Sp1 and Sp3 transactivate the human lipoprotein lipase gene promoter through binding to a CT element: synergy with the sterol regulatory element binding protein and reduced transactivation of a naturally occurring promoter variant

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Abstract **Lipoprotein lipase (LPL) is a key enzyme in lipoprotein and energy metabolism and, therefore, regulation of its expression could have an important bearing on these processes. We have identified an evolutionarily conserved 5′-CCTCCCCCC-3′ motif (from −91 to −83, CT element) in the human LPL gene promoter, deletion or mutation of which caused approximately 70–80% decrease in promoter activity. We found that Sp1 and Sp3 in THP-1 nuclear protein extracts bind specifically to this element. Co-transfection with Sp1 and Sp3 expression plasmids transactivated the LPL promoter via the CT element in *Drosophila* SL2 cells devoid of Sp proteins. Sp3 moderately repressed Sp1-mediated LPL promoter activation when both were co-expressed in SL2 cells. Furthermore, co-expression of an active sterol regulatory element binding protein (SREBP-1), with Sp1, but not with Sp3, synergistically activated the LPL promoter in SL2 cells. We previously reported a naturally occurring T→G substitution at position −93 of the human LPL promoter which reduces promoter activity by 40–50% in transient transfection assays. In this study, we showed that this substitution results in reduced binding affinity to Sp1/Sp3 and in diminished transactivation by Sp1/Sp3 alone and by the synergistic action of Sp1 and SREBP-1. In conclusion, recruitment of Sp1/Sp3 by the CT element may play an important role in expression of the human lipoprotein lipase gene. Synergistic transcriptional activation by Sp1 and SREBP-1 may provide a mechanism for cross-talk between cholesterol and triglyceride metabolic pathways.**— Yang, W-S., and S. S. Deeb. **Sp1 and Sp3 transactivate the human lipoprotein lipase gene promoter through binding to a CT element: synergy with the sterol regulatory element binding protein and reduced transactivation of a naturally occurring promoter variant. *J. Lipid Res.* 1998.** 39: **2054–2064.**

Supplementary key words transfection • gelshift • THP-1 • *Drosophila* SL2

Lipoprotein lipase (LPL) is a key enzyme in lipoprotein and energy metabolism. LPL dimers bind to glycosaminoglycans on the vascular endothelial surface and hydrolyze the core triglycerides of circulating chylomicrons and very low density lipoproteins. The resulting fatty acids are either utilized by peripheral tissues (e.g., skeletal muscle) as a source of energy or re-esterified to triglycerides (in adipose tissue) for storage (1). Deficiency of LPL results in extreme hypertriglyceridemia in familial chylomicronemia syndrome, whereas reduced levels of LPL are associated with familial hypertriglyceridemia, familial combined hyperlipidemia, hypoalphalipoproteinemia, and post-prandial dyslipidemia (2). LPL may also function as a ligand for cell surface glycosaminoglycans and lipoprotein receptors, such as the LDL receptor-related protein, to facilitate lipoprotein uptake by a variety of cell types (2, 3). Moreover, in vitro studies indicated that LPL may enhance lipoprotein retention in the subendothelial matrix (3) and promote atherogenesis (4). Local expression of LPL may also promote lipid uptake by macrophages and smooth muscle cells within the artery wall, thus leading to the formation of lipid-laden foam cells. Therefore, expression of LPL by macrophages within the arterial wall was proposed to be atherogenic (2, 3).

LPL is synthesized in a variety of tissues, including adipose tissue, cardiac and skeletal muscle, lactating mammary gland, and macrophages (5). Its expression is regulated at the transcriptional or post-transcriptional levels by the nutritional status, hormones, cytokines, and growth factors (5). For example, LPL activity in adipose tissue was shown to increase after a 6-h insulin/glucose infusion in human subjects (6). Various *cis*-DNA elements have been identified by functional analysis of the human LPL promoter. Two motifs, LP-α (between −702 and −666) and

Abbreviations: LPL, lipoprotein lipase; SREBP, sterol regulatory element binding protein; LDLR, low density lipoprotein receptor; FAS, fatty acid synthase; ACC, acetyl coenzyme A carboxylase; EMSA, electrophoretic mobility shift assay; CT element, 5′-CCTCCCCCC-3′.

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LP-β (between −468 and −430), which bind hepatic nuclear factor (NF) 3-like proteins, were suggested to contribute to differentiation-dependent promoter activity during adipogenesis of 3T3-F442A cells (7). An NF-1-like binding site (between −517 and −491) was implicated in post-natal extinction of LPL expression in rat liver (8). A silencer element (from −169 to −152) was shown to suppress basal promoter activity in HeLa and CHO cells, even though the latter cell type expresses endogenous LPL (9). Fatty acids, fibrates as well as the new class of anti-diabetic agents, thiazolidinediones, have been shown to induce rat LPL gene expression in liver and adipose tissue through their action on the peroxisome proliferator response element (from −169 to −157) of the LPL promoter (10). In the proximal promoter, the octamer binding protein Oct-1 (from −46 to −39) (11, 12), and nuclear factor Y (NF-Y) (from −65 to −61) (13, 14) binding motifs have been shown to be critical for basal promoter activity. Interestingly, it was demonstrated that tumor necrosis factor alpha, which reduces adipose tissue LPL activity, eliminates binding of NF-Y and Oct-1 to the LPL promoter (13). Mutagenesis of the TATA-like element (from −27 to −23) did not affect promoter activity (14).

The sterol regulatory element binding proteins (SREBP), originally shown to regulate the transcription of some genes in the cholesterol metabolic pathway (e.g., low density lipoprotein receptor; 15), were recently demonstrated to regulate transcription of certain genes in the fatty acid biosynthetic pathway such as fatty acid synthase (FAS) and acetyl coenzyme A carboxylase (ACC) (16, 17). The murine homologue of SREBP-1c, adipocyte determination and differentiation factor-1 (AAD1), partially induced the differentiation of NIH-3T3 cells into adipocytes and activated expression of the LPL gene (18). Overexpression of active SREBP-1a or SREBP-1c in transgenic mice resulted in induction of LPL mRNA in the liver (19, 20). These results suggest that SREBP may regulate transcription of the LPL gene. One important feature of promoter activation by SREBP-1 is that synergy with the other transcription factors, such as Sp1, is required for maximal activity (16, 17, 21).

In this study, we show that an evolutionarily conserved 5′-CCTCCCCCC-3′ motif (from −91 to −83, henceforth referred to as the CT element) is important for basal promoter activity and that it binds to and is transactivated by Sp1 and Sp3. Moreover, Sp1 but not Sp3 acts synergistically with SREBP-1 to activate the LPL promoter. We also show that the naturally occurring T(−93)G promoter substitution (12, 22) results in reduced binding affinity of Sp1/Sp3 to the CT element and in diminished transactivation of the LPL promoter by Sp1 and Sp3 and by Sp1 in synergy with active SREBP-1.

MATERIALS AND METHODS

Plasmid construction

Human LPL promoter nested deletion constructs, ranging in length between 7.2 kb and 235 bp (**Fig. 1**), were generated by cloning the DNA fragments having the same 3′ end at position +135 with respect to the transcription start site into the luciferase reporter vector, pXP1 (ATCC #: 37576, ref. 23). These DNA fragments were derived from genomic cosmid clone HLPLG-83-2 (24), making use of the unique restriction sites *Sst* I, *Bam* HI, *Nco* I, *Pst* I, *Xba* I, *Spe* I, and *Sma* I in the 5′ flanking sequences and a *Nae* I site at position +135 of exon 1. The 7.2 kb *Sst* I-*Nae* I promoter fragment was cut out from genomic clone HLPLG-83-2, blunt-ended, and cloned into the *Sma* I site of plasmid pXp1. The 4.2 kb *Bam* HI-*Nae* I fragment was cut out from the same clone, blunt-ended, and cloned initially into the EcoRV site of plasmid KSSV. Nested deletions of this 4.2 kb promoter segment were generated by digestion with restriction enzymes that cleave at one site in the 5′ multiple cloning region and at another within the promoter, followed by isolation of the desired fragment by agarose gel electrophoresis and religation. *Nco* I, *Pst* I, *Xba* I, *Spe* I, and *Ava* I were used to generate the 3.0, 1.8, 0.8, 0.555, and 0.235 kb promoter constructs, respectively. These nested promoter segements were then cleaved with *Hind* III at the 3′ end and the respective enzyme at the 5′ end and cloned directionally into pXp1.

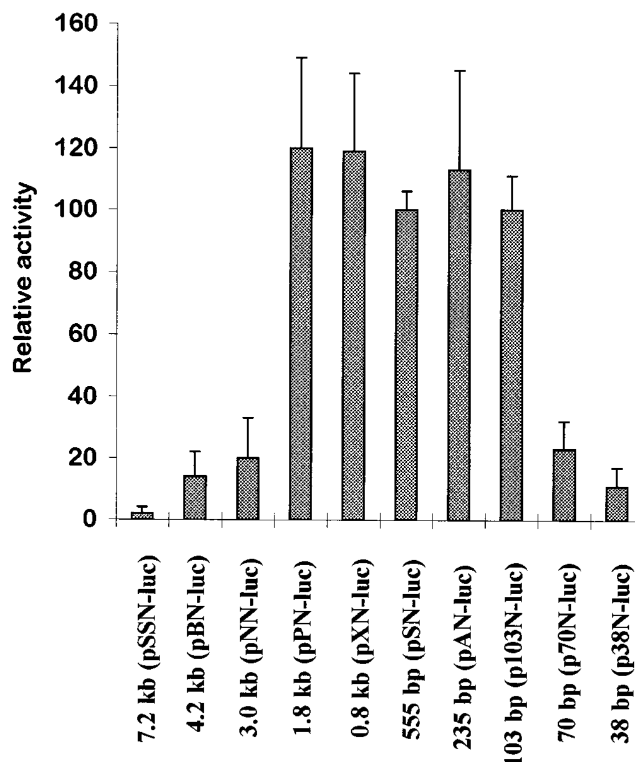

Fig. 1. Activity of LPL promoter deletion constructs in THP-1 transient transfections. Plasmids containing the luciferase reporter gene driven by variable lengths of the LPL promoter sequences as indicated were transfected into THP-1 cells. The numbers represent the lengths of promoter fragment starting from the transcription start site. All constructs also contain the first 135 bp of 5′-untranslated region of exon 1. Luciferase activity of pSN-luc (−555) was arbitrarily assigned as 100. The data represent the means and standard errors (SE) of 5 independent transfections. All values in transfection experiments were corrected for transfection efficiency by co-transfection with a lacZ reporter plasmid driven by the CMV promoter as described in Materials and Methods.

Proximal promoter deletion constructs, containing 103, 70, and 38 bases (Fig. 1) were generated as follows: DNA fragments were amplified by PCR using pAN-luc (Fig. 1) as template and

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TABLE 1. Oligonucleotide primers used for PCR, EMSA, and in vitro mutagenesis

Primers	Position	Sequence
R16	+135	5′-atccaagcttGATGGCTGAGC-3′
R18	+20	5′-caccaagcttGAGGGGGAATCGAGTCTGAC-3′
R17	−103	5′-cgcggtaccTGAATTTAGGTCCCTCCCCC-3′
R19	−70	5′-cgcggtaccTATAGCCAAATAGGTGATGAG-3′
R21	−38	5′-gcggtcgacACCTCATCACCTATTGGCT-3′
CT-1	−97	5′-tcgaTAGGTCCCTCCCCCCAACTT-3′
CT-1C	−78	5′-tcgaAAGTTGGGGGGAGGGACCTA-3′
R-M1	−97	5′-tcgaTAGGTCCC**GAATT**CCAACTT-3′
R-M2	−97	5′-tcgaTAGGTCCC**G**CCCCCCAACTT-3′
NF-kB		5′-TGACAGAGGGACTTTCCGAGAGGA-3′

The sequences of the human LPL gene are shown in upper case. Restriction sites and spacer sequences added to the 5′ ends of primers for cloning purposes are printed in lower case. Altered bases in R-M1 and R-M2 are indicated in bold and underlined. The numbers under position indicate the location of the most 5′ nucleotide of the LPL gene sequences of each primers in the LPL gene.

three sets of primers, R17/R16, R19/R16, and R21/R16 (**Table 1**). The fragments were digested with restriction enzymes (the 5′ ends of the primers contain restriction sites) and cloned directionally into pXP1. The 3′ ends of these promoter constructs also started at position +135.

For mutational analysis, three constructs (p97/20-luc, pM1-luc, and pM2-luc), containing the LPL promoter sequences from nucleotide −97 to +20 with either wild-type or variant CT elements, were generated as follows. DNA fragments were amplified by PCR, using p103/20-luc plasmid DNA (described in ref. 12) as template and primer sets CT-1/R18, R-M1/R18 and R-M2/R-18 (Table 1). The fragments were phosphorylated and cloned into the *Sma* I site of pXP1. The base substitutions within the CT element in pM1-luc and pM2-luc are indicated in Table 1.

Three constructs were generated to test the effect of the orientation of the CT element on promoter activity. Primers CT-1/CT-1C (Table 1) were annealed and cloned into the *Xho* I site immediately 5′ upstream to the LPL promoter sequences in p70N-luc (Fig. 1). Constructs p1CT(+)-70N-luc, p1CT(−)-70N-luc and p2CT(−)-70N-luc contain one copy of CT element in the native orientation, one copy in the opposite orientation, and two copies in the opposite orientation, respectively.

The constructs containing LPL promoter sequences from −144 to +20 with either T or G at position −93 have been described (22). These constructs were used as templates for PCR to generate competitor DNA for competition EMSA, and were also used in co-transfection experiments in SL2 cells. The expression plasmids pPac-0, pPac-Sp1, pPac-Sp3, pPac-SREBP1, and γF-gal used in cotransfection of SL2 cells were kindly provided by Drs. R. Tjian, G. Suske, T. F. Osborne, and P. P. Di Nocera, respectively, and have been described (21, 25–27).

All plasmid constructs generated in our laboratory were sequenced to verify the orientation and sequence of inserts. Plasmid DNA for transfection experiments was prepared using a Qiagen Plasmid Kit, according to manufacturer's protocol (Qiagen Inc., Chatsworth, CA). All transfection experiments were repeated with at least two different preparations of DNA.

Cell culture and transient transfection

The human monocytic leukemia cell line, THP-1 (ATCC: TIB 202) was cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 2 mm l-glutamine as described (28). Transient transfection of THP-1 cells by the DEAE–dextran method has been described in detail (12, 22). The mouse myoblast cell line, C2C12-F3 (kindly provided by Dr. M. Horowitz, University of Washington) (29) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% FCS and 2 mm l-glutamine. 3T3-F442A cells (kindly provided by Dr. M. Reina, University of Barcelona) (30) were cultured in DMEM supplemented with 10% FCS, 2 mm l-glutamine, 8 mg/L biotin, and 15 nm HEPES. HeLa (ATCC: CCL 2) and HepG2 cells (ATCC: HB 8065) were cultured in DMEM supplemented with 10% FCS and 2 mm l-glutamine. C2C12-F3, 3T3-F442A, HeLa, and HepG2 cells were transfected using lipofectin–DNA complexes following the manufacturer's protocol (Life Technologies Inc., Gaithersburg, MD). C2C12 and HeLa cells were incubated with DNA/lipofectin mixture for 4–6 h, and 3T3-F442A and HepG2 cells were incubated with the mixture for 16–20 h. *Drosophila* SL2 cells (ATCC: CRL 1963, ref. 31) were cultured in Shields and Sang M3 insect medium (Sigma, St. Louis, MO) supplemented with 10% FCS (Sigma, tested for insect cell culture) and 2 mm l-glutamine. Transient transfection of SL2 cells was performed using the calcium phosphate method as described (32). The cells were incubated with DNA/Ca^{2+} phosphate precipitates for 2 days until harvest. Equimolar concentrations of plasmids carrying different promoter constructs were used in all transfection assays. Plasmids pnCMVlacF (in mammalian cells) and γFgal (in SL2 cells), both driving expression of β-galactosidase, were co-transfected with LPL promoter constructs in order to correct for differences in transfection efficiency. Cell lysates were assayed for luciferase activity, β-galactosidase activity, and protein concentration as described before (12, 22).

Preparation of crude nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts were prepared from THP-1 cells according to a modification of Dignam's method (33). Approximately 40 million THP-1 cells were incubated in the lysis buffer containing 0.2% NP-40 for 10–15 min in order to achieve optimal lysis of the plasma membrane. Protease inhibitors were added to the buffers just before use (final concentrations: 0.5 mm dithiothreitol, 0.25 mm phenylmethylsulfonyl fluoride, 1.0 μg/ml leupeptin, 18 KIU/ml aprotin; Sigma, St. Louis, MO). The nuclear protein extracts were aliquoted and stored at −70°C until use.

Electrophoretic mobility shift assays (EMSA) were performed as follows. Double-stranded oligonucleotide (CT-1/CT-1C in Table 1) was labeled with [α-^{32}P]dCTP by the Klenow filling-in reaction as described (34). Approximately 0.1 pmol of the labeled probe was incubated with 2 μg of nuclear extract in a 16-ml reaction mixture (0.06% NP-40, 6.0 mm $MgCl_2$, 80 mm NaCl, 40 mm KCl, 30 mm HEPES, 10 mm EDTA, 10% glycerol, and 2 μg of poly-dIdC). After 15–30 min of incubation at room temperature, the DNA–protein complexes were fractionated on a 6% nondenaturing polyacrylamide gel (gel thickness, 1.5 mm; model SE 250, Hoefer, San Francisco, CA) at 50 volts for 2.5 to 5 h in 0.5× TBE at room temperature or on a 5% polyacrylamide gel (gel thickness, 3 mm) at 200 volts for 5 h in 0.5× TBE at 4°C. The concentration of competitor DNA was measured with TKO 100 minifluorometer (Hoefer, San Francisco, CA), following the manufacturer's protocol. The radioactivity of retarded DNA–protein complexes in competition EMSAs was quantified by phosphoimager analysis (model 400S, Molecular Dynamics, Sunnyvale, CA).

To identify the transcription factors in EMSA, the DNA–protein binding reactions were assembled essentially as described above followed by addition of 20 ng of specific polyclonal antibodies against Sp1, Sp2, Sp3, or Sp4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). To neutralize the antibodies, 200 ng of Sp1 or Sp3 neutralization peptides (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was incubated with 20 ng of cognate antibodies

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at 4°C overnight before addition to the binding reactions. After approximately 1 h of incubation at room temperature, the binding reaction mixtures were electrophoresed on a 5% nondenaturing polyacrylamide gel (gel thickness, 3 mm) at 200 volts in 0.5× TBE for 5 h at 4°C.

RESULTS

A regulatory element (5′-CCTCCCCCC-3′) located between position −103 and −70 is critical for activity of the human LPL promoter

The Oct-1 (−46 to −39) and NF-Y (from −65 to −61) binding sites were previously shown to be critical for activity of the human LPL promoter (11–14). We now show that a third *cis*-element is also critical for its activity. A series of 5′ nested promoter deletions (Fig. 1) directing expression of the luciferase reporter gene were compared for activity in transient transfections of the human monocytic leukemic cell line THP-1, which expresses endogenous LPL (28). As shown in Fig. 1, promoter constructs containing 3 kb, 4.2 kb, and 7.2 kb had much lower activity than that with 1.8 kb, indicating the presence of negative regulatory element(s) in the region between 1.8 kb and 3.0 kb from the transcription start site. Segments of the proximal LPL promoter, ranging in length between 1.8 kb and 103 bp, had approximately the same activity (Fig. 1). A significant loss in activity (77%, $P < 0.001$, *t*-test) was observed as the result of deleting the sequences between −103 and −71, suggesting the presence of at least one critical positive regulatory element in this region (Fig. 1). Deletion of the region between −70 and −39, which contains the Oct-1 and NF-Y sites (11–14), caused a further decrease in activity (52%; two-tailed *t*-test: $P = 0.051$, one-tailed: $P = 0.025$; Fig. 1).

Alignment of the proximal promoters of the human (24), mouse (35), and chicken LPL genes (36) revealed that the only conserved sequence within the region between −103 and −71 is a 5′-CCTCCCCCC-3′ motif (from −91 to −83, **Fig. 2**). To further investigate the role of this CT element in basal LPL promoter activity, we assessed the activity of a promoter construct (pM1-luc) with an altered CT element (changed from wild-type: CCTCCCCCC to M1: CCGAATTCC). This alteration reduced promoter activity to 23 ± 2% in transient transfection of THP-1 cells (**Fig. 3**). This residual activity was equivalent to that observed upon deleting the sequences from −103 to −71 (Fig. 1), suggesting that the CT element may be the only sequence of functional importance within this region. The same alteration also caused reductions in promoter activity to 23 ± 3% in C2C12-F3 (mouse myoblast) cells, 7 ± 3% in 3T3-F442A (mouse pre-adipocyte), 24 ± 5% in HepG2 (human hepatocellular carcinoma), and 11 ± 2%

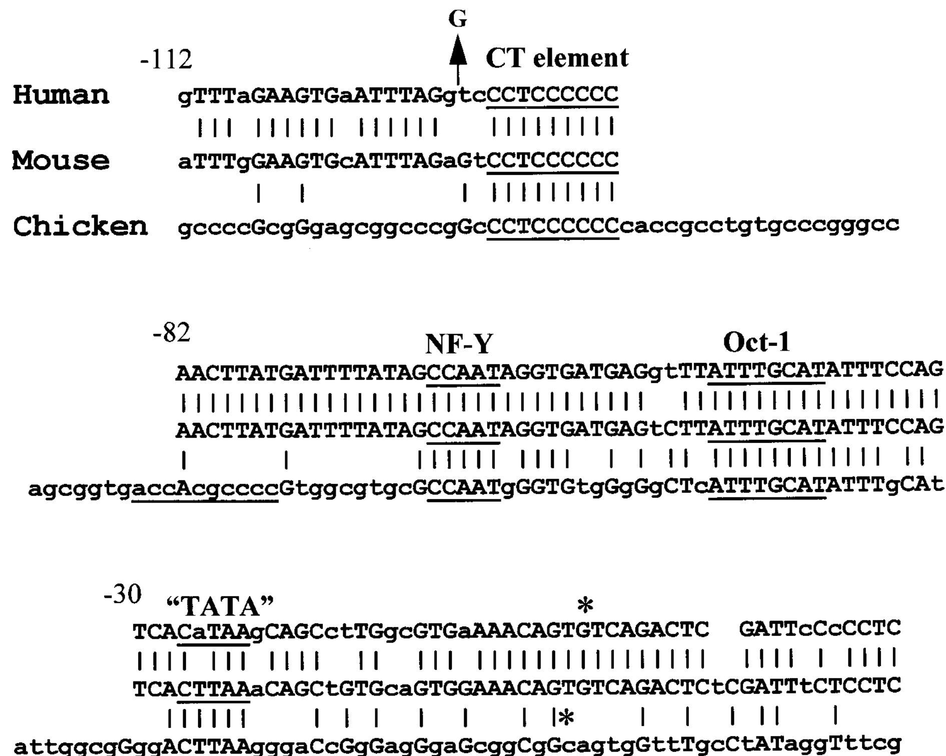

Fig. 2. Sequence alignment of the proximal promoters of the human, mouse, and chicken LPL genes. The CT element, NF-Y, Oct-1 binding sites, and putative TATA box are underlined. The GC box located between the CT element and NF-Y site in the chicken LPL promoter is also underlined. The major transcription start sites are indicated with asterisks above the specific bases. The T→G substitution at −93 is also indicated. The major transcription start sites of the human and mouse LPL gene are at the same position. Numbering of the human LPL gene sequence is according to ref. 24.

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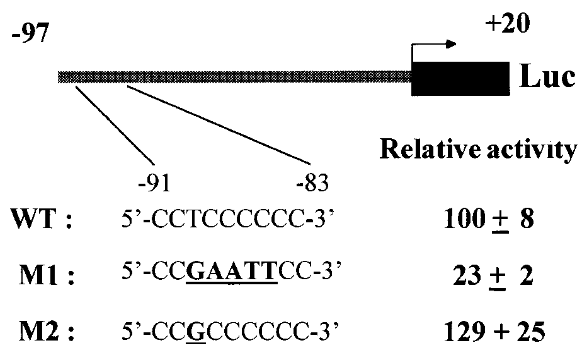

Fig. 3. Activity of the LPL promoters with the wild-type or variant CT element in THP-1 cells. Plasmid DNA, containing the luciferase reporter gene driven by the LPL promoter sequences (from −97 to +20) with either wild-type or variant CT elements, was transfected into THP-1 cells. The altered bases within the CT element in pM1-luc and pM2-luc are indicated in bold and underlined. Luciferase activity of the wild-type promoter construct was arbitrarily set to 100. The data represent the means and SE of two separate transfection experiments, each in triplicate. All values in transfection experiments were corrected for transfection efficiency.

in HeLa cells (human epitheloid carcinoma) (data not shown).

Because the sequence of the CT element is similar to the consensus sequence of Sp1 binding sites (5′-G/AC/T C/TCCGCCCC/A-3′, ref. 37), we also generated a variant promoter plasmid, pM2-luc, with a T to G substitution at position −89 which basically changed the CT element into a canonical Sp1-binding motif (GC box). The activity of this promoter variant was 129% of wild-type ($P = 0.04$, *t*-test) in THP-1 transient transfection assays (Fig. 3).

We next investigated whether the orientation of the CT element is important for promoter activity. A 20-bp double-stranded oligonucleotide containing the CT element (CT-1/CT-1C in Table 1) was cloned 5′ upstream to the human LPL promoter in p70N-luc (Fig. 1) which was devoid of the CT element. A single copy of the CT element in its native orientation increased the promoter activity of p70N-luc by 3.2-fold (data not shown). One and two copies of the CT element in opposite orientation enhanced the activity of p70N-luc by 6.0- and 8.3-fold, respectively (data not shown). However, it is noteworthy that the CT element in these reconstructed promoters was not in its natural context in terms of flanking sequences (contained only 6 nucleotides 5′ and 5 nucleotides 3′ to the conserved CT element of the LPL promoter) and distance (7 nucleotides further upstream) from the major transcription start site (24). Nevertheless, these results indicate that the 20-mer oligonucleotide containing the CT element has enhancer activity.

The CT element of the LPL promoter binds Sp1 and Sp3

Using a radiolabeled double-stranded oligonucleotide containing the CT element (CT-1/CT-1C in Table 1), we investigated whether this CT element bound nuclear proteins from THP-1 cells in electrophoretic mobility shift assays (EMSA). Four major bands of DNA–protein complexes were observed (**Fig. 4A**). Using the unlabeled CT element oligonucleotide as competitor, the upper two bands (bands 1 and 2) decreased in intensity as the concentrations of the competitor increased (Fig. 4A). By contrast, a 50-fold molar excess of double-stranded oligonucleotide recognized by NF-κB (NF-κB in Table 1 and its complement, ref. 33) did not compete with the CT element oligonucleotide for binding nuclear proteins (Fig. 4A). Therefore, bands 1 and 2 represented specific DNA–protein interactions. Patterns of EMSA similar to those with THP-1 nuclear extracts were observed using nuclear extracts from C2C12, 3T3-F442A, HepG2, and HeLa cells (data not shown).

In order to confirm the specificity of these DNA–protein interactions, we performed a second competition EMSA experiment using DNA fragments of the LPL promoter (−97 to +20) with either a wild-type or an altered CT elements as competitors. A DNA fragment with the altered CT element (CCGAATTCC, Fig. 3), PCR-amplified from pM1-luc, competed much less effectively than the wild-type sequence (Fig. 4B). As expected, a DNA fragment, PCR-amplified from pM2-luc (Fig. 3), the CT element of which was changed into a canonical Sp1-binding GC box (CCGCCCCCC), competed effectively with the wild-type CT element (Fig. 4B). The close correspondence between results of transfection assays (Fig. 3) and EMSA (Fig. 4B) strongly suggests that the nuclear proteins revealed by bands 1 and 2 in EMSA may be transcription factors that activate the LPL promoter via the CT element.

In view of the sequence similarity between the CT and Sp1-binding motifs (GC-box), and of the results of transfection assays and competition EMSA which showed that the GC box (in pM2-luc) was at least as active as the CT element (Fig. 3 and Fig. 4B), we investigated whether the nuclear proteins that bind to the CT element are known members of the Sp family (38, 39). Using a 3 mm-thick gel in EMSA, we were able to resolve the uppermost band (band 1 in Fig. 4A and 4B,) into two bands (bands 1A and 1B in Fig. 4C). Using a polyclonal antibody against Sp1, band 1A was upshifted to a higher molecular weight (Fig. 4C, lanes 2–6 and 10). Preincubation of the anti-Sp1 antibody with Sp1 neutralizing peptides abolished the upshift (Fig. 4C, lane 7). When anti-Sp3 was added alone or together with anti-Sp1 to the EMSA reactions, the intensity of bands 1B and 2 diminished significantly but no supershifted bands were observed (Fig. 4C, lanes 5 and 8) which could be due either to the low levels of Sp3 or to interference by the antibody with binding to the olignucleotide probe. Preincubation of the anti-Sp3 antibody with Sp3 neutralization peptides restored bands 1B and 2 (Fig. 4C, lanes 9 and 10). On the other hand, addition of anti-Sp2 and anti-Sp4 had no apparent effect on the EMSA patterns (Fig. 4C, lanes 4 and 6). These results indicate that Sp1 and Sp3, respectively, constitute the major and minor components of the CT element binding proteins.

Sp1 and Sp3 transactivate the human LPL promoter via the CT element

We next performed co-transfection experiments in the *Drosophila* cell line SL2, which was shown to be deficient in

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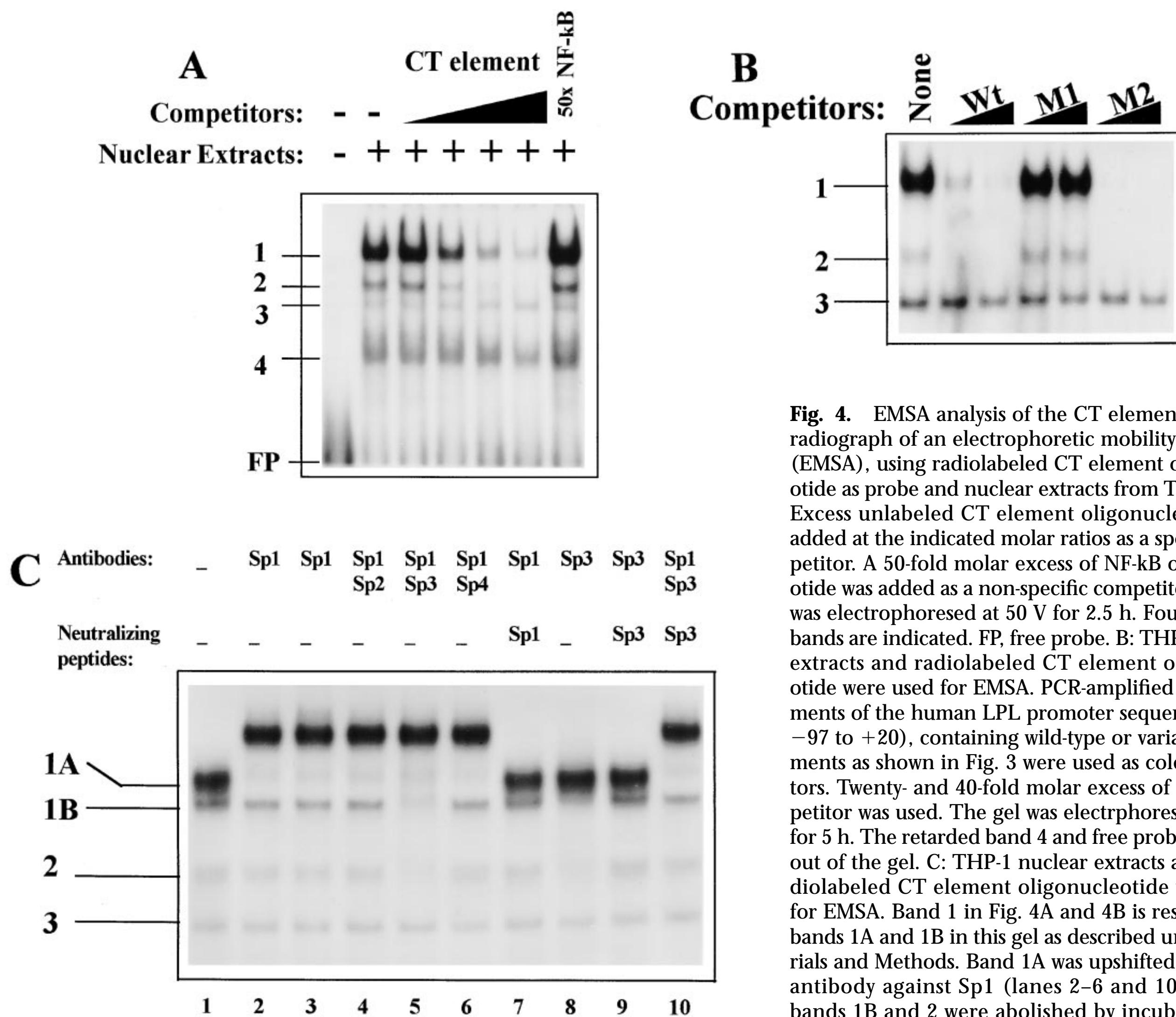

Fig. 4. EMSA analysis of the CT element. A: Autoradiograph of an electrophoretic mobility shift assay (EMSA), using radiolabeled CT element oligonucleotide as probe and nuclear extracts from THP-1 cells. Excess unlabeled CT element oligonucleotide was added at the indicated molar ratios as a specific competitor. A 50-fold molar excess of NF-kB oligonucleotide was added as a non-specific competitor. The gel was electrophoresed at 50 V for 2.5 h. Four retarded bands are indicated. FP, free probe. B: THP-1 nuclear extracts and radiolabeled CT element oligonucleotide were used for EMSA. PCR-amplified DNA fragments of the human LPL promoter sequences (from −97 to +20), containing wild-type or variant CT elements as shown in Fig. 3 were used as cold competitors. Twenty- and 40-fold molar excess of each competitor was used. The gel was electrphoresed at 50 V for 5 h. The retarded band 4 and free probe were run out of the gel. C: THP-1 nuclear extracts and the radiolabeled CT element oligonucleotide were used for EMSA. Band 1 in Fig. 4A and 4B is resolved into bands 1A and 1B in this gel as described under Materials and Methods. Band 1A was upshifted by adding antibody against Sp1 (lanes 2–6 and 10) and the bands 1B and 2 were abolished by incubation with anti-Sp3 antibody (lanes 5 and 8). Incubation of cognate neutralizing peptides with anti-Sp1 (lane 7) and anti-Sp3 (lanes 9 and 10) offset these effects of the antibodies. This EMSA was performed as described under Materials and Methods except that 40 ng of anti-Sp1, instead of 20 ng, was used in the third lane. The retarded band 4 and free probe are not shown in this part of the gel.

the Sp family of transcription factors (25, 38). The aims of these experiments were, first, to test whether Sp1 could transactivate the human LPL promoter directly through the CT element, and second, to determine whether Sp3 is a transcriptional activator or repressor of the LPL promoter. Earlier reports had suggested that Sp3 might be a transcription repressor for a number of genes (26, 40–43). LPL promoter constructs with wild-type (p97/20-luc) or mutant CT element (pM1-luc) were transfected into SL2 cells together with either an Sp1 (pPac-Sp1) or an Sp3 (pPac-Sp3) expression plasmid. For control, the LPL promoter constructs were co-transfected with pPac-0, which contains only the *Drosophila* 5C actin promoter without Sp1 or Sp3 cDNA (25). Activity of the wild-type LPL promoter increased approximately 6- and 3.5-fold when co-transfected with pPac-Sp1 and pPac-Sp3, respectively (**Fig. 5A**). In contrast, the mutant CT element promoter (pM1-luc) was not activated by co-transfection with either Sp1 or Sp3 expression vector (Fig. 5A). These results suggest that activation of the human LPL promoter by Sp1 and Sp3 is mediated directly by the CT element.

Sp1 and Sp3 co-modulate LPL promoter activity

Co-transfection with the Sp3 expression vector was consistently observed to activate the LPL promoter less effectively than that with the Sp1 expression vector, suggesting that Sp3 is less potent in transactivating the LPL promoter. Hagen et al. (26) observed that the less potent Sp3 represses Sp1-mediated transcriptional activation by competing for the same binding site. We asked whether this is also true for the LPL promoter. Reciprocal competition experiments were performed to address this question. If the potency of Sp3 is equivalent to or greater than that of Sp1 in transcriptional activation, co-transfection of Sp3 would not decrease Sp1-mediated promoter activation, irrespective of its expression level. Co-transfections with a

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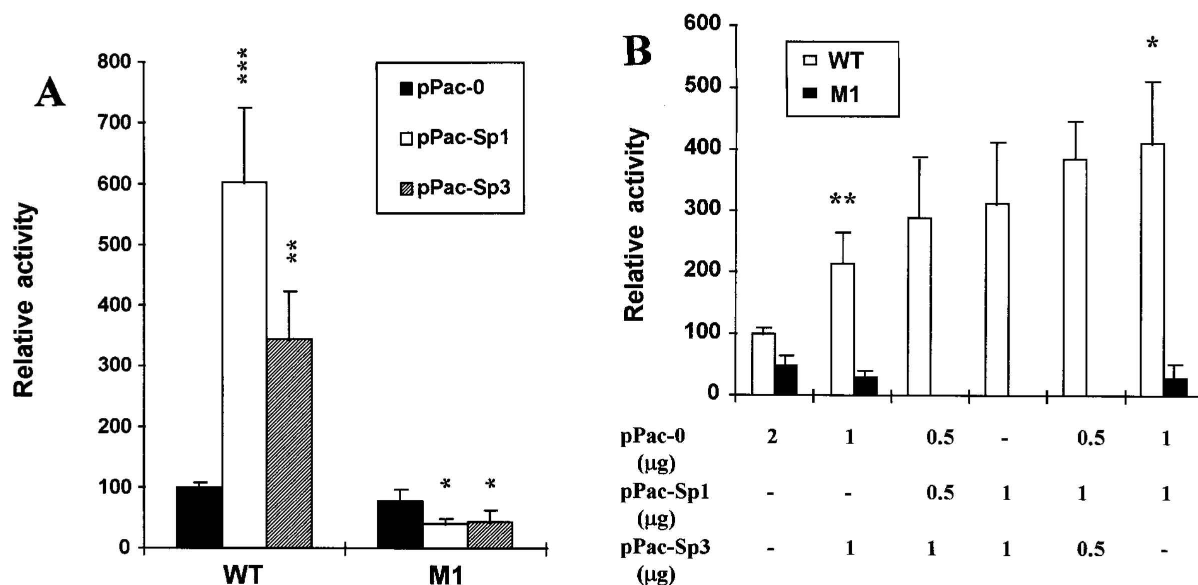

Fig. 5. Activation of wild-type or mutant LPL promoters by Sp1 and/or Sp3 in SL2 cells. A: Five µg of p97/20-luc (wild-type) or pM1-luc (mutant CT element) was transfected into SL2 cells with 1 µg of pPac-0 (no insert), pPac-Sp1 or pPac-Sp3. ***, $P < 0.001$ in comparison with pPac-0; **, $P < 0.01$ in comparison with pPac-0. $P < 0.02$ in comparison with pPac-Sp1; *, $P < 0.05$ in comparison with pPac-0. B: Reciprocal competition between Sp1 and Sp3 was performed by co-transfection of variable amount of pPac-0, pPac-Sp1 and/or pPac-Sp3 into SL2 cells with 2.5 µg of p97/20-luc. The amounts of pPac-0, pPac-Sp1, and pPac-Sp3 used in each transfection are indicated below the graph. **, $P <$ 0.02 in comparison with pPac-0 or pPac-Sp1; *, $P < 0.01$ in comparison with pPac-0. Luciferase activity of p97/20-luc, co-transfected with pPac-0, was arbitrarily set to 100. All the data represent the means and SE of two transfection experiments, each performed in duplicate. All values were corrected for transfection efficiency. Statistical analyses were performed with *t*-test.

variable amount of pPac-Sp3 and a fixed amount of pPac-Sp1 decreased Sp1-mediated promoter activation (Fig. 5B). In contrast, Sp3-mediated promoter activation was further enhanced by co-transfections with a variable amount of pPac-Sp1 (Fig. 5B). The relative promoter activity was found to be linearly correlated with the relative DNA amount of Sp1 and Sp3 expression plasmids used in these experiments (correlation coefficient $r = 0.82$, $P = 0.0001$, data not shown). This indicates that Sp1 and Sp3 may co-modulate LPL promoter activity.

Sp1 but not Sp3 acts synergistically with the sterol regulatory element binding protein-1 (SREBP-1) in activating the LPL promoter

Earlier studies suggested that SREBP may activate transcription of the LPL gene (18–20). Furthermore, an Sp1 site was shown to be required for maximal transcriptional activation of the LDL receptor (LDLR), fatty acid synthase (FAS), and acetyl coenzyme A carboxylase (ACC) by SREBP-1 (16, 17, 21). Here, we show that co-transfection with the wild-type LPL promoter construct (p97/20-luc) along with plasmids that express either Sp1 or active SREBP-1 (amino acids 1–490) resulted in an 8- and 2-fold activation, respectively (**Fig. 6**). Co-transfection with both the SREBP-1 and Sp1 expression plasmids synergistically activated the wild-type LPL promoter by approximately 18-fold (Fig. 6). In contrast, the LPL promoter construct with the mutant CT element (pM1-luc) was not synergistically activated by co-exlpression with Sp1 and SREBP-1. As opposed to Sp1, co-transfection with the Sp3 and SREBP-1 expression plasmids did not have any significant synergistic effect on activity of the LPL promoter (Fig. 6).

The naturally occurring T(−93)G promoter substitution results in reduced binding to Sp1/Sp3 and in diminished transactivation by Sp1/Sp3 alone or in synergy with SREBP-1

We previously screened the human LPL promoter for sequence variants among individuals with familial combined hyperlipidemia and coronary artery disease as well as normal controls (12, 22). Three of the observed single-base substitutions are located in close proximity to the CT element. Two of these substitutions (a T→G at −79 and a G→T at −95) did not affect LPL promoter activity, whereas the T→G substitution at −93 reduced the promoter activity to approximately 50–60% of wild-type in transient transfections of THP-1 and C2C12 cells (22). Therefore, we investigated whether the T→G substitution at −93 influences Sp1/Sp3 binding to the CT element. A competitor DNA fragment, containing the human LPL promoter sequence from −144 to +20 with G at −93, competed with the CT element oligonucleotide for Sp1/Sp3 binding less effectively than that with the wild-type sequence in EMSA experiments (**Fig. 7**). For band 1 (Sp1+ Sp3), the ratios for densities produced by competition with T competitor to that with G at position −93 were 0.53, 0.62, and 0.50 when 5×, 10×, and 20× of unlabeled competitor were added, respectively. For band 2 (Sp3), the corresponding values were 0.50, 0.43, and 0.47. These results indicate that Sp1 and Sp3 may bind less effectively to the LPL promoter with G than with T at −93.

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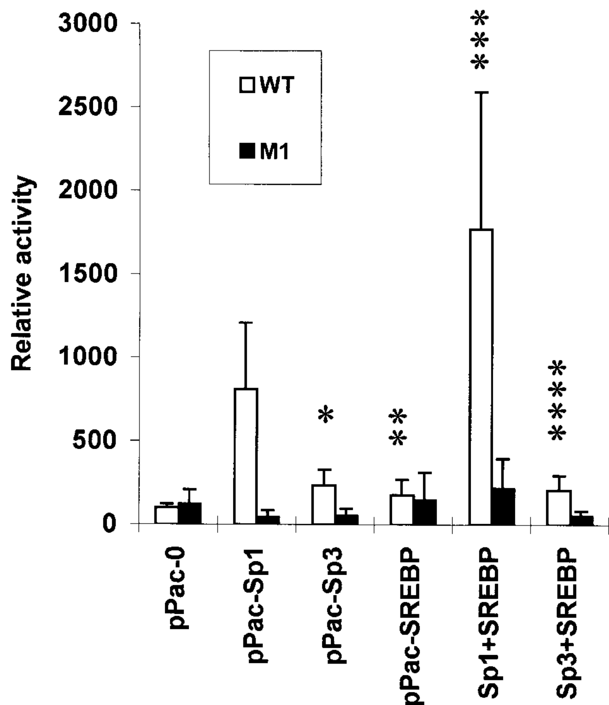

Fig. 6. Synergy between SREBP and Sp1, but not Sp3 in activation of the LPL promoter in SL2 cells. LPL promoter with either wild-type (p97/20-luc) or mutant (pM1-luc) CT element of 2.5 μg was transfected into SL2 cells with 0.5 μg of the expression plasmids as indicated. The data are the means and SE of four transfection experiments, each in triplicate. All values were corrected for transfection efficiency by co-transfection of a lacZ reporter plasmid as described under Materials and Methods. The activity of the p97/20-luc co-transfected with pPac-0 was arbitrarily set to 100. *, $P < 0.005$ in comparison with pPac-0; **, $P < 0.01$ in comparison with pPac-0; ***, $P < 0.005$ in comparison with pPac-Sp1; ****, $P < 0.002$ in comparison with pPac-0; $P = 0.42$ in comparison with pPac-Sp3 and $P = 0.44$ in comparison with pPac-SREBP.

We next performed co-transfection experiments in SL2 cells in order to determine whether the LPL promoter with G at position −93 is transactivated less efficiently by Sp1 and Sp3. The G(−93)-allele promoter was activated to a lesser extent than the T(−93)-allele when co-transfected with 20 or 50 ng of the Sp1 or Sp3 expression plasmids (**Fig. 8A**). Co-transfection with higher amounts of pPac-Sp1 or pPac-Sp3 (500 ng and 100 ng) transactivated both alleles almost equally (Fig. 8A), consistent with the observation that the two alleles differ in their affinity to Sp1/Sp3. We next investigated whether synergistic activation by Sp1 and active SREBP-1 is influenced by the T(−93)G substitution. When co-transfected with 20 ng of the pPac-Sp1 and 500 ng of the SREBP-1 expression plasmids, LPL promoter activity of the G(−93)-allele was significantly lower than that of the T(−93) allele (68% of T(−93), Fig. 8B). However, the two LPL promoter alleles were transactivated to the same extent by SREBP-1 alone (Fig. 8B). These results indicate that the T(−93)G substitution at −93 in the LPL promoter results in reduced binding affinity to Sp1/Sp3 and in diminished transactivation by Sp1 and Sp3 as well as to synergistic activation by Sp1 and SREBP-1.

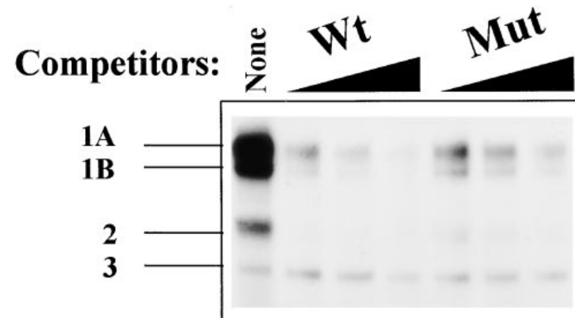

Fig. 7. EMSA analysis of the natural promoter variant T(−93)G. THP-1 nuclear extracts and radiolabeled CT element oligonucleotide were used for EMSA. PCR-amplified DNA fragments of the promoter (from −144 to +20) with either T or G at −93 were used as cold competitors (5-, 10-, and 20-fold of molar excess of each). Band intensities were determined by phosphorimage analysis. Band 1A represents Sp1, bands 1B and 2 represent Sp3, and band 3, which is not competed with unlabeled competitor, represents a non-specific DNA protein complex. This gel is a sample of two independent experiments.

DISCUSSION

Previously, two *cis*-DNA elements were shown to be critical for basal promoter activity of the human LPL gene. One is the Oct-1 binding site, between −46 and −39 (11, 12), and the other is the NF-Y binding site between −65 and −61 (13, 14). Here, we have identified a third *cis*-element, the CT element located between −91 and −83. The ubiquitous transcription factors Sp1 and Sp3 specifically bind to this element and activate transcription of the human LPL promoter.

CT elements have been reported to bind Sp1 and Sp3 in promoters of a variety of genes, such as the LDL receptor gene (44). Other nuclear proteins have also been shown to bind such CT elements. For example, an element containing 5 imperfect repeats of 5′-CCCTCCCCA-3′ in the promoter of the human c-*myc* proto-ocogene was shown to bind Sp1/Sp3, the myc-associated zinc finger protein (45) and two single-stranded DNA binding proteins hnRNP K (46) and CNBP (47). As we used only double-stranded oligonucleotide probes for EMSA, we cannot exclude the possibility that single-stranded DNA binding proteins, such as hnRNP K and CNBP, may bind to the CT element and modulate the LPL promoter activity.

Several novel Sp1-related transcription factors (Sp2, Sp3, and Sp4) have been described (38, 39). Unlike Sp1 and Sp4, Sp3 was observed to act either as a transcriptional activator or as a repressor (26, 40–43). Our results indicate that Sp3 is a transcriptional activator of the human LPL promoter. There is evidence that Sp3 may act as an activator of promoters containing a single copy of the Sp3 binding site and as a repressor of promoters with multiple copies of Sp3 binding site (42). Our finding that the CT element is the only Sp3 binding site in the proximal 97 base pairs of the human LPL promoter appears to be consistent with this observation.

Overexpression of SREBP-1c in stably transfected NIH-

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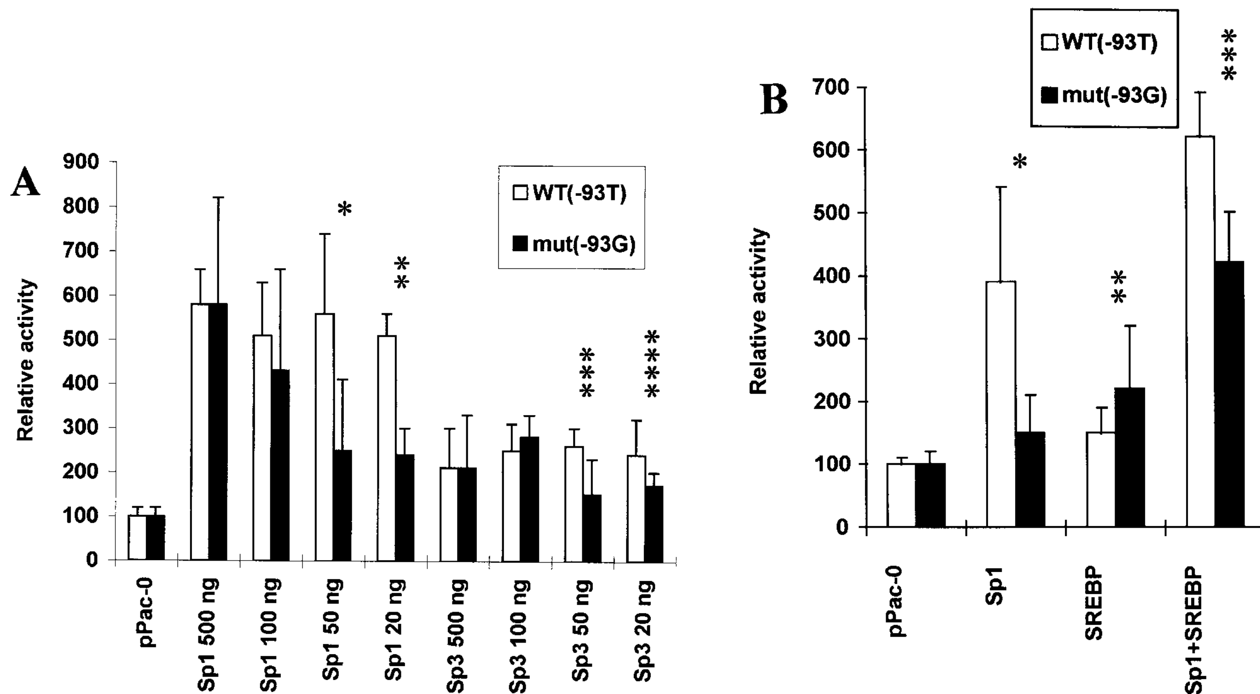

Fig. 8. Reduced Sp1/Sp3 transactivation and synergistic activation by Sp1/SREBP of the LPL promoter by the T(−93)G substitution in SL2 cells. A: T(−93) or G(−93)-allele promoter (2.5 μg) was co-transfected into SL2 cells with 500, 100, 50 ng or 20 ng of pPac-0, pPac-Sp1 or pPac-Sp3 as indicated. Luciferase activity of the T(−93)-allele and G(−93)-allele promoters co-transfected with pPac-0 was arbitrarily set to 100. The data represent the results from two to four transfection experiments, each in duplicate. All values were corrected for transfection efficiency. *, $P < 0.02$; **, $P < 0.0001$; ***, $P < 0.05$; ****, $P = 0.056$ (two-tailed), $P = 0.028$ (one-tailed *t*-test). B: T(−93) or G(−93)-allele promoter of 2.5 μg was co-transfected into SL2 cells with 20 ng of pPac-Sp1 and/or 500 ng of pPac-SREBP. Variable amounts of pPac-0 were used to make the total co-transfected DNA amount to 520 ng. Luciferase activity of the T(−93)-allele and G(−93)-allele promoters co-transfected with pPac-0 was arbitrarily set to 100. The data represent the results from two transfection experiments, each in triplicate. All values were corrected for transfection efficiency. *, $P < 0.005$; **, $P = 0.12$, ***, $P < 0.001$.

3T3 cells and SREBP-1a and -1c in transgenic mice was shown to increase the steady-state levels of LPL mRNA (18–20), suggesting a potential role of SREBP-1 in modulation of the LPL gene transcription. Bennet et al. (16), Lopez et al. (17), and Sanchez, Yieh, and Osborne (21) showed that Sp1 and SREBP-1 synergistically activate the LDLR, FAS and ACC gene promoters. We showed that the LPL promoter is also synergistically activated by SREBP-1 and Sp1, but not Sp3. In contrast, Sp3 was shown to substitute for Sp1 in synergistic activation (with SREBP-1) of promoters of the LDLR, FAS and ACC genes (48). However, we have not been successful in demonstrating synergy between Sp3 and SREBP-1 in activation of the LDLR promoter in our laboratory (data not shown). The reason for this discrepancy remains to be investigated. Nevertheless, our results indicate that regulation of the cholesterol and triglyceride metabolic pathways may, at least in part, be coordinated through the action of Sp1 and SREBP-1 on the LPL promoter (16, 17, 21, 48).

SREBP-1 binds to the sequence motif 5′-ATCACC/GCCAC-3′, called the sterol regulatory element (SRE) in the LDLR promoter (21). An inverted SRE-like element, 5′-TTCACGCCAT-3′ is found in the proximal LPL promoter (from −15 to −8). Although the central 8 bases are identical to those of the LDLR SRE, this element contains only one of the CAC direct repeats (underlined) that are known to be required for binding SREBP and for transcriptional activation (15). Analysis of this region of the LPL promoter by site-directed mutagenesis and functional assays will be required to identify the SRE.

Several lines of evidence suggest that Sp1/Sp3 binding motifs may also mediate regulation of transcription by other factors, such as phorbol esters (49), cAMP (50), and lipopolysaccharide (51). However, we found that the Sp1/Sp3 binding site (CT element) in the human LPL promoter was not necessary for activation by phorbol esters, cAMP, or lipopolysaccharide in THP-1 cells (data not shown).

In contrast to the human and mouse proximal LPL promoters, that of chicken contains one GC box in addition to the CT element. (Fig. 2) (52). Transient transfection assays in chicken primary adipocytes showed that both the GC box and the CT element had enhancer-like activity (52).

Results of transient transfection of THP-1 cells with nested deletions of the LPL promoter (Fig. 1) indicate the presence of one or more negative regulatory elements in the region between 1.8 and 3.0 kb from the transcription start site. A similar result was obtained upon transfection of C2C12 cells (R. Peng and S. S. Deeb, unpublished observations) and of 3T3 L1 and HepG2 cells (11).

Promoter mutations of the human LPL gene have been suggested to underlie partial LPL deficiency in patients

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with familial combined hyperlipidemia (FCHL) (12, 22). Among these, a T→G substitution at position −93 is most prevalent. The frequency of the G(−93) allele is 0.008 in 183 unselected Caucasians (22). Interestingly, we found its frequency to be 0.36 among African Americans and 0.51 among Gambians (T. Li, J. D. Brunzell, and S. S. Deeb, unpublished results). Here, we show that the T→G substitution at position −93 results in moderately reduced binding of Sp1/Sp3 to the CT element and in a proportionately diminished response of the LPL promoter to activation by Sp1/Sp3 as well as to the synergistic action of Sp1 and SREBP. These results, together with those of the transient transfection experiments reported previously (22), suggest that the T to G substitution has a moderate negative effect on LPL promoter function in vitro. However, its effect on the level of LPL mRNA in adipose tissue and macrophages, in vivo, remains to be assessed. The presence of the T −93 G substitution two bases upstream of the 5′ end of the conserved CT element does not preclude its participation in regulation of the LPL promoter by Sp1/Sp3. In vivo DNA footprinting analysis indicated that footprints of GC or CT elements include a few flanking bases (53).

In collaboration with B. J. Nodestgaard's group of the University of Copenhagen, we found that the LPL promoter allele with G at position −93 to be associated with mild elevation in plasma triglyceride levels among 9,085 men and women from the general Danish population, and among 940 patients with ischemic heart disease (H. H. Wittrup, A. Tybjaerg-Hansen, R. Steffensen, S. S. Deeb, J. D. Brunzell, G. Jensen, and B. G. Nordestgaard, unpublished results). This result is consistent with the our finding that this promoter allele is less active than the T(−93) allele.

In conclusion, an Sp1/Sp3 binding site in the human LPL promoter is important not only for its basal promoter activity, but also for the synergistic transcriptional activation with SREBP. The naturally occurring T(−93)G substitution partially affects binding affinity of Sp1/Sp3 to this site, thus compromising transcription from the LPL promoter.

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