A Role for Sp1 in Transcriptional Regulation of Phosphatidylethanolamine N-Methyltransferase in Liver and 3T3-L1 Adipocytes *

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Running Title: Transcriptional regulation of PEMT

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3 The abbreviations used are: PC, phosphatidylcholine; C/EBP, CAAT element binding protein; ChIP, chromatin immunoprecipitation assay; CT, CTP: phosphocholine cytidylyltransferase; EMSA, electrophoretic mobility shift assay; ER, estrogen receptor; FABP, fatty acid binding protein; MLV, murine leukemia virus; PE, phosphatidylethanolamine; PEMT, PE N-methyltransferase; PPAR, peroxisome proliferator activated receptor.

Phosphatidylcholine (PC) is made in all nucleated mammalian cells via the CDP-choline pathway. Another major pathway for PC biosynthesis in liver is catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT). We have now identified 3T3-L1 adipocytes as a cell culture model that expresses PEMT endogenously. We have found that PEMT mRNA and protein levels increased dramatically in 3T3-L1 cells upon differentiation to adipocytes. 5’ deletion analysis of the PEMT promoter-luciferase constructs stably expressed in 3T3-L1 adipocytes identified a regulatory region between -471 and -371bp (relative to the transcriptional start site). Competitive and super-shift assays demonstrated binding sites for transcription factors Sp1, Sp3 (-408 to -413) and YY1 (-417 to -420). During differentiation of 3T3-L1 cells to adipocytes, the amount of Sp1 protein decreased by ~50% just prior to activation of PEMT. Transduction of 3T3-L1 adipocytes with retrovirus containing Sp1 cDNA demonstrated that Sp1 inhibited PEMT transcriptional activity. Similarly, shRNA directed against Sp1 in 3T3-L1 adipocytes enhanced PEMT transcriptional activation. Chromatin immunoprecipitation assays confirmed
that Sp1 binds to the PEMT promoter and this interaction decreases upon differentiation to adipocytes. These experiments directly link increased PEMT expression in adipocytes to decreased transcriptional expression of Sp1. In

Phosphatidylcholine (PC)\(^3\) is the predominant phospholipid of all cell membranes and of the circulating blood lipoproteins (1). In mammalian species, there are two major pathways for PC synthesis. The majority of PC is formed by the CDP-choline pathway (1) which is regulated by the activity of CTP:phosphocholine cytidylyltransferase (CT) and for which a dietary source of choline is required. In the liver substantial amounts of PC can also be synthesized by phosphatidylethanolamine N-methyltransferase (PEMT) (2,3). PEMT is a small integral membrane protein (22 kDa) which catalyzes three sequential methylations of phosphatidylethanolamine (PE) using \(\text{S-adenosylmethionine}\) as a methyl donor (4,5). PEMT contributes approximately 30 % of total hepatic PC synthesis whereas the enzymes of the CDP-choline pathway produce the remaining 70 % (4,6-8). Despite being the smaller contributor to hepatic PC production in the liver, the significance of the PEMT pathway was underscored when \(\text{Pemt}\)\(^{-/-}\) mice were fed a choline-deficient diet which attenuated the CDP-choline pathway. The mice developed severe liver pathology and died within 5 days (9). PC derived from PEMT has been shown to have an important role in hepatic lipid metabolism, the transcriptional regulation of \(\text{Pemt}\) gene expression is not well understood. The \(\text{Pemt}\) gene is localized to mouse chromosome 11 and spans 35 kb; it contains 7 exons and 6 introns (18). The promoter lacks a TATA box but contains a GC-rich region that is characteristic of many TATA-less gene promoters (19). \(\text{Pemt}\) gene expression is regulated in both a tissue-dependent and developmental manner (4,20). It was previously shown that PEMT expression is activated during the perinatal period in rat liver (20). Recently, it has also been shown that \(\text{Pemt}\) gene expression is induced by estrogen in human and mouse primary hepatocytes (21). In the present study we have identified 3T3-L1 adipocytes as a cell culture model that expresses PEMT endogenously. Thus, the aim of the study was to elucidate transcription factors and respective cis-acting elements that regulate gene expression of \(\text{Pemt}\) in 3T3-L1 adipocytes.

EXPERIMENTAL PROCEDURES

Materials- Dexamethasone, 3-isobutyl-1-methylxanthine, bovine insulin, tamoxifen and mithramycin A were purchased from Sigma (St. Louis, MO, USA). The luciferase vectors, pGL3 Basic, and pGL3 Control vectors containing the cDNA for \(\text{Photinus pyralis}\), the pSV-\(\beta\)-galactosidase vector, the dual luciferase reporter assay system and the
β-galactosidase assay system were obtained from Promega (Madison, WI). The pBK-CMV vector was purchased from Stratagene (La Jolla, CA), pCL-ECO was purchased from IMGENEX (San Diego, CA) and pBabe-puro obtained from Addgene (Cambridge, MA). Lipofectamine™ Plus, lipofectamine™ 2000, DMEM (Dulbecco’s modified Eagle’s Medium), alpha MEM (Minimum Essential Medium), and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, California). Anti-Sp1 antibody, anti-Sp3 antibody, anti-SREBP-1 and anti-IgG antibodies were purchased from Santa Cruz biotechnology, anti-protein disulfide isomerase (PDI) antibody was purchased from Stressgen Biotechnologies (Victoria, B.C., Canada), anti-YY1 antibody was from Cedarlane Laboratories Ltd. (Burlington, ON., Canada) and anti-TBP antibody was obtained from Upstate Biotechnology (Lake Placid, NY). The polyclonal antibody directed against the C-terminal end of rat PEMT was raised in rabbit in our laboratory (4).

Cell Culture and Differentiation of NIH 3T3-L1 Fibroblasts- Cos-7 African green monkey kidney cells, C3H10T1/2 mouse embryonic fibroblasts, and NIH 3T3-L1 fibroblasts (A.T.C.C.) were cultured in high-glucose Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). Hepa-1c1c7 mouse hepatoma cells were cultured in Alpha-MEM, 10% (v/v) FBS and H2.35 mouse hepatocytes were cultured in high-glucose DMEM, 5% (v/v), FBS and 200 nM dexamethasone. All cell lines were obtained from A.T.C.C. and grown in medium supplemented with penicillin G (100 units/ml) and streptomycin (100 units/ml) in a 5% CO2 humidified incubator at 37°C. To induce differentiation, NIH 3T3-L1 fibroblasts were cultivated in growth medium until confluent. Two days after reaching confluency, cells were induced to differentiate by the addition of 0.25 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 3 µg/ml insulin for 3 days. Cells were then incubated in normal growth media until differentiated. Medium was replaced every 2 days. Cells were considered adipocytes when > 90% had large cytoplasmic lipid droplets.

Adult mouse cultured primary hepatocytes were isolated from male Pemt+/+ mice with a mixed genetic background of 129/J and C57BL/6J using the collagenase perfusion technique as previously described (22). The cells were plated at a density of 0.5 X 10^6 on collagen-coated 60-mm dishes in DMEM supplemented with 4% FBS, 100 nM dexamethasone, and 1 nM insulin.

Animal Care- All animal procedures are performed in accordance with the University of Alberta Animal Policy and Welfare Committee, which adheres to the principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences (CIOMS). Male C57BL/6J mice (Jackson Laboratory, Bar Harbour, ME) were 8-12 weeks old and maintained on standard chow diet containing 6% (w/w) fat and 0.02% (w/w) cholesterol (LabDiet). Tamoxifen (Sigma) was dissolved at a concentration of 0.2 mg/ml in sesame oil containing 1% benzyl alcohol. The mice were injected subcutaneously with either tamoxifen 0.5 mg/kg of mouse weight or vehicle 2.5 µl (sesame oil, 1% benzyl alcohol) / g of mouse weight. The mice were injected at the same time on five consecutive days and anesthetized 4 h following the final injection.

Immunoblot Analyses- Cell lysates (50 µg) from NIH 3T3-L1 cells during differentiation were heated for 10 min at 90°C in 62.5 mM Tris-HCl (pH 8.3), 10% glycerol, 5% (v/v) 2-meaptoethanol, 1%
SDS, and 0.004% bromophenol blue. The samples were electrophoresed on a 10% SDS-polyacrylamide gel in 25 mM Tris-HCl (pH 8.5), 192 mM glycine, and 0.1% SDS buffer. The proteins were transferred to polyvinylidene fluoride (PVDF) by electroblotting in transfer buffer (125 mM Tris-HCl, pH 8.3), 960 mM glycine, 10% (v/v) methanol). Following transfer, the membrane was incubated overnight at 4°C with 5% skim milk in 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.1% Tween 20 (T-TBS) followed by 1 h at room temperature or overnight at 4°C with antibody raised against the protein indicated. Anti-PEMT, and anti-Sp1 antibodies were diluted (1:5000) in 1% milk, T-TBS. Anti-PDI and anti-TBP antibodies were diluted (1:4000) in 4% bovine serum albumin, T-TBS. Immunoreactive proteins were detected using enhanced chemiluminescence system (Amersham Biosciences) according to the manufacturer’s instructions.

**RNA Isolation and PCR Analysis**- Total RNA was isolated from liver tissue or cultured cells using the Trizol® reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. First-strand cDNA synthesis from 2 µg of total RNA was performed using SuperScript™ II reverse transcriptase (Invitrogen) primed by oligo(dT)$_{12-18}$. PCR was performed using the indicated gene specific primers given in Table 1. Amplicons were visualized with ethidium bromide on an agarose gel or measured using real-time quantitative PCR using the Rotor-gene 3000 instrument (Montreal Biotech) and analysed with the Rotor-Gene 6.0.19 program (Montreal Biotech).

**Plasmid Construction**- The Pemt promoter region -1000 to +130 was obtained by PCR amplification using mouse liver genomic DNA as a template. We used a numbering system based on the transcriptional start site (+1) previously determined (18). The primers (forward 5’-CGGGGTACCCTCCGCAGCTGCAATGGGTCAATGTTCTTCTT-3’) and (reverse 5’-CCCAAGCTTGGGCAGACGGAAGTGGTAATG-3’) were designed to contain restriction enzyme sites for Hind-III and Kpn-I. The promoter region was purified from agarose gel using the Qiax II gel extraction kit (Qiagen Inc., Mississauga, Ontario Canada) according to the manufacturer’s instructions, and directionally ligated into double-digested (Hind-III, Kpn-I) pGL3 Basic to generate -1000 Luc. All other promoter sections were generated by PCR using -1000 Luc as a template and then double-digested and ligated into pGL3 Basic as previously outlined. The reverse primer remained the same with various forward primers for -471 Luc (5’-CGGGGTACCCTCCGCAGCTGCAATGGGTCAATGTTCTTCTT-3’), -371 Luc (5’-CGGGGTACCCTCCGCAGCTGCAATGGGTCAATGTTCTTCTT-3’), -271 Luc (5’-CGGGGTACCCTCCGCAGCTGCAATGGGTCAATGTTCTTCTT-3’), -171 Luc (5’-CGGGGTACCCTCCGCAGCTGCAATGGGTCAATGTTCTTCTT-3’) and -71 Luc (5’-CGGGGTACCCTCCGCAGCTGCAATGGGTCAATGTTCTTCTT-3’).

The cDNA for full length mouse Sp1 was obtained by RT-PCR. Briefly, total RNA was obtained from mouse liver and reverse transcribed with SuperScript™ II primed by oligo(dT)$_{12-18}$. Primers used in the subsequent PCR amplification of Sp1 cDNA were forward (5’-CGGGGTACCCTCCGCAGCTGCAATGGGTCAATGTTCTTCTT-3’) and reverse (5’-CGGGGTACCCTCCGCAGCTGCAATGGGTCAATGTTCTTCTT-3’) containing restriction enzyme sites for Eco-RI. The PCR product was digested with Eco-RI and gel purified prior to being ligated into pBabe.puro.
DNA oligonucleotides for the synthesis of shRNA were synthesized by the University of Alberta DNA Core Facility. For each specific gene we designed DNA oligonucleotides which are predicted to form a stem-loop structure when transcribed to RNA. The sequence of the forward DNA oligonucleotide included the unique nucleotide target sequence in both the sense and antisense orientation, separated by a spacer sequence (5'-TTCAAGAGA-3') and flanked by Bgl-II and Hind-III restriction site overhangs. The target sequence specific for Sp1 was (5'-GGCTGCTACCCCAACTTAC-3'). As a negative control we used a sequence that did not have homology with expressed genes and is referred to as Scrambled (5'-ACTACCGTTGTTATAGGTGGT-3') (23).

After annealing, the double-stranded DNA was directionally cloned into pSuper.retro.puro (OligoEngine, Seattle, WA) double-digested with Bgl-II and Hind-III.

Site-directed mutagenesis was applied to introduce a mutation of the Sp1 binding element in the -471 Luc plasmid. For that purpose, the core sequence of the Sp1-element (5'-GGGAGG-3') was replaced with (5'-GGGAAA-3') using the QuickChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA). The resulting construct was termed -471MSp1 Luc. The identity of the all generated plasmids was confirmed by sequencing.

Nuclear Extract Preparations and Electrophoretic Mobility Shift Assays (EMSAs)- Nuclear extracts from mouse liver and 3T3-L1 cells were prepared as described (24,25). Promoter-derived oligonucleotides (40 bp) were synthesized by the University of Alberta DNA Core Facility. Complimentary oligonucleotides (1 nmol of each) containing 5’ overhangs were heated at 90°C for 10 min in 100 µl of annealing buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl and 1 mM EDTA). The annealing mixtures were slowly cooled to room temperature and 50 pmol of double-stranded oligonucleotide were labelled by filling in end recessed 3’ ends using Klenow fragment in the presence of [α-32P]dCTP. For each binding reaction (40 µl), 2 µg of Poly(dI-dC)•Poly(dI-dC), 20 µl of a 2X binding buffer (12.5 mM HEPES, pH 7.9, 25 mM KCl, 2 mM MgCl, 0.07% Triton X, 13.3% glycerol, 5 mM dithiothreitol), 20 µg of nuclear extract and labelled probe (150,000 cpm) were incubated for 1 h at 4°C. For competitive EMSAs, 100-fold molar excess of non-labelled double-stranded oligonucleotides were incubated with nuclear extracts for 15 min at 4°C before addition of labelled probe. Commercially available antibody (2 µg) was added to the binding reactions 15 min before the labelled probe for super-shift assays. Binding reactions were terminated by the addition of 8 µl of gel loading buffer (30%, v/v, glycerol, 0.25%, w/v, bromophenol blue, 0.25%, w/v, xylene cyanol). The protein-DNA complexes were separated on a 8% or 7% (for some competitive/super-shift assays) non-denaturing polyacrylamide gel electrophoresis with Tris borate-EDTA buffer system (89 mM Tris, 89 mM Borate, 2 mM EDTA, pH 8.3) at 4°C and detected by autoradiography of a dried gel.

Stable and Transient Transfections- 3T3-L1 stable cell lines were generated by transfecting pre-adipocytes at 30% confluency in 100 mm dishes with 10 µg luciferase reporter construct and 1 µg pBK-CMV containing the neomycin cassette, using the calcium phosphate precipitation method (26). Stable cell lines were selected with Geneticin® (G-418 sulfate, Invitrogen) and confirmed by PCR amplification using commercially available primers RVP3 and GLP3 (Promega, Corp.)
which flank the multiple cloning site. The copy number and level of differentiation of each clone was determined by PCR. To measure luciferase activity samples were collected from 3T3-L1 stable cell lines at various time points during the differentiation period. Luciferase activity was assayed using commercial kits on a microbeta liquid scintillation counter (PerkinElmer) and normalized to protein concentration. Cultured cell lines (H2.35, Hepa1c1c7, Cos-7 and C3H10T1/2 (2 x 10^5/60 mm dish) and freshly isolated adult mouse hepatocytes in primary culture (5 X 10^5, 60 mm dish), were transiently transfected with 1 µg luciferase reporter construct and 1 µg pSV-β-galactosidase using Lipofectamine™ 2000 (hepatocytes) or Lipofectamine™ Plus reagent (cell lines). After 20-48 h, cell extracts were assayed for luciferase activity and normalized to β-galactosidase activity to adjust for transfection efficiency.

Retroviruses- The packaging human renal epithelium cell line 293T/17 was transiently transfected with the murine leukemia virus (MLV) packaging vector (pCL-ECO), an expression retroviral vector (pBabe.puro, or pSuper.retro.puro) using Lipofectamine™ Plus reagent (cell lines). After 24 h, the conditioned medium was filtered (0.45 µm filters), supplemented with polybrene (8 µg/ml) and used to infect 3T3-L1 cells stably transfected with -471 Luc. Transduced 3T3-L1 cells were selected with medium containing 2.5 µg/ml of puromycin (Sigma) for 48 h. 3T3-L1 cells were cultured and differentiated as described above.

Chromatin Immunoprecipitation (ChIP) Assays- 3T3-L1 pre-adipocytes and adipocytes were fixed with 1% formaldehyde (27). Cells were washed with cold PBS twice and resuspended in 0.8 ml radioimmunoprecipitation assay (RIPA) buffer containing 0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10 mM Tris-HCl, pH 8, supplemented with protease inhibitor cocktail (Sigma). The cells were sonicated (30 sec x 4) using a Sonicator Ultrasonic Processor XL (Misonix Farmingdale, NY) followed by centrifugation at 10,000 x g for 10 min at 4°C. The supernatants were collected and diluted with RIPA buffer to 0.2 µg/µl DNA. Protein G-Sepharose beads (Sigma) were pre-treated with 0.1 mg/ml sonicated salmon sperm and 1 mg/ml BSA for 2 h at 4°C. Immunoprecipitation was performed by incubating 1 ml of diluted supernatant (200 µg DNA) with 4 µg anti-Sp1, anti-YY1 or anti-IgG antibodies for 30 min at room temperature. Then the pre-treated protein G-Sepharose beads (50 µl, 50% with 1X RIPA), 5 µg sonicated salmon sperm and 10 µg BSA were added and the immunoprecipitation was continued for 16 h at 4°C. The protein G Sepharose beads were washed 3 times with 1X RIPA, 3 times with 1X RIPA supplemented with 1 M NaCl, 2 times with LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, 500 mM NaCl, pH 8) and twice with TE buffer (20 mM Tris-HCl, pH 8, 1 mM EDTA). The protein-DNA complexes were eluted from the beads at room temperature for 15 min each with 200 µl 1.5% SDS for 15 min, followed by 150 µl 0.5% SDS. The fractions were combined and incubated at 68°C for 16 h to reverse crosslinks. Protein was digested with proteinase K and DNA extracted by phenol/chloroform followed by ethanol precipitation. To detect the Pemt promoter, the following primers were used for standard PCR: forward: (5’-TTCCGGTAAAGGAACCTGACC-3’) and reverse: (5’-GAACATTCCGCTGGGCAATTCC-3’).
Statistical Analysis- Values are expressed as means ± standard error mean (SEM). A significant difference between means was determined using the unpaired Student’s two-tailed t-test for most analysis. To compare differences between multiple groups one way analysis of variance (ANOVA) using tukey post-hoc analysis or ANOVA on the ranks using kruskal-wallis post-hoc analysis were used where appropriate. A probability of $P<0.05$ was considered significant.

RESULTS

3T3-L1 Cells Express PEMT mRNA Upon Differentiation to Adipocytes- Despite the important role that PEMT plays in hepatic lipid metabolism very little is known about its transcriptional regulation. Therefore, the goal of this study was to identify promoter elements and trans-acting factors that regulate expression of the Pemt gene. Previous research on this topic was impeded by the lack of immortalized cell lines which express PEMT. We found very low levels of PEMT mRNA in several immortalized cell lines including the monkey kidney cell line Cos7, the mouse hepatoma cell lines H2.35, Hepa-1c1c7 and the mouse embryonic fibroblast cell line C3H10T1/2 (Fig. 1A). PEMT is highly expressed in the liver making primary cultures of mouse hepatocytes an obvious choice for studies on gene expression. However, PEMT mRNA levels are initially high in primary cultures of mouse hepatocytes (Fig. 1A), expression decreases rapidly within 16 h of isolation. These results are consistent with the de-differentiation of hepatocytes that occurs in culture (28). When each cell type was transiently transfected with luciferase reporter construct -471 Luc containing the mouse Pemt promoter (-471 to +130) the luciferase activity was lower than that from the promoter-less parent pGL3 basic (Empty) plasmid (Fig. 1B). Since, luciferase activity was detected at high levels in each cell type when the reporter gene was under the control of a strong SV40 promoter (pGL3 control), the low level of luciferase activity was not due to inefficient transfection efficiency. Therefore, the low level of Pemt gene expression observed in both immortalized cell lines and primary cultures of mouse hepatocytes was not sufficient to investigate promoter function.

However, PEMT mRNA is expressed in 3T3-L1 mouse embryonic fibroblasts following adipocyte differentiation (Figs. 2A and B). Analysis of the level of PEMT mRNA by PCR before and after differentiation demonstrated that PEMT mRNA was barely detectable in pre-adipocytes and increased dramatically (~20 fold) following differentiation to adipocytes (Fig. 2B). PEMT mRNA levels remained low during the early stages of differentiation and increased markedly by day 4 during the late stage of adipocyte differentiation. This process is characterized by the expression of Ap2/FABP4 and follows increased expression of mRNAs encoding PPARγ and C/EBPα (Fig 2B). Consistent with the increase in PEMT mRNA during 3T3-L1 differentiation, we found that luciferase activity from the Pemt promoter increased when 3T3-L1 fibroblasts stably expressing PEMT-luciferase constructs differentiated into adipocytes (Fig. 2C). In contrast, luciferase activity was lower in adipocytes than pre-adipocytes when 3T3-L1 cells stably expressing pGL3 basic lacking a promoter upstream of the luciferase reporter gene were differentiated. These data establish that 3T3-L1 adipocytes provide a suitable cellular model for identification of elements in the Pemt gene using promoter-activity assays.

The Region -471/-371 Is Important for Activation of PEMT mRNA Expression- To
identify section(s) of the mouse Pemt promoter which may be important for regulating activation of the Pemt gene, we generated 3T3-L1 clones which stably express different sections of the mouse Pemt promoter. Serial deletions from the 5’ end of the Pemt promoter were constructed and ligated into the luciferase reporter gene pGL3 basic and stable cell lines were created. Luciferase activity was measured before (day -2), and after (day 7) differentiation to adipocytes. Two clones (clone -1, clone -2) were analyzed for each promoter length and normalized for cell protein, copy number and level of adipocyte differentiation. Compared to the promoter-less parent vector (pGL3-Basic), transfection of -471 Luc into pre-adipocytes resulted in no change in luciferase expression (Fig. 3). Following differentiation of 3T3-L1 cells into adipocytes, the -471 Luc construct exhibited a 20- to 30-fold increase in luciferase expression. Deletion to -371 bp resulted in the absence of any measurable promoter activity, suggesting that transcription factor binding sites located in the region between -471 and -371 bp may be important for regulating the activation of Pemt gene expression. Since, this promoter region is more active in 3T3-L1 adipocytes than in pre-adipocytes, subsequent experiments focused on characterization of the sequence between -471 and -371 of the mouse Pemt promoter.

Identification of Sp and YY1 Binding Sites Within the Activation Region of the Pemt Promoter- In an attempt to identify the cis-elements responsible for regulating the activation of Pemt gene expression we utilized electromobility shift assays (EMSA). We used thirteen overlapping DNA oligonucleotides from the -471/-371 region to identify the sections which bind nuclear proteins. Each oligonucleotide was labelled with 32P, and incubated with nuclear extract isolated from 3T3-L1 adipocytes. Three different protein-DNA complexes were generated as indicated by the appearance of bands labelled “a” through “c” (Fig. 4A). DNA oligonucleotides which contained the Pemt promoter region -426 to -402 generated all three DNA-protein complexes.

Comparison of the binding patterns produced with nuclear extracts from mouse liver or 3T3-L1 cells revealed that mouse liver generates three protein-DNA complexes (d-f) which are not detected with nuclear extract from 3T3-L1 cells (Fig. 4B). When nuclear extract isolated from mouse liver was incubated with the thirteen overlapping DNA oligonucleotides, those which contained the Pemt promoter region -426 to -402 generated all six DNA-protein complexes (Fig. 4B). Together these results indicate that nuclear proteins which regulate the trans-activation of the Pemt gene expression might bind the -426 to -402 promoter section. Comparison of the -426 to -402 sequence of the Pemt promoter with the TRANSFAC database revealed that this region contains two candidate binding sites, one for the SP family of transcription factors and one for YY1, a member of the gli-Kruppel class of zinc finger transcription factors (29).

The Transcription Factors Sp1, Sp3 and YY1 Bind to the Mouse Pemt Promoter- The Sp transcription factor family includes several members (Sp1, Sp2, Sp3 and Sp4) that share significant sequence homology and recognize GC boxes with similar specificity (30). To determine which of these proteins bind to the Pemt promoter, we performed super-shift assays with antibodies specific for individual Sp proteins. Antibodies specific for Sp1 were added to an EMSA reaction with the 32P-labelled Pemt -441/-402 promoter fragment and nuclear extracts isolated from 3T3-L1 cells (Fig. 5A) and mouse liver (Fig. 5B). In the absence of
antibody, incubation of $^{32}$P-labelled Pemt-441/-402 promoter fragment with nuclear extracts isolated from 3T3-L1 cells generates three different protein-DNA complexes (a-c) (lane 6 and 16, Fig. 5A). Upon addition of Sp1 specific antibody to the EMSA reaction the mobility of complex a was retarded such that a new upper band appeared in lane 2 (left panel, Fig. 5A). The accompanying decrease in the intensity of complex a in the presence of anti-Sp1 antibody was detected when complexes were resolved using a 6% non-denaturing polyacrylamide gel (lane 12, right panel, Fig. 5A). The addition of anti-Sp3 antibodies decreased the intensity of complexes a and b with an accompanying super-shifted complex (lane 4 and 14). The disappearance of more than one band is likely due to the different isoforms of Sp3 (115-kDa, 80-kDa and 78-kDa)(30); the experimental conditions might be unable to resolve the protein-DNA complexes generated by Sp1 from those of full length Sp3. We further illustrated that both complex a and b contain Sp family members by adding oligonucleotides corresponding to the consensus GC box (GGGGCGGGG) to EMSA reactions. In the presence of the consensus GC box, formation of both complexes was prevented (lane 1 and 11). Together, these results indicate the presence of Sp1 in complex a and Sp3 in complexes a and b. Antibodies raised against Sp2 did not alter the mobility or formation of any complex (lanes 3 and 13). Sp4 in the mouse is primarily in the brain and thus is not considered to regulate expression in the liver (30).

Next, we investigated whether the Pemt promoter interacted with the nuclear protein YY1. Addition of antibodies specific for YY1 to the EMSA reaction reduced the mobility of complex c so that it co-migrated with complex a (lane 7 and 17, Fig. 5A). The addition of the unlabelled competitor YY1 consensus sequence reduced the appearance of complexes c-f (lanes 9 and 19, Fig. 5A), consistent with results presented in Fig 5 indicating that the YY1 binding site is important for generating several complexes. However, the super-shift with anti-YY1 (lanes 7 and 17, Fig. 5A) suggests that only complex c contains the YY1 protein. Since similar DNA-transcription factor complexes containing Sp1, Sp3 and YY1 are formed with both 3T3-L1 (Fig. 5A) and liver (Fig. 5B) nuclear extracts, similar mechanisms may regulate the Pemt promoter in these cells.

Inhibition of Sp1 Binding to the Mouse Pemt Promoter Increases Pemt Reporter Activity—Next we determined whether Sp1 and/or YY1 protein bound to the Pemt promoter in vivo. We performed chromatin immunoprecipitation assays (ChIP) using pre-adipocytes and adipocytes to determine the temporal binding pattern of each transcription factor. Sp1 and YY1 were detected at the Pemt promoter before and after differentiation of 3T3-L1 cells (Fig. 6). Significantly fewer Pemt promoter fragments were pulled down by immunoprecipitation of Sp1 in 3T3-L1 adipocytes than in pre-adipocytes (Figs. 6A and B), indicating that less Sp1 had bound to the Pemt promoter. In contrast, similar amounts of YY1 protein were present at the Pemt promoter in either pre-adipocytes or adipocytes (Figs. 6C and D). The temporal binding pattern of Sp1 to the Pemt promoter is consistent with a regulatory role for Sp1 as an inhibitor of Pemt gene transcription.

To obtain further support for the proposal that reduction of Sp1 binding at the Pemt promoter increases promoter activity we used the cell permeable agent mithramycin to inhibit the binding of Sp1 to the DNA. Mithramycin A binds to GC-rich DNA sequences, thereby precluding the binding of nuclear proteins including Sp1 (31). When adipocytes stably expressing -
471 Luc were treated with 10 µM mithramycin A for 24 h, luciferase activity was significantly increased compared to that in vehicle treated cells (Fig. 7A). The mithramycin A-mediated increase in promoter activity was accompanied by reduced formation of complex the Sp1-Pemt promoter complex (complex a) in EMSA experiments (Figs. 7B). The amount of Sp1-Pemt complexes generated (complex a) was normalized to the amount of YY1-Pemt promoter complexes (complex c) in an attempt to account for variation in gel loading (Fig. 7C) In addition, significantly fewer Pemt promoter fragments were pulled down by immunoprecipitation of Sp1 in 3T3-L1 adipocytes treated with mithramycin A than with vehicle (Figs. 7D and E ), indicating that less Sp1 had bound to the Pemt promoter. Together, these results suggest that decreased Sp1 binding promotes increased Pemt promoter activity.

Expression of the Mouse Pemt gene is regulated by SP1 in 3T3-L1 Adipocytes-

To determine whether Sp1 directly inhibits transcription from the mouse Pemt promoter, Sp1 was over-expressed using a retrovirus containing Sp1 cDNA in pre-adipocytes which stably express the Pemt promoter construct -471 Luc. Over-expression of Sp1 significantly reduced the ability of the Pemt promoter construct -471 Luc to drive luciferase activity in both 3T3-L1 pre-adipocytes and adipocytes (Figs. 8B and C), indicating that Sp1 is a negative regulator of the Pemt promoter. To investigate whether reduction of Sp1 protein increased Pemt promoter activity, we constructed retroviruses that contained short hairpin sequences corresponding to Sp1. A 50% decrease in Sp1 protein significantly increased luciferase activity from -471 Luc in 3T3-L1 adipocytes compared to the scrambled RNAi control (Figs. 8D to F). Thus, Sp1 inhibits Pemt promoter activity independent of the differentiation status of the cells. However, the reduction of Sp1 was insufficient to induce Pemt gene transcription in pre-adipocytes.

To further address the functional significance of Sp1-mediated regulation of Pemt gene expression during 3T3-L1 differentiation, the temporal expression of Sp1 and PEMT were evaluated by immunoblot analysis (Fig. 9). The reduction of Sp1 protein levels during 3T3-L1 differentiation is consistent with release of Sp1-mediated inhibition of Pemt gene transcription and thus activation in adipocytes.

Inhibition of the Mouse Pemt Promoter by Tamoxifen is Mediated by Sp1-

We next examined whether Sp1 binding regulates Pemt gene expression in response to an estrogen receptor modulator. It was recently shown that tamoxifen, a common breast cancer treatment drug, inhibits expression of PEMT in rodents (32). To determine whether we could reproduce this tamoxifen-mediated effect in mice we injected animals for 5 consecutive days with either vehicle (sesame oil, 1% benzyl alcohol) or tamoxifen (0.5 mg/kg/day). Analysis of hepatic PEMT mRNA levels by qPCR revealed that following tamoxifen treatment the mRNA level of PEMT was significantly reduced compared to the vehicle (Fig. 10A). When adipocytes stably expressing -471 Luc were treated with either 1 µM or 10 µM tamoxifen for 7 days, luciferase activity was significantly decreased compared to that in vehicle treated cells (Fig. 10B). The tamoxifen-mediated decrease in promoter activity was accompanied by increased formation of the Sp1-Pemt promoter complexes (complex a) in EMSA experiments (Figs. 10 C and D). The amount of Sp1-Pemt promoter complexes generated (complex a) was normalized to the amount of YY1-Pemt gene transcription.
promoter complexes (complex c) in an attempt to account for variation in gel loading (Fig. 10 D). In addition, significantly more Pemt promoter complexes were pulled down by immunoprecipitation of Sp1 in 3T3-L1 adipocytes treated with tamoxifen than with vehicle (Figs. 10 E and F), indicating that more Sp1 had bound to the Pemt promoter.

To confirm that tamoxifen was inhibiting Pemt promoter activity via the Sp1 binding site we mutated the GC-element (5’-GGGA A-3’) by replacing two guanine nucleotides with adenine (5’-GGGA A-3’). We demonstrated that this mutation prevented Sp1 binding to the Pemt promoter in EMSA experiments. The -442 to -402 section of the Pemt promoter was 32P-labelled and incubated with nuclear extract to generate the three different protein–DNA complexes previously observed (Fig. 11A). When we 32P-labelled the -441 to -402 section of the Pemt promoter containing a mutation within the Sp1 binding site (MSp1) the formation of Sp1 containing complexes was prevented, indicating the inability to bind Sp1 protein (Fig. 11A). When adipocytes stably expressing -471MSp1 Luc were treated with tamoxifen there was no change in luciferase activity compared to the vehicle control (Fig. 11B). Therefore, the tamoxifen-mediated decrease in Pemt promoter activity requires the Sp1 binding site. Together, these results establish that tamoxifen inhibits Pemt gene expression by promoting Sp1 binding to the promoter.

DISCUSSION

Sp1 is an inhibitor of Pemt gene transcription- Sp1 is responsible for recruiting basal transcriptional machinery to TATA-less promoters (33-35) as well as acting as an activator or an inhibitor at target promoters (36-39). We have found that Sp1 regulates Pemt gene expression in 3T3-L1 cells by acting as a transcriptional repressor. Sp1 has been previously shown to promote repression of target genes by recruiting histone deacetylases both directly (37) and indirectly (36,40,41) to specific promoters. Typically Sp1 physically interacts with several nuclear proteins in a dynamic complex to perform transcriptional repression (42) as well as de-repression and activation (40). The complexity of Pemt gene expression during 3T3-L1 adipogenesis was apparent from our observation that reduction in Sp1 protein was not sufficient to release repression of Pemt gene transcription in pre-adipocytes. It is likely that Sp1 is only part of the transcriptional machinery which cooperatively inhibits Pemt gene expression, thus modification of additional repressors might limit regulation by Sp1. This mechanism could potentially include the transcription factors YY1 and Sp3 since they physically interact with Sp1 in some cell types (43,44). However, we determined that the amount of YY1 binding at the Pemt promoter remained similar before and after activation of Pemt gene transcription. Thus, suggesting that the abundance of YY1 protein does not regulate PEMT expression in adipocytes. The activation of PEMT in 3T3-L1 adipocytes might also be promoted upon binding of an activation complex which is expressed during adipogenesis. Although the decrease in Sp1 is required for Pemt gene expression during differentiation, the finding that a decrease in Sp1 is not sufficient precludes the likelihood that decreasing Sp1 in other immortalized cell models or primary mouse hepatocytes will promote Pemt gene expression. There is also evidence for additional regulation upstream of the -471 bp section of the mouse Pemt promoter. When we extended the length of PEMT promoter upstream of the luciferase reporter gene from -471 bp to -1000 bp (Fig. 3) transcriptional activation was prevented suggesting additional repressor binding sites.
Implications for Transcriptional Regulation of the Pemt gene in Mouse Liver-

Our results demonstrate that elements of the transcriptional regulation of the Pemt promoter may be conserved between mouse liver and 3T3-L1 cells. In gel-shift assays Sp1, Sp3 and YY1 proteins from mouse liver nuclear extract bound to the Pemt promoter. Sp1 regulates the transcription of other genes involved in hepatic lipid metabolism (45) including PC metabolism (23,38). Sp1 regulates CT expression during the cell cycle, and another enzyme in the CDP-choline pathway, choline kinase may also be regulated by Sp1 because two putative binding sites have been recently identified (46). The CDP-choline pathway is typically up-regulated in the liver when PEMT activity is repressed, for example during hepatic regeneration (47) and growth (20,48,49). The inverse transcriptional regulation of Pemt gene expression and the CDP-choline pathway could potentially be achieved with the contribution of Sp1. For example, such co-ordinate regulation might promote the synthesis of PC via the CDP-choline pathway during rapid proliferation and through the PEMT pathway in quiescent cells. We observed the highest levels of Sp1 protein in 3T3-L1 cells during rapid proliferation which is consistent with previous reports of cell-cycle-dependent regulation of Sp1 (50,51). Thus, Sp1-mediated repression of Pemt gene expression may be part of a cellular response to promote growth.

We have observed three additional transcription factor-Pemt promoter complexes in the EMSAs with nuclear extract from liver but not with nuclear extract from 3T3-L1 cells, suggesting that liver specific expression of the Pemt gene might, in part, be controlled by these factors. We have been unable to identify these factors but have ruled out binding sites for NF1, Chop, C/EBP, GATA, SF1, MycMax, HNF1, HNF3/6, LXR, GR, STAT, PPAR, SMAD, CHREBP, NFKβ, NFA, NKX2.5 and SREBP (data not shown). Since tamoxifen binds estrogen receptors (ER) (52) and recent evidence has suggested that estrogen activates Pemt gene transcription (21), we introduced a double-stranded oligonucleotide containing the estrogen receptor element in gel mobility experiments but this also did not alter the binding pattern of nuclear factors to the Pemt promoter.

Alternatively, our data established that the Sp1 binding site was required for tamoxifen-mediated inhibition of Pemt promoter activity. In support of this finding it is known that estrogen and tamoxifen can affect expression from genes lacking a classical ER element (53-55). Specifically, it has been determined that only 11% of genes regulated by estrogen/estrogen receptor modulators (e.g. contain a classical ER element (53). The remainder of genes appear to be regulated by Ap1, Sp1, FOXA1 or nuclear factor κB promoter sites (53). Gene regulation by non-classical ER signalling events is complex including physical interactions between ER and other transcription factors (54,56), ER-mediated changes in expression levels of regulatory factors (57,58), and post-translational modification of nuclear proteins via intracellular signalling cascades (e.g. cAMP/PKA, MAPK/PI3K) (55,59,60). While the upstream signalling events remain to be elucidated our results clearly establish that activation of ER by tamoxifen promotes the assembly of a Sp1 inhibitory complex at the Pemt promoter.

In conclusion, the present study demonstrates that the -471 to +130 section of the Pemt promoter is essential for Pemt gene expression in 3T3-L1 adipocytes, and established that Sp1 transcriptionally regulates Pemt gene expression in adipocytes under native conditions and in response to tamoxifen treatment.
Acknowledgments- We thank Vernon Dolinsky, Chieko Aoyama, David Shields, Jean Vance and Claudia Banchio, for helpful advice and discussion.

REFERENCES


### Table 1: PCR primer sequences

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The abbreviations for the mRNAs are: PEMT, phosphatidylethanolamine N-methyltransferase; CYC, cyclophilin; PPAR, peroxisome proliferator activated receptor; C/EBP, CAAT element binding protein; FABP4, fatty acid binding protein 4.
Figure Legends

FIGURE 1. Expression of PEMT in various cell types. (A) Total RNA was extracted from, COS-7 (COS), C3H10T1/2 (C3H), H2.35, Hepa-1c1c7 (Hepa) cells and primary mouse hepatocytes, reverse-transcribed to cDNA, and Pemt and cyclophilin (CYC) mRNAs were detected by PCR. Amplicons were visualized with ethidium bromide on a 2% agarose gel. Duplicate samples of isolated primary mouse hepatocytes were collected 4 and 16 h post isolation. Total RNA was isolated from the liver of adult male PEMT wild type (WT) and knockout (KO) mice for positive and negative controls respectively. A non-template control (NTC) represents a negative control for PCR in the absence of cDNA template. Results are from a single experiment which was repeated once with similar results. (B) Cells were transiently transfected in duplicate with -471 Luc, the empty pGL3 basic vector as a negative control (empty) or the pGL3 control vector containing the SV40 promoter upstream of luciferase cDNA (+ control) as a positive control for transfection efficiency. Luciferase activity was measured 20-48 h post-transfection and expressed relative to β-galactosidase activity. Values represent the means ± SEM of three independent experiments.

FIGURE 2. Pemt mRNA is increased during 3T3-L1 differentiation to adipocytes. (A) Total RNA was extracted from triplicate cultures of 3T3-L1 pre-adipocytes and adipocytes, reverse-transcribed to cDNA, and Pemt and cyclophilin (CYC) mRNAs were detected by PCR. NTC is a non-template control (duplicate lanes). Amplicons were visualized with ethidium bromide on a 2% agarose gel. (B) 3T3-L1 fibroblasts were differentiated into adipocytes and cells were collected on the indicated days. Day 0 indicates two days post-confluence and the start of the differentiation protocol. Total RNA was isolated, reverse-transcribed to cDNA and Pemt, FABP4, PPARγ, and C/EBPα mRNA levels measured by qPCR. 18S RNA levels were measured by qPCR by generating a single stranded DNA template using the reverse primer. mRNA and 18s RNA levels were normalized to cyclophilin using a standard curve. All data are means ± SEM from three separate independent experiments. ND refers to non-detectable amplicon within the indicated sample. (C) 3T3-L1 fibroblasts were stably transfected with -471 Luc or the empty pGL3 basic vector (Empty) as a negative control. Luciferase activity was measured in pre-adipocytes and adipocytes and normalized to cellular protein. Values represent means ± SEM of three separate independent experiments (*, P<0.05).

FIGURE 3. Mouse Pemt promoter region -471/+130 bp is activated during 3T3-L1 differentiation. 3T3-L1 cells stably expressing various lengths of the Pemt promoter upstream of the luciferase reporter gene were generated and differentiated to adipocytes. Cells were collected before (-2) and following (+7) differentiation to adipocytes and luciferase activity was measured and normalized to cellular protein. The results are reported as fold induction at day 7 compared with luciferase activity at -2 days. Two stable cell lines (clone -1 and clone -2) are shown for each plasmid construct. The negative control was an empty pGL3 basic vector (Empty). Values represent means ± SEM of four independent experiments (*, P<0.05).

FIGURE 4. EMSA analysis of binding of nuclear extracts to Pemt promoter. Sections of the mouse PEMT promoter 40 bp in length were labelled with 32Pi and incubated without (-) or with (+) nuclear extract (NE) isolated from (A) 3T3-L1 adipocytes or (B) mouse liver. The resulting DNA-protein complexes (a to f) were separated on 8% polyacrylamide gels and visualized by
autoradiography. The results are representative of three independent experiments with similar results. In (B) where the nuclear extract is primarily from mouse liver, A refers to nuclear extract isolated from 3T3-L1 adipocytes.

FIGURE 5. **Super-shift analysis of protein binding to the Pemt promoter using EMSA.** The -441/-402 section of the mouse Pemt promoter was labelled with $^{32}$P, and incubated without (-) or with (+) nuclear extract from (A) 3T3-L1 cells or (B) mouse liver. Unlabelled oligonucleotides containing the consensus sequence for YY1, the Sp family of transcription factors, or sterol response element binding protein (SREBP) were added to the binding reaction at 100-fold molar excess, as indicated. Antibodies raised against Sp1, Sp2, Sp3, YY1 or SREBP were used in binding reactions as indicated. Resulting DNA-protein complexes (a to f) and super-shifted complexes (indicated by arrow heads) were separated using 8% (left panel) or 7% (right panel) polyacrylamide gels and visualized by autoradiography. The results are representative of three independent experiments with similar results.

FIGURE 6: **ChIP analysis of Sp1 binding to the Pemt promoter.** (A) *In vivo* association of Sp1 with the Pemt promoter. Protein-DNA complexes were immunoprecipitated from lysates of 3T3-L1 pre-adipocytes (P) and adipocytes (A), without or with antibodies (AB) to IgG or Sp1. The Pemt promoter was detected by PCR. DNA purified from sonicated cell lysates was used as a positive control (input). (B) Results of densitometric analysis of bands from three independent experiments using Sp1 antibody and are means ± SEM (*, $P<0.05$). (C) *In vivo* association of YY1 with the Pemt promoter. Protein-DNA complexes were immunoprecipitated from lysates of 3T3-L1 pre-adipocytes (P) and adipocytes (A), without or with antibodies (AB) to IgG or YY1. The Pemt promoter was detected by PCR. DNA purified from sonicated cell lysates was used as a positive control (input). (D) Results of densitometric analysis of bands from three independent experiments using YY1 antibody and are means ± SEM.

FIGURE 7: **Analysis of mithramycin A-mediated inhibition of Sp1 binding to the Pemt promoter.** (A) 3T3-L1 stably expressing -471 Luc were differentiated to adipocytes and treated with the indicated concentration of mithramycin A (in 1% dimethylsulfoxide) or vehicle in 10% FBS, DMEM for 24 h. Luciferase activity was normalized to cellular protein. Values are means ± SEM of three independent experiments (*, $P<0.05$). (B) 3T3-L1 adipocytes were incubated with vehicle (V) or mithramycin (M, 10 µM) for 24 h. Cells were then washed twice with phosphate-buffered saline. Nuclear extract was obtained and incubated with $^{32}$P-labeled Pemt promoter (-441/-402). The resulting DNA/protein complexes (a to c) were separated on an 8% polyacrylamide gel. A 100-fold molar excess of unlabelled DNA oligonucleotide containing the Sp family consensus sequence was added to the binding reaction as indicated. (C) Results of densitometric analysis of bands from three independent EMSA experiments are shown as a ratio of intensity of complex a (Sp1) to complex c (YY1) (*, $P<0.05$). (D) *In vivo* association of Sp1 with the Pemt promoter. Protein-DNA complexes were immunoprecipitated from lysates of 3T3-L1 adipocytes treated with vehicle (V) or mithramycin (M, 10 µM) for 24 h using antibodies (AB) to IgG or Sp1. The Pemt promoter was detected by PCR. DNA purified from sonicated lysates was used as a positive control (input). (E) Results of densitometric analysis of band from three independent experiments using Sp1 antibody and are means ± SEM (*, $P<0.05$).
FIGURE 8. Analysis of influence of Sp1 on Pemt promoter/luciferase activity. (A) 3T3-L1 pre-adipocytes stably expressing the pGL3 basic vector without (pGL3 basic) or with the Pemt promoter section -471 to +130 bp (-471 Luc) upstream of the luciferase reporter gene were treated with retrovirus generated from either an empty pBabe vector (Empty, black bars) or pBabe.puro containing cDNA encoding Sp1 (Sp1, white bars). Cell lysates from 3T3-L1 fibroblasts transduced with the indicated virus were immunoblotted with antibodies raised against Sp1 and PDI (protein disulfide isomerase). Densitometric analysis of immunoblots is shown below the immunoblot. Values represent means of three separate independent experiments. Luciferase activity was measured and normalized to cellular protein in 3T3-L1 pre-adipocytes (B) and adipocytes (C). (D) 3T3-L1 pre-adipocytes stably expressing the pGL3 basic vector without (pGL3 basic) or with the Pemt promoter section -471 to +130 bp (-471 Luc) upstream of the luciferase reporter gene were transduced with retrovirus generated from either pSuper.retro. containing scrambled- (Scrambled RNAi) or Sp1- (Sp1 RNAi) shRNA sequences. Luciferase activity measured in pre-adipocytes (E) and adipocytes (F) and normalized to cellular protein. Values are means ± SEM of three independent experiments (*, P <0.05).

FIGURE 9: Analysis of Sp1, PEMT and TBP protein levels by immunobloting. 3T3-L1 fibroblasts were differentiated into adipocytes. Day 0 represents 2 days post-confluence and the starting point of the differentiation protocol. Cells were harvested at the indicated times (top row of numbers). 3T3-L1 homogenates (50 µg protein) were separated on 0.4% SDS/10% polyacrylamide gels, electroblotted on to PVDF membranes, and probed with antibodies raised against Sp1, PEMT or TBP (TATA binding protein). The results are representative of three independent experiments. The numbers below each gel represent the mean of densitometric analysis of three immunoblots relative to the amount of TBP detected. HeLa whole cell lysate (50 µg, Santa Cruz Biotech.) was used as a positive control for the indicated nuclear proteins.

FIGURE 10: Tamoxifen inhibits PEMT expression by increasing Sp1 binding. (A) Total RNA was extracted from the liver of adult male PEMT wild type mice injected subcutaneously with either tamoxifen (0.5mg/kg/day in sesame oil, 1% benzyl alcohol) or vehicle for 5 consecutive days. The RNA was reverse transcribed to cDNA, and Pemt mRNA levels were detected by qPCR. mRNA levels were normalized to cyclophilin using a standard curve. Data represents means ± SEM (*, P<0.05, n=5-7 mice). (B) 3T3-L1 stably expressing -471 Luc were differentiated to adipocytes and treated with the indicated concentration of tamoxifen (in 1% dimethylsulfoxide) or vehicle in 10% FBS, DMEM for 7 days. Luciferase activity was normalized to cellular protein. Values are means ± SEM of three independent experiments (*, P<0.05). (C) 3T3-L1 adipocytes were incubated with vehicle (V), or tamoxifen (T, 10 µM) for 7 days or mithramycin (M, 10 µM) for 24 h. Cells were then washed twice with phosphate-buffered saline. Nuclear extract was obtained and incubated with 32P-labeled Pemt promoter (-441/-402). DNA/protein complexes (a-c) were separated on an 8% polyacrylamide gel. A 100-fold molar excess of unlabelled DNA oligonucleotide containing the Sp family consensus sequence was added to the binding reaction as indicated. (D) Densitometric analysis of results of three independent EMSA experiments are shown as a ratio of intensity of complex a (Sp1) to the intensity of complex c (YY1) (*, P<0.05). (E) In vivo association of Sp1 with the Pemt promoter. Protein-DNA complexes were immunoprecipitated from lysates of 3T3-L1 adipocytes treated with vehicle (V) or tamoxifen (T, 10 µM) for 7 days using antibodies (AB) to IgG or Sp1. The Pemt promoter was detected by PCR. DNA purified from sonicated lysates was used as a positive
control (input). (F) Results of densitometric analysis of band from three independent experiments using Sp1 antibody and are means ± SEM (*, P<0.05).

FIGURE 11: Tamoxifen requires the Sp1 binding site to inhibit Pemt gene expression
(A) The -441/-402 section of the mouse promoter containing the wild-type GC-element (Wt) or the mutated GC-element (MSp1) was $^{32}$P$_{i}$-labelled and incubated without (-) or with nuclear extract from mouse liver (+). Resulting DNA-protein complexes (a to c) were separated using an 8 % polyacrylamide gel and visualized by autoradiography. The results are representative of three independent experiments. (B) 3T3-L1 adipocytes stably expressing -471 Luc or -471MSp1 Luc (containing a mutation in the Sp1 binding site) were differentiated to adipocytes and treated with 10 $\mu$m tamoxifen (in 1 % dimethylsulfoxide) or vehicle in 10 % FBS, DMEM for 7 days. Luciferase activity was normalized to cellular protein. Values are means ± SEM of three independent experiments (*, P<0.05 compared to 3T3-L1 adipocytes stably expressing -471 Luc treated with vehicle; #, P<0.05, compared to 3T3-L1 adipocytes stably expressing -471 Luc treated with vehicle or tamoxifen, ANOVA).
Figure 1

A

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B

- **Hepa-1c1c7**
- **H2.35**
- **Cos-7**
- **C3H10T1/2**
- **Hepatocytes**

![Bar graph showing luciferase β-galactosidase levels](image)
**Figure 5A**

### 3T3-L1

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*Images showing gel electrophoresis with bands marked as a, b, and c.*
## Figure 5B

| Liver | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|-------|---|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|
| Anti-SREBP | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| SREBP oligo | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - |
| Anti-YY1 | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| YY1 oligo | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Anti-Sp3 | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Anti-Sp2 | - | + | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - |
| Anti-Sp1 | - | + | - | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| Sp1 oligo | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Nuclear Ext. | + | + | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

[Images of gel blots showing bands labeled Sp1/3 ss, YY1 ss, Sp1 ss, and YY1 ss with arrows indicating lanes and bands.]
Figure 7

A. Luciferase Activity

B. Sp1 Oligo

C. Sp1 binding

D. Input, No AB, IgG, Sp1

E. Sp1 at PEMT Promoter

* Significant difference
Figure 8

A. Sp1/PDI

- Empty
- Sp1

Sp1/PDI

pBabe

B. Pre-adipocytes

- Empty
- Sp1

Luciferase Activity

pGL3 basic -471 Luc

C. Adipocytes

- Empty
- Sp1

Luciferase Activity

pGL3 basic -471 Luc

D. Sp1/PDI

- Scrambled
- Sp1

pSuper.retro (RNAi)

E. Pre-adipocytes

- Scrambled
- Sp1

Luciferase Activity

pGL3 basic -471 Luc

F. Adipocytes

- Scrambled
- Sp1

Luciferase Activity

pGL3 basic -471 Luc
Figure 11

A

\[
\begin{array}{c|cccc}
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\text{c} & (\text{YY1}) & & & \\
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B

Luciferase Activity

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*P < 0.05

**P < 0.01
A role for Sp1 in transcriptional regulation of phosphatidylethanolamine N-methyltransferase in liver and 3T3-L1 adipocytes
Laura K. Cole and Dennis E. Vance

J. Biol. Chem. published online February 11, 2010

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