoB mRNA remains relatively constant under conditions in which the rate of apoB production changes significantly (13–15). Nevertheless, a number of laboratories have reported that the steady state level of hepatic apoB mRNA may change, although the magnitude of change is small. Indeed, in several studies a positive correlation between the levels of apoB mRNA and apoB secretion was observed (16–19), suggesting that the rate of apoB synthesis can influence the magnitude of VLDL secretion. This hypothesis is supported by recent studies in transgenic animals, which demonstrated that overexpression of the human apoB gene was associated with substantially increased plasma LDL concentrations (20–23).

The potential relationships in humans between polymorphisms in the apoB gene and the plasma levels of apoB-containing lipoproteins have been evaluated in a large number of studies (see for example refs. 24–34). Several reports documented associations between polymorphisms of the apoB gene locus and LDL cholesterol levels, but not one of these associations has been consistently observed in a large number of studies. Indeed, none of the common polymorphisms analyzed thus far has been generally accepted as a genetic marker of hypercholesterolemia. Moreover, it is largely unknown whether any of the apoB polymorphisms are linked directly to changes in apoB metabolism. It is therefore not clear whether variation in apoB gene transcription plays a role in the regulation of plasma LDL concentration in humans. To address this question we have screened the proximal promoter of the human apoB gene in search for a common genetic variant with distinct effects on the transcriptional activity of the gene. Here we report the discovery of a C to T substitution at position –516 of the promoter of the apoB gene. We provide evidence that this common genetic variant influences the basal rate of transcription of the apoB gene in the liver and is associated with a significant increase in the plasma LDL cholesterol concentration in middle-aged men. These observations provide the first human evidence in support of the hypothesis that variation in the rate of transcription of the apoB gene may affect the plasma LDL cholesterol concentration at physiological levels.

METHODS

Subjects

A total of 179 men with a first myocardial infarction before the age of 45 were included in this study. They belonged to a consecutive series of native Swedish patients who were admitted to the 10 hospitals in the greater Stockholm area with a coronary care unit. The patients were subsequently referred to the Karolinska Hospital for metabolic, hemostatic, and cardiological investigations. All patients were examined 4–6 months after the acute event, when it was expected that acute-phase reactions due to the myocardial infarction had declined. During the study period, 198 male survivors of a first myocardial infarction were analyzed at the Karolinska Hospital. Exclusion criteria for this study were the presence of concomitant disorders, like severely impaired renal function, arteritis, collagenosis, and diabetes mellitus, and a history of alcohol abuse or other forms of addiction. On the basis of these criteria the total population was reduced to 179 patients.

The control group comprised two subsets. One subset consisted of 186 healthy men with the same age distribution as the patients. The other subset was composed of 156 healthy men, all 50 years of age. All the men were selected at random from a register containing all permanent residents in Stockholm County. Of those initially invited, 81% agreed to participate in the research program. All the men were interviewed to exclude individuals with a history of cardiovascular disease. Additional exclusion criteria were identical to the ones used for the selection of the patients. Thus, the control subjects, like the patients, were all of Swedish extraction.

The study was approved by the ethics committee of the Karolinska Hospital, and all subjects had given their informed consent to participation.

Blood sampling, biochemical methods, and DNA procedures

Blood sampling, preparation of plasma, and quantification of major fasting plasma lipoproteins were as described (35). For DNA procedures, nucleated cells from frozen whole blood were prepared according to Sambrook, Fritsch, and Maniatis (36), and DNA was extracted by a salting-out method (37). All subjects were genotyped for the apoE polymorphism, essentially as described by van den Maagdenburg et al. (38), except that 3% metaphore agarose (FMC BioProducts, Rockland, ME) was used for the separation of the PCR products. All DNA samples were tested on two occasions in a blinded fashion. Except for two administrative errors, complete agreement between the two assays was observed.

Gene sequencing

For the nucleotide sequencing of the promoter of the apoB gene, a 824 base pair (bp) section of the proximal promoter, spanning from position –847 to –23, was amplified by PCR using the forward primer 5′-TGCTTAGAAGGAAGGAAC and the reverse primer 5′-TTTATAGGACCGCGCGT. This PCR-fragment was used as template for further amplifications as part of the Taq DyeDeoxy Terminator Cycle sequencing system (Perkin-Elmer, Applied Biosystems Division, Foster City CA). Nested primers, designed on the basis of the published sequence of the promoter.
of the apoB gene, were used for the analysis of overlapping sections of 200–300 bp in both directions.

Genotyping

Genotyping for the −516C/T polymorphism was performed in all subjects using two different RFLPs with non-overlapping primer pairs for PCR amplification in order to enhance the reliability of the RFLP results. With the exception of administrative errors, complete agreement was observed between the two assays for all DNA samples analyzed. Examples of the assays are shown in Fig. 1. The first primer pair (designated A; Fig. 1, lanes 2–4) contained the forward primer 5'-GCTGGGTTTCTTGAAGACA and the reverse primer 5'-CAAGCCTCTCTCTGCTCTG, leading to a PCR product of 422 bp. The second primer pair (designated B; Fig. 1, lanes 5–7) was composed of the forward primer 5'-GGAAACCTAGAAGCTGGTGC and the reverse primer 5'-TCTTCAGATGACCCACCATG, generating a 430 bp fragment. The PCR-amplified fragments were digested with the restriction enzyme Ear I (New England Biolabs, Beverly, MA), followed by electrophoresis using 2% agarose. The conditions for genotyping were: PCR in a 25 μL reaction mixture containing 50–500 ng of genomic DNA, 1.2 μmol/L of each primer, 2.5 mmol/L MgCl₂, 10 mmol/L Tris-HCl, pH 9.0, 0.1% Triton X-100, 0.2 mmol/L of each dNTP and 1 unit of Taq polymerase. The reaction mixtures were incubated for 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 1 min, and extension at 72°C for 2 min. Digestion with Ear I was performed in 12 μL of the PCR product using 2 units of the restriction enzyme for 1 h at 37°C.

DNA constructs

Two sets of double-stranded oligonucleotides were constructed, constituting the 30 bp sequence around the polymorphic region of the −516C/T mutation, flanked by BamHI and BglII ends. The double-stranded oligonucleotides were ligated head to tail into a BamHI-digested HCAT vector (39). The correct sequence and orientation of the inserts were tested by DNA sequencing.

Electromobility shift assay (EMSA)

Nuclear extracts were prepared according to Alksnis et al. (40). All buffers were freshly supplemented with leupeptin (0.7 μg/ml), aprotinin (16.6 μg/ml), PMSF (0.2 μm), and 2-mercaptoethanol (0.33 μl/ml). The protein concentrations in the extracts were estimated by the method of Kalb and Bernlohr (41). Incubation for EMSA was conducted as described (42), and the reaction products were applied to 7% (wt/vol) polyacrylamide gel (80:1 acrylamide/N,N'-methylene-bisacrylamide weight ratio), whereafter electrophoresis was performed in 22.5 mm Tris/22.5 mm boric acid/0.5 mm EDTA buffer for 2.5 h at 200 V. Non-radioactive competitor DNAs, either identical, or of the opposite allelic variant, or of non-specific origin, were added in 100-fold excess of the labeled DNA.

Transient transfection assay

Human hepatoma (HepG2) cells were cultured in 90-mm dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. Confluent cells were transfected using the calcium-phosphate DNA coprecipitation method, essentially as described by Sambrook, Fritsch, and Maniatis (36). The pSV-b-galactosidase gene (Promega, Madison, WI) was cotransfected as an internal control. In all experiments, 5 μg of CAT-construct and 5 μg of β-galactosidase plasmid were added to the medium. CAT activity was analyzed using the method described in (36) and quantified using a phosphorimager. β-Galactosidase activity was determined as described by the supplier (Promega, Madison, WI). CAT levels were expressed in arbitrary units after standardization for β-galactosidase activity. All constructs were tested in triplicate in 8 independent transfection experiments.

Statistical methods

Logarithmic transformation was performed on all skewed variables to obtain a normal distribution before statistical computations and significance testing were undertaken. Differences in continuous variables between groups were tested by Student’s unpaired two-tailed t test. Allele frequencies were compared by gene counting and χ² analysis. Differences in lipoprotein traits according to −516C/T genotype were assessed using either one-way or two-way analysis of variance, in the latter case apoE genotype, body mass index (BMI), and age were used as covariates.

RESULTS

Detection of a common C to T substitution at position −516 of the apoB promoter

An 824 bp section of the proximal promoter of the apoB gene was sequenced in both directions using DNA samples from 10 subjects with a broad range of plasma LDL cholesterol concentrations. No differences compared with the published nucleotide sequence (43) were found. However, a C to T substitution at position −516 was observed in several subjects. RFLP assays were developed for the detection of the −516C/T polymorphism (see below), and the nucleotide sequence of the promoter of the apoB gene was determined in DNA samples from three additional subjects who were homozygous for the rare −516T allele. No additional polymorphisms were detected when a total of 10 alleles with the −516T variant were analyzed.

Fig. 1. Genotyping of the −516C/T polymorphism. The PCR fragments A (lanes 2–4) and B (lanes 5–7) were used to analyze subjects with the −516C/C (lanes 2 and 5), the −516C/T (lanes 3 and 6), and the −516T/T (lanes 4 and 7) genotypes. The PCR fragment A generated a band of 422 bp or bands of 306 and 116 bp, while the PCR fragment B generated bands of 339 and 91 bp or bands of 206, 133, and 91 bp, after digestion with the restriction enzyme Ear I. Lanes 1 and 8 contain a 123 bp ladder as molecular weight marker. The negative image of the ethidium bromide-stained agarose gel is shown. For more experimental detail, see Methods.
Allele-specific binding of one or more nuclear proteins

The $-516C/T$ polymorphism is located in the middle of footprint E described by Paulweber et al. (44). We therefore explored the possibility that the C to T substitution might affect the interaction with nuclear proteins, using the EMSA. The binding characteristics of a 30 bp DNA fragment containing either the $-516C$ or the $-516T$ site of the apoB promoter was evaluated using nuclear extracts derived from HepG2 cells. As indicated by the arrows in Fig. 2, two protein-DNA complexes were found to be associated with the $-516C$ allele, which were not detectable when the $-516T$ allele was analyzed. Occasionally, we noted that the upper band shown in Fig. 2 was split in two bands with slightly different mobility. Competition studies showed that a 100-fold excess of unlabeled $-516C$ fragment substantially reduced the interaction of the labeled $-516C$ fragment with the nuclear proteins (Fig. 3, lane 3). In contrast, no effect was observed with a 100-fold excess of unlabeled $-516T$ fragment on the interaction of labeled $-516C$ fragment with the nuclear proteins (Fig. 3, lane 4). Taken together, the results of the EMSA studies provided strong evidence for a distinct difference between the $-516C$ and the $-516T$ fragments regarding the specific binding of one or more nuclear proteins.

$-516C/T$ polymorphism increases the transcription of the apoB gene

Transfection studies in HepG2 cells were conducted to explore whether the $-516C/T$ polymorphism influences the rate of transcription of the apoB gene. The CAT activities were compared between constructs harboring two tandemly arranged 30 bp fragments of the apoB promoter containing either the $-516C$ or the $-516T$ site. As shown in Fig. 4, significantly higher CAT activities were observed for the $-516T$ construct as compared to the $-516C$ construct ($+41\%$, $P < 0.05$). This indicates that the C to T substitution at the $-516$ position of the apoB promoter increases the rate of transcription of the apoB gene. However, it is noteworthy that the effect of the $-516C/T$ polymorphism on the rate of transcription is relatively small. Indeed, we failed to find a significant difference in CAT activities between constructs containing single copies of the 30 bp fragments with either the $-516C$ or the $-516T$ site.
were found when 766 bp promoter constructs with either the −516C site or the −516T site were tested in transfection experiments. However, in all of these experiments, a trend towards higher CAT activities for the −516T constructs as compared to the −516C constructs was noted (data not shown).

Association between the −516C/T polymorphism and the plasma LDL cholesterol level

The relationships of the −516C/T polymorphism to the plasma levels of apoB-containing lipoproteins were analyzed in two populations of apparently healthy middle-aged men. The data for the two groups were pooled, while similar allele frequencies and comparable relationships with lipoprotein levels were found in both groups. In all, 10 control subjects were excluded from the analysis, as these individuals displayed uncommon features known to markedly influence plasma LDL cholesterol concentrations (exclusion criteria: BMI > 35 (n = 4), VLDL cholesterol concentration > 2.0 mmol/L (n = 3), and E2/2 genotype (n = 3)). As shown in Table 1, the −516T allele was associated with significantly increased LDL cholesterol levels as compared to the −516C allele. The association was graded, e.g., subjects homozygous for the −516T allele had a greater increase in LDL cholesterol concentration as compared to subjects who were heterozygous for the −516T allele. No associations were observed between the −516C/T polymorphism and plasma VLDL cholesterol or HDL cholesterol levels (Table 1). In addition, no association was found between the −516C/T polymorphism and plasma triglyceride level or VLDL triglyceride concentration (data not shown). To account for other factors known to be related to plasma LDL cholesterol concentration, a two-way analysis of variance was performed using three variables as covariates: apoE genotype, BMI, and age. The adjusted mean LDL cholesterol levels were 3.38, 3.56, and 3.77 mmol/L for the −516C/C, −516C/T, and −516T/T genotypes, respectively (P < 0.03). Thus, the results of this analysis confirmed that the −516T allele has a significant, independent relationship to plasma LDL cholesterol levels.

TABLE 1. Association between the −516C/T mutation and plasma lipoprotein levels

<table>
<thead>
<tr>
<th>Subjects (n)</th>
<th>−516C/C</th>
<th>−516C/T</th>
<th>−516T/T</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n)</td>
<td>157</td>
<td>147</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>0.42 ± 0.26</td>
<td>0.44 ± 0.30</td>
<td>0.42 ± 0.32</td>
<td>0.86</td>
</tr>
<tr>
<td>LDL</td>
<td>3.53 ± 0.81</td>
<td>3.77 ± 0.90</td>
<td>3.94 ± 0.81</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HDL</td>
<td>1.26 ± 0.35</td>
<td>1.21 ± 0.30</td>
<td>1.26 ± 0.33</td>
<td>0.51</td>
</tr>
<tr>
<td>Patients (n)</td>
<td>76</td>
<td>72</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>0.97 ± 0.67</td>
<td>1.01 ± 0.77</td>
<td>0.96 ± 0.39</td>
<td>0.93</td>
</tr>
<tr>
<td>LDL</td>
<td>4.30 ± 0.93</td>
<td>4.50 ± 1.33</td>
<td>4.79 ± 1.08</td>
<td>0.16</td>
</tr>
<tr>
<td>HDL</td>
<td>0.97 ± 0.21</td>
<td>0.97 ± 0.22</td>
<td>0.91 ± 0.23</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Values are mean ± SD. P values were calculated by ANOVA with the Scheffe post hoc test.

The observed relationship between the −516C/T polymorphism and the plasma LDL cholesterol level raised the question of whether this mutation might constitute a risk factor for CHD. We therefore analyzed the impact of the −516C/T polymorphism on the plasma lipoprotein levels in young post-infarction patients, and compared the allele frequencies of the −516C/T polymorphism between the patients and control subjects. The same criteria were used for the selection of the patients as were used for the selection of the control subjects, with the exception of an upper limit of 4.0 mmol/l instead of 2.0 mmol/l for the VLDL cholesterol concentration. In all, 7 patients were excluded (BMI > 35 (n = 4), VLDL cholesterol > 4.0 mmol/l (n = 3)). As shown in Table 1, qualitatively comparable results were obtained for the postinfarction patients as for the control subjects when the relationship between the −516C/T polymorphism and plasma LDL cholesterol levels was evaluated. Again, a graded association between the −516T allele and LDL cholesterol level was observed. However, the positive relationship between the −516T allele and the LDL cholesterol concentration did not reach the level of statistical significance, primarily due to the inclusion of subjects with markedly elevated plasma LDL cholesterol concentrations. After two-way analysis of variance using apoE genotype, BMI, and age as covariates, the adjusted mean LDL cholesterol concentrations were 3.96, 4.12, and 4.51 mmol/l for the −516C/C, −516C/T, and −516T/T genotypes, respectively (P = 0.08).

A comparison was made between the postinfarction patients and the control subjects regarding allele frequencies of the −516C/T polymorphism and several established risk factors for CHD. As expected, significantly increased BMI, VLDL cholesterol, and LDL cholesterol levels and a decreased HDL cholesterol concentration were found in the patients as compared to the control subjects (Table 2). No significant difference in the allele frequencies of the −516C/T polymorphism was observed between the two groups, although there was a distinct trend towards a higher frequency of the −516T allele in the postinfarction population. Indeed, further analysis re-
revealed a significant difference in allele frequencies when the group comparison was restricted to subjects with E3/3 genotype and plasma LDL cholesterol concentrations below 6.0 mmol/L. These selection criteria were based on the well-known relationship between apoE genotype and plasma LDL levels (45). Moreover, it was assumed that a substantial proportion of subjects with plasma LDL cholesterol levels exceeding 6.0 mmol/L are characterized by monogenic disorders of lipoprotein metabolism that are unrelated to the −516C/T polymorphism. In this subgroup analysis, the frequencies of the rare −516T allele were 0.30 and 0.38 in the control subjects and patients, respectively (P < 0.05). This underlines the potential role of the −516C/T mutation as a risk factor for CHD.

### DISCUSSION

In this study we made an initial evaluation of the physiological significance of the C to T substitution at position −516 of the promoter region of the apoB gene. Three basic observations were made. First, in EMSA studies a distinct difference in the binding of nuclear factors was observed between 30 bp fragments containing either the −516C or the −516T site. Second, transfection studies provided evidence for an increased basal transcription rate of constructs containing the −516T site as compared to constructs containing the −516C site. Third, a significant association was found between the −516C/T genotype and fasting plasma LDL cholesterol concentration in apparently healthy middle-aged men, and a similar trend was noted in young survivors of a first myocardial infarction. It seems reasonable to assume that these three observations are interrelated and part of a sequence of events starting at the level of the apoB promoter and ultimately leading to increased plasma LDL cholesterol levels. We thus propose that the −516C/T polymorphism affects the binding of one or more hepatic nuclear factors to the promoter of the apoB gene, resulting in an increase in apoB expression in hepatocytes and an enhanced secretion of apoB-containing lipoproteins by the liver, ultimately leading to increased plasma LDL cholesterol levels. Whereas it must be stressed that several components of this hypothesis have not been tested experimentally, this working model is compatible with all data presented in this report.

The proximal promoters of 10 different alleles containing the −516T site were sequenced in order to uncover potential additional polymorphisms linked to the −516C/T polymorphism. As no additional promoter polymorphisms were found, it is likely that the observed association with the plasma LDL cholesterol concentration is primarily related to the −516T allele. We cannot formally exclude the possibility that other mutations linked to the −516T allele may influence the metabolism of apoB containing lipoproteins. Indeed, during the course of this study we noted that the −516C/T polymorphism and the insertion/deletion polymorphism (46) in the signal peptide of the apoB gene are in strong linkage disequilibrium. The signal peptide length polymorphism is caused by the deletion (SP24) of three hydrophobic amino acids from the core of the 27-residue-long peptide allele (SP27) (47). However, there are two lines of evidence that indicate that the −516C/T polymorphism, and not the signal peptide polymorphism, is of primary physiological significance in man. First, when expressed in yeast, the SP24 signal sequence variant confers a secretion defective phenotype as compared to the wild-type SP27 signal sequence (48). This suggests a possible association between the SP24 signal variant and reduced plasma LDL cholesterol concentrations. However, almost all in vitro studies have failed to detect a relationship between the SP24 signal variant and a decreased plasma LDL cholesterol concentration. In contrast, most of these studies provide evidence that the SP24 signal variant is associated to an increased plasma LDL cholesterol level (see for example refs. 32–34). As the SP24 signal variant is linked to the −516T allele, and the −516T allele is associated with an increased transcription rate of the apoB gene, it seems more plausible that the −516T allele is of primary importance for raising plasma LDL cholesterol levels. Second, in the present study a statistically significant relationship between the −516T allele and plasma LDL cholesterol levels was observed. As the SP24 signal variant is linked to the −516T allele, it was not surprising that we also found an association between the SP24 signal variant and the plasma LDL cholesterol concentration, but this relationship did not reach the level of statistical significance. In fact, in both the control population and the postinfarction patients, we noted a weaker relationship between plasma LDL cholesterol level and SP genotype as compared to the −516C/T genotype, which again argues in favor of a physiological role of the −516T allele. Taken together, it is likely that the −516C/T polymorphism is of primary functional significance for the observed association with the plasma LDL cholesterol concentration.

In this study we found no association between the −516C/T polymorphism and the plasma VLDL cholesterol concentration. Indeed, more extensive analysis of the plasma concentrations of different subfractions of triglyceride-rich lipoproteins failed to uncover any significant relationships with the −516C/T genotype. This indicates that the −516C/T polymorphism primarily affects plasma LDL cholesterol levels and is not associated with differences in the plasma levels of triglyceride-rich lipoproteins. These observations appear paradoxical, in view of the...
precursor-product relationship between VLDL and LDL. However, overexpression of apoB in transgenic animals leads to a similar change in the lipoprotein pattern: a prominent increase in plasma LDL concentration without marked changes in plasma VLDL levels (20–23). This indicates that substantial changes can occur in vivo in the rate of synthesis of triglyceride-rich lipoproteins without notable effects on the plasma VLDL levels. In addition, it is well known that the intra-individual variation in the plasma VLDL cholesterol concentration is large, whereas the intra-individual variation in the plasma LDL cholesterol level is relatively small. This phenomenon will reduce the chance of finding an association between a genetic variant and plasma VLDL levels. In general, it appears that the plasma VLDL level is an inadequate marker for the analysis of the metabolism of triglyceride-rich lipoproteins. More sophisticated methods will be required to evaluate the possible role of the −516C/T polymorphism in the metabolism of triglyceride-rich lipoproteins.

The −516C/T polymorphism is a common genetic variant in Sweden with a frequency of the rare allele of approximately 0.30. While the −516T allele was associated with increased plasma LDL cholesterol levels, we tested the hypothesis that the −516T allele may be associated with an increased risk of CHD. A suggestive increase in frequency of the −516T allele was observed amongst the postinfection patients, but this difference did not reach the level of statistical significance when the original groups were compared. However, the frequency in allele frequency became statistically significant when the analysis was restricted to individuals with apoE3/3 genotype and plasma LDL cholesterol concentrations below 6.0 mmol/l. Needless to say, caution should be exercised when interpreting the results of subgroup analyses, as these invariably lead to reductions in group size and are usually based on selection criteria that favor a positive outcome. In view of the fairly limited effect of the −516T allele on the plasma LDL cholesterol concentration, it will be necessary to analyze quite large populations to finally establish whether the −516T allele is a risk factor of CHD in the conventional epidemiological sense.

The regulation of the rate of synthesis of apoB-containing lipoproteins by the liver has been analyzed extensively in the past decade, predominantly using cell biological model systems (2, 11). The general consensus which has emerged from these studies is that the secretion of apoB-containing lipoproteins is predominantly regulated at the posttranscriptional level. However, recent studies in transgenic animals have challenged this conclusion. It was shown in both mice and rabbits that overexpression of the human apoB gene was associated with substantially increased plasma LDL concentrations (20–23). A positive correlation between transgene copy number and plasma LDL concentration was observed, suggesting that increased apoB gene expression can result in enhanced secretion of apoB-containing lipoproteins. This conclusion is corroborated by a study on transfected hepatoma cell lines expressing recombinant human apoB, which demonstrated an important effect of the level of apoB mRNA on the synthesis and secretion of apoB-containing lipoproteins (19). The results from the present study further extend these observations and provide evidence in humans that variation in the rate of transcription of the apoB gene affects the plasma LDL cholesterol concentration at physiological levels.

The level of apoB gene expression in the liver appears to be tightly controlled by a large number of nuclear proteins. Regulatory elements that enhance or reduce apoB expression have been mapped not only to the proximal promoter region of the apoB gene (44, 49–51), but also to regions in the second intron (52) and the 5′ distal portion (53) of the apoB gene. There is evidence that a large number of nuclear proteins bind to regulatory elements of the apoB gene and can influence the expression of apoB (54). Among these are C/EBP (55) and members of the steroid hormone receptor superfamily, such as HNF-4, ARP-1, EAR-2, and EAR-3 (56). However, the nature of the nuclear proteins interacting with most of the regulatory elements of the apoB gene has not been defined. Indeed, little is known regarding the relative importance of the different regulatory elements on the overall rate of apoB expression. In view of the complexity of this system, it seems likely that other genetic variants, in addition to the −516C/T polymorphism, may influence apoB expression and thereby affect plasma LDL cholesterol concentrations and the risk of CHD.

In all, the C to T substitution at position −516 represents a common, functional polymorphism in the promoter region of the apoB gene. The rare −516T allele significantly increases the plasma level of LDL, the main cholesterol-containing lipoprotein, and may confer a genetically determined increased risk of CHD.

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