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A novel cholesteryl ester transfer protein promoter polymorphism
(−971G/A) associated with plasma high-density lipoprotein cholesterol levels

Interaction with the TaqIB and −629C/A polymorphisms

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Abstract

The plasma cholesteryl ester transfer protein (CETP) plays a key role in reverse cholesterol transport (RCT) by mediating the transfer of cholesteryl ester (CE) from high-density lipoprotein (HDL) to atherogenic ApoB-containing lipoproteins, including VLDL, IDL and LDL. We describe a new polymorphism located at position −971 in the human CETP gene promoter, which corresponds to a G/A substitution at a potential AaI restriction site. The relationship between the −971G/A polymorphism, plasma lipid parameters and plasma CETP concentration was evaluated in the Etude Cas-Témoins de l’Infarctus du Myocarde (control–myocardial infarction cases) cohort, and revealed that the −971G/A polymorphism (A allele frequency: 0.491) was significantly associated with both plasma high-density lipoprotein cholesterol (HDL-C) levels and CETP concentration (P = 0.006 and 0.009, respectively). Subjects with genotype −971GG displayed both low HDL-C levels and high plasma CETP concentration, while genotype −971AA subjects displayed the inverse relationship. Evaluation of potential interactions between the −971G/A and the −629C/A or TaqIB polymorphisms demonstrated that the −971G/A polymorphism interacts significantly with both plasma high-density lipoprotein cholesterol (HDL-C) levels and CETP concentration (P = 0.0014 and 0.012, respectively), but does not affect plasma CETP concentration. These results clearly suggest that the interaction between the 971G/A polymorphism and either the −629C/A or TaqIB polymorphisms on plasma CETP concentration is different than that implicated in HDL-C levels. Transient transfection of HepG2 cells revealed that the −971G/A polymorphism did not modulate transcriptional activity of the human CETP gene promoter. The −971G/A promoter polymorphism therefore constitutes a non-functional marker. Furthermore, the observed effects of the −971G/A polymorphism on both plasma CETP concentration and HDL-C levels are due to functional variants in linkage disequilibrium with it. Our findings strongly suggest the existence of as yet unidentified functional polymorphisms in the CETP gene promoter that could explain the association between

Abbreviations: CETP, cholesteryl ester transfer protein; ECTIM, case–control study of myocardial infarction (Etude Cas-Témoins de l’Infarctus du Myocarde); HDL, high-density lipoproteins; HDL-C, high-density lipoprotein cholesterol.

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specific polymorphisms of the CETP gene and both plasma HDL-C and CETP concentrations. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Cholesteryl ester transfer protein; High-density lipoprotein cholesterol; Polymorphism; Myocardial infarction

1. Introduction

The hypothesis that high-density lipoprotein cholesterol (HDL-C) is protective towards atherosclerosis was first proposed by Barr et al. in 1951 [1]. The protective action of HDL may reflect the role of HDL particles in the reverse cholesterol transport (RCT) pathway [2]. Indeed, HDL particles are involved in the uptake of cholesterol from peripheral tissues and its transport back to the liver for excretion. Plasma cholesteryl ester transfer protein (CETP) plays a key role in RCT by mediating the transfer of cholesteryl esters (CEs) from HDL to atherogenic ApoB-containing lipoproteins [3]. However, the relationship between plasma CETP levels and cardiovascular risk remains controversial. CETP deficiencies caused by mutations of the CETP gene are frequently associated with high HDL-C levels [4,5]. Moreover, subjects who lack plasma CETP activity may display increased cardiovascular risk despite elevated plasma HDL concentration [6,7]. Conversely, transgenic mice overexpressing CETP display a reduction in HDL-C levels [8,9] and develop premature atherosclerosis [10].

The human CETP gene contains ~25 kbp and is composed of 16 exons [11,12]. Several polymorphisms, each corresponding to single base substitutions, have been identified in the promoter [13,14], in the coding [15] or intronic [11,16–18] regions of the CETP gene. Most of them have been reported to be associated with plasma CETP and HDL-C levels [17,19,20]. However, no relationship between allelic variation at the CETP locus and plasma HDL-C levels has been detected in nuclear families by sibling-pair linkage analysis [21,22]. The TaqIB polymorphism identified in the first intron of the CETP gene is associated with plasma CETP concentration [17,19,20,23] and HDL-C levels [17,19,23–29]. The relationship between plasma CETP mass and HDL-C levels has been reported to be modulated by environmental factors, such as obesity, smoking [25], alcohol consumption [30] or hypertriglyceridaemia [23]. Recently, it was demonstrated that the TaqIB polymorphism is associated with progression of coronary atherosclerosis independently of plasma HDL-C levels [31]. Moreover, the effects of the TaqIB polymorphism on plasma CETP and HDL-C are independent [17,23], suggesting the presence of at least two functional variants [17]. Recently, a polymorphism located at position −629 in the upstream region of the CETP gene promoter has been reported to modulate the transcriptional activity of the CETP gene in vitro [13]. In addition, this polymorphism is significantly associated with plasma CETP and HDL-C levels.

In the present study, we have identified a new polymorphism at position −971 (G/A) in the promoter of the CETP gene, which is significantly associated with both plasma CETP concentration and HDL-C levels. However, in vitro studies revealed that the −971G/A polymorphism did not modulate the transcriptional activity of the human CETP gene promoter. In addition, we observed a significant interaction between the −971G/A polymorphism and both the TaqIB and −629C/A polymorphisms on plasma HDL-C but not on plasma CETP levels, thereby suggesting that the mechanism(s) of the interaction between the 971G/A polymorphism and either the −629C/A or the TaqIB polymorphism on plasma CETP concentration is different than that implicated in HDL-C levels.

2. Materials and methods

2.1. Study population

The design of Etude Cas-Témoins de l’Infarctus du Myocarde (ECTIM), a case–control study on myocardial infarction, was described in 1992 [32]. Recent extensions of the recruitment were carried out in the United Kingdom in Belfast and Glasgow [33]. These two centres recruited both men and women, whereas only men were recruited in France (recruitment centres in Lille, Strasbourg, Toulouse). The results reported here are based on samples of subjects selected from populations covered by the WHO MONItoring in Cardiovascular disease (MONICA) registers of these five centres. Cases (n = 858; 759 males, 99 females) aged 25–64 years for men and 25–69 years for women, were recruited over a 6-month period (2 years for women in Belfast) after the index of myocardial infarction. Controls (n = 972; 842 males, 130 females) of comparable age and sex were recruited from the lists of general practitioners in the same areas in the United Kingdom, and from the electoral rolls in France. Informed consent was obtained from all subjects.

2.2. Analysis of plasma lipid parameters

Lipid, lipoprotein and apolipoprotein analyses have been previously reported in detail [32]. Plasma CETP concentration was determined using a two-site immunoenzymatic assay as described [34].
2.3. Identification of gene polymorphism by single strand conformation polymorphism and sequencing

Single strand conformation polymorphism (SSCP) was performed using DNA from 50 individuals randomly selected in the ECTIM population. A DNA fragment of 335 bp, corresponding to the promoter region from -710 to -1045 bp was obtained by using the polymerase chain reaction (PCR). The amplification was performed as follows: 100 ng of DNA was added in a 20 μl mixture containing 10 ng of each primer (the upstream primer: 5’-TGGGAAAACAGTGAGGGTCA-AG-3’ and the downstream primer: 5’-TCTGTGGC- ATTTCAATTCTG-3’), 0.25 μCi of [32P]dCTP (3000 Ci/mmol; Amersham, Les Ulis, France), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatine and 0.5 U Taq polymerase (Appligene, Illkirch, France). The reaction was carried out on a Peltier Thermal Cycler (PTC-200) and comprised of 3-min denaturation at 94 °C followed by 30 cycles of a 30-s denaturation at 94 °C, 30-s annealing at 56 °C and a final 1-min extension at 72 °C.

Following the PCR, 12 μl of a solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol were added to the PCR product. Samples were denatured for 5 min at 70 °C and immediately placed on ice. Samples (5 μl) were loaded onto a 5% acrylamide gel (acrylamide/bis-acrylamide 19:1) in the presence and in the absence of 10% glycerol. Electrophoresis was carried out at room temperature at 15 W for 4 h for gels devoid of glycerol and at 4 W overnight for gels with glycerol. Following electrophoresis, gels were transferred to 3MM paper (Whatman, Ivry sur seine, France), dried and exposed to Hyperfilm MP (Amersham) overnight.

DNA from individuals corresponding to different SSCP patterns was amplified as described above in the presence of a 2 mM concentration of each non-radio labelled dNTP. The PCR product was purified using the PCR QIAquick Purification kit (QIAGEN, Courtaboeuf, France) and cloned into a pGEM*-T vector (Promega, Charbonnières, France). The resulting plasmid was sequenced by the method of Sanger et al. [35] using the T7 Sequenase version 2.0 kit (Amersham).

2.4. Genotyping

The single point polymorphism identified at position -971 in the human CETP gene promoter is associated with the presence (−971G) or absence (−971A) of a cleavage site for the restriction enzyme AvaI. Genotyping of the ECTIM population, with the exception of samples from Glasgow, was carried out by enzymatic digestion. Following DNA amplification as described above, PCR products of 335 bp were digested overnight with 1 U of the restriction enzyme AvaI at 37 °C according to the manufacturer’s instructions (New England Biolabs, Saint Quentin en Yvelines, France). Digestion products (5 μl) were separated on a 5% acrylamide gel (acrylamide/bis-acrylamide 19:1) by electrophoresis at room temperature at 15 W for 4 h. Following electrophoresis, gels were transferred to 3MM paper (Whatman), dried and exposed to Hyperfilm MP (Amersham) overnight. Digestion of the PCR products of a 335-bp fragment containing a −971A by AvaI generated two fragments of 191 and 142 bp whereas three fragments of 191, 72 and 71 bp were generated when a G was present at position −971.

Genotyping of the ECTIM population from Glasgow was performed more recently using allele specific oligonucleotides (ASO). The PCR product of 335 bp was denatured in 200 μl of 1.5 M NaCl and 0.5 M NaOH, and blotted onto nylon membranes (Hybond + ; Amersham). Specific oligonucleotides for each allele were labelled with 25 μCi of [γ-32P]ATP (3000 Ci/mmole; NEN Life, Paris, France) by T4 polynucleotide kinase (Promega) at 37 °C for 30 min. Membranes were incubated overnight in the presence of 30 pmol of labelled probe (probe C: 5’-CCCTAGTCGGGAGTTTGAC-3’ and probe T: 5’-CCCTAGTCTGAGTTGCAC-3’) at melting temperature −5 °C (59 and 57 °C for probe C and T, respectively), washed at room temperature in 1× SSC for 3 min and then incubated for 3 min in 0.5× SSC at a melting temperature of −3 °C (61 and 59 °C for probe C and T, respectively). Membranes were exposed to Hyperfilm MP (Amersham) overnight.

2.5. DNA constructs

A 1090-bp DNA fragment corresponding to the region from +36 to −1054 of the CETP promoter was amplified by PCR from individuals homozygous for either the −971A or −971G allele. The sequence of the upstream primer was 5’-TCTGAGCTCTGGGA- AACAGTGAGGTCA-CCCGCC-3’; the sequence of the downstream primer was 5’-AGCAGATCTGGTTTACAGCGACTGTTGTAAGTG-3’. These primers contain sequences for the generation of a SacI restriction enzyme site in the upstream primer and a BglII restriction enzyme site in the downstream primer on PCR amplification. The thermal cycle profile was as described above with an annealing step for 30 s at 62 °C and 0.5 U of Taq polymerase (Stratagene, Saint Quentin en Yvelines, France). The PCR products were purified using the PCR QIAquick Purification kit (QIAGEN) and subcloned in a pGEM*-T vector (Promega). Then a 1077-bp fragment was isolated from pGEM*-T by enzymatic digestion with both SacI and BglII (New England Biolabs), purified by agarose gel electrophoresis and

cloned upstream of the luciferase coding region into the SacI/BglII digested pGL3 basic luciferase expression vector (Promega). The sequence of the insert was controlled by sequencing as described above and vectors were amplified and prepared by double equilibrium ultracentrifugation in caesium chloride. In a recent study, two new polymorphisms were identified in position −631 and −629 [13] in the human CETP gene promoter. Our two constructs −971GG and −971AA displayed a C and an A in position −631 and −629, respectively.

2.6. Cell culture and transient transfection experiments

HepG2 cells, obtained from the American Type Culture Collection (Rockville, MD) were grown at 37 °C in 5% CO2 in Dulbecco’s Modified Eagle’s Medium containing 10% foetal calf serum (GIBCO-BRL, Cergy Pontoise, France), 2 mM l-glutamine and 40 μg/ml gentamycin. Cells were seeded on six-well plates at 2.5 x 105 cells per well. After 48 h incubation, 3 μg of each CETP promoter construct was co-transfected with 0.5 μg of a β-galactosidase expression vector (pSV-βgal; Promega) using the Lipofectin Liposomal Reagent (GIBCO-BRL) in line with the manufacturer’s instructions. Twenty-four hours after transfection, the medium was replaced by fresh medium and the cells were incubated for an additional period of 16 h. Cells were harvested with 150 μl of Cell Culture Lysis Reagent (Promega). The lysate was centrifuged for 10 min at 14000 rpm in order to remove excess cellular fragments. Luciferase activity was measured on the supernatant using the Luciferase Assay System kit (Promega) in a 1420 VICTOR Multilabel counter (Wallac, EG and G Co), and β-galactosidase activity was measured using the β-galactosidase Enzyme Assay System kit (Promega). Transcriptional activity was expressed after normalization for β-galactosidase activity. Experiments were performed in triplicate and given values correspond to the mean from at least five independent experiments.

2.7. Electrophoretic mobility shift assay

HepG2 nuclear extracts were prepared from confluent 150-mm dishes as previously described by Dignam et al. [36] and stored at −80 °C before use. The protein concentration of nuclear extracts was determined using the bichinonic acid assay reagent (BCA: Pierce, Bezons, France). The electrophoretic mobility shift assay (EMSA) was performed thus: 20 bp synthetic oligonucleotides with either G or A at position −971 (−971A: 5′-GTGCGAATACTCAGGA-ACTAGGG-3′ and −971G: 5′-GTGCGAATACTCGGG-ACTAGGG-3′) were annealed with their respective complementary strand at 100 °C for 3 min in a solution containing 100 mM Tris–HCl (pH 7.5), 100 mM MgCl2, 13 mM EDTA, 13 mM spermidine and 20 mM DTT. Double strand probes were radiolabelled with 20 μCi of [γ32P] ATP (3000 Ci/mmol; NEN Life) by T4 polynucleotide kinase (Promega) at 37 °C for 30 min. Radiolabelled double strand probes (0.25 pmol) were incubated for 15 min on ice in a final volume of 20 μl in the presence of 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 3 mM MgCl2, 5 mM EDTA, 1 mM DTT, 5% glycerol, 2 μg poly(dI-dC), 4 mM spermidine, 1 μg BSA and 6 μg of nuclear extracts. In experiments which required the presence of unlabelled competitor, the latter was added to the mixture before the addition of the radiolabelled probe. After incubation, samples were loaded on a 6% acrylamide gel (acrylamide/bis-acrylamide 29:1). Electrophoresis was performed at room temperature at 200 V for 3 h and the gels were transferred to 3MM paper (Whatman), dried and exposed to Hyperfilm MP (Amersham) overnight.

2.8. Statistical analyses

Hardy–Weinberg equilibrium was tested in each subgroup of cases and controls from each centre by a χ2-test with 1 degree of freedom (d.f.). Allele frequencies were deduced from genotypic frequencies by gene counting. Linkage disequilibrium with other polymorphisms of the CETP gene was estimated using log–linear analyses [37] and the extent of the disequilibrium was expressed as D’ = D/Dmax or D/Dmin. The sign in front of D’ is positive, if frequent alleles are preferentially associated, and negative if a rare allele is preferentially associated with a frequent allele. For case–control comparison, controls with coronary heart disease (CHD) were excluded. The effect of −971G/A on lipid and CETP levels was tested assuming an additive effect of alleles (i.e. −971G/A was coded as a continuous variable, GG carriers were coded 0, GA coded 1, AA coded 2). Similarly, TaqIB and −629C/A were tested assuming additive effects of alleles for tests of interaction. Furthermore, for the test of third order interaction (1 d.f.) between alcohol consumption (≥ 75 g vs lower), TaqIB and −971G/A, these polymorphisms were tested as TaqIB/AA vs G+ and −971/AA vs G+, since the effect appeared to differ in AA homozygotes only. All these analyses were adjusted for age, sex, country and case–control status. Because of the positive skewness of the distribution, triglycerides, VLDL cholesterol (VLDL-C) and CETP mass were log-transformed for tests, but untransformed means are reported in the Section 3.
Table 1
Allele frequencies and pairwise linkage disequilibrium between −971G/A and other CETP polymorphisms in controls (women and men pooled) of the ECTIM study

<table>
<thead>
<tr>
<th>Position</th>
<th>Allele frequency</th>
<th>±D'</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C-631A (0.082)</td>
</tr>
<tr>
<td>−971G/A</td>
<td>0.590/0.491</td>
<td>−0.92*</td>
</tr>
</tbody>
</table>

NS, non-significant. Values in parenthesis indicate rare allelic frequency. Three groups of polymorphisms are in nearly complete association: TaqIB and C+8/in7T; A373P and R451Q; I405V and G+524T.

* P<0.001.
3. Results

3.1. Distribution and frequencies of the $-971G/A$ polymorphism

In order to identify new polymorphisms in the CETP promoter, the region from $-710$ to $-1045$ of the CETP promoter was analysed in 50 individuals from the ECTIM population by PCR/SSCP and sequencing. A new polymorphism (G/A) was identified at position $-971$ relative to the transcription start site. This polymorphism was associated with the presence ($-971G$) or the absence ($-971A$) of a restriction site for the enzyme AvaI. Genotyping was performed in the 1830 individuals of the ECTIM population by using either the enzymatic digestion or ASO as described under Section 2. There was no significant deviation from Hardy–Weinberg equilibrium in cases or controls from any centre. In the whole population studied, genotype AA represented 20.6 and 24.7% in cases and controls, respectively, whereas genotype GG accounted for 28.9 and 26.9% in cases and controls, respectively. No significant difference in genotype or allele frequencies between countries or between cases and controls was observed.

In controls of the ECTIM Study, the $-971A$ and $-971G$ alleles were found at frequencies of 0.491 and 0.509, respectively (Table 1). Linkage disequilibrium coefficients for pairs of polymorphisms in controls of the ECTIM study are presented in Table 1. There was a strong linkage disequilibrium between the $-971G/A$ polymorphism and the $-631A/C$, A373P and R451Q polymorphisms: $-0.92$, $+1$ and $+1$ ($P < 0.001$). A positive linkage disequilibrium was also observed between the $-971G/A$ polymorphism and the $-629C/A$ and TaqIB polymorphisms: $+0.52$ and $+0.49$ ($P < 0.001$).

3.2. Effect of $-971G/A$ polymorphism on plasma lipid profile and CETP concentration

The association of the $-971G/A$ polymorphism with plasma lipid levels was evaluated in the whole population of pooled CHD cases and controls since the results were not significantly different between groups. We observed that the $-971G/A$ polymorphism was significantly associated with plasma HDL-C levels ($P = 0.006$; Table 2). Plasma HDL-C levels were the highest in subjects with AA genotype ($51.3 \pm 0.8$ mg/dl) and the lowest in those with GG genotype ($48.7 \pm 0.8$ mg/dl). This effect was not modified by the level of alcohol consumption. After adjustment for TaqIB or $-629C/A$, the association remained significant. When we tested the interaction between the $-971G/A$ polymorphism and sex on plasma HDL-C levels, we observed that this latter was not significant. A marginally but significant effect was observed for both ApoAII and LpAI levels ($P = 0.016$ and 0.034, respectively). Interestingly, the significance of the association between the $-971G/A$ polymorphism and LpAI level was increased in non-hypercholesterolaemic controls ($P = 0.008$; $n = 421$); these subjects were not receiving a hypolipidaemic agent and displayed a LDL cholesterol (LDL-C) $< 180$ mg/dl. No significant association was observed between the $-971G/A$ polymorphism and total cholesterol, triglyceride, VLDL-C, LDL-C, ApoAI or ApoB levels.

Plasma CETP concentration was determined in 788 male subjects of the ECTIM population (Table 2). The $-971G/A$ polymorphism was significantly associated with the plasma concentration of CETP ($P = 0.009$). Indeed, plasma CETP levels were significantly increased in subjects with the GG genotype as compared with subjects displaying the AA genotype ($2204 \pm 72$ and $1972 \pm 79$ mg/l, respectively). These results were consistent with those observed for plasma HDL-C levels.

Table 2

| Lipid and lipoproteins levels (mg/dl) in the entire ECTIM population (women, men, cases and controls pooled) and CETP mass (µg/l) in 788 males according to CETP/−971 genotype |
|-----------------|------|-----------------|------|-----------------|------|-----------------|------|
|                 | AA   |     | AG   |     | GG   |     | P value |
| Total cholesterol | 231.7 (2.6) | 410 | 231.1 (2.0) | 889 | 233.0 (2.4) | 504 | NS |
| Triglycerides    | 153.9 (4.7) | 410 | 155.8 (3.6) | 889 | 157.6 (4.3) | 504 | NS |
| VLDL-C           | 29.3 (1.5) | 290 | 29.1 (1.2) | 587 | 30.7 (1.4) | 339 | NS |
| LDL-C            | 152.9 (2.3) | 410 | 152.3 (1.8) | 889 | 154.7 (2.1) | 504 | NS |
| HDL-C            | 51.3 (0.8) | 410 | 49.8 (0.6) | 889 | 48.7 (0.8) | 504 | 0.006 |
| ApoB             | 133.5 (2.2) | 410 | 134.6 (1.6) | 889 | 137.2 (2.0) | 504 | NS |
| ApoAI            | 151.0 (1.4) | 410 | 148.3 (1.1) | 889 | 148.1 (1.3) | 504 | NS |
| ApoAII           | 34.9 (0.68) | 249 | 33.9 (0.46) | 494 | 33.1 (0.5) | 297 | 0.016 |
| LpAI             | 44.6 (0.8) | 249 | 43.5 (0.6) | 494 | 42.4 (0.7) | 297 | 0.034 |
| CETP             | 1972 (79) | 186 | 2103 (57) | 378 | 2204 (72) | 224 | 0.0094 |

All values were adjusted for age, sex, country and case-control status. Values are means $\pm$ SEM. NS, non-significant.
levels and vice versa. However, the HDL-C levels is associated with low plasma CETP concentration. Indeed, the genotype associated with high plasma HDL-C levels appeared to be confounded by the effect of the −971G/A polymorphism on HDL-C levels (P < 0.0014). Indicated values correspond to the mean value of plasma HDL-C level.

3.3. Effect of −971G/A polymorphism on both plasma HDL-C and CETP levels in relation to the −629C/A and TaqIB polymorphisms

In a recent study, a new polymorphism in the CETP promoter (−629C/A) was identified by some of us and shown to be functional in vitro [13]. In addition, this polymorphism was significantly associated with both plasma CETP concentration and HDL-C levels. Therefore, we investigated the putative interaction between the −971G/A and −629C/A polymorphisms on plasma HDL-C levels. As shown in Fig. 1, the significant effect of the −971G/A polymorphism on plasma HDL-C concentration appeared to be confined to the −629AA carriers (P = 0.0014). The highest plasma HDL-C levels (54.6 ± 1.1 mg/dl) were seen in subjects with the −629AA/−971AA genotype, whereas low plasma HDL-C levels (46.8 ± 1.2 mg/dl) were found in subjects with the −629AA/−971GG genotype. A significant association between the −971G/A polymorphism and plasma HDL-C levels was also observed with TaqIB (P = 0.012; data not shown), this latter polymorphism is in nearly complete positive linkage disequilibrium with the −629C/A polymorphism (D = +0.97). Interestingly, when this interaction was evaluated taking alcohol consumption into account in male controls, we found that among the TaqIBAA/−971AA carriers, HDL-C levels in the nine subjects consuming ≥75 g alcohol daily were significantly higher (86.3 ± 5.8 mg/dl, P = 0.0009 for interaction; Fig. 2). By contrast, no significant difference was detected in HDL-C levels in all genotype, in classes of alcohol intake inferior to 75 g/day.

No significant effect between the −971G/A and either the −629C/A (Fig. 3) or the TaqIB polymorphisms on plasma CETP concentration could be de-
Fig. 4. (A) Luciferase activity of \(-971GG\) and \(-971AA\) constructs in HepG2 cells. HepG2 cells were transiently transfected with the pGL3 basic vector containing 1077 bp of the CETP promoter (\(-971AA\) or \(-971GG\)). Luciferase activity is expressed in RLU after standardization for \(\beta\)-galactosidase activity. Values are mean \pm S.D. of the average of five independent experiments. (B) Analysis of binding by EMSA with nuclear extracts from HepG2 cells. 0.25 pmol of radiolabelled probes with either G or A at position 971 were incubated with 6 \(\mu\)g of nuclear extracts (lanes 1 and 4), in the presence of 100-fold excess of specific competitor (lanes 2 and 5) or non-specific competitor (lanes 3 and 6).

3.4. In vitro transcriptional activity of the \(-971G/A\) polymorphism

In order to evaluate the functionality of the \(-971G/A\) polymorphism, we carried out transient transfection experiments in HepG2 cells (Fig. 4(A)). For that purpose, we determined the activity of a 1077-bp fragment of the CETP promoter corresponding to the region from +32 to \(-1045\) bp with either G or A at position \(-971\), cloned into the pGL3 basic luciferase expression vector. In parallel, control experiments were performed using the pGL3 basic vector without an insert as a negative expression control and a pGL3 basic vector containing 138 bp of the proximal region of the human CETP gene promoter, which has been previously shown to be sufficient to obtain maximal in vitro expression of the CETP gene, as a positive expression control [38]. As expected, the pGL3 basic vector showed very low luciferase activity whereas the pGL3 basic vector containing 138 bp of the proximal region of the human CETP gene promoter was associated with a 3.5-fold increase in luciferase activity as compared to longer constructs (data not shown). Luciferase activity was not significantly different between the construct containing the G allele (\(-971GG; 166 \pm 19\) relative luciferase units (RLU)) and that with the A allele (\(-971AA; 153 \pm 28\) RLU).
3.5. Determination of the binding of nuclear proteins by EMSA

In order to determine whether DNA–protein interaction may occur in the CETP locus promoter region containing the −971G/A polymorphism, EMSAs were performed as described under Section 2 (Fig. 4(B)). A DNA–protein complex was observed with both −971G and −971A probes (lanes 1 and 4) and is indicated with an arrow. The DNA–protein complex was partially competed for the presence of a 100-fold excess of unlabelled −971G or −971A probes (lanes 2 and 5). By contrast, the DNA–protein complex was not removed in the presence of a non-specific competitor (lanes 3 and 6). These results suggested that a similar specific DNA–protein complex was formed with both the −971G and −971A probes, and indicated that equivalent proteins are involved in the formation of a DNA–protein complex for the two alleles at position −971 of the CETP gene promoter.

4. Discussion

We have presently identified a new polymorphism at position −971G/A in the human CETP gene promoter, which is significantly associated with both plasma CETP concentration and HDL-C levels, but was without effect on the in vitro transcriptional activity of this promoter. The −971G/A polymorphism significantly interacts with the functional −629C/A site and the TaqIB polymorphism on plasma HDL-C levels but not on plasma CETP concentration suggesting the existence of as yet unidentified functional polymorphisms in the CETP gene.

The −971G/A common polymorphism (A allele frequency: 0.491) is significantly associated with both plasma CETP concentration and HDL-C levels in the entire population (CHD cases and controls) of the ECTIM study. However, the observed association between the −971G/A polymorphism and plasma CETP concentration became insignificant after adjustment for the TaqIB and −629C/A polymorphisms. In addition, the effect of the −971G/A polymorphism on plasma HDL-C levels is not gender specific in our studied population whereas many studies suggested gender specific effects of CETP variants on plasma HDL-C levels [27,39]. However, our cohort comprised only 229 women from the United Kingdom (8-fold less than men). Our findings are in agreement with previous association studies which described a significant association between common variants of the CETP gene and both plasma CETP concentration and HDL-C levels [13,17,19,20] though other studies using sibling-pair linkage analysis detected no relationship between allelic variation at the CETP locus and plasma HDL-C levels in nuclear families [21,22]. It has been proposed that plasma CETP concentration is related to CETP activity [40] and that plasma CETP activity is inversely associated with plasma HDL-C levels. Our results for the association of both plasma CETP concentration and HDL-C levels with the −971G/A polymorphism are consistent with these findings. Indeed, subjects with genotype −971GG displayed both low HDL-C levels and high plasma CETP concentration and vice versa for subjects with genotype −971AA. However, transient transfection and electrophoretic mobility shift experiments revealed that the −971G/A polymorphism did not modulate the in vitro transcriptional activity of the human CETP gene promoter. These results indicate that the −971G/A polymorphism constitutes a non-functional marker and that the observed relationships of the −971G/A polymorphism on both plasma CETP concentration and HDL-C levels are therefore due to functional variants in linkage disequilibrium with the −971G/A polymorphism.

Pairwise linkage disequilibrium in controls of the ECTIM Study demonstrated that the −971G/A polymorphism is in complete linkage disequilibrium with the −631C/A, R451Q and A373P polymorphisms, and in moderate linkage disequilibrium with both the TaqIB and −629C/A polymorphisms. However, the −631C/A promoter polymorphism is associated with neither plasma CETP concentration nor plasma HDL-C levels [13]. The R451Q and A373P polymorphisms have been described in exons 15 and 12 of the CETP gene, respectively [15], and they were only associated with plasma HDL-C levels when considered jointly; by contrast, no association was observed when they were studied separately in univariate analysis [20]. These polymorphisms can therefore be excluded as putative functional candidates and thus they cannot explain the association between the −971G/A polymorphism and both plasma CETP concentration and HDL-C levels. The TaqIB polymorphism has been widely investigated and is associated with both plasma CETP concentration and HDL-C levels [17,19,23]. The effect of the TaqIB polymorphism on plasma HDL-C levels was modulated by alcohol consumption in the ECTIM study [17], the maximum effect being observed in subjects with the highest alcohol consumption (≥75 g/day). In the present study, we found a significant interaction between alcohol consumption and both the −971G/A and TaqIB polymorphisms on plasma HDL-C level. This finding should be interpreted with caution since the significance was mainly drawn from the nine high consumers carrying the TaqIB/AA and −971/AA genotypes.

At this time, the −629C/A polymorphism is the sole functional polymorphism described in the CETP gene promoter [13]. In addition, the −629C/A polymorphism is associated with both plasma CETP concentra-
tion and HDL-C levels. Thus, it would be the best candidate to explain the association between the −971G/A polymorphism and both the plasma HDL-C levels and/or CETP concentration. We observed a significant interaction between both the −971G/A and −629C/A polymorphisms on plasma HDL-C levels. In fact, the effect of the −971G/A polymorphism on plasma HDL-C levels was evident in −629AA carriers, thereby suggesting that the −971G/A polymorphism could modulate the high plasma HDL-C levels observed in −629AA carriers. In a recent study, Corbex et al. [20] observed in multivariate analysis, an association between a group of completely concordant polymorphisms (TaqIB, −629C/A, intron 7) and both plasma CETP and HDL-C concentrations. However, in our case, no interaction was detected between the −971G/A and either the −629C/A or TaqIB polymorphisms on plasma CETP concentration suggesting that the effect observed on plasma HDL-C levels is independent of the effect on plasma CETP concentration. This latter result was quite surprising since each of these polymorphisms have been shown to be associated with both plasma CETP and HDL-C levels. Nevertheless, we have to consider that plasma CETP concentration was only measured in 798 men, whereas plasma HDL-C levels were determined in the entire population with both women and men. Several studies [17,22,23] showed that the effects of the TaqIB polymorphism on plasma CETP and HDL-C are independent. Obviously, it is difficult to infer the underlying biological relationship between these polymorphisms. However, we may speculate that the −971G/A, −629C/A and TaqIB polymorphisms might be in linkage disequilibrium with an unknown variant located in the CETP gene which may modulate plasma CETP activity without affecting CETP mass. In this case, this variant would be specifically associated with plasma HDL-C level as a result of modulation of CETP-mediated CE transfer activity without effect on CETP concentration. This hypothesis would suggest that the effect of the interaction between the −971G/A and either the −629C/A or the TaqIB polymorphisms on both plasma CETP concentration and HDL-C would be independent. Nevertheless, we consider that it is more probable that an interaction between a polymorphism of the CETP promoter and plasma HDL-C levels would be expressed via CETP mass. In this case, the lack of interaction between the −971G/A and both −629C/A and TaqIB polymorphisms with plasma CETP concentration might be due to the fact that these polymorphisms are in linkage disequilibrium with a polymorphism located in a gene involved in the metabolism of HDL which may affect HDL-C levels without affecting plasma CETP levels. However, even if these hypotheses are conceivable, our interpretation of the data presented in the current study is limited to the conclusion that the interaction between the −971G/A polymorphism and either the −629C/A or the TaqIB polymorphism on plasma CETP concentration is different than that implicated in HDL-C levels.

In conclusion, we have demonstrated that the −971G/A, −629C/A and TaqIB polymorphisms located in the human CETP gene interact in order to modulate plasma HDL-C levels. Moreover, our data suggest the existence of as yet unidentified functional polymorphism(s) in the CETP gene promoter that could explain the association between polymorphisms of the CETP gene and both plasma HDL-C and CETP concentrations. Finally, our study emphasizes the key role of CETP in the modulation of plasma HDL-C levels hence supporting the suggestion that CETP may represent a major pharmacological target for therapeutic attenuation of atherosclerotic disease.

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