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Hepatic lipase promoter activity is reduced by the C-480T and G-216A substitutions present in the common *LIPC* gene variant, and is increased by Upstream Stimulatory Factor

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Abstract

The common −216G→A and −480C→T substitutions in the promoter region of the human hepatic lipase (*LIPC*) gene show high allelic association, and are correlated with decreased hepatic lipase activity and increased high-density lipoprotein cholesterol levels. To test the functionality of these substitutions, CAT-reporter assays were performed in HepG2 cells. *LIPC* (−650/+48) but not (−650/+61) promoter constructs showed transcriptional activity. *LIPC* (−650/+48) constructs with both −216A and −480T exhibited significantly lower promoter activity (−45%) than the wild-type form. Activities of −289/+48 constructs were not significantly affected by the −216G→A substitution. The −480C/T site lies within a binding region for Upstream Stimulatory Factor (USF). Gel-shift assays showed that the binding affinity of USF protein for HL specific oligonucleotides was decreased four-fold by the −480C→T substitution. However, promoter activity of the −650/+48 constructs was not significantly affected by the −480C→T substitution alone. Co-transfection of HepG2 cells with USF^{43} cDNA yielded a similar dose-dependent increase in activity of all −650/+48 constructs; the absolute difference in promoter activity increased but the relative difference between the variant promoter forms was maintained. Our studies demonstrate that the common *LIPC* promoter variation is functional, which explains the association of the −480T allele with a lower hepatic lipase activity in man. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Hepatic lipase promoter; Gene polymorphism; Hepatic lipase gene expression; Upstream Stimulatory Factor; Transcriptional regulation; Transient transfection

1. Introduction

Human hepatic lipase (HL; triacylglycerol lipase, EC 3.1.1.3) is an extracellular liver enzyme with phospholipase A_1 and triacylglycerol hydrolase activity that plays an important role in plasma lipid transport [1]. HL is involved in the remodeling of high-density lipoproteins (HDL), thereby facilitating cholesterol transport from peripheral tissues to the liver [2–5]. HL also accelerates liver uptake of chylomicron remnants, either by changing the exposure of apoE on these particles and increasing their binding to apoE-recognizing receptors [6], or by acting as a ligand protein for chylomicron remnants [7,8]. Studies in humans and genetically modified animals have shown that HL deficiency is generally associated with elevated HDL cholesterol levels, increased triglyceride levels in HDL and low density lipoprotein (LDL), presence of β-VLDL, and impaired metabolism of post-prandial triglyceride-rich lipoproteins [9–13], which are all considered to be risk factors for premature atherosclerosis. Indeed, coronary artery disease has been reported for subjects with HL deficiency [12,13].

Mutations in the coding part of the human hepatic lipase gene (*LIPC*) that lead to a decreased expression

Abbreviations: CDP, CCAAT displacement protein; HDL, high density lipoprotein; HL, hepatic lipase; PCR, polymerase chain reaction; USF, Upstream Stimulatory Factor.

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of HL activity in post-heparin plasma are rare. We recently identified a common C → T substitution in the promoter region of the *LIPC* gene at nucleotide −480 [14,15], which has also been described as the −514C → T by Guerra et al. [16]. This base substitution is associated with a 15–50% lowering of post-heparin HL activity [15,17–19] and increased levels of HDL cholesterol and apoAI [15,16,19–21] or HDL and LDL triglycerides [18]. Moreover, it is associated with increased levels of remnant particles in plasma [21]. Interestingly, post-heparin plasma HL activity increased with fasting insulin levels in −480CC homozygotes but not in carriers of the −480T, suggesting that the base substitution may interfere with insulin responsiveness [15]. The −480C → T substitution is in strong linkage disequilibrium with three other base substitutions in the 5′-regulatory region of the *LIPC* gene, one more proximal and two more distal; they are therefore collectively designated as the −480C and T alleles [16]. The −480T allele frequency varies from 0.2 to 0.5 among Caucasian and African American populations [15,17,20], respectively.

Although an association was found between post-heparin plasma HL activity and the *LIPC* promoter variants, individuals of the same *LIPC* genotype show a broad range of HL activities that largely overlaps with the other genotypes [15,18,19,22]. This indicates that the differences in post-heparin plasma HL activity may be due to other factors that modulate expression of the *LIPC* gene, or to additional base variants in the gene. However, the functionality of these base substitutions has not been established. In the present study, we tested whether the difference in HL expression is causally related to the base substitutions in the *LIPC* promoter by studying its effect on in vitro promoter activity.

2. Materials and methods

2.1. Generation of wild-type and mutant promoter-CAT constructs

For in vitro promoter studies, the 5′-flanking region of the *LIPC* gene was cloned into reporter plasmids in front of the chloramphenicol *O*-acetyltransferase (CAT) gene. The −1068 to +61 fragment of the wild-type human HL gene in the reporter plasmid pGCAT-A was kindly donated by Dr H. Will (University of Hamburg, Germany) [23]. Since the wild-type fragment in pGCAT-A showed very low promoter activity when transfected into human hepatoma cells [23], the HL promoter fragment was subcloned into another reporter plasmid, pCAT-basic (Promega, Leiden, The Netherlands). An upstream *Pst*I restriction site was introduced by PCR at position −650 using the primer 5′-AAC TGC AGT GGT CGC CTT TTC CCT ACC-3′, whereas a downstream *Xba*I restriction site was introduced either at position +61 using primer 5′-GCT CTA GAC CCG GGG TCC AGG CTT TCT TGG-3′ or at position +48 using primer 5′-GCT CTA GAC TTG GTA ATT TCT GAA GCC-3′ (restriction sites underlined). After digestion with *Pst*I and *Xba*I, the PCR products were cloned into pCAT-basic upstream of the CAT reporter gene. Similarly, a −289/+48 promoter fragment was cloned into pCAT-basic after the introduction of a *Hin*dIII site at position −289 and an *Xba*I site at position +48 of the HL gene by PCR using the upstream primer 5′-CGG AAG CTT AGG CAG CCA CGT G-3′ and the *Xba*I-primer described above, respectively.

For site-directed mutagenesis, the 1.1 kb wild-type promoter fragment was subcloned into pBluescript KS$^-$ (Stratagene, La Jolla, CA, USA) using *Hin*dIII and *Sst*I. The −216A and −480T mutants were created independently using the Transformer™ site-directed mutagenesis kit (Clontech, Palo Alto, CA, USA). The mutagenic primers used were 5′ GCT CCT TTT GAC ATG GGG GTG AAG GG-3′ and 5′-CAC AGT AGC TTT AAA TTG ATT AAT TTG G-3′, respectively; the selection primer 5′-CCC TCG AGG TGC ACG GTA TCG-3′ was chosen to disrupt the unique *Sal*I site in pBluescript (mutations underlined). Subsequently, the mutant HL promoter fragments were subcloned into pCAT-basic as described above. Different combinations of the −480C/T and −216G/A variants were generated by appropriate exchange of 424 bp *Apa*I-*Xba*I fragments using an internal *Apa*I site.

All inserts in pCAT-basic were verified by cycle sequencing using the Thermo Sequenase dye terminator sequencing kit (Amersham Pharmacia Biotech, UK) and the ABI 377 sequencer. Plasmid DNA's used for transfections were isolated with the Wizard Midiprep System (Promega, Leiden, The Netherlands). All oligonucleotides were custom-made by Eurogentec (Seraing, Belgium). Restriction enzymes were from Boehringer Mannheim (Germany).

2.2. HepG2 transfections assays

Twenty-four hours before transfection, HepG2 cells were plated in 60 mm culture dishes at 20% confluency in Dulbecco's modified Eagle's medium (ICN, Costa Mesa, CA, USA) supplemented with 10% fetal calf serum (Gibco BRL, Breda, The Netherlands). Three hours before transfection, the medium was refreshed. DNA transfections were performed by calcium phosphate co-precipitation using 10 μg of the CAT-construct and 0.4 μg of a RSV-β-galactosidase expression vector per dish. In co-expression studies, the indicated amounts of the recombinant plasmid pCX-USF (a kind gift from Dr R.G. Roeder, Rockefeller University, New York, USA) were included, which contained cDNA

encoding the 43-kDa human Upstream Stimulatory Factor (USF) isoform under the control of the CMV-promoter [24]. In each experiment, parallel transfections with promoter-less pCAT-basic and SV40 promoter-driven pCAT promoter-vector (Promega) were included as negative and positive controls, respectively. At 48–72 h after transfection, cell extracts were prepared and the amount of CAT and β-galactosidase antigen was determined by ELISA using kits from Boehringer Mannheim. Promoter activity was expressed as pg CAT/ng β-galactosidase to correct for differences in cell number and transfection efficiency. All transfection experiments were repeated at least once with independent plasmid DNA preparations.

2.3. Electrophoretic mobility shift assays

Oligonucleotides used were 5′-GTC AGC TCC TTT TGA CA(C/T) GGG GGT GAA GGG-3′ and 5′-CTT TTC CCT TCA CCC CC(A/G) TGT CAA AAG GAG C-3′, which contained either the polymorphic −480C or T of the *LIPC* promoter at a central position; the oligonucleotides 5′-GGT GTA GGC CAC GTG ACC GGG TGT AAG CTT-3′ and 5′-GGA AGC TTA CAC CCG GTC ACG TGG CCT ACA-3′ represent the USF binding site in the adenovirus major late promoter (AdML) (overlapping sequences underlined) [25]. Of each pair, one oligonucleotide was end-labeled using [γ-^{32}P]ATP (Amersham, UK) and polynucleotide kinase (Boehringer Mannheim). The labeled oligonucleotides were annealed with excess unlabeled complementary oligonucleotide by slow cooling from 95°C to room temperature. Double-stranded competitor oligonucleotides were prepared in parallel in the absence of labeled nucleotides. Of the labeled oligonucleotides, 4.5 fmol (10 000–25 000 dpm) were incubated for 30 min at room temperature with different amounts of purified calf brain USF protein [26] (a kind gift from Dr M. Timmers, University of Utrecht, The Netherlands) in a final volume of 10 μl of 10 mM Hepes/KOH buffer (pH 7.6) containing 60 mM KCl, 1 mM EDTA, 1 mM DTT, 4% Ficoll, 100 μg/ml poly(dI/dC) and 0.25 mg/ml of BSA. In some experiments, 1 μl of anti-human USF^{43} (C-20; Santa-Cruz Biotechnology, CA, USA) was added just prior to the labeled oligonucleotide. Thereafter, protein–DNA complexes were separated in a 4% (29:1) polyacrylamide gel in 0.5 × TBE electrophoresis buffer (44 mM Tris, 44 mM boric acid, 0.1 mM EDTA, pH 8.0), and the dried gel was exposed to an autoradiographic film.

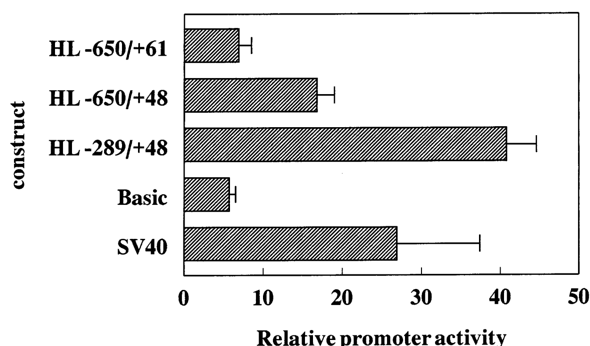

Fig. 1. Effect of 3′- and 5′-deletions on HL promoter activity in HepG2 cells. Expression studies were performed in HepG2 cells using CAT-constructs harboring the indicated regions of the wild-type human HL gene. Both negative (pCAT-basic) and positive (SV40-CAT) controls were included. Promoter activity was expressed as the amount of CAT expression relative to β-galactosidase expression. Data represent mean ± S.D. for three independent experiments, each performed in triplicate.

2.4. Statistics

Differences were tested statistically by one-way ANOVA followed by the Student–Newman Keuls-test, and were considered significant at $P < 0.05$.

3. Results

3.1. Effect of the HL promoter variants on transcriptional activity

To test for the functionality of the −216 and −480 base substitutions, we initially prepared promoter-CAT constructs of the human HL promoter region from −650 to +61. This 3′-end corresponds to the *Sma*I-restriction site used by others for subcloning into reporter plasmids [22,27,28]. Upon transient transfection of human hepatoma HepG2 cells, these constructs expressed low promoter activity just slightly above the promoter-less reporter vector (Fig. 1). Since a further lowering of promoter activity is hard to assess, we sought to design constructs with a higher starting activity in vitro. A 3′-deletion construct containing the −650 to +48 region showed a markedly increased promoter activity with levels three- to five-fold higher than pCAT-basic. A further increase in promoter activity to levels well above that of the SV40-promoter was obtained by an additional 5′-deletion from −650 to −298. We therefore, decided to use the *LIPC* (−650/+48)- and *LIPC* (−298/+48)-containing CAT-constructs for further experiments.

The −216G→A mutation was introduced into the *LIPC* (−298/+48)-containing CAT-constructs by site-directed mutagenesis, and the effect on promoter activity was tested by transient transfection of HepG2 cells. Constructs with either −216G or A showed similar promoter activities (n.s., $n = 8$) (Fig. 2A). When the −216G→A and −480C→T mutations were introduced collectively into *LIPC* (−650/+48)-containing constructs, thus representing the two common alleles,

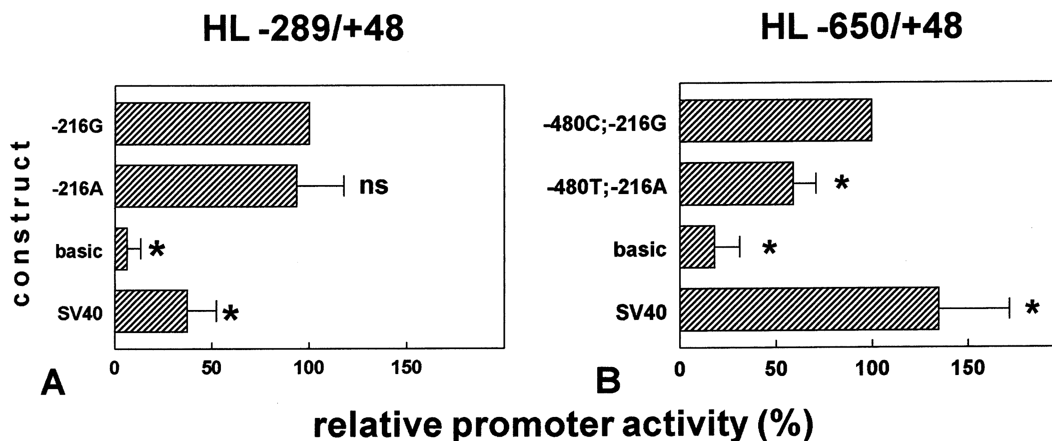

Fig. 2. Effect of base substitutions on HL promoter activity in HepG2 cells. Expression studies were performed in HepG2 cells using CAT-constructs harboring the $-289/+48$ region (panel A) or the $-650/+48$ region (panel B) of the human HL gene. Both negative (pCAT-basic) and positive (SV40-CAT) controls were included in each experiment. The CAT/β-galactosidase ratios obtained with the wild-type CAT constructs were set at 100%. Data represent mean ± S.D. for five to eight independent experiments, each performed in triplicate. The asterisk indicates a statistically significant difference from the wild-type construct ($P<0.05$).

transcriptional activity was reduced to almost half of that of the wild-type construct ($56\pm13\%$, $n=7$, $P<0.05$) (Fig. 2B). As these two base substitutions are almost completely linked, we conclude that the common promoter polymorphism in the *LIPC* gene is functional.

3.2. Differential binding of USF to HL −480C and −480T oligonucleotides

As the −216G→A substitution did not affect promoter activity of the short construct, we assumed that −480C→T substitution was responsible for the reduced promoter activity of the longer construct. A database search for homology with binding sites for known transcription factors [29] revealed that the −480C→T substitution disrupts a consensus binding site for USF, a potentially insulin-responsive transcription factor involved, among others, in the regulation of lipogenesis [25,30]. To confirm the effect of the −480C→T substitution on USF binding, we performed electrophoretic mobility shift assays using double-stranded oligonucleotides harboring either the −480C or the −480T sequence, and USF protein purified from calf brain [26]. Binding conditions were optimized with oligonucleotides that contain a perfect consensus sequence for USF binding (AdML). An amount of USF protein was chosen that produced an approximately 50% shift of the AdML oligonucleotides. Under these conditions, a similar gel shift was observed with the −480C oligonucleotide, but the extent of binding was markedly less than with the AdML oligonucleotides (Fig. 3A). Co-incubation with anti-USF[43] peptide antibodies completely prevented the gel shift and induced a supershift, thereby confirming the involvement of USF[43] protein (not shown). In contrast, the USF-dependent mobility shift was almost absent with the −480T oligonucleotide. In competition experiments using ^{32}P-labeled −480C oligonucleotide, unlabeled −480T oligonucleotide was able to completely abolish the gel-shift but this required levels that were four-fold higher than with unlabeled −480C oligonucleotide (Fig. 3B). These observations demonstrate that USF binds to this region of the HL gene, and indicate that the affinity of binding to −480T is markedly lower than to −480C oligonucleotides. Hence, the −480C→T substitution interferes with binding of USF.

3.3. Effect of USF over-expression on HL promoter activity

To test the effect of USF on transcriptional activity, the *LIPC* ($-650/+48$) promoter-CAT constructs were transfected into HepG2 cells together with an expression vector containing human USF[43] cDNA. Promoter activities of both the wild-type and the double-mutant constructs gradually increased with co-transfection of increasing amounts of USF-encoding plasmids (Fig. 4). The difference in promoter activity between both constructs was more pronounced in the USF-transfected than in the control cell cultures. In five independent experiments, the activity of the double mutant was $66\pm26\%$ of that of the wild-type construct (mean ± S.D., $n=5$, $P<0.05$) at the highest dose of USF-encoding plasmid used. Here, transcriptional activity of both the wild-type and double-mutant constructs was increased by a factor of 8.9 ± 2.1 and 9.2 ± 2.7 (mean ± S.D., $n=5$, n.s.), respectively. Hence, despite the different binding affinities for USF, promoter activities were similarly sensitive to transactivation by USF.

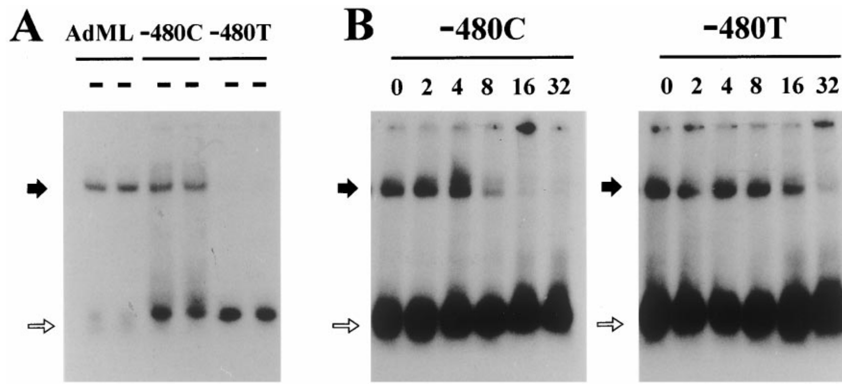

Fig. 3. Gel shifts of the −480C/T oligonucleotides by purified USF protein. Gel shift assays were performed with 30-bp oligonucleotides centered around the polymorphic −480 site of the human HL gene containing either C or T at this site, and with a 30-bp oligonucleotide containing the USF consensus binding site from the AdML. Of a preparation of USF protein highly purified from calf brain [26] (a gift from Dr M. Timmers), 0.1 μl was used throughout. In A, USF was incubated with the radiolabeled oligonucleotides indicated before electrophoretic separation and autoradiography. The specific radioactivity of the two HL-specific oligonucleotides was taken three-fold higher than of the AdML oligonucleotide to facilitate visualization. In B, radiolabeled wild-type HL −480C oligonucleotides were incubated with USF in the presence of increasing amounts of unlabeled competitor, either wild-type HL −480C (left panel) or mutant HL −480T (right panel). Numbers indicate the molar excess of unlabeled over labeled oligonucleotides. The positions of the bound and unbound oligonucleotides in the autoradiograms are indicated by filled and open arrowheads, respectively.

We then tested the separate effects of the −480C→T and −216G→A mutations on promoter activity and USF transactivation of the *LIPC* (−650/+48) promoter-CAT constructs. Compared with the wild-type (−480C; −216G) promoter construct, the −480C→T substitution alone did not significantly affect expression of the CAT gene in control HepG2 cells or in the USF-transfected cells (Fig. 5). The −216G→A mutation alone reduced transcriptional activity in control HepG2 cells (Fig. 2B), but this did not reach statistical significance when the unprocessed data were tested by ANOVA ($n=7$). When the data in each experiment were first expressed as percentage of the parallel wild-type construct, however, the promoter activity of the −480C; −216A construct tested significantly lower than the wild-type construct and not statistically different from the double-mutant construct. In USF-transfected cells, the effect of the −216G→A substitution alone was no longer apparent ($n=4$). Collectively, these data suggest that the −216G→A substitution is more effective than the −480C→T substitution in the longer promoter constructs, though it does not affect promoter activity of the short constructs in the absence of the −480 region. The effect of the −216G→A substitution on promoter activity may be overcome by USF over-expression, provided that there is a high-affinity binding site for USF at position −480.

4. Discussion

Several groups have shown that the −480T allele of the *LIPC* gene is associated with a 15–50% lower post-heparin plasma HL activity compared with the −480C allele [15,17–19]. In these alleles, the −480C→T substitution is strongly linked to the −216G→A substitution, and to two other polymorphisms further upstream in the gene. We confirmed that in our previously reported population of normocholesterolemic CAD patients [15], the −480C→T substitution is almost completely associated with the *Dra*I and *Ava*II variants at −216 and −676, respectively (unpublished data, 1999). In reporter assays using the human hepatoma HepG2 cells, the combination of the −480C→T and −216G→A

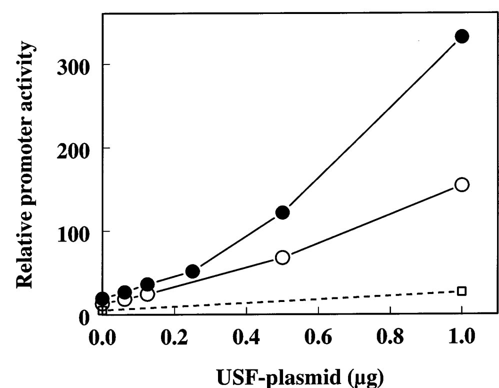

Fig. 4. Effect of co-expression of USF on HL promoter activity in HepG2 cells. HepG2 cells were co-transfected with HL promoter-CAT constructs and the indicated amounts of the human USF[43] expression vector pCX-USF. Reporter plasmids (10 μg) used were the HL(−650/+48) CAT-constructs harboring either the wild-type (−480C; −216G) or the double-mutant (−480T; −216A) promoter region (closed and open circles, respectively). The effect of co-transfection is also shown for the pCAT-basic (squares). Promoter activities are expressed relative to that of the SV40-promoter. Data are means of duplicate transfections, and are representative for two similar experiments.

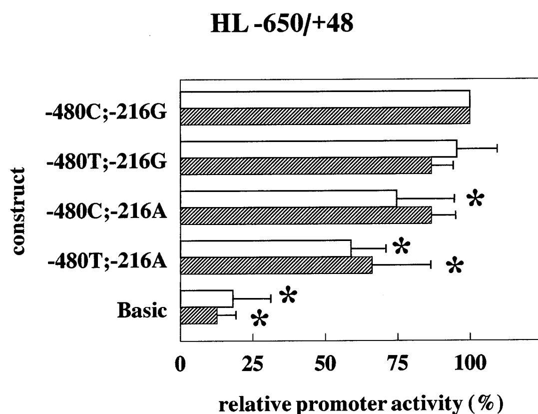

Fig. 5. Effect of base variations on HL promoter activity in HepG2 cells. Expression studies were performed in HepG2 cells using the indicated CAT-constructs harboring the −650/+48 region of the human HL, as described in the legends to Fig. 2. Cells were co-transfected without (open bars) or with 1.0 μg of pCX-USF (hatched bars). Promoter activities are expressed relative to that of the wild-type promoter CAT constructs. Data represent mean ± S.D. for three to five independent experiments each performed in triplicate. The asterisk indicates a statistically significant difference from the wild-type construct ($P < 0.05$).

substitutions results in a reduction of *LIPC* promoter activity by 40–50%, which is in good agreement with the in vivo effect of this polymorphism on hepatic lipase expression. This observation demonstrates that the common polymorphism of the *LIPC* promoter is functional and can be explained solely by the combined effect of the −480C→T and −216G→A substitutions. However, an additional contribution of the two other base variants further upstream cannot be ruled out at present.

From the data presented here, the reduction in promoter activity cannot be unambiguously attributed to either one or the other base substitution alone. The −480C→T substitution alone did not significantly reduce promoter activity of the −650/+48 constructs either in control or in USF-over-expressing HepG2 cells. The −216G→A substitution alone did not affect the activity of the −289/+48 promoter fragment. One explanation for our data would be that each of the two base substitutions has a small effect on transcription, which when combined add up to statistically significant levels under the conditions tested. Alternatively, the effect of the −216G→A substitution may only become apparent in combination with the −480C→T substitution, or vice versa. Our data suggest that the −216G→A substitution alone may be effective in the −650/+48 constructs, i.e. in the presence of the −480 region. In addition, the effect of the −216G→A substitution on promoter activity may be overcome by USF over-expression, provided that there is a high-affinity binding site for USF at position −480. This suggests that the proteins that bind to these loci interact with each other.

Several regulatory elements have been reported in the 5′-regulatory region of the human *LIPC* gene, some of which include the −480C/T and the −216G/A positions [27,28]. According to the Transfac DataBank [29], the −216G→A substitution affects the consensus binding site for c-Myb and CDP, which have no obvious relevance to lipid or lipoprotein metabolism. Interestingly, the −480C→T substitution disrupts one of the potential USF binding sites present in the promoter region of the *LIPC* gene. In vitro gel shift assays confirmed that this substitution reduced the binding of USF to this locus. The transcription factors USF^{43} and USF^{44} are expressed in various mammalian tissues including liver, and have recently been implicated in the regulation of lipogenic genes such as L-PK, Spot14 and fatty acid synthase [25,30]. In rat livers, the expression of the USF^{43} protein was markedly upregulated under conditions of high plasma glucose and insulin levels [25]. In vitro, *LIPC* promoter activity increased several-fold upon co-transfection of the HepG2 cells with USF^{43} cDNA. Potentially, an increased liver expression of USF^{43} in conditions with elevated plasma glucose and/or insulin, such as occur post-prandially and in hyperinsulinaemia, may lead to enhanced transcription of the hepatic lipase gene. Some evidence for the regulation of HL expression in humans by glucose and/or insulin has been presented. Post-heparin plasma HL activity in men is positively correlated with plasma insulin levels in response to an oral glucose load [31,32]. We recently reported that post-heparin plasma HL activity positively correlated with fasting plasma insulin levels in coronary artery disease patients homozygous for the −480C allele [15]. This correlation was not found in patients that were carriers of the −480T allele. In vitro, however, the −480C→T substitution alone did not significantly reduce promoter activity of the −650/+48 constructs either in control or in USF-overexpressing HepG2 cells, despite the reduced ability to bind USF protein. The relative increase in promoter activity by USF over-expression was independent of the presence of a C or a T at position −480. These observations suggest that the in vitro USF effect is mediated by transactivation through other elements, possibly through one of the additional USF consensus sites present in the *LIPC* (−650/+48) promoter fragment.

In summary, we show here for the first time that the common variation in the *LIPC* promoter is functional. Therefore, the reduced HL activity found in carriers of the −480T allele is likely due to reduced transcription. The molecular mechanism and the transcription factors involved remain to be established.

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