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Functional Significance of Lipoprotein Lipase *Hind*III Polymorphism Associated with the Risk of Coronary Artery Disease

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Abstract

Lipoprotein Lipase (LPL) plays a pivotal role in lipid metabolism by hydrolyzing triglyceride (TG) rich lipoprotein particles. Abnormalities in normal LPL function are associated with the risk of coronary artery disease (CAD). A number of genetic variants have been identified in the LPL gene that affects different functions of the LPL protein. A common *Hind*III polymorphism in intron 8 (T/G) of the LPL gene has been found to be associated with altered plasma TG and HDL-cholesterol, and CAD risk in several studies, but its functional significance is unknown. It has been shown that certain intronic sequence contain regulatory elements that are important for transcription and translational regulation of a gene. In this study we tested the hypothesis that this polymorphism affects the binding site of a transcription factor that regulates the transcription of LPL gene. Electrophoretic mobility shift assays revealed that the *Hind*III site binds to a transcription factor and that the mutant allele has lower binding affinity than the wild type allele. Transcription assays containing the entire intron 8 sequence along with full-length human LPL promoter were carried out in COS-1 and human vascular smooth muscle cells. The mutant allele was associated with significantly decreased luciferase expression level compared to the wild type allele in both the muscle (3.394 ± 0.022 vs. 4.184 ± 0.028; $P=4.7 \times 10^{-6}$) and COS-1 (11.603 ± 0.409 vs. 14.373 ± 1.096; $P<0.0001$) cells. In conclusion, this study demonstrates for the first time that the polymorphic *Hind*III site in the LPL gene is functional because it affects the binding of a transcription factor and it also has an impact on LPL expression.

Keywords

Lipoprotein lipase; *Hind*III polymorphism; electrophoretic mobility shift assay; luciferase reporter gene assay; coronary artery disease

1. Introduction

Lipoprotein lipase (LPL) plays a pivotal role in lipid metabolism by hydrolyzing triglyceride (TG) -rich lipoproteins. Many diseases, including coronary artery disease (CAD),

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atherosclerosis and obesity seem to be directly or indirectly related to abnormalities in LPL function [1–3]. A large number of variants have been identified in the LPL gene, of which 80% occur in coding regions while the rest are in noncoding regions [4–5]. Most of these functional LPL variants are rare, as they are mostly restricted to families with LPL deficiency or are found only in isolated geographic regions [3].

Several common LPL genetic variants have been widely studied, and among them *Hind*III in intron 8 (T→G at position 481), and S447X in exon 9 are of particular interest because of their common occurrence (25% and 9% of the less common alleles, respectively in most populations) and their associations with plasma lipid profile [6–11] and susceptibility to CAD [10–15] in several studies, although some inconsistent results have also been reported [16–18]. Since the *Hind*III polymorphism is located in the middle of intron 8, it is not considered to be functional but rather in linkage disequilibrium with a putative functional variant. Some groups, including ours, have attempted to find such a functional variant and identified coding SNPs, including D9N, N291S and S447X [6,19–21]. Among these, only the S447X was common with a frequency of about 9% in Caucasians. The 447X allele results in a premature truncation of LPL by two amino acids and thus has been considered functional. However, the *in vitro* data on the effect of the S447X mutation on LPL function is equivocal. Compared to the wild type, the LPL activity associated with the X447 mutation was higher in one, lower in another, but comparable in three studies [reviewed in 22]. In association studies, although the X447 allele has been reported to be associated with a favorable lipid profile in some studies [7–8,22] the results have not been confirmed in other studies [15]. Similarly, the association of the S447X polymorphism with CAD is inconsistent [10–18]. In a meta analysis the association of the S447X polymorphism with TG/HDL-C and CAD was found to be small compared to three other LPL amino acid polymorphisms examined [23] or it was gender-specific [24]. Most likely, the S447X polymorphism has some direct effect on plasma TG and HDL-C, but this does not explain all the effect associated with the *Hind*III polymorphism.

Because of these findings, a logical next step is to postulate that either the *Hind*III polymorphism is functional by itself or the regulatory elements in the 5’ or 3’ regions contain functional mutations for which *Hind*III acts as a marker, a notion which has been favored by several other investigators as well [9–10,20,23]. The rationale that *Hind*III could be functional is supported by an early study suggesting the presence of regulatory elements important for LPL expression in introns 8 or 9 of the LPL gene [25]. The goal of this study was to test the hypothesis that the *Hind*III polymorphic site is functional.

2. Materials and Methods

2.1. Plasmid DNA Constructs for Luciferase Assay

Initially, the entire intron 8 was sequenced from 14 homozygous subjects for the *Hind*III wild type allele (TT genotype). One sample that carried no other sequence variation was chosen for subcloning and site-directed mutagenesis. A DNA fragment of 1,180 bp containing the entire LPL intron 8 fragment (1,029 bp) plus 151 bp flanking sequence harboring the wild type (T) *Hind*III site was amplified using intron 8 PCR primers containing the *Xba*I restriction site: Forward primer (LPLXBAI-F 5’-3’) TCTAGACTTTAGCTGGTCAGACTGGTG; Reverse primer: (LPLXBAI-R) AGATCTTTCACAAATACCGCAGGTG. The wild type PCR product was then ligated to the pCR II-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) using the supplier's standard procedure. The full-length LPL promoter (1,537 bp) was inserted into the *BamH*I restriction site upstream of the intron 8 insert. The size and orientation of the DNA insert and fidelity of DNA polymerase was confirmed by restriction analysis and DNA sequencing. Site-directed mutagenesis of this wild type clone was performed using Stratagene’s QuikChange® Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) to create the mutant type *Hind*III/G site as per the manufacturer’s protocol. The fragments

containing 5' LPL promoter and intron 8 insertion of either wild (T) or mutant (G) *Hind*III polymorphic site were then inserted upstream of the firefly *Luc* reporter gene into the promoter-less pGL3-basic vector (Promega, Madison, WI, USA) using appropriate restriction enzymes followed by ligation with T4DNA ligase (New England Biolabs, Inc., Beverly, MA, USA), as described elsewhere [26]. Positive clones with full-length insertions of both promoter and intron 8 sequences were confirmed by restriction analysis. Identification of wild (T) and mutant (G) *Hind*III alleles in the intron 8 region was confirmed by both restriction analysis and DNA sequencing.

2.2. Transient Transfection and Dual-Luciferase Assay

The wild (T) and mutant (G) type chimeric-firefly Luc constructs (4μg) were used to transiently transfect human vascular smooth muscle (Lonza [Cambrex], Walkersville, MD) and COS-1 cells along with an internal control vector, Renilla Luciferase (Rluc) vector (pRL-CMV) to assess their ability to drive transcription of the luciferase gene using LipofectAMINE (Gibco/BRL). Human vascular smooth muscle cell line was maintained in SmGM-2 smooth muscle media with 5% fetal bovine serum. COS-1 cells were maintained in DMEM with 10% fetal bovine serum, 2mM glutamine, 100μ/ml penicillin and 100 mg/ml streptomycin at 37 °C under 5% CO_2. After 48 hours of transfection, cells were washed twice with PBS and collected for the measurement of luciferase activity to determine the level of transcription. The transfected cells were lysed in lysis buffer (Promega, Madison, WI, USA) and the lysates used for the measurement of luciferase activity using Dual-luciferase Reporter (DLR) assay system (Promega, Madison, WI, USA) by TD 20/20 luminometer (Turner Design, Sunnyvale, CA, USA), as described elsewhere [26]. We co-transfected the LPL-reporter gene constructs with Rluc vector, which served as internal control to normalize difference in transfection efficiencies, while pGL3-basic vector with no insert was served as a negative control. Actual Luc activity was calculated as the ratio of firefly to *Renilla* Luc activity for each experiment.

2.3. Electrophoretic Mobility Shift Assay (EMSA)

Two double-stranded 30-mer oligonucleotides (wild type—CTA TAG GAT TTA AAG CtT TTA TAC TAA ATG; mutant—CTA TAG GAT TTA AAG CgT TTA TAC TAA ATG) corresponding to each variant (wild type or mutant) were prepared (Operon, Alameda, CA). Non-radioactive and radioactive EMSAs were used. For non-radioactive assay, 1.75 pmol of each oligo or commercially available nuclear factor consensus oligos (TFIID, AP2, AP1, SP1, OCT1 and CREB) were incubated with 2 μl (~20 ng) TATA binding protein (TBP) (Promega or Santa Cruz Biotechnologies) in 2 μl TBP 4x buffer [40% glycerol, 80 mM Tris-HCl (pH 8.0), 320 mM KCl, 40 mM $MgCl_2$, 8 mM DTT], and 6 μl ddH_2O at room temperature for 15 minutes. 1 μl of 10x loading buffer [250 mM Tris-HCl, pH 7.5, 40% glycerol, 0.2% bromophenol blue] was added to the reaction and the mixture was loaded on 6% polyacrylamide retardation gel (Novax). The gel was run at 25 °C constant temperature in 0.5x TBE buffer with constant voltage of 250V for 15 minutes. The gel was then stained with a 1:10000 diluted SYBR Gold (BioProbes) for 10 minutes and photographed. For the radioactive competitor assay, the wild type oligonucleotide was 5-end-labeled with γ-^{32}P ATP and purified using the QIAquick Purification Kit (QIagen, Valencia, CA). Same concentrated, non-radioactive competitor DNA (wild type or mutant oligos) were added 1x, 3x, 5x, 10x, 20x, and 50x; excess volumes of the labelled probe. The mixture of unlabelled and labelled oligos were incubated with 2 μg aorta smooth muscle cell nuclear extracts (Geneka, Toronto, Canada) for 20 minutes at room temperature in biding buffer [1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50mM NaCl, 10 MM Tris-HCl (pH=7.5), 20% glycerol]. The protein complexes were then separated on 6% non-denaturing polyacrylamide gel at 120 volts for 2 hours, and the gel was dried and autoradiaographed overnight.

2.4. Statistical Analysis

A matrix-based algorithm [27] was used to identify putative regulating elements in intron 8 of the LPL gene. The luciferase activity of each construct was calculated as a mean ± SD value of three experiments in triplicate after adjusting the transfection efficiency by normalizing them with the *Rluc* control value. Differential luciferase expression between the wild type and mutant type was determined using Student's T-test.

3. Results

3.1. Identification of Regulatory Elements in Intron 8

We performed a computer-based analysis to identify putative regulatory elements in intron 8 of the LPL gene using a matrix-based algorithm. Intron 8 harbors seven putative regulatory elements, including the consensus sequence of TATA, which is affected by the *Hind*III polymorphism (Table 1). The presence of consensus sequences for well known vertebrate-encoded transcription factors in intron 8 (Sp1, GATA, C/EBP, and TATA) indicates that intron 8 may participate in the LPL transcription.

3.2. Effect of the *Hind*III Polymorphism on Reporter Gene Expression

Wild (T) and mutant (G) type constructs were tested for their effects on promoter activity by cotransfecting COS-1 and human vascular smooth muscle cells along with the control vector (pR1-CMV), which was used to adjust the transfection efficiency within different sets and experiments. The smooth muscle cells were used for cell-specific expression, while COS-1 cells were used as a control cell line for comparison purpose. The promoter activity of each vector was determined by the dual luciferase assay system that revealed about 23% lower promoter activity associated with the mutant type allele (G) as compared to the wild type allele (T) in both vascular smooth muscle cells (3.394 ± 0.022 vs. 4.184 ± 0.028; $P=4.7 \times 10^{-6}$) (Figure 1a) and COS-1 cells (11.603 ± 0.409 vs. 14.373 ± 1.096; $P<0.0001$) (Figure 1b).

3.3. Electrophorectic Mobility Shift Assay (EMSA)

To confirm our hypothesis that the *Hind*III site in intron 8 is involved in binding with a transcription factor, we performed both non-radioactive and radioactive competitor EMSAs. We postulated that since the *Hind*III site is part of a putative TATA box, it will likely have a binding with TATA binding protein (TBP). Our non-radioactive assay showed that a subunit of TFIID or TBP that recognizes TFIID consensus sequence (lanes 3 and 6 on Figure 2a), is bound to the *Hind*III wild type and mutant type oligos. The results also showed that the TBP has higher affinity for the *Hind*III wild type (T oligo, lane 1) than the mutant type (G oligo, lane 2) (Figure 2a). The binding between the *Hind*III oligos and TBP was specific as TBP did not bind to consensus sequences for other nuclear factors such as AP2, SP1, AP1, OCT1 and CREB (lanes 4, 7 to 11).

In order to further distinguish the binding affinity between the T and G alleles, we performed competition mobility shift assay by using the non-labelled oligos (both wild type and mutant) to compete with radioactive-labelled oligo. As LPL is abundantly expressed in smooth muscles, we used aorta smooth muscle cell nuclear extract in this experiment. The results show that the *Hind*III/T or G oligonucleotides formed a DNA-protein complex with aorta smooth muscle cell nuclear extract (Figure 2b). By using increasing amounts of unlabeled competitor oligos (T or G allele) to compete with the labelled T oligo, we found that the T allele had a higher binding affinity compared with the G allele (Figure 2b, DNA-protein complex are less visible in lanes 5–7 compared to the ones in lanes 12–14). Identical allele-dependent differences in binding were observed on repeating EMSA three times. The EMSA data indicate that the

*Hind*III sequence binds regulatory protein and that the wild type T allele has a higher affinity for binding compared with the mutant G allele.

4. Discussion

The objective of this study was to test the hypothesis that the *Hind*III polymorphic site in intron 8 of the LPL gene is functional. Our computer-based analysis on the presence of regulatory elements in intron 8 as well as functional studies of EMSA and reporter gene assays, strongly suggest that the *Hind*III polymorphic site is functional. We identified 7 putative regulatory elements in intron 8, including the consensus sequence of TATA that is affected by the *Hind*III polymorphism. To confirm our hypothesis that the *Hind*III site in intron 8 is involved in binding with a transcription factor, we constructed wild type and mutant type oligos (T→G) and examined their binding with purified human TATA binding protein (TBP or TFIID). We postulated that since the *Hind*III site is part of putative TATA box, it will more likely bind with TBP. The TBP not only bound with the *Hind*III wild type (T) and mutant type (G) oligos, there was a differential binding with the two alleles such that the binding affinity for TBP was higher with the wild type allele than the mutant type. Furthermore, the binding between the *Hind*III oligos and TBP was specific, as TBP did not bind to consensus sequences for other nuclear factors (see Fig. 2; lanes 4, 7–11). Our competition mobility shift assays with the two *Hind*III oligos in aorta smooth muscle cell nuclear extracts also confirmed the finding that the *Hind*III sequence binds regulatory factors (most likely TBP) and that this binding is affected by the *Hind*III sequence variation such that the mutant type allele (G) has reduced binding compared to the wild type allele. Finding a functional TATA box in the middle of an intron is unusual and to our knowledge has not been reported in the literature before. We suspect that this motif somehow contributes to the regulation of the LPL gene. Additional molecular work is necessary to work out the exact function of this intronic motif.

Further evidence that the *Hind*III polymorphic site is functional comes from our luciferase reporter gene assay performed in human smooth muscle cells and COS-1 cells. In both cell types, the mutant G allele was associated with a modest 20% lower transcription activity than the wild type T allele. This modest effect (20% difference in promoter activity) is predicted from a common variant and is similar to the effect size of 18–24% difference in promote activity reported earlier for a common LPL promoter variant (−93 T/G) [28]. These data strongly suggest that human LPL promoter controls LPL expression in conjunction with regulatory elements present in intron 8 and confirm the original observation made by Enerbäck et al. [25]. Both positive and negative regulatory elements have been identified not only in the 5’ region [29], but also in the intragenic and 3’ regions [25] of the LPL gene. Previato et al. [29] localized a single positive element (−368 to −35) and a negative element (−724 to −565) in the 5’ region by assessing the LPL transcription in 3T3-L1 adipocyte or HepG2 cells. Using 3T3-F442A preadipocyte cells, Enerbäck et al. [25] identified two major positive elements at −430 to −400 and −196 to −168, and two major negative elements at −4 kb to −827 and −239 to −196. The construct containing the −4 kb to −827 deletion was associated with an 8-fold drop in promoter activity. Using *DNase*I hypersensitivity assay, which detects sites of altered chromatin structure that correlate with transcriptional regulation, Enerbäck et al. [25] identified two enhancer regions present in introns 8 or 9 and in the 3’ UTR of the LPL gene, which counteracted the negative effect of the −4 kb to −827 construct. The identification of regulatory elements in the LPL intronic regions is analogous to that described for several other genes which harbor regulatory elements in their introns [30–35].

The lower transcription activity and lower binding of TBP associated with the *Hind*III mutant G allele may appear counterintuitive because this allele is associated with protection from CAD risk and/or with favorable lipid profile possibly due to higher LPL activity. However, we do not have the LPL activity data available in this study. Previously one study has shown a non-

significantly higher LPL activity among *Hind*III mutant carriers (n=15) than the homozygotes of the wild type (n=16) in postheparin plasma (9.21 ± 4.6 vs. 8.11 ± 4.16) [9]. However, the postheparin plasma LPL activity studies may not fully reveal the impact of the *Hind*III polymorphism on LPL activity as exemplified by the inconsistent findings with the S447X polymorphism. To date eight studies have analyzed the impact of the S447X polymorphism on postheparin LPL activity and only three studies found significant association [22,36–37] but the other five did not [15,38–41]. Even one study that found a significant association with a haplotype carrying the 447X mutant allele suggested that other variants in the 3'UTR of LPL are responsible for the increase in LPL activity rather than the S447X variant [37]. Thus the inclusion of the LPL activity data in this study might have not provided a conclusive answer about the functional impact of the *Hind*III polymorphism on LPL activity because the postheparin plasma reflects the total LPL activity and not tissue-specific activity. Likewise the *in vitro* measurement of LPL catalytic activity or mass may also be inconclusive as reflected in equivocal findings with the S447X polymorphism [22].

A possibility is that the effect of the *Hind*III polymorphism on LPL expression is influenced by other tightly linked functional variants that also affect LPL expression. In addition to the presence of potential transcriptional regulatory elements in the 3' region, translational regulatory elements in the 3' region of the LPL gene have also been identified [42–44]. A recent study suggests that regulatory polymorphisms are complex and sensitive to modifiers with multiple *trans*-acting or environmental factors affecting their function [45]. Multiple *cis*-acting sites may affect the contribution of a variant to the expression of a gene and if they are not examined together then *in vitro* experiments would not represent the true *in vivo* effect of a variant. If the *Hind*III site indeed increases LPL activity by increasing LPL expression in conjunction with other unknown *cis*-acting regulatory sequences in or around the LPL gene then they need to be examined together. Our constructs contained only the LPL promoter and the entire intron 8 sequence with a caveat that additional unknown *cis*-acting regulatory sequences may be required to understand the full spectrum of the LPL expression. Furthermore, LPL gene expression is sensitive to hormonal regulation of insulin. For example, it has been shown that LPL mRNA increase significantly in brown adipose tissue after insulin injection to fasting rats [46]. On the other hand, LPL activity is reduced under insulin resistance state because the ability of insulin to up-regulate LPL gene is impaired in insulin resistant individuals, leading to hypertriglyceridemia [47]. It is conceivable that insulin differentially affects the allele-specific effect of the *Hind*III polymorphism in individuals with different state of insulin sensitivity. Notwithstanding its true *in vivo* effect, our consistent in vitro results in two different cell systems (muscle and COS-1) along with EMSA results provide compelling evidence that the *Hind*III polymorphic site is functional.

In conclusion, we provide new information about the functional significance of the *Hind*III polymorphism and our data in conjunction with previous genetic association data reinforce the concept that this is a common functional variant with a modest effect for a common trait.

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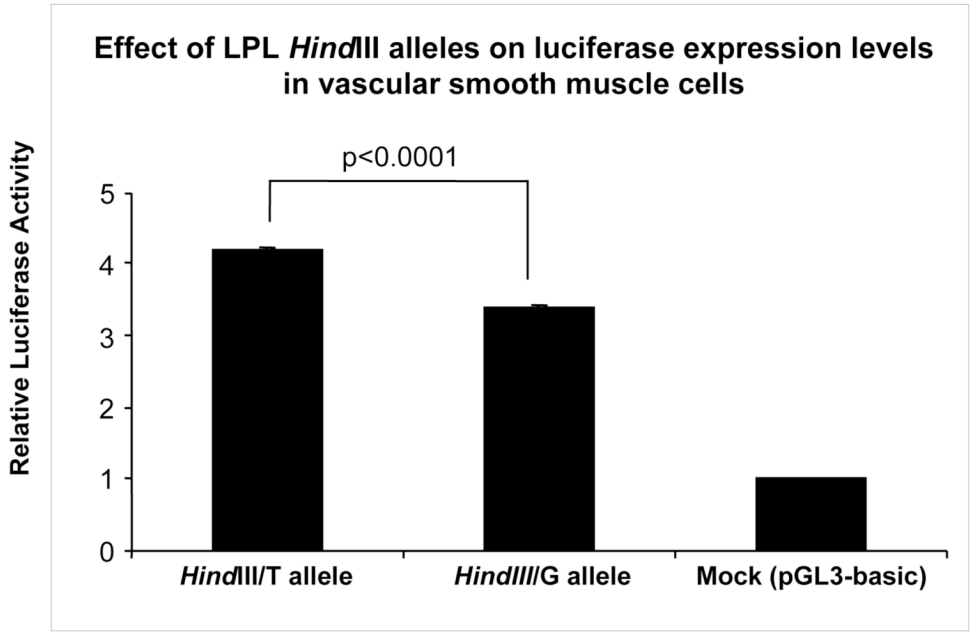

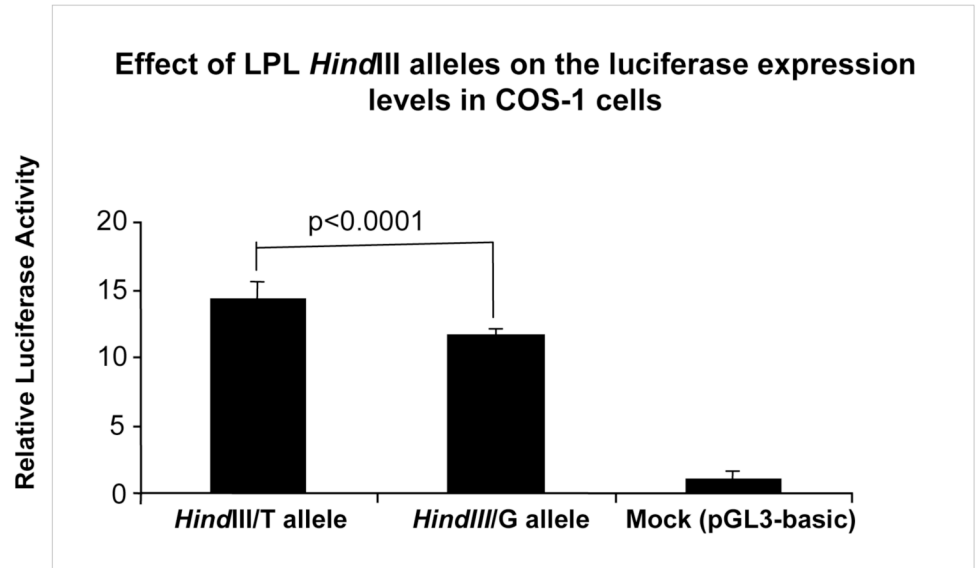

Figure 1.

Effect of the *Hind*III mutation on reporter gene expression in vascular smooth muscle cells and COS-1 cells. The effect of *Hind*III mutation on promoter activities was measured as the mean of the firefly Luc levels, which was adjusted with the *Renilla* Luc levels. The results presented are from three independent clones for each construct in triplicate, and each error bar represents the standard error.

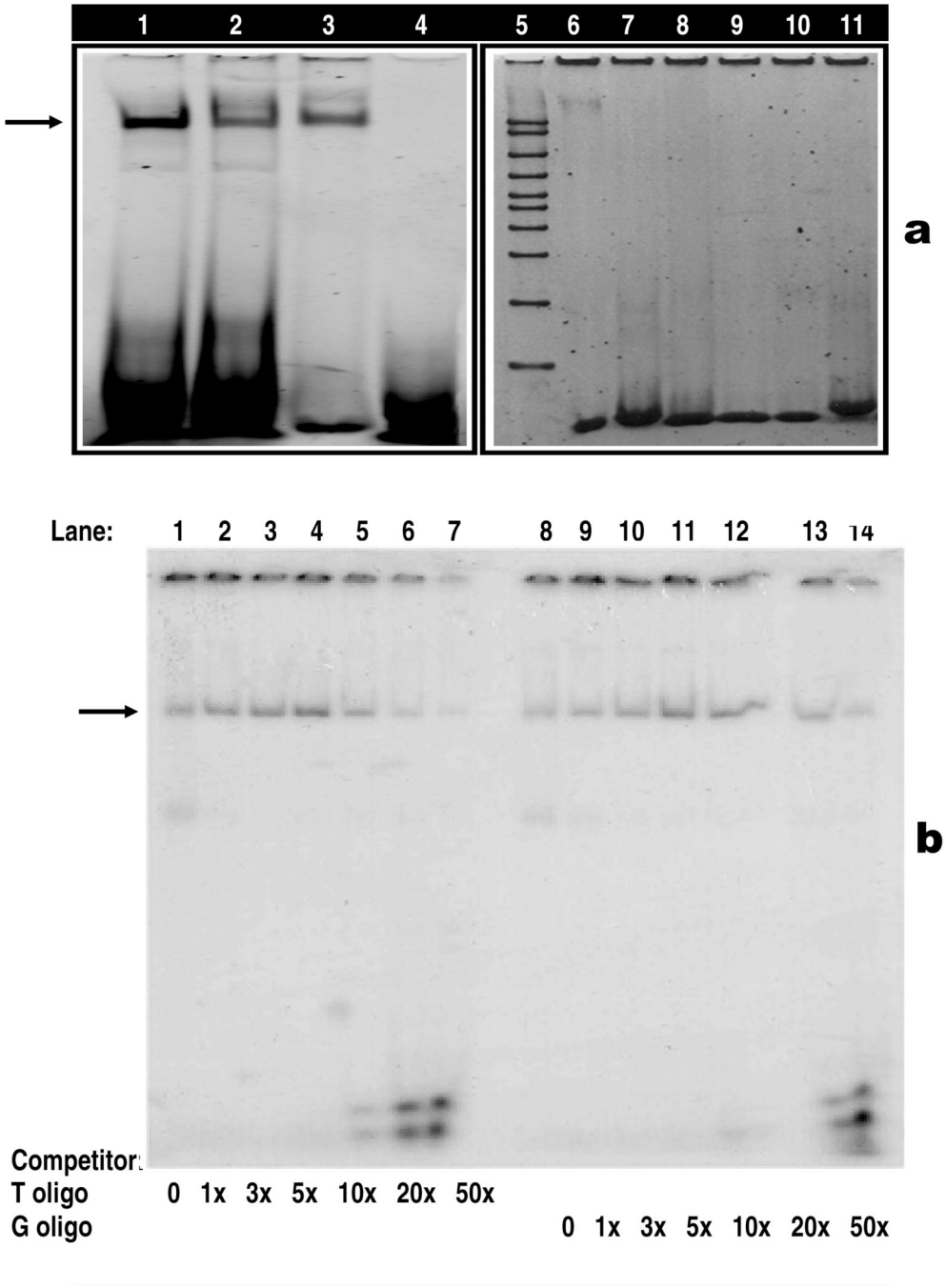

Figure 2.

a: Non-radioactive electrophoretic mobility shift assay (EMSA). Arrow head indicates the specific DNA-protein binding (lanes 1–3, 6) or no DNA-protein binding (lanes 7–11). Binding of *Hind*III wild type (lane 1) and mutant type (lane 2) with purified human TATA binding protein (TBP). Lanes 1 and 2 contain identical amounts of *HindIII* oligos and TBP, but the affinity of TBP is higher for the *Hind*III wild type (lane 1) than the mutant type (lane 2). Lanes 3 and 6 are positive controls showing the binding of TBP with TFIID consensus oligo. Purified TBP used in Lane 3 was obtained from Promega, whereas purified TBP used in lane 6 comes from Santa Cruz Biotechnologies and this may explain the difference in the intensity between the two bands. Lanes 4, 7–11 are negative controls showing no binding of TBP with consensus

oligos, including AP2 (lanes 4 and 7), SP1 (lane 8), AP1 (lane 9), OCT1 (lane 10) and CREB (lane 11). Lane 5 contains 50 – 2000 bp marker.

b: Radioactive competitor EMSA for LPL/*Hind*III T→G polymorphism. Each sample contains a mixture of 5 μg of nuclear extract derived from aorta smooth muscle cells and a 30-mer ^{32}P-labeled LPL/*Hind*III oligonucleotide containing the T allele. The arrowhead indicates the specific DNA-protein complex associated with the LPL/*Hind*III T→G polymorphic site. Competition assay performed by adding excess cold oligonucleotides containing either the T allele (lanes 2–7) or the G allele (lanes 9–14). Lanes 1 and 8: no competitor, lanes 2–7 have increasing amounts of T oligo competitor (1x, 3x, 5x, 10x, 20x and 50x, respectively); lanes 9–14 have increasing amounts of G oligo competitors (1x, 3x, 5x, 10x, 20x and 50x, respectively).

Table 1

Putative Regulatory Elements in Intron 8 of the Human LPL Gene.

Element	Consensus Sequence	Nucleotide Start Position of the Element*
Lmo2	CAGG	62*
Sp1	GGCG	196
MZF-1	GGGG	204, 664
GATA	GATA	372, 770
TATA	TAAA	481 (*Hind*III site), 489
C/EBPβ	GCAA	526
Ap-1	TGAC	600

* The number of consensus nucleotide from the start of intron 8 sequence.