New Functional Promoter Polymorphism, CETP/\(-629\), in Cholesteryl Ester Transfer Protein (CETP) Gene Related to CETP Mass and High Density Lipoprotein Cholesterol Levels

Role of Sp1/Sp3 in Transcriptional Regulation

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Abstract—A new polymorphism located at position \(-629\) (CETP/\(-629A/C\)) in the promoter of the cholesteryl ester transfer protein (CETP) gene is described. The \(-629A\) allele was associated with lower CETP mass \((P<0.0001)\) and higher high density lipoprotein cholesterol \((P<0.001)\) than the \(C\) allele in a sample of 536 control subjects from the ECTIM study. Transfection studies in HepG2 cells with a luciferase expression vector incorporating a 777-bp fragment of the CETP promoter and containing either \(A\) or \(C\) at position \(-629\) showed significantly lower luciferase activity with the promoter fragment of the \(A\) allele \((25\%, P<0.05)\). By gel-shift assay, DNA-protein interactions were evaluated in nuclear extracts of HepG2 cells with the use of 2 probes (\(A\) or \(C\) probe) composed of 20 bp of the promoter sequence surrounding the polymorphic site. Two specific complexes of distinct migration rate were identified with the \(A\) and the \(C\) probe. Competition with an excess of oligonucleotide containing the Sp1 consensus binding site showed that a protein(s) of the Sp transcription factor family was implicated in complex formation with the \(A\) probe but not with the \(C\) probe. Incubation with specific antibodies indicated that Sp1 and Sp3 bound specifically to the \(A\) probe. We introduced mutations in the \(-629\)-Sp1 binding site to test its functionality and to define the characteristics of transcription factor binding. We showed, by gel-shift assay, that no nuclear proteins bound to the mutated sequence. Transient transfection of HepG2 cells revealed that the expression of the mutated fragment was significantly increased compared with that of the \(A\) promoter fragment \((25\%, P<0.05)\). The mutated fragment displayed the same activity as that of the \(C\) promoter. These results indicate that Sp1 and/or Sp3 repress CETP promoter activity, whereas nuclear factors binding the \(C\) allele are without effect on promoter expression. (Arterioscler Thromb Vasc Biol. 2000;20:507-515.)

Key Words: cholesteryl ester transfer protein ■ gene polymorphisms ■ transcription factors ■ ECTIM ■ cardiovascular disease

Several prospective epidemiological studies have shown that elevated levels of HDL cholesterol (HDL-C) constitute an independent negative risk factor for coronary heart disease.\(^1\) Cholesteryl ester transfer protein (CETP) plays a central role in the reverse transport of cholesterol from peripheral tissues to the liver and in the remodeling of plasma lipoproteins by promoting the transfer of cholesteryl esters from HDL to LDL and VLDL lipoproteins.\(^2,3\) The critical role of CETP in lipoprotein metabolism is illustrated by the high levels of HDL-C observed in patients with genetic CETP deficiency.\(^4\) In addition, elevated levels of CETP activity may contribute to an increased risk of coronary artery disease by reducing the cholesterol content of HDL relative to LDL and VLDL\(^5\) and by promoting the formation of atherogenic, small, dense LDL in hyperlipidemic patients.\(^6\) Several common polymorphisms have been described in the CETP gene,\(^7-10\) most of which are associated with plasma CETP mass and HDL-C levels.\(^9,11,12\) However, these associations are complex. The effects of the CETP TaqIB polymorphism on plasma CETP mass and HDL-C appear to be independent.\(^10,13\) In addition, the strength of the relation between polymorphisms in the CETP gene and HDL-C levels may be affected by environmental factors. For example, the strength of the association was reduced by obesity and smoking\(^14,15\) and enhanced by mild hypertriglyceridemia and alcohol intake.\(^16,17\) Moreover, CETP activity can be modulated independently of variation in CETP mass by the metabolic state and especially by levels of TG-rich lipoproteins.\(^13,15,18\)

Only rare mutations, prevalent in the Japanese population, appear to affect either the secretion or the functionality of
CETP: these involve splicing defects, introns 10 and 14,\textsuperscript{19–21} nonsense mutations within exons 2,\textsuperscript{2,2} 6,\textsuperscript{2,3} 9,\textsuperscript{2,4} and 10,\textsuperscript{2,5} and a missense mutation within exon 15,\textsuperscript{2,6} D442G, which is close to the active site of the enzyme. This latter mutation leads not only to reduction in CETP biosynthesis but also to diminution in the specific activity of the enzyme. Conversely, none of the frequently occurring polymorphisms described to date have been demonstrated to be functional; most of these are located within intronic regions (introns 1, 7, 8, 9, and 10),\textsuperscript{7,8,10} and there is no evidence that they affect the splicing of the CETP mRNA. The possibility that intron sequences may be involved in the regulation of CETP promoter activity cannot be excluded. However, none of these polymorphisms has been demonstrated to be implicated in such mechanisms. Only 1 common variant within exon 14 has been described in the coding region,\textsuperscript{9} and it leads to alteration in the primary structure of the protein (Ile405Val). However, this structural change does not affect the specific activity of CETP.\textsuperscript{17} Some of these polymorphisms are associated with CETP mass and HDL-C level and could represent markers for at least 1 functional variant in linkage disequilibrium with them. A previous investigation\textsuperscript{10} has suggested that such a variant is not located in the coding sequence of the CETP gene. We have recently identified 2 new polymorphisms in the 5′-flanking region of the CETP gene at positions −629 (CETP/−629) and −631 (CETP/−631) from the start of the translation.\textsuperscript{2,7} The CETP/−629 polymorphism is tightly concordant with the TaqIB polymorphism and could account for the associations found between the latter polymorphism and plasma CETP mass and HDL-C level. The results of transfection experiments that we report in the present study demonstrate that this polymorphism is functional, with the A allele displaying lower promoter activity than the C allele. Moreover, gel-shift assays reveal that the Sp1 and Sp3 transcription factors bind to the A but not the C allele and act as repressors of promoter activity.

Methods

Study Population

The population of the ECTIM study, a case/control study of myocardial infarction, has already been described in detail.\textsuperscript{2,8} In the present study, which is focused on plasma CETP and HDL-C, only data in controls are reported to avoid bias that might result from possible changes due to myocardial infarction. Briefly, controls were men aged 25 to 64 years who were randomly sampled from the population of Belfast (Northern Ireland), Lille (northern France), Strasbourg (eastern France), and Toulouse (southwestern France). All participants were of European ancestry for at least 2 generations. Informed consent was obtained from all subjects. A set of questionnaires was completed that included details of personal history (including the presence of cardiovascular disease), drug intake (not including dose), cigarette smoking, and alcohol consumption. The present analysis, which is focused on HDL-related lipid variables, includes subjects receiving lipid-lowering drugs. However, the results were unaffected by exclusion of subjects treated with lipid-lowering drugs (data not shown).

Analysis of Plasma Lipid Parameters

The assays for determination of lipoprotein parameters and especially HDL-C, together with detailed analysis of lipid variables, have been reported earlier for the ECTIM study.\textsuperscript{2,9} CETP mass was measured in frozen samples stored at −80°C by a 2-site immunoenzymatic assay.\textsuperscript{2,9} Missing measurements are the consequence of transportation or storage problems; subjects not included did not differ from those included for any relevant parameter.

Detection of Gene Polymorphisms by SSCP and Sequencing of 5′-Flanking Region

For polymerase chain reaction (PCR)/single-strand conformational polymorphism (SSCP) analysis, 20 individuals were randomly selected among controls in the Belfast and Strasbourg populations. Genomic DNA was prepared from white blood cells by phenol extraction. A fragment of 820 bp of the upstream sequence of the CETP gene\textsuperscript{2,10} was divided into overlapping fragments of ≈300 bp and enzymatically amplified by use of specific oligonucleotides. Each amplification was performed by using 250 ng of DNA in a total volume of 50 μL containing 10 mmol/L Tris-Cl, 1.5 mmol/L MgCl\textsubscript{2}, 0.1% Triton X-100, 0.2 mg/mL BSA, 200 μmol/L dNTPs, 25 pmol of each primer, and 0.2 U Taq polymerase (ATGC). For the SSCP analysis, 0.3 μCi of [α-\textsuperscript{32}P]dCTP was added to the mixture. Thereafter, PCR products were diluted 2-fold in a solution containing 95% formamide, 10 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol. After denaturation at 94°C for 5 minutes, the samples were placed on ice, and a 4-μL sample was loaded onto nondenaturing 6% acrylamide gels (acrylamide to bis-acrylamide ratio 39:1). Electrophoresis was performed at room temperature at 40-W constant power for 6 hours in the absence or presence of 7.5% glycerol in the gels. The gels were subsequently dried and autoradiographed overnight.

DNA from patients with different SSCP migration patterns was reamplified by PCR with unlabelled primers. PCR products were then purified and sequenced by the method of Sanger et al\textsuperscript{3,1} with use of a direct sequencing kit (Perkin-Elmer). All samples of DNA that were analyzed differed only by the presence of A or C at the position of the considered polymorphism (−629 or −631). However, when 2 published sequences, which concern 360 and 3500 bp of the promoter (Agellon et al\textsuperscript{3,2} and Oliveira et al\textsuperscript{3,0} respectively) are considered, several discrepancies were noted at position −310 (we and Oliveira et al reported T, and Agellon et al reported A), at position −323 (we and Agellon et al reported G, and Oliveira et al reported C), and at position −266 (we and Oliveira et al reported 6A, and Agellon et al reported 5A).

Allele-Specific Oligonucleotide Hybridization

Genotyping of the CETP/−631 and the CETP/−629 polymorphisms was performed in all subjects participating in the ECTIM Study by use of allele-specific oligonucleotides.\textsuperscript{3,3} The 2 polymorphisms were detected on the same amplified fragment (262 bp) by use of the following probes: upper, GCATAACAGTTCACACAAC; lower, TGTCCTGACTGTAGTATT (annealing temperature 55°C). The probes used and the assay conditions are described in Table 1. After enzymatic amplification, 1/5 of the PCR product was denatured in 150 μL of 0.5 mol/L NaOH and 1.5 mol/L NaCl with 10 μL of 0.05% bromophenol solution and blotted onto nylon membranes (Hybond N+, Amersham). Four oligonucleotides were used to detect the 2 polymorphisms: (1) TGTTACCAACCCAGAG, (2) GTATACCCCGCAGT, (3) TGTTACACCCCGAG, and (4) TGTTATACACCCAGAG (the polymorphic nucleotides are underlined and in bold). Hybridization temperatures were 47°C, 49°C, 45°C, and 47°C, respectively. Oligonucleotides 1 and 2 were used to detect haplotypes CC and CA, and oligonucleotides 3 and 4 were

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**TABLE 1. Allele Frequencies and Pairwise Linkage Disequilibrium Coefficients**

<table>
<thead>
<tr>
<th>Position</th>
<th>Allele Frequency</th>
<th>CETP/−629A</th>
<th>Tag1B</th>
</tr>
</thead>
<tbody>
<tr>
<td>CETP/−631C/A</td>
<td>0.918/0.082</td>
<td>−1.00</td>
<td>−0.95</td>
</tr>
<tr>
<td>CETP/−629A/C</td>
<td>0.469/0.531</td>
<td>⋯</td>
<td>+0.95</td>
</tr>
<tr>
<td>Tag1B</td>
<td>0.596/0.404</td>
<td>⋯</td>
<td>⋯</td>
</tr>
</tbody>
</table>

All linkage disequilibria were significant at \( P<0.001 \).
used to detect haplotypes AA and AC. Each allele was detected after preincubation of the membranes for 2 hours with 50 pmol of unlabeled oligonucleotide probe for the other allele, followed by incubation for 4 hours with 10 pmol of the labeled probe specific for the studied allele. The membranes were washed twice at room temperature in 1× SSC for 5 minutes followed by 10 minutes in 0.5× SSC at the hybridization temperatures.

Plasmid Construction
A 777-bp fragment (extending from the translation start site to −745 bp) of the CETP promoter was amplified by PCR from individuals homozygous for either the −629A or −629C allele by use of oligonucleotides designed to create Nhel (5′) and BgIII (3′) cutting sites. The digested fragments were cloned between the unique Nhel and BgIII sites of the pGL3 basic luciferase expression vector (Promega). In the same manner, we generated a control vector in which the luciferase gene was driven by 138 bp (relative to the translation start site) of the CETP promoter. Indeed, a previous study showed that this fragment was sufficient to promote maximum activity of the CETP promoter in transfected HepG2 cells.

Thereafter, the integrity of inserts was verified for all constructs by sequencing, and a large amount of each vector was prepared by double equilibrium ultracentrifugation in cesium chloride.

Generation of Mutations
Mutagenesis was conducted with use of the Gene Editor Site-Directed Mutagenesis System (Promega). The following synthetic oligonucleotide, containing 2 mismatched bases (underlined bases), was used to introduce mutations at positions −627 and −626 of the CETP gene promoter: 5′ GGCGTGATACCACCGAGAGTTAT TatTTTTATGC 3′. The −629A construct was used as the matrix. The presence of the mutations was confirmed by sequencing.

Cell Culture and Transfections
HepG2 cells were obtained from the American Type Culture Collection and maintained in culture in DMEM supplemented with 10% FBS (Bio-Whittaker), L-glutamine (2 mmol/L) and gentamycin (40 μg/mL) at 37°C, 5% CO2. Cells were transfected using Lipofectin Liposomal reagent (GIBCO-BRL) according to the manufacturer’s protocol. To account for variable transfection efficiency, 3 μg of each CETP promoter construct was cotransfected with 0.5 μg of a β-galactosidase expression plasmid (pSV-βgal, Promega). After 24 hours of incubation, the medium was removed, fresh medium was added, and the cells were incubated for an additional 16 hours. Cells were harvested in Cellular Lysis Buffer (Promega), cell debris was pelleted by centrifugation, and the supernatant was used for assays. Luciferase activities were measured in a 1420 VICTOR Multilabel counter (Wallac, EG and G Co) with luciferin reagent (Promega). The β-galactosidase activities were determined by a colorimetric method (Promega), and values were used to normalize variability in transfection efficiency. Data were averaged from at least 3 independent experiments performed in triplicate with 2 different preparations of DNA.

Electrophoretic Mobility Shift Assay
HepG2 nuclear extracts were prepared from confluent 150-mm dishes by the method described by Dignam et al. Aliquots of nuclear extracts were stored at −70°C. The protein concentration was determined by bichinchoninic acid protein assay reagent BCA (Pierce). For electrophoretic mobility shift assay (EMSA), 0.25 pmol of [32P]-end-labeled double-strand oligonucleotide (1×10⁶ cpm) was mixed with 6 μg of nuclear factors in a final volume of 20 μL containing 10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 3 mmol/L MgCl₂, 0.5 mmol/L EDTA, 1 mmol/L dithiothreitol, 5% glycerol, 2 μg poly(dI-dC)·poly(dI-dC), 4 mmol/L spermidine, and 1 μg BSA. The appropriate competitor was added to the reaction mixture before the addition of the end-labeled probe. When indicated, 1 μg of rabbit affinity-purified polyclonal antibody raised against Sp1 or Sp3 (Santa Cruz Biotechnology) was incubated with nuclear extracts for 30 minutes on ice before the addition of the probe. The anti-Sp1 and anti-Sp3 antibodies correspond to epitopes at amino acids 436 to 454 and 676 to 695 of human proteins. Finally, samples were incubated for 15 minutes on ice, loaded onto a 6% polyacrylamide gel, and electrophoresed at 200 V for 3 hours. The protein-DNA complexes were visualized by autoradiography of the dried gels on Hyperfilm MP (Amersham, Life Science) at −70°C.

Oligonucleotides for the A or C probe were as follows: for −629A, 5′GGCGTTGATACCCACCAGGTTATT 3′; for −629C, 5′GGCGTTGATACCCCGAGAGTTAT 3′.

When oligonucleotides including mutations in the −629A probe were used, the positions of the mutations are shown in Figure 4. Double-strand Sp1 and Egr consensus oligonucleotides were purchased from Promega and Tebu, respectively. A synthetic oligonucleotide containing the PuF consensus binding site was synthesized as described by Ji et al. The nonspecific competitor had the following sequence: 5′ TGTCGAAATGCACACTAGAA 3′.

Statistical Analysis
Pairwise linkage disequilibrium coefficients were estimated by using log-linear model analysis. The extent of the disequilibrium is reported as the ratio of the unstandardized coefficients to their minimal/maximal value (D’). The sign in front of the coefficients indicates whether the linkage disequilibrium is positive (rare alleles preferentially associated) or negative (rare allele preferentially associated with frequent allele). Mean levels of plasma CETP mass and HDL-C were compared across genotypes by ANOVA, and possible interactions between genotypes and between genotypes and quantitative variables, such as alcohol consumption, on dependent variables were also tested (SAS/Proc GLM). Hardy-Weinberg equilibrium was tested by a χ² test with 1 df.

Results
Localization and Frequencies
A fragment of 820 nucleotides of the CETP promoter (from the translation start site to −793 bp) was screened for polymorphisms in 20 individuals by using PCR/SSCP and sequencing. Two new polymorphisms (C/A) were identified at positions −629 and −631 relative to the transcription start site. These polymorphisms were analyzed in all participants of the ECTIM study. Genotype frequencies of both polymorphisms were in Hardy-Weinberg equilibrium. The frequency of the CETP/−631A allele was 0.082, and that of the CETP/−629A allele was 0.469 (Table 1). Allele frequencies for the CETP/−629 polymorphism were homogeneous across the populations of the ECTIM study, whereas the frequency of the CETP/−631 polymorphism increased from Belfast to Toulouse (Table 2).

Effects of CETP Polymorphisms on Plasma CETP Mass and HDL-C
The effects of the 2 polymorphisms on plasma CETP mass and HDL-C levels (536 and 668 subjects, respectively) are shown in Table 2. The CETP/−629 polymorphism was significantly associated with plasma concentrations of both CETP mass and HDL-C. Subjects homozygous for the −629C allele displayed higher CETP mass and lower HDL-C concentrations than subjects homozygous for the −629A allele (30% [P<0.001] and 12% [P<0.01], respectively). Subjects heterozygous for the polymorphism (−629C/A) displayed intermediate values. Conversely, no significant correlation was observed between the CETP/−631 poly-
morphism and either plasma CETP mass or HDL-C concentration. This lack of significant association was unaffected when the CETP/−629 polymorphism was taken into account in the statistical analyses. No association was observed between the CETP polymorphism and other lipid variables, including VLDL or LDL cholesterol, plasma cholesterol, apoB, or triglyceride concentrations (data not shown). Interestingly, as we reported before, no correlation was observed between plasma CETP mass and HDL-C. As expected from the tight association between the CETP/−629 and CETP/TaqIB polymorphisms and from our former results concerning CETP/TaqIB, a strong interaction between the CETP/−629 polymorphism and alcohol consumption on plasma HDL-C was detected (result not shown).

Functionality of CETP/−629C/A Polymorphism

To determine whether the CETP/−629 polymorphism might influence the activity of the CETP promoter, transient transfections of HepG2 cells were performed with 4 plasmids: a negative control consisting of the pG3 basic vector lacking an insert, a 138-bp plasmid containing the proximal region upstream from the translation site as a positive control, and 2 other constructs containing a 777-bp fragment (from translation site to −745 bp) with either A or C at position −629 (termed p−629A and p−629C, respectively). As expected, the negative control resulted in low levels of luciferase activity. The highest expression was obtained with the 138-bp fragment of the CETP promoter, as already described. Luciferase activity produced by vectors containing the long promoter fragment (777 bp) represented ~30% of the activity obtained with the short fragment (~138 bp, data not shown). The results obtained after transfection with the 2 CETP allelic promoter fragments are shown in Figure 1. Luciferase activity after cell transfection with the plasmid containing the A allele, p−629A, was significantly lower than that observed with the C allele, p−629C (2960±308 versus 3834±397 related luciferase units, respectively; P<0.05), with activity of the A allele representing ~75% of that of the C allele. This finding is consistent with the low plasma CETP mass observed in subjects homozygous for the A allele.

EMSA of CETP Promoter Fragment Surrounding −629C/A Polymorphism

To investigate protein-DNA interactions in the vicinity of the polymorphism, EMSA was performed by use of nuclear extracts from HepG2 and synthetic oligonucleotides spanning the sequence of the CETP gene promoter from −641 to −617 bp and containing either A or C at position −629. Interaction with the A probe in the absence of competitor resulted in the formation of 4 bands (Figure 2, lane 1). Two bands, designed AI and AII, were specifically competed by a molar excess of unlabeled A probe (Figure 2, lanes 3 and 4) but not by a nonspecific competitor (Figure 2, lane 2). In the same manner, 4 retarded complexes were observed after incubation of nuclear extracts with the C probe in the absence of competitor (Figure 2, lane 8). Two of them, designed CI and CII, disappeared after competition with a molar excess of unlabeled C probe (Figure 2, lanes 12 and 13) but not after competition with an unrelated oligonucleotide (Figure 2, lane 9). Moreover, a molar excess of unlabeled C or A probe was only a poor competitor of retarded complexes formed with the other radiolabeled probe: A (Figure 2, lanes 5 and 6) or C (Figure 2, lanes 10 and 11). Taken together, these results indicate that 2 specific complexes having different migration rates were formed with the 2 probes and suggest that different proteins are involved in the formation of complexes with the A or C probe.

The promoter sequence surrounding the −629 polymorphism displayed similarities with the inverted form of a nonconsensus Sp1 binding site, hereafter termed −629-Sp1.

![Figure 1. Expression studies of the CETP gene promoter polymorphism. HepG2 cells were transfected with the pGL3 basic vector containing 777 bp of each promoter allele (p−629A and p−629C) as described in Methods. The results are expressed as mean±SD of the average of 3 separate experiments with DNA from 2 different plasmid preparations. RLU indicates relative luciferase units. Luciferase activity was normalized to β-galactosidase activity. Statistical analyses were performed by the standard t test.](attachment:image)
Figure 2. EMSA of HepG2 nuclear proteins that interact with the 2 probes overlapping the −629 polymorphism. Left panel: interaction with the −629A probe. Right panel: interaction with the −629C probe. 0.25 pmol of each end-radiolabeled probe was incubated with 6 μg of nuclear extracts in the absence of competitor (lanes 1 and 8) or in the presence of a molar excess of unlabeled non-specific competitor oligonucleotide (lanes 2 and 9). −629A oligonucleotide (lanes 3, 4, 10, and 11), −629C oligonucleotide (lanes 5, 6, 12, and 13), and consensus Sp1 oligonucleotide (lanes 7 and 14). The specific retarded complexes are indicated by Al and AII for the −629A probe and by CI and CII for the −629C probe. NS indicates non-specific complexes.

Subsequently, we investigated whether any protein of the Sp1 transcription factor family might be implicated in complex formation. A synthetic oligonucleotide containing the Sp1 consensus binding site was used as competitor. The 2 complexes formed with the A probe, AI and AII, were entirely competed by an excess of Sp1 oligonucleotide (Figure 2, lane 7), whereas a partial competition was observed with CI, and no competition was observed with CII (Figure 2, lane 14). To confirm the participation of a protein of the Sp1 family in the formation of a specific complex, we used 2 antibodies specific for either human Sp1 or human Sp3 (Figure 3). The antibody against human Sp1 significantly decreased the Al complex (Figure 3, lane 2), whereas the antibody specific for human Sp3 partially abolished AI but totally abolished AII (Figure 3, lane 3). When antibodies to Sp1 and Sp3 were incubated together with nuclear extracts, complexes AI and AII were entirely disrupted and supershifted (Figure 3, lane 4). Formation of complexes CI and CII was not affected by incubation with antibodies to either Sp1 or Sp3 (Figure 3, lanes 7 and 8). Incubation with preimmune serum did not affect the formation of any complex formed with either the A or C probe (Figure 3, lanes 5 and 9). These results strongly suggest that Sp1 and Sp3 are the 2 nuclear factors implicated in the formation of the 2 specific complexes observed with the A probe.

Curiously, in the presence of the C probe, a complex was observed just below the CI band and especially in the presence of the nonspecific competitor (Figure 2, lane 9). This complex appeared to migrate at the same position as the AII complex and disappeared after competition with an excess of unlabeled A probe (Figure 2, lanes 10 and 11) but not after competition with the C probe (Figure 2, lanes 12 and 13). An excess of Sp1 oligonucleotide did not compete for the formation of this complex, indicating that no protein of the Sp family is implicated in the formation of this complex.

Further analysis of the promoter sequence surrounding the −629 polymorphism revealed similarities with 2 other consensus binding sites (or their inverted form) that could potentially bind the PuF/nm23H2 and Egr transcription factor families. To identify the factor(s) involved in the formation of CI and CII complexes, synthetic oligonucleotides containing either the PuF or the Egr consensus binding sites were used as competitors. The PuF probe competed with the retarded complex, CII. However, the incubation of nuclear extracts with an antibody specific to human nm23H2 (the protein that binds the PuF site) failed to disrupt either the CI or CII complexes (data not shown). Furthermore, a synthetic oligonucleotide containing the Egr consensus binding site did not compete for formation of CI or CII (data not shown).

Functional Relevance of −629-Sp1 Binding Site

To determine whether the −629-Sp1 binding site was implicated in the regulation of CETP promoter activity, we performed transfection experiments in HepG2 cells by use of the CETP promoter plasmid containing the A allele promoter fragment in which the nonconsensus Sp1 binding site was disrupted. We first generated an oligonucleotide that was mutated in the −629-Sp1 binding site to eliminate the fixation of Sp1 and Sp3 and that contained an A at position −629; in this way, binding of unidentified proteins to the −629C probe was avoided (Figure 4A). This mutated oligonucleotide (−629AM) was tested by EMSA (Figure 4B). When used as a competitor, a 100-fold molar excess of mutated oligonucleotide had no effect on the specific retarded complexes, AI and AII, formed with the −629A probe (Figure 4B, lane 3). By contrast, the same molar excess of unlabeled wild-type −629A completely prevented complex formation (Figure 4B, lane 2). When mutated oligonucleotide was used as a probe, incubation with HepG2 nuclear extracts in the absence of competitor resulted in the formation of 2 complexes having the same migration rate as the nonspecific complexes observed after incubation with the wild-type −629A probe (Figure 4B, lanes 4 and 1, respectively). A 100-fold molar excess of unlabeled mutated oligonucleotide did not affect the formation of these 2 complexes (Figure 4B, lane 5), thereby confirming their nonspecificity. As expected, under our EMSA conditions, the mutated oligonucleotide...
failed to specifically bind any transcription factors. These mutations were then introduced by site-directed mutagenesis into the p2629A vector and the CETP-mutated promoter plasmid (p2629AM) used to transfect HepG2 cells. As expected, A allele (p2629A) promoter activity was significantly lower than C allele (p2629C) promoter activity (225%, P<0.05; Figure 4C). Compared with the wild-type plasmid (p2629A), the mutated plasmid (p2629AM) resulted in a significant increase of promoter activity (25%, P<0.05; Figure 4C). Plasmid p2629AM displayed promoter activity similar to that of the p2629C plasmid containing the C allele promoter sequence (Figure 4C). These findings suggest that the binding of the 2 transcription factors, Sp1 and Sp3, to the −629-Sp1 binding site accounts for differences detected in promoter activity between the A and the C alleles.

Discussion

In the present study, we have described the first functional polymorphism in the promoter region of the CETP gene. Indeed, we detected 2 new polymorphisms in the 5′-flanking region of this gene, both corresponding to a C/A substitution at positions −631 and −629. The specific association between the polymorphism and plasma CETP mass and HDL-C was investigated in control samples from the ECTIM study. The −631 polymorphism was relatively uncommon (A allele frequency 0.079) and unrelated to CETP mass or HDL-C. However, the low frequency of the CETP/−631 polymorphism could introduce a bias in the interpretation of this result. Conversely, the −629 polymorphism was common (A allele frequency 0.469) and significantly associated with plasma CETP mass and HDL-C levels. The change in CETP mass observed with the −629 polymorphism (30%) resembled that observed for the TaqIB polymorphism (30%) in the same population (27%). In addition, the CETP/−629 polymorphism was in almost complete linkage disequilibrium with other known polymorphisms and especially with the TaqIB polymorphism. Considered with the fact that this polymorphism was located in the promoter of the gene, our data strongly suggest that CETP/−629 represents a functional variant. Indeed, our results revealed that the −629 polymorphism exerts a significant effect on transcriptional activity in reporter gene assays. The promoter fragment containing the A allele displayed lower activity than did that containing the C allele (3078 ± 308 versus 3834 ± 397 related luciferase units, respectively). This observation is consistent with the low CETP mass observed in subjects homozygous for the A allele. The promoter activity of the −629A allele was 25% lower than that of the C allele. This degree of reduction can be related to the 28% lower mass of plasma CETP observed in subjects homozygous for the A allele. Similar alterations in promoter activity “in vitro” have already been described for a polymorphism in the promoter of the lipoprotein lipase gene. HepG2 cells have been commonly used to study regulation of the CETP gene promoter; however, it is of
interest to further evaluate this polymorphism in other cell types as well as in animal models “in vivo.”

Two specific complexes, AI and AII, were identified when HepG2 nuclear extracts were incubated with the A probe. In the presence of an antibody specific for human Sp1, the band intensity of the high-M, complex, AI, was significantly reduced. Whereas in the presence of an antibody specific for human Sp3, the band intensity of this complex, AI, was slightly decreased, and the low-M, complex, AII, disappeared entirely. The molecular mass of Sp1 is 109 kDa, whereas Sp3 has been reported to be present in cells as 2 isoforms of 115 and 80 kDa. In the presence of the 2 antibodies, both complexes were supershifted, indicating that only Sp1 and Sp3 are involved in their formation. Thus, supershift data indicate that AI may correspond to complexes formed with Sp1 and the higher molecular weight isoform of Sp3 and that AII may correspond to complexes formed with the lower molecular weight isoform of Sp3. Together, these results strongly suggest that nuclear factors Sp1 and Sp3 bind specifically to the A allele. The basic recognition unit of the Sp family transcription factors is a motif with a consensus sequence 5′-GGGCGG-Pu-Py-3′, known as a GC box. Furthermore, GA and GT boxes (or their inverted forms, CT and CA elements) can also bind Sp proteins with similar specificity in the promoters of a variety of genes. Such observations are consistent with our results, because the promoter sequence surrounding the A allele closely resembles the inverted form of a GT box. Conversely, neither Sp1 nor Sp3 bound to the C allele; this finding is in agreement with a recent study showing that the transversion of the central C to G (or G to C for the inverted form) is critical in the formation of DNA-protein complexes.

When we introduced mutations in the -629-Sp1 binding site to avoid the binding of Sp1 and Sp3 and to avoid creation of new complexes with other DNA binding proteins, the activity of the mutated CETP promoter fragment was significantly increased compared with that of the A wild-type fragment. These data strongly suggest that binding of Sp1 to the -629 site is associated with lower transcriptional activity of the reporter gene and indicate that the -629-Sp1 binding site is functional. Previous studies have identified several binding sites for nuclear factors in the proximal sequence of the CETP gene promoter; these include Sp1 at position -38, in juxtaposition to the TATA box, and an apoA-1 regulatory protein-1 (ARP-1) site located between -118 and -93 bp. The proximal 580 bases of the promoter contain several potential binding sites for sterol response element binding protein-1 (SREBP-1) and at least 1 potential binding site for CCAAT-enhancer binding protein (C/EBP). Interestingly, ARP-1 can act either as a repressor or as an inducer of CETP gene transcription, depending on the presence or the absence of the promoter region between -636 and -300 bp and including the -629-Sp1 binding site. A distal element, which could correspond to the -629-Sp1 binding site, may therefore modulate CETP expression. On the other hand, it has been shown that SREBP-1 and C/EBP activate CETP gene expression. In addition, Sp1 can interact synergistically with a number of other nuclear factors and particularly with SREBP-1 and C/EBP. In vivo, transcription factors binding to distal regulatory elements can interact with transcription factors binding to sequences within the proximal region. Indeed, a recent study involving in vitro transient transfection has revealed an interaction between 2 transcription factors, Sp1 and GATA 1. Together, these data suggest that despite its distal position, the -629-Sp1 binding site may modulate transcriptional activity of the CETP gene by synergistic interaction with other nuclear factors, including ARP-1, SREBP-1, and C/EBP.

In the present study, transfection experiments showed that Sp1/Sp3 acted as repressors of promoter activity. It is generally admitted that Sp1 stimulates transcription and that Sp3 represses Sp1-mediated transcriptional activation. However, several studies suggest that gene regulation by Sp transcription factors is more complex than previously assumed. There is some evidence that Sp1 may itself decrease transcriptional activity when bound to certain Sp1 elements, depending on its local interactions. On the other hand, it has been demonstrated that Sp3 is a bifunctional protein containing independent repressor and activator domains. Such dual-function regulation is dependent on the promoter and the cellular context. In addition, it appears that the level of Sp3 or the nuclear Sp1/Sp3 ratio may play a role in regulating promoter transcription activity. Moreover, the Sp1/Sp3 ratio differs between cell types. From our results, the mechanism by which Sp1 and/or Sp3 represses CETP gene promoter activity cannot be precisely defined. Further investigations and especially cotransfection studies with vectors expressing either Sp1 or Sp3 in Drosophila SL2 cells, which lack endogenous Sp activity, would be necessary to define the molecular mechanism involved.

We did not identify nuclear factors binding the C allele. Nonetheless, the nm23H2 and Egr transcription factors were excluded. However, the mutated promoter fragment (which did not bind any transcription factors in the region of the polymorphism) expressed activity similar to the promoter fragment containing the C allele, strongly suggesting that nuclear factors binding the C allele do not affect promoter activity. One hypothesis may be that undetermined nuclear factors binding the C allele prevent the binding of Sp1 and Sp3 without themselves modulating promoter activity. On the other hand, we cannot exclude an action of these nuclear factors in another metabolic context or in vivo.

The CETP/-629 polymorphism is significantly associated with plasma CETP mass and HDL-C levels. However, no correlation was observed between CETP mass and HDL-C, thereby indicating that the 2 associations were independent. Clearly, our results show that the -629 polymorphism is functional and that it modulates not only the transcription rate of the CETP promoter but also plasma CETP mass. It has been suggested that CETP may be in excess in the plasma of normolipidemic subjects, such that cholesterol ester transfer rates are determined by plasma triglyceride concentrations; by contrast, CETP may be limiting in hypertriglyceridemic patients, in which case, transfer rates may be determined by plasma CETP concentration. The association between the -629 polymorphism and HDL-C could be, at least in part, the consequence of alterations in plasma CETP mass, especially in dyslipidemic individuals. The lack of correlation between CETP mass and HDL-C could reflect the contribution of metabolic and environmental factors to the modulation of HDL-C. However, it has been shown that CETP deficiency may be associated with the prevalence of cardiovascular
disease despite marked elevation of HDL-C, especially in hypertriglyceridemic patients. In addition, CETP activity appears to inhibit the progression of atherosclerosis in hypertriglyceridemic mice. Finally, a recent study has shown a significant relation between variation at the CETP gene locus and the progression of coronary atherosclerosis that is independent of plasma HDL-C levels. Clearly, then, the CETP gene may exert effects on cardiovascular risk that are independent of HDL-C levels. Thus, further knowledge of the molecular mechanisms that modulate the expression of the CETP gene is essential.

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References


New Functional Promoter Polymorphism, CETP−629, in Cholesteryl Ester Transfer Protein (CETP) Gene Related to CETP Mass and High Density Lipoprotein Cholesterol Levels: Role of Sp1/Sp3 in Transcriptional Regulation

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