A Role for Sp1 in the Transcriptional Regulation of Hepatic Triacylglycerol Hydrolase in the Mouse*

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† The abbreviations used are: BTEB, basic transcription element binding; EMSA, electromobility shift assay; GFP, green fluorescent protein; bHLH, basic helix-loop-helix; TG, triacylglycerol; TGH, triacylglycerol hydrolase; VLDL, very low density lipoproteins.
Microsomal triacylglycerol hydrolase (TGH) hydrolyzes stored triacylglycerol in cultured hepatoma cells [Lehner and Vance (1999) Biochem J., 343, 1-10]. We studied expression of TGH in murine liver and found both protein and mRNA increased dramatically at 27 days after birth. Nuclear run-on assays demonstrated this was due to increased transcription. We cloned 542 bp upstream of the transcriptional start site of the murine TGH gene. Electrophoretic mobility shift assays demonstrated enhanced binding of hepatic nuclear proteins from 27-day-old mice to the murine TGH promoter yielding 3 differentially migrating complexes. DNase I footprint analysis localized these complexes to two distinct regions: site A contains a putative Sp binding site and site B contains a degenerate E box. We transfected primary murine hepatocytes with a series of 5'-deletion constructs upstream of the reporter luciferase cDNA. Positive control elements were identified in a segment containing site A. Competitive electromobility shift assays and supershift assays demonstrated that site A binds Sp1 and Sp3. Transcriptional activation assays in Schneider SL-2 insect cells demonstrated that Sp1 is a potent activator of the TGH promoter. These experiments directly link increased TGH expression at the time of weaning to transcriptional regulation by Sp1.
Mammalian carboxylesterases (E.C. 3.1.1.1) are serine esterases constituting a family of isoenzymes that are implicated in several physiological processes. Multiple forms of carboxylesterase have been identified in mammalian tissues and some have been shown to differ in biochemical, immunological, and genetic properties [for review see (1) and references therein]. Carboxylesterase activity is widely distributed in mammalian tissues with the highest levels being present in liver microsomes (2). The ability of carboxylesterases to hydrolyze various xenobiotoics and endogenous substrates such as esters, thioesters, or amide bonds indicate that the known functions of these enzymes are mainly for drug metabolism and detoxification of harmful chemicals (1). Some carboxylesterases have been found to preferentially hydrolyze lipids such as long-chain acyl-CoA (3), cholesteryl ester (4), and triacylglycerol (TG) (5). Some carboxylesterases are thought to participate in transport of fatty acids across the endoplasmic reticulum membrane or in maintenance of membrane structure.

In the liver, mobilization of stored TG to supply fatty acids for metabolic energy occurs via an initial hydrolysis of stored TG and subsequent re-synthesis of TG as a component of very low density lipoproteins (VLDL) [for review see (6)]. Although the enzymes catalyzing de novo synthesis of TG are associated with endoplasmic reticulum membranes, approximately 70% of the TG that is secreted by hepatocytes in VLDL has been calculated to arise from a cytosolic storage pool whereas only 30% is derived from de novo TG synthesis (7, 8). Hormone-sensitive lipase, while fundamental in adipose tissue lipid metabolism and overall energy homeostasis, is not expressed in liver in
sufficient quantities to account for the rate of lipolysis of stored TG and subsequent VLDL secretion (7).

Lehner and Verger (5) reported the purification of a member of the carboxylesterase family from porcine liver, microsomal triacylglycerol hydrolase (TGH), capable of hydrolyzing long-, medium- and short-chain TGs in vitro (5). Further detailed characterization of TGH with respect to its intracellular location, developmental expression, and tissue and species specificity strongly supported a role for TGH in the lipolysis of cytoplasmic TG, some of which is used for assembly into VLDL (9). Stable expression of rat TGH cDNA in McArdle RH7777 cells, a rat hepatoma cell line that lacks TGH and is defective in VLDL secretion, demonstrated unambiguously that TGH does indeed hydrolyze stored TG (10).

In our previous studies we observed increased expression of rat TGH mRNA and protein in liver at the time of weaning, coincident with an enhanced ability to secrete VLDL (9). The aim of the present study was to determine whether the increased expression of TGH seen at the time of weaning is linked to transcriptional regulation and to identify potential transcription factors and cis-acting DNA elements that might mediate the observed developmental expression of TGH in liver.
EXPERIMENTAL PROCEDURES

Isolation of Protein and RNA - Livers samples from mice of various ages were washed with ice-cold phosphate-buffered saline and homogenized using a Polytron homogenizer (Brinkman Instruments, Ontario, Canada) for 30 s at low speed in 250 mM sucrose, 25 mM Tris-HCl (pH 7.4), and 5 mM EDTA to yield 20% (w/v) crude extract. Unbroken cells and cellular debris were removed by 10 min centrifugation at 500 x g. Protein concentrations of total liver homogenates (supernatants) were determined by the micro BCA method (Pierce) using bovine serum albumin as a standard. For RNA isolation, livers were immediately frozen in liquid nitrogen prior to extraction of total RNA using Trizol reagent (Gibco BRL, Life Technologies) according to manufacturer’s instructions.

Immunoblot Analysis — Homogenate protein (50 g) or nuclear protein (10 g) from murine liver was heated for 3 min at 90°C in 62.5 mM Tris-HCl (pH 6.4), 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 1.05% SDS, and 0.004% bromophenol blue. The protein samples were electrophoresed on a 10% SDS-polyacrylamide gel in 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 0.1% SDS buffer. The proteins were then transferred to nitrocellulose by electroblotting in transfer buffer (25 mM Tris-HCl (pH 8.3), 192 mM glycine, 20% (v/v) methanol). Following transfer, membranes were incubated for 1 h at room temperature or overnight at 4°C with 5% skim milk in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20 (T-TBS), then incubated for 1 h with antibody raised against the specified protein. Depending on the primary antibodies, the
secondary antibodies used were goat anti-rabbit IgG, rabbit anti-goat IgG, or goat anti-
mouse IgG (Pierce) and were detected using the enhanced chemiluminescence system
(Amersham Pharmacia Biotech) according to the manufacturer’s instructions. Where
indicated, primary and secondary antibodies were stripped from the membrane by
incubation with 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.7) for 30
min at 50°C and the blot was re-probed with another antibody. The primary antibodies
used were affinity purified anti-TGH polyclonal antibody (5), anti-actin monoclonal
antibody (Sigma, clone no. AC74, cat. no. A 5316), and from Santa Cruz Biotech,
polyclonal anti-Sp1 (sc-59-G), anti-Sp2 (sc643,), anti-Sp3 (sc-644), anti-E47 (sc-763x,),
and anti-YY1 (sc-1703x,). In some cases, primary incubations with anti-Sp antibodies
also contained competitive antigenic peptides to identify antigenically related proteins.
These peptides were SP1(PEP2)P (sc-59P), SP2(K-20)P (sc-643P) and SP3(D-20)P
(sc-644P) from Santa Cruz Biotech.

Northern Blot Analysis -- Equivalent amounts of total RNA (2.5 g) were
electrophoresed in a 1.2% agarose/formaldehyde gel and transferred to HyBond-N+
membrane. TGH mRNA was detected by incubation of the membrane with a [32P]-
labeled, single-stranded anti-sense oligo designated pTGHII (5’-
GAGCAAAAGTTGGCCCATTATTTCACTACCATTGTGCTGA-3’). This oligonucleotide
was derived from the rat TGH cDNA sequence that is 90% identical to the
Corresponding murine cDNA, and does not align with other carboxylesterases from the
same gene family. Probes for glyceraldehyde-3-phosphate dehydrogenase (G3PDH)
were prepared by RT-PCR with murine liver total RNA with Superscript II (Gibco, BRL)
according to the manufacturer’s instructions. The primers used were G3PDH1A (5’-GAGCCAAACGGGTCATCATC-3’) and G3PDH2B (5’-CATCACGCCACAGCTTTCCA-3’) which were designed to amplify a 232 bp fragment. The PCR product was used as a template for Random Primers DNA Labeling System (Gibco, BRL) according to the manufacturer's instructions. Northern hybridizations were visualized by autoradiography.

**PCR-Based Nuclear Run-on Assay** - This assay was performed essentially as described (11). Briefly, highly purified and transcriptionally active nuclei were prepared from murine liver according to the methods of Marzluff and Huang (12). Freshly prepared or frozen/thawed nuclei (200 l) were split into two aliquots and incubated for 30 min at 30...C in 20% glycerol, 30mM Tris-HCl (pH 8.0), 2.5 mM MgCl₂, 150 mM KCl, 1 mM DTTand 40 U of RNasin (Promega) with or without 0.5 mM (each) ribonucleoside triphosphates (+/- rNTPs). After 30 min, nuclei were lysed by the addition of 200 l of 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 4.0), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. Yeast tRNA (20 g) was added and RNA was extracted by the acid-guanidinium-thiocyanate method then resuspended in water treated with diethylpyrocarbonate. RT-PCR was performed as described above. The primers used for TGH were EX6F (5-CACTGCTGCTCTGATTACAACAG-3) and EX10R (5-GCGACCACCTGGAATCATATTC-3). The primers used for G3PDH were G3PDH1A and G3PDH2B. Because the run-on products are not labeled, newly transcribed mRNA in isolated nuclei was detected following nuclear transcription initiated by the addition of exogenous rNTPs (+ rNTPs). Nuclear transcription reactions lacking exogenous rNTPs
(- rNTPs) were included to control for mRNAs synthesized by endogenous rNTPs in isolated nuclei.

Cloning of the Murine TGH Promoter and Plasmid Construction — Genomic sequences containing the putative proximal murine TGH promoter were identified by Southern blot analysis of BAC clone 313P24 that contains the complete murine TGH gene (13). Briefly, BAC DNA was digested with EcoRI and a single 6 Kb DNA fragment that contained exon 1 was identified by Southern blotting using a murine TGH exon 1-specific probe: ATGCAGCTCTACCCTCTGATATG. This fragment was subcloned and sequenced by gene walking using overlapping primers to determine sequences upstream of the transcriptional start site. The sequence of the murine TGH promoter was confirmed by sequencing both strands of the DNA. A genomic fragment encoding the murine TGH promoter region spanning -542 and +112 was cloned into the T/A cloning site of pCR2.1 TOPO (Invitrogen) according to the manufacturer’s instructions to create pCR (-542/+112). The promoter region was excised from pCR 2.1 TOPO by restriction digestion with KpnI and XbaI. The insert was purified from an agarose gel (Qiagen gel elution kit) and directionally ligated into pCI (Promega) that had been subjected to the same restriction digestion and gel purification. The cloned fragment was released from pCI by restriction digestion with KpnI and SmaI, gel purified, then directionally inserted into the luciferase reporter vector, pGL3Basic (Promega) that had also been digested by KpnI and SmaI and gel purified to generate -542Luc. Luciferase reporter plasmids containing serial deletions at the 5′-end of the cloned promoter fragment were generated by standard PCR methods using -542Luc as template and
GL primer 2 (Promega) as the reverse primer with each of the following forward primers: TGGCTGCTGCTGTCTGCTTT (-313Luc); CTGAATTGAGGTGAGAG (-276Luc); TGAGTACTGCGGCACTG (-198Luc); TAGTGGGCGTGGCTTG (-154Luc); GAGCTCTTTGGAAGGAAGGAG (-116Luc); CTGAGCTGTTGGAGCAAGAC (-48Luc); and TGGTCCACAACAGA (-10Luc). In each case, the sequence of the forward primer started with CGCGGTACC (KpnI site in bold). Each of the PCR products was directionally cloned into pGL3Basic following restriction digestion of each with KpnI and SmaI.

Vectors enabling expression of recombinant Sp proteins were obtained from Dr. R. Tjian (pPacSp1 and pPac0) (14) and Dr. J. Noti (pPacSp3) (15). The plasmid enabling expression of green fluorescent protein (GFP) in insect cells [copia (XhoI)-eGFP] was kindly provided by Dr. John F. Elliott (University of Alberta).

The cDNA for full-length Th1 (16) was obtained by RT-PCR. Briefly, total RNA was obtained from hearts isolated from 1 day-old mice (as described below for liver) and reverse transcribed with Superscript II reverse transcriptase (Gibco, BRL) and oligo dT15 antisense primer. Primers used in subsequent PCR were 5' - ATGAACCTCGTGGCAGCTAC-3' and 5' - TCACTGGTTAGCTCCAGCGCCCA-3'. The PCR (650 bp) product was cloned into the T/A cloning site of pCR2.1 TOPO. Th1 and GFP were excised from PCR2.1 TOPO and copia (XhoI)-eGFP respectively using XhoI and BamHI and the Th1 cDNA fragment was directionally ligated downstream of the copia promoter in place of GFP to generate a Th1 expression plasmid, copia-Th1. Copia(XhoI) was generated by blunt-end self-ligation after excision of GFP with XhoI.
and *Bam*HI. Cloned inserts of all transfection constructs were confirmed by DNA sequencing (University of Alberta DNA Core Facility).

**Cell Culture, Transfections, and Reporter Assays** — Murine hepatic cells were isolated by collagenase perfusion and transiently transfected with TGH promoter luciferase reporter constructs (2 g) using a cationic liposome technique (17). Reporter assays were performed as recommended (Promega) and luciferase activity was normalized to protein.

SL2 cells (American Type Culture Collection) were plated at a density of 2-3 x 10^6 cells/35-mm dish in Schneider's Drosophila medium (Gibco, BRL) containing 10% (v/v) heat-inactivated fetal bovine serum and transfected by a standard calcium phosphate co-precipitation method (18). Each plate received 5 g of 154Luc or reporter plasmid, pGL3Basic, with or without 5 g Sp expression plasmid (pPacSp1, pPacSp3, or pPacO as a control) and 5 g Th1 expression plasmid (copia(XhoI) as a control). All plates received 5 g of copia(XhoI)-eGFP as a control for transfection efficiency. Total plasmid DNA was conserved (20 g) for each transfection. *In vivo* fluorescence measurements were carried out on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with an argon laser. The sample temperature was maintained at 25…C. SL2 cells expressing GFP were excited at 488 nm. Fluorescence emission was detected at 525 nm. For quantification of GFP fluorescence, SL2 cells were grown using the same conditions as for the luciferase assays. Background fluorescence of SL2 cells was determined at excitation wavelength of 488 nm using the vector copia(XhoI) which does not contain the GFP coding region. Cell pellets were resuspended in phosphate-
buffered saline (58 mM Na$_2$HPO$_4$, 17 mM NaH$_2$PO$_4$, 68 mM NaCl, pH 7.4). Data analysis was performed using CELLQuest 3.1 (Becton Dickinson, San Jose, CA) and the ratio of fluorescence intensity to cell density was taken as a measure for GFP expression levels. Luciferase activities were normalized to transfection efficiency as determined by GFP expression. Statistical analysis was performed using a one-way Anova test followed by a post hoc Student-Newman-Keuls test. Values of $p$ less than 0.05 were taken to be significant.

**Preparation and Dephosphorylation of Nuclear Extracts** - Nuclear extracts from murine liver were prepared as described (19). Dephosphorylation of extracts was performed in 25 mM Hepes(pH 7.5), 37 mM KCl, 50 mM MgCl$_2$ at 30°C for 5 min, then 15 min on ice with calf intestinal alkaline phosphatase (1 unit/50 g of nuclear extract). Dephosphorylation reactions were terminated by the addition of a mixture of inhibitors to final concentrations of 10 mM NaF, 10 mM sodium vanadate, 10 mM potassium pyrophosphate, and 5 mM sodium phosphate. For mock dephosphorylation reactions, inhibitors were added to the extract before the 30°C incubation.

**Electrophoretic Mobility Shift Assays**- Distal (-542/-371), medial (-370/-68) and proximal (-67/+112) promoter fragments were released from pCR(-542/+112) by restriction digestion with Blpl/NotI, HindIII/Blpl, and HindIII respectively. Promoter fragments were purified from 2% agarose gels using Qiaex II Gel Extraction Kit (Qiagen Inc., Mississauga, Ontario) according to the manufacturer’s instructions and labeled by filling in ends using Klenow fragment in the presence of [$\alpha$-32P]dCTP. Promoter-derived
oligonucleotides were synthesized by the University of Alberta Core DNA Facility. Complimentary oligonucleotides (10 nmol of each) were heated at 70°C in 100 l annealing buffer (10 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 1 mM EDTA). After 10 min, annealing reactions were slowly cooled to room temperature and 5 pmole of double-stranded oligonucleotide was 5’-end labeled using T4 kinase (Boehringer-Manheim) and [γ-32P]-ATP (NEN-Dupont). The sequences of oligonucleotides used in EMSAs are as follows (coding strand): -1565’-CCTAGTGGGCGTGGCTTGG-3’ (site A); -125 5’-ACACCCAGAGAGCTCTTT-3’ (site B); and 5’-ATTCGATCGGGGCGGCGAGC-3’ (Sp consensus oligonucleotide). For each binding reaction (40 l), 4 g poly(dI-dC)-poly( dl-dC), 8 l of a 5 X binding buffer (100 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 250 mM NaCl, 5 mM EDTA, 50% glycerol, 0.5% Nonidet P40, 5 mM DTT), 2.5 g nuclear extract and labeled probe (20,000 cpm) were incubated at room temperature for 30 min. Competitive EMSAs were carried out under identical conditions except that a 50-molar excess of non-labeled double stranded oligonucleotides or 2 g of commercially available antibody was added to the binding reactions 15 min prior to the addition of labeled probe. Binding reactions were terminated by the addition of 4 l of gel loading buffer (30% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol). Protein-DNA complexes were resolved on 3.5% (for labeled promoter fragments) or 6% (for labeled double stranded oligos) non-denaturing polyacrylamide gel electrophoresis with Tris-borate-EDTA buffer system (45mM Tris, 44.5 mM borate, 1 mM EDTA, pH 8.0) at 4°C and were detected by autoradiography. Specific antibody-protein interactions were observed as super-shifted or immuno-depleted complexes.
In vitro DNase I Footprinting - The sense strand of the TGH medial promoter fragment was labeled by filling in the HindIII site using Klenow fragment in the presence of [α-32P]dCTP. Labeled DNA (20,000 cpm) was incubated for 30 min at room temperature with 10 g of poly(dI-dC)-poly(dI-dC) and 10, 25, and 50 g of nuclear protein extract from 37-day-old murine liver in a final volume of 100 l (20 mM Tris-HCl, pH 7.4, 2.2 mM MgCl2, 0.2 mM CaCl2, 50 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Nonidet P40, 1 mM DTT). To determine the optimal conditions, a titration was performed for each probe using increasing concentrations of DNase I for the same amount of nuclear extract. DNase I (5-10 milliunits) in DNase I buffer (1 mM MgCl2, 1 mM DTT, 20 mM KCl) was added for 5 min at room temperature. DNase I digestions were terminated by the addition of 200 l of DNase I stop buffer (200 mM NaCl, 30 mM EDTA, 1% SDS, 100 g/ml tRNA) and 2 l of proteinase K (25 g/l). After a 15 min incubation at 37°C, reaction mixtures were extracted twice with phenol/chloroform/isoamylalcohol and were then ethanol precipitated before analysis on a 5% polyacrylamide, 7 M urea sequencing gel.

RESULTS

Postnatal expression of TGH in murine liver.

Immunoblot analysis of proteins from livers of mice of various ages demonstrates that TGH protein levels increase dramatically between 20 and 27 days after birth (Fig. 1A). TGH mRNA expression in these livers was evaluated by Northern blot hybridization of
total RNA with a murine TGH-specific oligonucleotide as probe (Fig. 1B). TGH mRNA was detected as early as 1 day after birth with maximum expression at 27 days.

TGH transcription was detected with a modified nuclear run-on assay based on RT-PCR (Fig. 1C). Amplicons for both TGH and G3PDH were generated by RT-PCR of RNA obtained from isolated nuclei in which nuclear transcription proceeded in the absence of exogenously supplied rNTPs. Due to the method of isolation of the nuclei, it is unlikely that either amplicon represents cytoplasmic RNA contamination. Namely, lysing the cells in an iso-osmotic solution followed by purification of nuclei by sedimentation through a dense solution of sucrose (2M). It is also unlikely that amplification occurred from genomic DNA contamination or immature, unprocessed hnRNA since the PCR primers were designed so that they spanned four introns of the murine TGH gene (13). The only amplicons detected were those of the expected sizes based on completely spliced cDNA. Indeed, intact splicing machinery can be recovered from nuclei prepared with techniques similar to those employed here (20). Therefore mature transcripts for both TGH and G3PDH are present in the nucleus in low abundance, and are available to act as functional templates for RT-PCR. In livers from 7-day old mice, no TGH transcription was observed (Fig. 1C; + rNTP) above that for transcripts formed in the nuclei prior to nuclear isolation or with endogenous NTPs (Fig. 1C; - rNTP). In liver from 27-day old mice, TGH transcripts were much more readily detected when isolated nuclei were supplied exogenous rNTPs. All PCR reactions were terminated while products in the + rNTP reactions were still accumulating. These data suggest that the lower levels of cytosolic TGH mRNA (Fig. 1B) seen in livers of 7-day old mice might be due to a lower level of transcriptional activity from the TGH gene.
While TGH protein and mRNA expression appear to be temporally spaced, indicating regulation at a post-transcriptional level, the nuclear run-on assay presented here indicates that in the liver, postnatal expression of TGH mRNA is regulated at the level of transcription.

Functional assays of murine TGH promoter activity in primary hepatocytes.

To investigate the transcriptional regulation of the murine TGH gene, a 6 kb DNA fragment derived from a BAC clone, previously demonstrated to contain the entire murine TGH gene (13), was sequenced. This DNA fragment contains exon 1, most of intron 1 and extends 3 kb upstream of the transcription start site and presumably contains the promoter. Fig. 2 shows 542 bp of the 5' sequence flanking exon 1. This sequence is 59% identical to the rat (4), and 46% identical to the previously cloned human (22) promoter. No TATA box was found to precede the transcriptional start site. Potential binding sites for transcription factors were identified by searching the TRANSFAC database with the murine sequence using the Matinspector program (23). These sites include three Sp1 binding sites, a NF-1 and two SRE-like sequences. Interestingly, several of these binding sites are also present in the human and rat promoter sequences suggesting that we have cloned a functional promoter with evolutionarily conserved transcriptional regulatory patterns. Previous evidence for sterol regulation of TGH transcription in the rat has been reported (24, 25), suggesting that the SRE-like elements are functional. We tested whether or not the cloned sequence had promoter activity by designing fusion constructs of the 5'-flanking sequence linked to the luciferase gene. In addition, several constructs were made in
which 5-segments of the murine TGH gene were cloned upstream of the luciferase gene, and their promoter activities were tested in transient transfection assays in primary hepatocytes obtained from adult mice. A schematic illustration of the deletion constructs is shown in Fig. 3A.

The construct with the largest 5-extension was —542Luc followed by —313Luc. Transient transfection into primary murine hepatocytes revealed a reproducible reduction of luciferase activity (~50%) upon deletion of the sequence spanning -154 to —117 (Fig. 3B), suggesting that positive control elements reside in this segment. Others have observed that reporter constructs containing this conserved Sp consensus sequence from orthologous rat and human TGH promoters activate transcription while their elimination reduces promoter activity (21, 22).

Interaction of hepatic nuclear proteins with the murine TGH promoter.

A genomic fragment containing the 5 proximal region of the murine TGH gene (-542 to +112 of exon 1) was cut by restriction enzyme digestion to yield three smaller non-overlapping fragments designated as the distal (-542 to -371), medial (-370 to -65) and proximal (-64 to +112) promoter regions. Each of these promoter regions was evaluated for their ability to bind proteins from nuclear extracts prepared from 7- and 27-day old mice in an EMSA (Fig. 4). The medial promoter region exhibited enhanced protein binding with nuclear extracts prepared from 27-day old compared to 7-day old murine liver yielding three major differentially migrating protein-DNA complexes, C1, C2, and C3. Similar migrating complexes with the medial promoter region and nuclear extract from 7 day-old mice was also visible, albeit with considerably longer exposure.
times or with increased nuclear protein in the binding assay (not shown). While longer exposure times, or increased nuclear protein in the binding assay, also revealed weak protein-DNA complexes with distal and proximal promoter regions (not shown), identical migration profiles were observed with nuclear extracts from 7- and 27-day old murine livers for each of these regions.

**DNase I footprinting of the medial promoter region of the murine TGH gene.**

To identify the cis-elements within the medial promoter region that bind hepatic nuclear proteins, the medial promoter region was subjected to DNase I footprint analysis (Fig. 5A). Two protected regions within the medial promoter were observed using nuclear extracts from 27-day old murine livers. Footprint 1 extends from —156 to —138 (site A), and footprint 2 from —125 to —108 (site B). Sequence alignment of the 5'-proximal promoters of the murine, human, and rat TGH genes demonstrates that the protected regions corresponding to sites A and B have been evolutionarily conserved (Fig. 5B). Furthermore, DNase I footprinting of the human TGH proximal promoter using nuclear extracts derived from adult murine liver and HeLa cells also resulted in protection of the conserved site A (not shown). Thus, the nuclear proteins that bind to this sequence may have an important functional role in TGH expression along phylogenetic lines.

**The transcription factors Sp1 and Sp3 bind to the murine TGH promoter.**

Comparison of the DNA sequences for site A with a data base of transcription factor binding sites (23) revealed that this site contains a canonical binding site for Sp1, a ubiquitously expressed transcription factor required for the constitutive and inducible
expression of multiple genes (26). The Sp1 gene family includes at least four distinct but closely related proteins, Sp1, Sp2, Sp3 and Sp4, all of which recognize GC boxes with similar specificity and affinity (26). To establish the identity of the protein(s) binding to the medial promoter region of the TGH gene, we performed super-shift assays with antibodies specific for individual Sp proteins (Fig. 6). Antibodies specific for Sp1 were added to an EMSA reaction with the 32P-labeled TGH medial promoter fragment and nuclear extract from 27-day old murine liver. The mobility of the C2 complex was retarded so that the super-shifted C2 complex co-migrated with the C1 complex. These results indicate the presence of Sp1 in the C2 complex. The addition of antibodies specific for Sp3 did not result in super-shifted complexes but rather prevented the formation of both C1 and C3 complexes. The anti-Sp3 antibody used in this study is not directed against the DNA-binding domain of Sp3 and therefore is not expected to disrupt direct Sp3-DNA interactions. Sp3 may indirectly promote protein binding to the medial fragment, perhaps via protein-protein interactions, yielding the C1 and C3 complexes. Antibodies against Sp2 did not alter the mobility or formation of any of the three complexes. Sp4 expression in the mouse is limited to the brain (27) and, therefore, is not a candidate protein for binding to the murine TGH promoter in the liver.

It is likely that C1, C2 and C3 complexes arise from protein binding at sites A and B since each of these sites was protected from DNase I digestion by binding to protein (Fig. 5). In an attempt to assign the cis-element(s) responsible for protein binding in each of the 3 complexes, double stranded oligonucleotides corresponding to sites A and B were synthesized and evaluated in EMSAs for their abilities to compete with the medial fragment for protein binding (Fig. 7A). A 50-fold molar excess of unlabeled
double stranded oligonucleotides corresponding to site A resulted in abrogation of all three complexes as did unlabeled Sp consensus oligonucleotides (Sp) which contains a canonical Sp1 site (28). On the other hand, a double stranded oligonucleotide corresponding to site B did not compete with any of the proteins that bind to the medial promoter fragment. Two implications arise from these results. First, Sp nuclear proteins likely bind to site A. Second, binding of protein to site B requires Sp binding at site A.

Binding of sites A and B to nuclear proteins from murine liver was examined directly. As shown in Fig. 7B, labeled site A double stranded oligonucleotides formed only weak protein-DNA complexes with nuclear proteins from 7-day old murine liver, whereas equivalent amounts of nuclear proteins from 27-day old murine liver resulted in enhanced binding to site A, reminiscent of that seen with the medial fragment but yielding two distinct band shifts (s and f) rather than three. Both site A and Sp consensus double stranded oligonucleotides bound nuclear proteins from 27-day old murine liver more readily than with equivalent protein from 7-day old murine liver. In addition, both site A and the Sp oligonucleotides bound nuclear proteins resulting in the same number of band shifts with similar mobilities. These observations suggest that similar Sp proteins bind site A and the Sp consensus oligonucleotides. The supershift assay with labeled medial fragment (Fig. 6) supports a role for binding of Sp1 and Sp3 to site A. Furthermore, the nuclear protein(s) responsible for the observed band shifts may be limiting in the livers of 7-day old mice.

A double stranded oligonucleotide corresponding to site B did not bind nuclear protein (Fig 7B). This finding is consistent with the observation that a 50-fold molar excess of
unlabeled site B oligonucleotide failed to compete with the medial fragment of the TGH promoter for binding nuclear protein (Fig 7A). Protection of site B is likely the consequence of direct protein binding since binding of nuclear protein to site A yields differentially migrating complexes that account for two out of the three protein-DNA complexes seen with the medial promoter. However, the possibility that DNase I protection of site B resulted from altered secondary structure of DNA induced by protein binding at the adjacent site A cannot be discounted.

Super-shift assays shown above demonstrate that both Sp1 and Sp3 nuclear proteins are involved in binding to the medial fragment of the murine TGH promoter (Fig 6). Similar super-shift assays were performed to demonstrate specific interaction with site A (Fig. 7C). Addition of an antibody specific for Sp1 in an EMSA reaction with $^{32}$P-labeled site A double stranded oligonucleotide and nuclear extract from 27-day old murine liver super-shifted only a fraction of the slower migrating complex (s). However, this complex was completely super-shifted by including 10-fold more anti Sp1 into the super-shift assay (not shown). Addition of an antibody specific for Sp3 super-shifted the faster migrating complex (f). Identical super-shift profiles were obtained with labeled Sp consensus oligonucleotides (not shown), consistent with the band shifts observed by direct Sp1 and Sp3 binding in other studies (26). Taken together, these data suggest that both Sp1 and Sp3 are capable of binding to the site A element.

**Immunoblot analysis of candidate transcription factors that bind to site A and site B of the murine TGH promoter.**
One mechanism that might confer increased binding of nuclear transcription factors to site A of the murine TGH promoter at the time of weaning is increased hepatic expression of Sp1 and/or Sp3. We examined the levels of Sp1 and Sp3 protein by immunoblot analysis of nuclear extracts isolated from the livers of 7- and 27-day old mice. As shown in Fig. 8A, low molecular weight proteins were detected with anti-Sp1 antibody in both nuclear extracts. The amount of proteins that specifically cross-reacted with the anti-Sp antibodies was diminished when a peptide against which the antibody was raised was included in the incubation. The proteolysis of Sp1 appeared to be specific since the same nuclear extracts contained immuno-reactive Sp3 having only those molecular weights that would be expected to arise from alternate translational start sites. Interestingly, we observed proteolysis of Sp1, but not Sp3, in nuclear extracts obtained from brain, heart, kidney, and spleen (not shown). Nuclear extracts from livers of 7- and 27- day-old mice contained full-length Sp1 and smaller polypeptide bands with similar profiles and abundance. The levels of Sp3 were also similar in both nuclear extracts. The differences between the levels of Sp1 and Sp3 in day 7 and day 27 hepatic nuclear extracts were not an artifact of the nuclear extract preparation since the levels of the nuclear protein YY1, a ubiquitously expressed transcription factor that interacts with a number of key regulatory proteins (e.g. TBP, TFIIB, TAFII55, Sp1, and E1A) were not affected (29). Therefore, it is not likely that enhanced binding of these two factors at day 27 is due to alteration in the abundance of Sp1 and Sp3.

Altered levels of phosphorylation of Sp1 result in changed DNA binding activity (30-34). Since the levels of Sp1 and Sp3 in the liver were found to be similar at days 7 and 27, we considered the possibility that phosphorylation modulates binding of these
factors to the TGH promoter. Fig. 7C shows that nuclear extracts that had been
dephosphorylated with calf intestinal alkaline phosphatase exhibit reduced binding of
both Sp1 and Sp3 to site A indicating a role for phosphorylation in the binding of Sp
proteins to the TGH promoter.

Comparison of the DNA sequences for site B with a data base of transcription factor
binding sites (23) revealed that this site contains a degenerate E box with the expected
half sites for E47 and Th1. E47 is a widely expressed member of the E family of basic
helix-loop-helix (bHLH) proteins. These factors play important roles in differentiation
processes as diverse as skeletal myogenesis, neurogenesis, and hematopoiesis and
function as either homodimers or heterodimers with other classes of bHLH proteins to
modulate gene expression. For example, Myo D (a muscle-specific bHLH protein)
partners with E proteins, and the heterodimers bind avidly to consensus (CANNTG) E
box motifs that are functionally important elements in the upstream regulatory
sequences of many muscle-specific terminal differentiation genes. Th1 was identified as
a member of a family of bHLH transcription factors using a modified two-hybrid screen
of a murine embryo cDNA library with the Drosophila E protein daughterless, a
Drosophila counterpart of mammalian E proteins with specificity very similar to that of
E47 (16). Interestingly, like TGH, Th1 has a developmentally regulated expression
pattern that is both stage- and tissue-specific; Th1 mRNA is expressed in the heart and
certain neural crest derivatives during embryogenesis and in adult tissue expression is
specific to gut and liver (16). We hypothesized that during the suckling period a lack of
E47 and Th1 might account for the decreased binding of nuclear factors to the B site of
the TGH promoter and perhaps for the decreased expression of TGH.
As shown in Fig. 8B, E47 is increased in hepatic nuclear extracts derived from 27-day old mice. This increase is not due to the amount of protein loaded since the nuclear levels of YY1 were similar at day 7 and day 27. However, we could not demonstrate an interaction of E47 with the medial promoter by immuno-depletion of nuclear extracts or super-shift assays (not shown), suggesting that E47 does not interact with site B as a heterodimer with ThI. The possibilities that Th1 interacts with site B as a monomer or as a homodimer cannot be eliminated.

*Transactivation of the murine TGH promoter in Drosophila Schneider S2 cells*

Previous reports have documented that Sp1 and Sp3 can interact with each other and, in cooperation with other nuclear transcription factors, either synergize or antagonize each other’s activity at any given DNA-binding site (26). Since Sp1 binds to site A and Sp3 is involved in protein binding at site B, we next determined whether or not there was a functional interaction between Sp1 or Sp3 with ThI, a potential candidate for binding to site B. For this purpose, expression plasmids for each of these nuclear transcription factors were co-transfected into SL2 cells, and the activity of (-154)Luc was monitored (Fig. 9). Co-expression of Sp1 expression plasmid resulted in a 10-fold increase in luciferase activity \( (p<0.005) \) indicating that Sp1 is a potent activator of the TGH promoter. By contrast, co-expression of the luciferase construct with the Sp3 expression plasmid had neither a stimulatory nor an inhibitory effect on transactivation. Co-expression of the luciferase construct with equivalent amounts of both Sp1 and Sp3 expression vectors (not shown) did not affect Sp1-stimulated promoter activity indicating that Sp3 does not compete with Sp1 for site A. It is noteworthy that
this lack of trans-activation potential of Sp3 is not due to lack of expression of Sp3 as determined by immunoblot analysis (not shown). Transfection of Th1 expression plasmid did not by itself affect promoter activity but, when co-transfected, attenuated Sp1-stimulated promoter activity (p<0.005) and positively cooperated with Sp3 to trans-activate the promoter above basal activity, albeit modestly (p<0.05) (Fig. 9).

DISCUSSION

Enhanced interaction of hepatic nuclear proteins from weaned mice with a putative Sp binding site and a degenerate E box motif within the murine TGH proximal promoter.

Marked changes in energy metabolism occur during the transition from fetal to postnatal life and throughout the weaning period. The lipoprotein profile of rats is not completely developed until about 4 weeks after birth, corresponding to the onset of weaning (35). In addition, rat hepatocytes from fetal and suckling rats secrete substantially lower quantities of TG and apoB than do hepatocytes from adult rats (36). In this study we have demonstrated that TGH mRNA expression is induced in murine liver at the time of weaning (see Fig. 1B). Nuclear run-on analysis (Fig. 1C) revealed that this up-regulation occurs mainly at the level of transcription. Since apoB mRNA is as abundant in 18-day fetal liver as at any subsequent period of hepatic development (37) we postulated that the developmental increase in TGH expression may be a necessary event in the ontogeny of VLDL assembly/secretion. Hence, we investigated the regulation of TGH expression by analyzing cis-regulatory elements in the TGH promoter and their corresponding transcription factors. These analyses revealed the
formation of three specific complexes with a fragment (-370 to -65) of the TGH promoter that were enhanced in nuclear extracts prepared from murine adult liver. DNase I footprinting experiments localized these complexes to two adjacent, yet distinct, cis-elements; a putative Sp binding site and a site containing a degenerate E box with putative half sites for E47 and Th1.

*Sp1 and Sp3 interact with the murine TGH proximal promoter.*

The TGH proximal promoter lacks a TATA motif. In the absence of a TATA box, mechanisms other than direct recruitment of TATA-binding proteins have been implicated in initiating formation of the basal transcription complex. In general, GC-rich promoters are usually considered to be a target for regulation by zinc finger transcription factors, and TATA-less promoters have been shown to be particularly sensitive to regulation by the Sp family of proteins (38-40). Sp1, originally identified as a cellular transcription factor necessary for SV40 gene expression, is a ubiquitous nuclear protein that activates the transcription of a wide variety of cell-type specific genes, including the myeloid specific integrin gene CD11β (41), the monocytic specific gene CD14 (42), the liver specific gene encoding α1 acid glycoprotein (43), and the α2 integrin gene (44). Further DNA binding studies identified a family of zinc-finger (His2-Cys2) transcription factors that includes Sp1, Sp2, Sp3, Sp4, and two distantly related proteins termed BTEB (basic transcription element-binding) and BTEB2 (26). These new members of the Sp family recognize GC boxes with specificities and affinities that are similar to those for Sp1. The apparent independence of members of the Sp family from requiring the classic binding proteins to activate transcription, together with the abilities of Sp
factors to associate with individual components of the basal transcriptional machinery, suggest that the Sp family of transcription factors regulate TATA-less promoters by bypassing selective steps in assembly of the core transcription machinery. The predilection of the Sp family of transcription factors to regulate TATA-less promoters is also evident in the case of the TGH promoter. Results of the deletional analysis indicate that the sequence between —156 and —117 of this promoter contains positive regulatory *cis* elements. DNase I footprint analysis and computer assisted analysis of the intervening sequence revealed the presence of a canonical binding site for the Sp family of proteins. EMSA experiments established that the Sp consensus element, which we termed site A, preferentially bound Sp1 and Sp3 from liver nuclear extracts obtained from adult, weaned mice rather than from suckling mice. In addition, three specific protein-DNA complexes were formed with a fragment spanning —373 to —65 of the TGH promoter. Whereas we were able to determine that Sp1 protein was present in one of these protein-DNA complexes (C2), the identity of the proteins in the fastest- and slowest-migrating (C3) complexes remains unknown. However, the observation that an antibody against Sp3 prevented formation of C1 and C3 rather than super-shifting them implicates an indirect role for Sp3 in stabilizing the C1 and C3 protein-DNA complexes, perhaps by protein-protein interactions. The results of the competition experiments with Sp consensus sequence oligonucleotides suggest that the DNA binding specificity of the protein(s) in the C1 and C3 complexes is similar to that of the Sp family of proteins. BTEB is a protein of smaller size than Sp1 with DNA binding specificity similar to that of Sp1. Thus, BTEB or a related protein is a candidate for the protein involved in the
formation of the C1 and C3 complexes. The precise role of the proteins involved in formation of these complexes awaits the identification of these proteins.

Evidence that phosphorylation modulates Sp binding to the murine TGH proximal promoter.

Although Sp1 is ubiquitously expressed, recent evidence suggests that Sp1 expression (45), binding affinity (46), and post-transcriptional modifications (47, 48) might be modulated to confer tissue-specific and developmental regulation of target genes (49). Using immunoblot analysis, we have demonstrated that the levels of Sp1 and Sp3 in liver nuclear extracts from 7- and 27-day old mice are the same and therefore increased amounts of these proteins do not account for the increased binding to the TGH promoter. Several studies have implicated a role for phosphorylation of Sp proteins in modulating both their DNA binding activity and trans-activation potential. For example, phosphorylation of Sp1 by DNA-dependent protein kinase is induced by Sp1 binding to HIV-1 Tat protein in vitro and phosphorylation of Sp1 by this kinase has been correlated with changes in Sp1-directed transcription in vivo (30). Other reports suggest that Sp1 is an in vivo target for phosphorylation by protein kinase A leading to increased DNA binding and transcription activity of some cAMP-responsive genes (31, 32). In renal carcinoma cells, Sp1 physically interacts with protein kinase Cζ and co-expression of Sp1 with protein kinase Cζ increases Sp1-mediated transcription. A dominant-negative protein kinase Cζ mutant has been shown to interact with Sp1 but Sp1-mediated transcriptional activity was not increased (33). Induction of the rat ornithine decarboxylase gene by serum requires promoter regions that contain multiple Sp1
binding sites and serum stimulation activated Sp1 binding activity 3- to 12-fold in a rat fibroblast cell line, without an increase in the quantity of Sp1 protein. Treatment of the extracts with potato acid phosphatase drastically reduced the induction of DNA binding activity suggesting that phosphorylation of Sp1 is necessary for increased DNA binding in response to serum stimulation (34). In this study, we provide evidence suggesting that Sp binding to the TGH promoter at the time of weaning is modulated by phosphorylation since treatment of nuclear extracts with calf intestinal alkaline phosphatase abrogated binding to the site A element as well as formation of the C1, C2 and C3 complexes with the medial fragment of the TGH promoter.

Sp1 is a potent activator of the murine TGH promoter in SL2 cells.

In general, Sp1 functions as a transcriptional activator. By contrast, Sp3 is a bifunctional protein with independent domains that can both activate and repress transcription. The predominant function of Sp3 depends upon both the promoter and the cellular milieu (50). Our results indicate that there is a differential sensitivity of the TGH promoter to these two proteins with Sp1 being a potent activator and Sp3 having no effect on reporter activity in SL2 cells. From our EMSA studies, it is clear that Sp3 can bind to the site A element when presented outside of the context of the TGH promoter. However, we could not demonstrate a direct interaction of Sp3 with the medial fragment of the TGH promoter but rather an indirect role for Sp3 in the binding of nuclear proteins to the TGH promoter. It is possible that the SL2 cell line lacks the endogenous proteins required for mediating an indirect Sp3-induced trans-activation or trans-repression of the TGH promoter.
Evidence for an indirect interaction of Sp3 with the murine TGH promoter.

TGH gene expression displays a distinct ontogenic pattern with expression being minimal after birth and throughout the suckling period but increasing significantly at the time of weaning. The paucity of TGH during the suckling period correlates with the inability of mice to secrete significant levels of VLDL until the time of weaning. The DNase I footprint analysis of the medial promoter fragment revealed two cis-elements within the medial fragment of the TGH promoter that bind nuclear proteins from livers obtained from weaned animals. This study revealed that one of these cis-elements, site A, binds Sp1 yielding the C1 complex. We also show that Sp1 acts as a potent activator of TGH promoter activity in SL2 cells. It is likely that binding of protein to site B is responsible for the C1 and C3 complex. Furthermore, binding of protein at this site may indirectly require Sp3 since both an antibody to Sp3 and an Sp consensus oligonucleotide prevents C1 and C3 complex formation. Studies have revealed that three Sp3 variants of molecular sizes 115, 80, and 78 kDa are abundantly expressed in a broad range of tissues (51) and we show by immunoblot analysis that these variants are present in the liver nuclear extracts used. The involvement of any two of these Sp3 variants with a protein(s) that interacts at site B could give rise to the two differentially migrating protein-DNA complexes, namely C1 and C3.

Th1 modulates Sp-induced TGH promoter activity in SL2 cells.

Computer assisted analysis of site B indicates that this site contains a degenerate E box with putative half sites for E47 and Th1 transcription factors. While the expression
of both of these proteins is increased in adult liver, hetero-dimerization of Th1 with E47 at site B is unlikely since an antibody against E47 did not affect the migration of the C1, C2 or C3 complexes. Our trans-activation studies in SL2 cells demonstrate only a minor role for Th1 in modulating Sp-induced promoter activity. However, we cannot eliminate the possibility that SL2 cells contain endogenous proteins that could compete with Th1 for binding to site B.

In summary, we identify and partially characterize the promoter-regulatory region controlling expression of the transcript of the murine TGH gene. Our studies establish that the 5'-flanking region of the murine TGH gene exhibits promoter activity. We identify and characterize a positive cis-regulatory element in the TGH promoter that interacts with Sp1. We also define an adjacent second cis element in the TGH promoter capable of binding nuclear proteins from adult murine liver. Although we are uncertain which transcription factor(s) binds this element, we suggest that binding of protein to this element is modulated by Sp3. A role for Sp1 and Sp3 in the ontogenic profile of TGH gene expression in the liver is suggested by the interactions of these proteins with the TGH promoter.

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Richard Lehner is a Scholar and Dennis Vance a Medical Scientist, of the Alberta Heritage Foundation for Medical Research

REFERENCES


Figure Legends

Fig. 1. **Developmental expression of hepatic microsomal TGH.** (A) Immunoblot analysis of TGH. Proteins of liver homogenates (20 g) prepared from mice of various ages (C is for control, rat liver microsomes) were resolved on 12% SDS-polyacrylamide gels, electroblotted onto nitrocellulose membranes and probed with anti-TGH antibody. Membranes were stripped and re-probed with an antibody directed against β-actin. These results represent one of 3 experiments. (B) Northern blot analysis of TGH mRNA. Total RNA (2.5 g) was extracted from livers of mice of the indicated ages, separated on a 1.2% agarose/formaldehyde gel, transferred to a HyBond-N+ nylon membrane and probed with [³²P]-labeled TGH oligo pTGHII. As a control, the expression of glycerol-3-P dehydrogenase (G3PDH) was also measured. (C) In vitro nuclear transcription reaction. Intact nuclei were isolated from livers of 7- and 27-day old mice (d7 and d27, respectively) and nuclear transcription reactions performed with (+NTPs) or without (-NTPs) ribonucleoside triphosphates. Following transcription, nuclei were lysed and hnRNA isolated. Transcripts encoding TGH and G3PDH were detected by RT-PCR using gene specific primers: EX6F (sense) and EX10R (antisense) for TGH mRNA and G3PDH1A (sense) and G3PDH2B (antisense) for G3PDH. PCR products were analyzed by agarose gel electrophoresis and sequences were confirmed by sequencing. These results are representative of 3 experiments performed with individual nuclei preparations.

Fig. 2. **Nucleotide sequence in the vicinity of the transcription start site for the murine TGH gene.** The 542-bp DNA sequence preceding exon 1 (lower case) is
shown. Putative binding sites for transcription factors are shown in boldface. The transcription start site is indicated by +1. The translational start codon is underlined and in bold. DNA sequences demonstrated to bind proteins (sites A and B) are underlined.

Fig. 3. **Promoter activity of TGH-luciferase gene chimeras transiently transfected into murine primary hepatocytes.** (A) Localization of promoter fragments is shown and putative transcription factor consensus sites are indicated. Numbers indicate the relative positions with respect to the start of transcription. (B) Nested 5'-deletion promoter fragments were cloned upstream of the luciferase coding region in pGL3Basic and transiently transfected into murine primary hepatocytes. Luciferase activity was normalized to equal amounts of protein. Data represent the means – SD of three separate experiments, each performed in triplicate.

Fig. 4. **Binding of nuclear extracts to TGH DNA (-542/+112) by EMSA analysis.** Liver nuclear extracts were prepared from 7- and 27-day old mice (d7 and d27, respectively) and used in binding assays with each of 3 32P-labeled promoter fragments: distal (-542/-371), medial (-370/-68), and proximal (-67/+112). Protein-DNA complexes were resolved on 3.5% non-denaturing PAGE with Tris-borate-EDTA buffer and were detected by auto-radiography. These results are representative of 5 independent experiments, each using freshly isolated nuclear extracts.

Fig. 5. **DNase I footprint analysis of the medial (-370/-68) fragment of the murine TGH promoter.** (A) Nuclear proteins (0, 10, 20 and 50 g) from the livers of 27-day old
mice were incubated with single-end labeled medial fragment of the murine TGH promoter. DNase footprint analysis was performed. Sequencing reactions (A/G and C/T) were performed using a DNA sequencing kit (Sigma, Seq-1) in parallel. Labeled DNA fragments were resolved on a 5% polyacrylamide, 7 M urea sequencing gel and observed by auto-radiography. Nuclear proteins enhanced the footprint of a GC-rich element -156 to —138 (site A) and -125 to —108 (site B) in a dose-dependent fashion. These results are representative of 3 individual experiments each using freshly isolated nuclear extracts. (B) Alignment of protected murine TGH sites A and B with rat and human TGH promoters.

Fig. 6. Supershift analysis of protein binding to the TGH promoter using EMSA.

Hepatic nuclear extracts were prepared from 7- and 27-day old mice (Day 7 and Day 27, respectively) and used in binding assays with the 32P-labeled medial (-370/-68) promoter fragment with (+) or without (-) antibodies against Sp1 (Anti-Sp1), Sp2 (Anti-Sp2) or Sp3 (Anti-Sp3). Protein-DNA complexes and super-shifted complexes were resolved on 3.5% non-denaturing PAGE with Tris-borate-EDTA buffer and were detected by auto-radiography. Three differentially migrating complexes, C1, C2 and C3, are indicated by arrowheads. These results are representative of 5 independent experiments, each using freshly isolated nuclear extracts.

Fig. 7. Analysis of protein-DNA interactions at site A and site B using competitive EMSAs. (A) Band shift assays were performed with hepatic nuclear extracts from 27-day old mice using the medial (-370/-68) promoter fragment as a probe. Unlabeled
oligonucleotides, derived from TGH sites A, B and Sp consensus oligonucleotides, were added to the binding assays as competitors. Results are representative of 5 independent experiments, each using freshly isolated nuclear extracts. (B) EMSAs were performed with liver nuclear extracts from 7- and 27-day old mice. Oligonucleotides derived from TGH sites A, B and Sp consensus oligonucleotides were used as probes. These results are representative of 5 different experiments each with freshly isolated nuclear extracts. (C) EMSAs were carried out with liver nuclear extracts from 27-day old mice with site A (-156 to —138) oligonucleotide as probe. Extracts were treated with calf intestine alkaline phosphatase (CIAP), with phosphatase and phosphatase inhibitors (CIAP + Pase Inh), or with antibodies against Sp1 (Anti-Sp1) or Sp3 (Anti-Sp3). These results are representative of 3 independent experiments, each using freshly isolated nuclear extracts.

Fig. 8. Immunoblot analysis of Sp1, Sp3 and E47 proteins in hepatic nuclear extracts. Hepatic nuclear proteins (10 g) were isolated from from 7- and 27-day old mice, resolved by 12% SDS-polyacrylamide gels, electroblotted onto nitrocellulose membranes and reacted with (A) anti-Sp1 (left panel), stripped then re-probed with anti-Sp3 (right panel). In each case, HeLa cell nuclear extracts (Promega) were loaded as a positive control. Parallel primary antibody incubations contained corresponding peptides as negative controls (+/- peptide, as indicated). These results are representative of at least 3 independent experiments, each using freshly isolated nuclear extracts. The arrow indicates normal migration of Sp1 or Sp3. The asterisks indicate proteolytic products of Sp1. (B) Immuno-blots for E47. Membranes shown in panels A and B were
stripped and re-probed for YY1 to control for protein loading. These results are representative of 3 independent experiments, each using freshly isolated nuclear extracts.

Fig. 9. Transactivation assays of -154Luc in Drosophila Schneider cells co-transfected with Sp1, Sp3 and/or Thil. —154Luc (5 g) and the indicated plasmid constructs (5 g of each) were co-transfected into Drosophila Schneider SL2 cells, and luciferase activity was measured. Luciferase-specific activity in cell homogenates was normalized for transfection efficiency monitored by co-transfection of a plasmid (5 g) expressing GFP and counting the number of cells expressing GFP relative to total number of cells as determined by flow cytometry. All transfections were performed with 20 g plasmid DNA and in all cases control plasmids were included. Data represent the means – S.E.of 3 independent experiments, each performed in duplicate. Statistical analysis was performed using a one-way Anova test followed by a post hoc Student-Newman-Keuls test. Values for p less than 0.05 were taken to be significant. *, p<0.005 compared with —156Luc; **, p<0.005 compared with pPacSp1; #, p<0.05 compared with pPacSp3.
Figure 5

A

Nuclear Protein (µg)
0 10 20 50

Site A
-156
-138

Site B
-125
-108

B

Site A Affinity

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Site B Site

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Figure 6

- + + + + + + + + Nuclear Extract
- - + - - - + - - Anti-Sp1
- - - + - - - + - - Anti-Sp2
- - - - + - - - + - - Anti-Sp3

Day 7 | Day 27

C1
C2
C3
Figure 9

Luciferase Activity (RLU/mg protein) normalized to GFP expression.
A role for Sp1 in the transcriptional regulation of hepatic triacylglycerol hydrolase in the mouse

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