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Functional characterization of the promoter for the mouse *SPTLC2* gene, which encodes subunit 2 of serine palmitoyltransferase

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Abstract

A series of luciferase reporter constructs was prepared from a 1035-bp fragment of mouse genomic DNA flanking the 5' -coding sequence for the *SPTLC2* subunit of serine palmitoyltransferase, the initial enzyme of *de novo* sphingolipid biosynthesis. The full-length DNA fragment promoted strong reporter gene expression in NIH3T3 cells while deletion and site-directed mutagenesis indicated that the proximal 335 bp contain initiator and downstream promoter elements, two proximal GC boxes that appear to stimulate transcription in a cooperative manner, and several additional elements whose activity cannot be accounted for by known factor binding sites. These findings provide insight into the control mechanisms for transcription of mammalian *SPTLC2*.

Keywords

sphingolipid biosynthesis; serine palmitoyltransferase; transcription; luciferase reporter construct

Abbreviations

SPT, serine palmitoyltransferase

1. Introduction

Serine palmitoyltransferase (SPT) catalyzes the condensation of L-serine and a fatty acyl-CoA (in most cases, palmitoyl-CoA) as the first unique reaction of sphingolipid biosynthesis *de novo* [1,2]. SPT is known to be essential for survival of cells in culture when the medium is depleted of sphingolipids (which otherwise can be provided by lipoproteins) [3,4], embryonic development [5,6], and some functions of adult cells since mutations in one of the two SPT subunits (*SPTLC1*) results in type I human hereditary sensory neuropathy [7–9]. Surprisingly little is known about the regulation of SPT expression considering that intermediates of the pathway are highly toxic [10] and sphingolipids perform important membrane functions,

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including raft formation [11], trafficking [12], and cell-cell and cell-matrix interaction [13], as well as regulate cell proliferation, migration, survival and other cell behaviors [14,15].

Mammalian SPT is (minimally) a heterodimer of two peptides encoded by the genes *SPTLC1* and *SPTLC2* (which correspond to the *LCB1* and *LCB2* genes of yeast) [16–23]. While most eukaryotic cells appear to possess the capacity for *de novo* sphingolipid biosynthesis, *SPTLC2* and *SPTLC1* expression varies significantly among mammalian tissues [19,20]. A number of physical and biochemical agents have been shown to elevate SPT mRNAs, including UVA or UVB radiation [24,25], endotoxin [26,27], inflammatory cytokines [26,27], nicotinamide [28], and others [1,2]. Both SPT genes have been mapped to specific loci in the mouse (*SPTLC1* on chromosome 13 and *SPTLC2* on chromosome 12) and human genomes (*SPTLC1* to 9q22.2, *SPTLC2* to 14q24.3-q31), and reference nucleotide sequences of the flanking regions have been determined [29–31]. The promoter elements and trans-acting factors that control *SPTLC2* and *SPTLC1* gene transcription, however, have not been identified.

Here, we report the cloning and the first functional analysis of the candidate promoter region upstream of the ATG translation start codon of mouse *SPTLC2*. The promoter region lacks a recognizable TATA motif but contains three GC-boxes, two of which appear to function cooperatively. Other potential control elements include an initiator (Inr) and downstream promoter element (DPE), a nuclear factor Y (NF-Y) recognition sequence, and several regions whose activity cannot be accounted for by known transcription factor binding sites.

2. Materials and Methods

2.1. Cell Culture and reagents

NIH3T3 cells (ATCC, Rockville, MD) were grown in Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with 100 U penicillin/ml, 100 µg streptomycin/ml, and 10% (v/v) fetal bovine serum (HyClone, Logan, UT). Cells were maintained at 37°C in an atmosphere containing 5% CO₂, and were transfected between passages 5 and 20.

2.2. Mouse *SPTLC2* genomic clones

Two independent clones containing portions of the mouse *SPTLC2* locus were obtained by screening 5x10⁵ plaques from a lambda EMBL3 SP6/T7 genomic library (Clontech, Palo Alto, CA) with a 430 bp *EcoR* I - *Sly* I restriction fragment derived from the 5' end of the murine *SPTLC2* cDNA described by Nagiec et al. (GenBank accession number U27455) [23]. The clones produced distinct restriction patterns, but nucleotide sequences including and adjacent to the region represented in the cDNA probe were the same in both cases. The complete sequences of a portion of the first exon and approximately 1 kb of 5'-flanking DNA were acquired from one clone by the automated dye-terminator method (Southwest Scientific Resources, Albuquerque, NM). Sequences spanning positions +87 through -955 bp relative to the first nucleotide of the RIKEN mouse *SPTLC2* cDNA clones G431001J02 (accession number BB865461) and G370001N18 (accession number BB851468) were amplified by polymerase chain reaction (PCR) from the lambda EMBL3 clone and from Swiss 3T3 fibroblast genomic DNA. Sense and antisense-strand amplification primers were 5'-TCCCCCGGGAACATAGAGGGTCACTCAG-3' and 5'-GGGGTACCAGACCCGGCAGCAGGAC-3', respectively, and both included 5' extensions containing restriction enzyme sites to facilitate cloning. Products were inserted into the pT7Blue[®] vector (Novagen, Madison, WI) and recombinants were screened by restriction enzyme analysis of the inserts. The nucleotide sequences of two candidate clones were determined by the Emory University DNA Sequencing Core Facility (Atlanta, GA). One clone spanned the entire target region with the exception of a 7 bp 3'-terminal deletion, but contained several 5' point mutations. The second was truncated by 159 bp at its 3' end but was otherwise

identical to the original genomic sequence. The two clones therefore were recombined at a common *Blp* I restriction site to produce a construct, designated pT7-SPT2pro, that contained wild type sequence extending from – 955 to +80.

2.3. Computer analysis of nucleotide sequences

Genomic sequences were analyzed for the presence of likely promoter elements using the programs TSSG and TSSW (Softberry, Inc., Mount Kisco, NY) and TFSEARCH (Parallel Application TRC Laboratory, RWCP, Japan). Alignments of nucleotide sequences were performed with the MegAlign program (DNASTAR, Inc., Madison, WI), using the Clustal W method. Slight adjustments to gaps were made based on visual inspection of the data.

2.4. Construction of SPTLC2 promoter deletion mutants

The insert of plasmid pT7-SPT2pro was subcloned into the pGL2-Basic reporter vector (Promega, Madison, WI) upstream of sequences encoding firefly luciferase, and the resultant construct was designated pGL2mSPT2pro– 955. Deletions of the *SPTLC2* promoter terminating at – 897, – 603, – 571, – 357, – 230, and – 34 were generated by restriction enzyme digestion and recircularization of this plasmid. Additional deletion mutants were produced by PCR amplification of the desired segment from pGL2mSPT2pro–955 and cloning of the product into pGL2-Basic. Sense strand primers were 5'-TCAACCCGGGCTAATTGGCTGTTCTTTCC-3' (–335); 5'-ATTATCCCGGGCTCCGCCTCTTCTCAACC-3' (–317); 5'-ATTACCCGGGTCTCAACCTGGACCAGTAGG-3' (–301); 5'-ATTACCCGGGAAGCTCCACCTACGACCTGG-3' (– 276); 5'-CTAGTTCCTCCGGGCTCCAATCAATTGCTCAG-3' (– 253); 5'-ATAACCCGGGTGGTCAATAGCCGATAGACC-3' (– 127); and 5'-ATAACCCGGGTTCCTGGTTTCGGAGAGGTG-3' (+2). The antisense strand primer for all reactions was 5'-TAATGGTACCGCGGCAGCAGGACAGG-3'. Each primer incorporated a 5' restriction enzyme site and 4-nucleotide extension to facilitate cloning of the products, and nucleotide sequences of promoter inserts were verified prior to use of constructs in transient expression experiments.

2.5. PCR-directed mutagenesis

Site-specific mutations of GC box sequences at positions – 51 and – 109 and CCAAT box sequences at – 248 and – 332 were created by a two-step PCR procedure [32]. Briefly, overlapping DNA fragments containing the desired mutations were generated in separate reactions where the antisense primer of one reaction was partially complementary to the sense primer of the other. The two products were then combined and used as templates for amplification of the entire target region. Nucleotide sequences of the final products were verified and restriction fragments containing mutations were substituted for the corresponding wild type fragments in the appropriate luciferase reporter constructs. Mutations were designed to alter nucleotides that are highly conserved or invariant in consensus sequences listed in the TRANSFAC database [33] while avoiding creation of potential binding sites for transcription factors. Nucleotide sequence changes (in the sense strand) were: 5'-GTGGCCCCGCCCTCG-3' to 5'-GTGGAAGTTAACTCG-3' (antisense GC box at –51); 5'-TAGACCCCCGCCCAT-3' to 5'-TAGACAGTTAACCAT-3' (antisense GC box at – 109); 5'-GGGCTCCAATCAATT-3' to 5'-GGGCTCGTTTCATTT-3' (sense CCAAT box at – 248); and 5'-CTCTAATTGGCTGTT-3' to 5'-CTCTAAACGCTGTT-3' (antisense CCAAT box at – 332).

2.6. Transfection of reporter constructs and assay of luciferase activity

Reporter constructs were transfected into NIH3T3 cells using Superfect reagent (Qiagen, Valencia, CA). Cells were seeded 24 h prior to transfection at a density of 1×10^5 per 35-mm well and were between 50 and 60% confluent at the time of DNA addition. Each well contained 10 μ l of Superfect reagent and 2 μ g of plasmid DNA in 0.7 ml of D-MEM + 10% fetal bovine serum. Plasmid mixtures consisted of 0.4 pmol of *SPTLC2* reporter construct, 0.05 pmol of pRL-TK (Promega), and pGEM7Zf(+) carrier (Promega) as required to bring to total mass of DNA to 2 μ g. The pRL-TK construct encodes *Renilla reniformis* (sea pansy) luciferase under control of the herpes simplex virus thymidine kinase promoter, and was included as an internal control for variations in the efficiency of DNA uptake. Transfection was allowed to proceed for 3 hours at 37°C, 5% CO₂, after which cells were washed twice with PBS and incubated with 2 ml of fresh growth medium for 24 h. Monolayers were washed once with PBS and cells were lysed by 15 min incubation with Passive Lysis Buffer (Promega). Lysates were subjected to a single round of freeze-thaw, centrifuged briefly to pellet cell debris, and assayed for firefly and *Renilla* luciferase activities using the Promega Dual Luciferase system with a Zylux Model FB12 luminometer (Zylux Corp., Maryville, TN).

3. Results

3.1 Cloning and characterization of the mouse *SPTLC2* promoter region

Two independent clones complementary to a 5'-terminal murine *SPTLC2* cDNA fragment were identified in screens of a mouse lambda EMBL3 SP6/T7 genomic library. Partial nucleotide sequence analysis confirmed that both included *SPTLC2* sequences surrounding and upstream of the ATG translation start codon. An 1100 bp segment of one clone, beginning with the first nucleotide of codon 18 and extending upstream, was sequenced to completion and determined to be identical in all but two positions to chromosome 12 data reported by the Mouse Genome Sequencing Consortium (GenBank accession number NT_039551). Although the site of transcription initiation in the murine *SPTLC2* gene has not been determined directly, comparison of genomic and cDNA sequence data suggests that the current mRNA reference sequence (accession number NM_011479) probably does not include the actual cap site. BLASTN alignment of *SPTLC2* exon 1 flanking sequences with the mouse EST database showed that 26 of the 30 best matches ($E \leq 5 \times 10^{-105}$) terminate between 9 and 36 bp further upstream. The most distal endpoint, corresponding to the first nucleotide of the cDNA inserts in RIKEN clones G431001J02 and G370001N18, lies within a sequence that fits the initiator (Inr) element consensus and matches the expected initiating nucleotide [34]. This position was therefore designated as +1 for purposes of characterizing the promoter.

The 5'-flanking sequences of the mouse *SPTLC2* gene contain potential binding sites for a number of transcription factors, including proteins involved in basal gene expression, cell differentiation, and enhancer-mediated responses (Fig.1). The proximal region lacks an obvious TATA element and, in general, is G/C-rich. Like many TATA-less promoters, the *SPTLC2* sequences contain a potential binding site (GC box) for the Sp1 family of transcription factors upstream of the Inr element (position - 51). There is also a five-nucleotide motif beginning at +27 that is similar in both sequence and distance from the Inr to the downstream core promoter element (DPE) required for efficient transcription from certain non-TATA promoters [35]. Comparison of mouse and human nucleotide sequences suggests that the *SPTLC2* promoter may be highly conserved in mammals. Positions - 1 through - 955 of the mouse gene are 73 percent identical to the corresponding human sequence, and a number of potential binding sites for basal and regulatory transcription factors are common to both. These include the GC box, Inr, and DPE motifs described above as well as two additional GC boxes at positions - 109 and - 313, and CCAAT-containing elements at - 24, - 132, - 251, and - 335. Much of the sequence that is similar or identical in the mouse and human promoters

lacks close matches to existing transcription factor consensus sequences, and its apparent conservation suggests the possible existence of novel regulatory elements in the *SPTLC2* promoter.

3.2 Basal transcription from the *SPTLC2* promoter is dependent on DNA elements downstream of position – 335

A 1035 bp fragment of *SPTLC2* genomic DNA containing 5' untranslated and upstream nucleotide sequences was subcloned into the pGL2-Basic luciferase reporter vector and transfected into NIH3T3 fibroblasts (Fig. 2). Luciferase expression from this plasmid was approximately 46,000-fold higher than the background level produced by a pGL2-Basic negative control, demonstrating the presence of a functional promoter in the insert. The approximate locations of basal transcription control elements in the promoter were determined by progressive deletion analysis of the parental pGL2mSPT2pro-955 construct. As shown in Fig. 2A, 5'-truncation from position –955 bp to –357 bp did not significantly alter reporter gene expression. This observation was supported by experiments with promoters terminating at –897, –603, and –571 bp, which also had activities that were indistinguishable from the –955 bp reporter construct (data not shown). Significant decreases in luciferase expression were observed when the promoter was truncated from –357 to –301, –301 to –230, –127 to –34, and –34 to +2 bp, indicating the presence of functional elements in these regions. No statistically significant change in activity occurred when the promoter length was decreased from 230 to 127 bp. Although luciferase expression in cells transfected with the +2 bp deletion mutant was less than 2 percent of the level obtained with the –955 bp construct, activity was consistently above the background observed in cells containing a promoterless vector, consistent with the presence of minimal recognition sequences for the core transcription machinery in the vicinity of the putative cap site.

A more detailed map of the distal promoter region was produced by analysis of a series of seven deletion mutants terminating between positions –357 and –230 (Fig. 2B). Truncation of the promoter to –335 bp, which eliminated a potential binding site for the transcription factor C/EBP β , did not significantly alter luciferase expression relative to the –357 bp control. Progressive decreases in activity were observed upon truncation from –335 to –317, –317 to –301, –276 to –253, and –253 to –230 bp, however. While some of these deletions removed sequences that closely match known consensus binding sites for transcription factors, no such motifs could be identified in the region between positions –301 and –253.

3.3 Two proximal GC boxes are functional components of the basal *SPTLC2* promoter

Truncation of the *SPTLC2* promoter region from –127 to –34 bp resulted in a ten-fold decrease in reporter gene expression and removed only two sequences that closely match known transcriptional control elements, a pair of GC boxes (potential Sp1 binding sites) located at positions –51 and –109. To investigate the role of these elements in basal promoter activity, we prepared a series of reporter plasmids in which one or both sites were mutated to alter the core consensus sequence from 5'-CCCGCC-3' to 5'-AGTTAA-3'. Mutation of the –109 GC box reduced the activity of a 955 bp promoter by 15 percent, while the effect of mutating the –51 site was somewhat greater, producing a 26 percent decrease in reporter gene expression (Fig. 3A). When both sequences were mutated, activity was reduced by 56 percent, an amount greater than the sum of the individual effects. These observations are consistent with involvement of both GC boxes in promoting transcription of the *SPTLC2* gene, and suggest that direct or indirect cooperativity may exist between proteins bound to the two sites. In the context of a promoter segment terminating at position –127, the –51 GC box mutation and the –109/–51 double mutation both reduced luciferase expression to a level equivalent to the –34 bp deletion mutant, demonstrating that the two GC boxes can account for all basal promoter activity associated with the –127 to –51 bp region (Fig. 3B). Surprisingly, cells transfected

with a construct in which only the – 109 site was mutated exhibited no luciferase expression above the background produced by a promoterless control plasmid. Nucleotide sequence analysis of the promoter and luciferase coding region of the – 127/GC_{–109} plasmid revealed no secondary mutations, and reporter gene expression from a co-transfected internal standard construct was normal, suggesting that cell toxicity or some other nonspecific factor was not responsible for the lack of enzyme activity. It is possible that alteration or absence of the – 51 GC box compensated in some way for a highly deleterious effect of the – 109 mutation, although the mechanism by which this would occur is unclear and similar behavior was not observed when the – 127 to – 955 bp sequence was present.

3.4 Distal CCAAT boxes in the *SPTLC2* promoter region may be targets for negative regulatory factors

Two of the upstream deletion mutations that reduced *SPTLC2* promoter activity eliminated copies of the CCAAT motif, one located in the sense DNA strand at – 248 bp and the other in the antisense strand at – 332 bp. Both CCAAT boxes are components of larger sequence elements similar to the consensus binding site for nuclear factor Y (NF-Y), a ubiquitous transcription factor that is known to interact with Sp1 [36]. To test if these elements are involved in basal expression of *SPTLC2*, derivatives of the pGL2mSPT2pro-955 reporter construct were prepared with changes in the essentially invariant central nucleotides of the CCAAT motif (5'-CCAAT-3' → 5'-CGTTT-3'). In contrast to results obtained in the deletion experiments, directed mutation of either CCAAT motif produced a 30–40 % increase in reporter gene expression (Fig. 4), which suggests that these sequences do not contribute to basal *SPTLC2* promoter activity but might act as binding sites for negative transcriptional regulators. It also raises the possibility that the – 335 to – 318 bp and – 253 to – 231 bp regions contain novel regulatory elements that account for the decrease in promoter activity observed in these mutants.

4. Discussion

This study provides insights into mechanisms that control basal expression of *SPT*. A 'minimal' luciferase reporter construct containing *SPTLC2* genomic sequences extending from +2 to +80 bp relative to the presumptive transcription initiation site exhibited low but consistently measurable levels of gene expression above vector background in NIH3T3 cells. While the precise location of the *SPTLC2* cap site has not been determined experimentally, comparison of mouse genomic and EST sequences clearly indicates that the region included in this construct is transcribed in a variety of tissues. Thus, sequences surrounding and immediately downstream of the initiation site are likely to contain elements that participate in recruiting TBP-associated factors (TAFs) or other components of the core transcription machinery. Consistent with this view, the mouse and human *SPTLC2* promoter regions include conserved matches to the initiator (Inr) consensus PyPyA_{–1}N(T/A)PyPy and a sequence 27 bp downstream that is similar to the downstream promoter element (DPE) consensus (A/G)G(A/T)(C/T)(G/A/C) [35]. A functional Inr and one or more downstream elements, such as the DPE or DCE (downstream core element) [37], are typically required for transcription of genes like *SPTLC2* that lack a TATA box, and it has been suggested that the pattern of these core elements may dictate a 'preference' for regulation by specific enhancers and activators [38]. The precise role of such sequences in basal and regulated *SPTLC2* transcription, however, remains to be determined.

The results of the deletion analyses indicate that the *SPTLC2* promoter has an upstream boundary at or near – 335 bp and consists of proximal and distal functional domains separated by approximately 100 nucleotides with no obvious role in promoter activity. The proximal domain extends to position – 127 and includes the putative Inr, a segment between positions +2 and – 34 that contains an inverted CCAAT box, and a pair of GC boxes that are likely

binding sites for members of the Sp family of transcription factors [39]. These GC boxes (or more properly the activators bound to them) appear to function cooperatively, based on the observation that mutation of both sites in the context of a 955 bp promoter had a more pronounced effect than the sum of the individual mutations (56 percent decrease vs. 15 and 26 percent decreases). Proteins bound to the GC boxes could also interact with transcription factors bound further upstream. This view is supported by our finding that the apparent synergism between the two GC elements disappeared when sequences upstream of position – 127 were absent. The pronounced decrease in reporter activity (≥ 90 percent) that occurred upon mutation of these sites in the – 127 bp promoter, however, indicates that Sp family activators or other proteins bound to the proximal GC boxes play a role in basal *SPTLC2* transcription that extends beyond mediating the effects of other factors.

The upstream portion of the *SPTLC2* promoter, consisting of sequences between positions – 230 and – 335, contains at least four functional DNA elements. One of these, located between – 301 and – 317 bp, maps almost precisely to a GC box and is a possible binding site for Sp1 or another Sp-family protein. The forward and inverted CCAAT motifs at – 248 and – 332 bp, respectively, are potential targets of the transcription factor NF-Y (also known as CCAAT-binding factor or CBF), although they are located further upstream than the position at which NF-Y typically acts [40]. The loss of promoter activity observed in 5' deletion mutations that eliminate the CCAAT-containing DNA segments is consistent with the binding of an activator; however, site-directed mutagenesis of the CCAAT sequences suggested that they are targets of transcriptional repressors. One explanation for these apparently conflicting observations is that sequence elements flanking the CCAAT motifs may act as transcription factor binding sites, or alternatively, that the CCAAT elements could be recognized by two or more regulatory proteins with opposing activities, whose binding is differentially influenced by upstream sequences that are present in the site-directed mutants and absent in the deletions. Interestingly, NF-Y is known to participate in both transcriptional activation and repression, and to regulate genes involved in cell cycle progression [41]. Given the demonstrated abilities of sphingolipid intermediates to influence cell proliferation and apoptosis, it is tempting to speculate that *SPT* gene transcription might be subject to cell cycle-dependent control by NF-Y or some other factor. The existence of such a relationship remains to be investigated, however. The fourth upstream element of the *SPTLC2* promoter lies between positions – 253 and – 276, a region that does not contain obvious matches to existing transcription factor consensus binding sites. This raises the possibility that basal transcription of the *SPTLC2* gene is dependent on one or more novel protein factors, or that a known factor may bind to a previously unrecognized sequence motif. Further mutational analysis of the promoter in concert with protein binding studies will be required to resolve these questions.

Although cDNA and genomic clones encoding the major enzymes of sphingolipid metabolism have been isolated from a variety of species, we are aware of only one previous study that addresses the functional organization of any of the corresponding promoters. Kolaczowski *et al.* (2004) demonstrated that pleiotropic drug-resistance (Pdr) pathway activation of the promoter for *LAC1*, one of two *Saccharomyces cerevisiae* genes required for dihydroceramide synthase activity, is dependent on binding of the zinc cluster transcription factor Pdr1p or Pdr3p to a pleiotropic drug resistance element (PDRE) [42]. The transcription factors Cb1p and Rox1p were implicated in negative regulation of the *LAC1* promoter, while the *SPTLC2* ortholog *LCB2* was found to contain a functional PDRE in its promoter region and was induced via the Pdr regulatory pathway. Existence of common transcriptional activators for dihydroceramide synthase and serine palmitoyltransferase would be consistent with a coordinate mechanism of gene regulation, but the broader significance of these findings is difficult to assess. First, the *LCB2* and *LAC1* promoter regions of *S. cerevisiae* differ significantly from those of homologous mammalian genes, and orthologs of Pdr1p/Pdr3p, Cb1p, and Rox1p have been identified primarily, if not exclusively, in fungi. Second, the number of genes involved in

mammalian dihydroceramide synthesis appears to be greater than in yeast, suggesting that the corresponding transcriptional regulatory pathways are likely to be more complex [43,44].

The data reported here provide a foundation for studying expression of the *SPTLC2* gene in metazoan species, including signal transduction pathways that alter serine palmitoyltransferase mRNA levels and the possibility of coordinate regulation with other genes involved in sphingolipid metabolism. Perhaps significantly, alignment of mouse and human *SPTLC1* cDNA sequences with their genomic counterparts indicates that the 5' flanking regions lack an obvious TATA motif and contain several GC-boxes. Further investigation will be required to determine if this structural similarity between the two SPT promoter regions reflects shared mechanisms of transcriptional control, and if expression of other sphingolipid metabolic genes is regulated by common subsets of nucleotide sequences and protein factors.

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Fig. 1.

Nucleotide sequence of the mouse *SPTLC2* promoter region. Murine genomic sequences are aligned with homologous sequences from human chromosome 14 (GenBank accession number AF111168). Gaps have been introduced where necessary to maintain alignment and identical sequences are shaded. Nucleotides are numbered relative to the presumptive transcription initiation site in the mouse gene (*see text*), and a sequence matching the initiator (Inr) element consensus PyPyA₊N(T/A)PyPy is labeled. A downstream sequence that matches the downstream promoter element (DPE) consensus at 4 of 5 positions is shown in parentheses. Labeled bars indicate the positions of potential transcription factor binding sites. Sites containing deviations from conserved nucleotides in the consensus sequence are shown in parentheses; potential binding sites with differences in nucleotides considered to be invariant, or nearly so, were ignored.

Fig. 2.

Sequence elements required for basal transcription are located within the first 335 bp of the mouse SPTLC2 promoter region. A: Luciferase reporter constructs containing promoter segments extending from +80 to the indicated 5' endpoints were transfected into NIH3T3 cells and luciferase activity was assayed after 24 h. Data were normalized to the reporter activity of a cotransfected pRL-TK plasmid and background luciferase activity determined by transfection of a promoterless vector was subtracted. Activities are expressed relative to the -955 bp promoter and results shown represent the means $\pm$ S.E.M. of at least three independent experiments. Decreases in promoter activity that are significant relative to the prior construct in the deletion series (single-factor Anova) are indicated by the appropriate P value. B: Promoter deletion mutants terminating between positions -230 and -357 were assayed as

described above. Locations of potential transcription factor binding sites in relationship to the deletion endpoints are indicated. Activities are expressed relative to the -357 bp promoter and results shown represent the means $\pm$ S.E.M. of at least nine independent experiments. Decreases in promoter activity that are significant relative to the prior construct in the deletion series (single-factor Anova) are indicated by the appropriate *P* value. Promoter activity of the -317 bp mutant was significantly different from the -357 bp control ($P < 0.05$) as well as from the -335 bp mutant.

Fig. 3.

Two consensus GC boxes at positions -51 and -109 account for basal *SPTLC2* promoter activity in the -34 to -127 bp region. A: NIH3T3 cells were transfected with luciferase reporter constructs containing the wild type -955 bp promoter or promoters containing mutations in the -109 bp, -51 bp, or both GC-boxes. Control cells were transfected with a -127 bp deletion mutant lacking the upstream promoter domain and a -34 bp mutant in which both GC-boxes are removed. Luciferase activity was assayed after 24 h and normalized to the reporter activity of a cotransfected pRL-TK plasmid. Background luciferase activity determined by transfection of a promoterless vector was subtracted and all activities are expressed relative to the -955 bp wild type (wt) promoter. Results shown represent the means $\pm$ S.E.M. of three independent

transfections. B: Cells were transfected with a -127 bp promoter deletion mutant containing the wild type sequence or the GC-box mutations described above. Control cells were transfected with the -357 bp or -34 bp promoter construct. Luciferase activity was assayed after 24 h and normalized to the reporter activity of a cotransfected pRL-TK plasmid. Background luciferase activity determined by transfection of a promoterless vector was subtracted and activities are expressed relative to the wild type -955 bp promoter. Results shown represent the means $\pm$ S.E.M. of three independent transfections.

Fig. 4.

Mutation of CCAAT sites at -332 and -248 increases *SPTLC2* promoter activity. NIH3T3 cells were transfected with luciferase reporter constructs containing the wild type (wt) -955 bp promoter or substitution mutations in the -248 bp sense strand or -332 bp antisense strand CCAAT sequences. Luciferase reporter activity was assayed after 24 h and normalized to the activity of a cotransfected pRL-TK plasmid. Background luciferase activity determined by transfection of a promoterless vector was subtracted from all values. Activities are expressed relative to the wild type -955 bp promoter and results shown represent the means $\pm$ S.E.M. of three independent transfections. Reporter gene expression from both mutants was significantly greater than from the wild type control ($P < 0.001$ in single-factor Anova) but the mutants were not significantly different from one another ($P > 0.10$).