Characterization of the Murine Fatty Acid Transport Protein Gene and Its Insulin Response Sequence*

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Fatty acid transport protein (FATP) was identified by expression cloning strategies (Schaffer, J. E., and Lodish, H. F. (1994) Cell 79, 427–436) and shown by transfection analysis to catalyze the transfer of long-chain fatty acids across the plasma membrane of cells. It is expressed highly in tissues exhibiting rapid fatty acid metabolism such as skeletal muscle, heart, and adipose. FATP mRNA levels are down-regulated by insulin in cultured 3T3-L1 adipocytes and up-regulated by nutrient depletion in murine adipose tissue (Man, M. Z., Hui, T. Y., Schaffer, J. E., Lodish, H. F., and Bernlohr, D. A. (1996) Mol. Endocrinol. 10, 1021–1028). To determine the molecular mechanism of insulin regulation of FATP transcription, we have isolated the murine FATP gene and its 5′-flanking sequences. The FATP gene spans ~16 kilobases and contains 13 exons, of which exon 2 is alternatively spliced. S1 nuclease and RNase protection assays revealed the presence of multiple transcription start sites; the DNA sequence upstream of the predominant transcription start sites lacks a typical TATA box. By transient transfection assays in 3T3-L1 adipocytes, the inhibitory action of insulin on FATP transcription was localized to a cis-acting element with the sequence 5′-TGTTTTTC-3′ from −1347 to −1353. This sequence is very similar to the insulin response sequence found in the regulatory region of other genes negatively regulated by insulin such as those encoding phosphoenolpyruvate carboxykinase, tyrosine aminotransferase, and insulin-like growth factor-binding protein 1. Fluorescence in situ hybridization analysis revealed that the murine FATP gene is localized to chromosome 8, band 8B3.3. Interestingly, this region of chromosome 8 contains a cluster of three other genes important for fatty acid homeostasis, lipoprotein lipase, the mitochondrial uncoupling protein 1 (UCP1) and sterol regulatory element-binding protein 1. These results characterize the murine FATP gene and its insulin responsiveness as well as present a framework for future studies of its role in lipid metabolism, obesity, and type II diabetes mellitus.

The plasma membrane in mammalian cells forms a physical barrier between the intracellular and extracellular environments. A variety of protein carrier systems have been identified which facilitate the movement of metabolites such as sugars and amino acids across the membrane (1–3). In contrast, the mechanism(s) allowing fatty acids to traverse the membrane are poorly understood. Kinetic analysis of the movement of fatty acids across membranes suggests two components: a high affinity, low capacity protein-mediated transport process and a low affinity, high capacity diffusional event (4). Although fatty acids are hydrophobic and are capable of rapidly diffusing through the lipid bilayer when present in high concentrations (5–7), a large body of evidence supports the presence of a protein-mediated carrier system that operates at low substrate concentrations. Because extracellular fatty acids are in equilibrium with albumin, and intracellular fatty acids associate with abundant cytoplasmic fatty acid-binding proteins, the concentration of free, unbound fatty acids traversing the plasma membrane is quite low, estimated to be in the nanomolar range or lower (4). These observations suggest that under normal physiological conditions, a significant fraction of fatty acid transport in animal cells occurs via a protein-mediated system.

Permeation of long chain fatty acids into mammalian cells demonstrates saturation kinetics (8–10), competitive inhibition (11), and is inhibited by prior treatment with phloretin and other protein-modifying agents (11–13). The molecular entities responsible for a protein-mediated fatty acid transport process have not been fully characterized, but several proteins have been identified by their affinities for long-chain fatty acids and are proposed to be either fatty acid transporters or components of a fatty acid transport complex (10,14–16). Employing expression cloning strategies as an alternative approach, Schaffer and Lodish (17) identified a novel protein, termed fatty acid transport protein (FATP)1 by virtue of its ability to elevate cellular long-chain fatty acid uptake when expressed in COS7 fibroblasts. FATP is a 63-kDa integral plasma membrane protein with four predicted transmembrane domains. It is expressed to the greatest extent in tissues exhibiting high levels of plasma membrane fatty acid flux such as muscle, heart, and adipose (17), whereas expression levels in other tissue types is considerably lower. FATP mRNA expression in 3T3-L1 adipocytes is increased during adipose conversion (17) and is under hormonal control (18). Northern blot analysis showed that

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** The abbreviations used are: FATP, fatty acid transport protein; PEPCK, phosphoenolpyruvate carboxykinase; IGFBP-1, insulin-like growth factor binding protein-1; IRS, insulin responsive sequence; HNF-3, hepatic nuclear factor-3; DMEM, Dulbecco’s modified Eagle’s medium; BAC, bacterial artificial chromosome; nt, nucleotide(s); RT-PCR, reverse transcriptase-polymerase chain reaction; PIPES, 1,4-piperazinediethanesulfonic acid; kb, kilobase pair(s); bp, base pair(s); PLE, phosphoenolpyruvate carboxykinase-like elements.

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FATP mRNA levels increased 5–7-fold during 3T3-L1 preadipocyte differentiation, whereas insulin down-regulated FATP mRNA levels 10-fold in cultured adipocytes (17, 18). Regulation of FATP expression by insulin is rapid, reversible and is exerted at the transcriptional level. In animal studies, FATP mRNA levels in murine adipose tissue increased 11-fold during short term fasting, consistent with the role of insulin as a negative regulator of FATP gene expression (18). Further supporting the effect of insulin on FATP expression, recent studies by Berk and colleagues (19) demonstrated that FATP mRNA levels were increased 5-fold in insulin-resistant Zucker rats when compared with control animals.

Insulin effects on gene transcription are believed to be mediated by modulating the activities of trans-acting transcription factors that bind to cis-acting DNA elements. A number of insulin responsive sequences (IRs) have been characterized (for review, see Ref. 20). Unlike other hormone responsive elements, the identification of a single consensus sequence for an insulin response element has been elusive. This complexity probably reflects the existence of multiple mechanisms by which insulin affects transcription. While most IRs have unique sequences that differ by several nucleotides, a number of genes that are negatively regulated by insulin share a common responsive sequence T/GA/TTTTT. Genes negatively regulated by insulin include those for phosphoenolpyruvate carboxykinase (PEPCK, Ref. 21), insulin-like growth factor binding protein-1 (IGFBP-1, Ref. 22), and tyrosine aminotransferase (FAS). For each of these genes, hormonal control of transcription follows a similar theme, glucocorticoid stimulation of gene transcription, a process inhibited by insulin. Interestingly, the IRS of these genes functionally coincides with the glucocorticoid response element. Hepatic nuclear factor-3 (HNF-3) is believed to be the accessory factor that mediates the inductive effects of glucocorticoids on gene transcription (24–26). However, HNF-3 does not directly mediate the action of insulin (25). The identity of the insulin response factor(s) is unknown, but it is speculated that this protein factor interferes with the binding/transactivation potential of HNF-3 and thereby inhibits the stimulatory effect of glucocorticoids.

To understand the regulation of FATP gene transcription in adipocytes, we report here the cloning and sequence analysis of the gene and its flanking sequences, the mapping of its chromosomal location and the characterization of its promoter. In addition, we have located an insulin responsive sequence which bears a striking similarity to that of the PEPCK, IGFBP-1, and tyrosine aminotransferase genes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**3T3-L1 preadipocytes were grown to confluence and induced to differentiate to adipocytes as described previously (27). Briefly, preadipocytes were cultured in DMEM, 10% calf serum and 2 days after reaching confluence, differentiation was induced by culturing cells in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 174 mM insulin, 0.5 mM methylisobutylxanthine, and 0.25 mM dexamethasone. Methylisobutylxanthine and dexamethasone were withdrawn after 2 days whereas insulin was withdrawn after four days. Differentiated cells were maintained in DMEM with 10% fetal bovine serum until adipocytes (days 9–12, referred to as mature adipocytes) were used for transfection experiments.

Transfections were performed by lipofection. Plasmids for transfection were purified by Qiagen Plasmid Midi Kit (Chatsworth, CA). Typically, for each of a 12-well culture plate, 500 ng of firefly luciferase plasmid pLuc-NR (Promega, Madison, WI), a control vector containing a sea pansy luciferase gene (Renilla reniformis) luciferase gene driven by a cytomegalovirus promoter. DNA in DMEM was complexed with Lipofectamine (Life Technologies, Gaithersburg, MD) at the ratio of 1.5 (w/v) for 45 min before addition to the cells. An equal volume of 20% fetal bovine serum in DMEM was added 4 h later. The transfection mixture was removed 20 h later and the cells were maintained in DMEM containing 10% fetal bovine serum for a further 48 h before lysis for reporter assays.

**Luciferase Assays—**Luciferase activities were assayed with the Dual Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's protocol. Brieﬂy, transfected cells were harvested once cells were in a 1:3 ratio of cell pellet to cell lysate. Cell pellets were lysed with 100 μl 1× Passive Lysis Buffer. Lysed cells were harvested by scraping with a rubber policeman. Firefly luciferase activities were normalized to sea pansy luciferase activities to adjust for transfection efficiency.

**Isolation of FATP Gene and Analysis—**A mouse genomic library in λEMBL-3 SP6/T7 (a gift from Dr. Harry Orr, University of Minnesota) was screened by using a 32P-labeled FATP cDNA as a probe. Positive clones were isolated and purified by three successive rounds. The bacterial artificial chromosome (BAC) clone was obtained from Genome Systems (St. Louis, MO).

Double-stranded sequencing was performed at the Obesity Center, University of Minnesota. All exons and exon-intron boundaries were sequenced. Intron sequences, except that of introns 3 and 7, were also determined. Plasmid DNA templates for sequencing were prepared using the Quantum Prep Plasmid Kit (Bio-Rad). The BAC DNA template was prepared by a modified protocol using Qiagen Plasmid Midi Kit (Chatsworth, CA). Nucleotide sequences were analyzed and assembled by the GCG sequence analysis programs Version 8.1 (Genetics Computer Group, Madison, WI) under UNIX emulation. Potential transcription factor-binding sites were identified using the SignalScan program (University of Minnesota). Southern blotting on mouse genomic DNA was performed by standard procedures (28). The blot was hybridized to full-length FATP cDNA or a 125-nucleotide exon 3-specific probe amplified by PCR using pBS77 × 1 as the template.

Probe for fluorescence in situ hybridization experiments was labeled digoxigenin-DUTP by nick translation. Hybridization with normal metaphase chromosomes from male embryonic stem cells was carried out in 50% formamide, 10% dextran sulfate, and 2× SSC. Fluorescence in situ hybridization-labeled antibody against was used for detection of signal and chromosomes were counterstained with 4',6-diamidino-2-phenylindole.

**RT-PCR—**Total RNA from 3T3-L1 adipocytes was reverse transcribed using avian myeloblastosis virus reverse transcriptase (Promega) and the primer, E4, which is complementary to a portion of exon 4 of the FATP gene. FATP cDNA was then PCR amplified using standard conditions and Taq polymerase (Promega). The primers used were E1 and E3, which anneal to exons 1 and 3 of the FATP gene, respectively. Primer sequences are as follows: E4, 5′-GCCGGCTCTGGTGTCCGCGGGCCGC-3′; E1, 5′-CTCAGAGGAGGACGACAGAG-3′; and E3, 5′-CGGGCGCGCGCGCGGTCGAGC-3′.

**S1 Nuclease Protection Assay—**A 383-nt single-stranded DNA probe, extending from the 3′ end of exon 1, was generated by primer extension. The primer PE3 (Fig. 3) was 5′ end-labeled with γ-32PATP by polynucleotide kinase reaction. DNA template was prepared by linearizing plasmid pBS77 × 1, which contains exons 1–3 and the upstream flanking sequences, with HindIII. DNA probe was synthesized by Klenow polyadenylated and purified by urea/polyacrylamide gel electrophoresis. Total RNA was isolated from 3T3-L1 adipocytes by the guanidinium thiocyanate method (29).

Reagents for S1 analysis were purchased from Ambion (Austin, TX). 10 μg of 3T3-L1 adipocyte total RNA or yeast tRNA was hybridized with 5 × 10^5 cpm end-labeled probe in a solution containing 80% formamide, 0.4 M NaCl, 40 mM PIPES, pH 6.4, and 1 mM EDTA for 12–16 h at 42 °C. Samples were then diluted 15-fold with ice-cold S1 nuclease digestion buffer to yield a final concentration of 0.2 M NaCl, 30 mM NaOAc, pH 4.5, 5 mM ZnCl2, and 0.05 μg/μl denatured salmon sperm DNA and digested with 300 units of S1 nuclease at 25 °C for 60 min. S1-resistant hybrids were analyzed by electrophoresis on a 6% polyacrylamide sequencing gel, and the protected probe was visualized by autoradiography.

**RNAase Protection—**An antisense RNA probe complementary to the FATP promoter (−131 to +85) was generated by in vitro transcription using T7 DNA polymerase. The template for the reaction was pBS-CIF digested with StyI. 10 μg of day 8 3T3-L1 DNA was dissolved in 30 μl of hybridization buffer (80% formaldehyde, 0.4 M NaOAc, 1 mM EDTA, 50 mM PIPES, pH 6.4) containing 5 × 10^5 cpm of antisense RNA probe. After denaturing at 95 °C for 5 min, samples were incubated at 45 °C overnight to allow annealing. 300 μl of RNAase digestion buffer (200 mM NaOAc, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5) was added with 1 unit of RNase ONE (Promega, Madison, WI) and incubated an additional 30 min at 37 °C. To terminate the digestion, 5 μl of Stop solution (10% SDS, 4 μg/ml yeast RNA) was added. Protected fragments were precipitated in ethanol and resolved on an 8% polyacrylamide/urea gel and...
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Organization of FATP Gene—Southern blot analysis was employed to characterize the FATP gene. Restriction endonuclease-digested mouse genomic DNA was probed with either the full-length cDNA or a 150-bp probe corresponding to the 5’-end of the coding region. Probing with full-length FATP cDNA revealed multiple positive species (Fig. 1A) whereas the 150-bp probe hybridized only to a single band on each lane (Fig. 1B). These results suggest that the FATP gene is unique in the genome and 10–30 kb in size.

The FATP cDNA (full-length) was used as a probe to screen a mouse genomic λEMBL3 library. Two clones, λ65 and λ77, were isolated after screening 9 × 10^6 plaques. Phage DNA was digested with SacI or XhoI and the genomic fragments subcloned into pBluescript II KS(+) for restriction mapping and sequencing. Restriction mapping and Southern blot analysis revealed that the two clones were non-overlapping and that λ65 contained the 3’-end of the cDNA while λ77 contained the 5’-end of the cDNA (Fig. 2). To obtain the intervening sequence, a bacterial artificial chromosome clone was isolated using a 3-kb BglII fragment from λ77 containing the first 3 exons. Southern blot analysis of the BAC clone revealed the identical pattern as with mouse genomic DNA, indicating that this clone contains the entire FATP gene (results not shown).

The FATP gene spans −16 kb and has 13 exons; two of which, exons 1 and 2, are upstream to the translation start site. Two cDNA species with differing 5’-untranslated region were detected by autoradiography.

**Plasmid Constructs**—A 4.9-kb XhoI fragment (containing exons 1–3) from λ77 was subcloned into the XhoI site of pBluescript II KS(+) (Stratagene, La Jolla, CA), giving rise to pBS77 × 1. A 6.9-kb SacI fragment from λ65 was also subcloned into the SacI site of pBluescript II KS(+), yielding plasmid pBS65HS. Fragments containing various lengths of the FATP promoter were amplified by PCR with restriction sites engineered for subcloning into the promoterless pGL3-Basic luciferase expression vector (Promega, Madison, WI). These PCR generated fragments were fully sequenced to check for mutations introduced by Taq polymerase. pCIF and pCIR, containing the sequence between −118/3 and −174, were constructed by ligating the fragment into the HindIII site of the vector in forward and reverse orientations, respectively. The same insert was cloned into pBluescript SK II(+) in the forward orientation to make pBS-CIF. pNH11 (−971/+84), pNH12 (−793/+84), pNH13 (−556/+84), pNH15 (−160/+84), and pNH16 (−30/−84) were constructed by ligating the corresponding fragments into the NheI and HindIII sites of pGL3-Basic. The two plasmids (CIFAG and CIFAS) that contain sequences −273/+84 and +53/+84 were generated by digesting CIF with BglII and SacI, respectively, followed by self-ligation. Construct I was generated by ligating a 7.8-kb fragment from λ77 into the SacI site of pGL3-Basic. In order to generate constructs II and III, construct I was digested with BglII; the 1.9- and 2.5-kb fragments were then subcloned into the BglII site of pNH13. Construct IV was made by subcloning a 2.6-kb NheI fragment from construct I into the NheI site of pNH13. The fragment in construct VI containing introns 1 and 2 was amplified by PCR, and subcloned into the NheI and XhoI sites of CIFAG. Construct VII was made by digesting construct IV with XhoI and EcoRI followed by self-ligation. Construct VIII was made in a similar fashion except MluI and EcoRI were used for digestion. Constructs IX and X were generated by digesting construct VIII with PvuII and PstI, respectively, followed by self-ligation. Constructs XI, XII, and XIII were generated by single-stranded mutagenesis using the Muta-Gen kit (Bio-Rad) with the oligos: 5’-ATGTTGACCTCCTACGCGTGGACATGTCGAG-3’, 5’-AAACATCGAGGCGAGCTCCTGACGATCAA-3’, and 5’-AAACATCGAGGCGAGCTCCTGACGATCAA-3’ respectively. These three constructs were sequenced to confirm the deletions as well as the junction sequences.

**Southern blot analysis of mouse genomic DNA. Panel A.** Full-length cDNA probe. Mouse genomic DNA (15 μg/lane) was digested overnight with BamHI (lane 1), BglII (lane 2), EcoRI (lane 3), HindIII (lane 4), and SacI (lane 5), respectively, and hybridized with full-length FATP cDNA probe. Panel B. These PCR-generated sites engineered for subcloning into the promoterless pGL3-Basic luciferase expression vector (Promega, Madison, WI).
sequences of these 2 cDNA clones with the genomic sequence revealed that while both cDNA clones contain the sequence of exon 1, the 91-nt sequence of exon 2 is absent from one class of cDNA clones, suggesting that the two different cDNA species arise from alternative splicing of exon 2.

The existence of two differentially spliced isoforms of FATP was verified by RT-PCR amplification of 3T3-L1 adipocyte RNA. A primer complimentary to a portion of exon 4 of the FATP gene was used to reverse transcribe total RNA. FATP mRNA was verified by RT-PCR amplification of 3T3-L1 adipocyte cDNA clones, suggesting that the two different cDNA species are expressed in adipocytes.

Intron Position Sequence Size
1 1287 bp
2 351 bp
3 336 bp
4 1287 bp
5 351 bp
6 336 bp
7 1287 bp
8 336 bp
9 1287 bp
10 336 bp
11 1287 bp
12 336 bp

A GT-rich downstream element, which is also important in defining the polyadenylation site (36), is located immediately 3' to sequence corresponding to the end of the cDNA clone. Another GT-rich element is found 25 nt downstream of the polyadenylation signal.

The proximal promoter sequence (Fig. 3) does not contain any canonical TATA consensus sequences. However, it possesses several features characteristic of a TATA-less promoter. The proximal promoter bears a high GC content (~65%) and contains multiple copies of Sp1-binding sites (37). In addition to a TATA box, consensus binding sites for C/EBP, NF-κB, and AP-2 are also found in this region.

RNA isolated from differentiated 3T3-L1 adipocytes was subjected to S1 nuclease protection analysis using a 387-nt single-stranded DNA probe generated by primer extension. Multiple bands were observed; the sizes of the three most predominant bands were 351, 336, and 351 bp.

2 J. E. Schaffer and H. F. Lodish, unpublished data.
protected fragments are 115, 130, and 145 nt, respectively (Fig. 4A). RNase protection assay (Fig. 4B) revealed the presence of three protected bands which mapped to the same region as those detected by S1 nuclease confirming the presence of multiple transcription start sites. The sizes of protected fragments were identical when experiments were repeated with different preparations of RNA, suggesting that the heterogeneity in size was not due to degradation of RNA.

**Chromosomal Localization of Murine FATP by Fluorescence in Situ Hybridization**—A 100-kb bacterial artificial chromosome clone spanning the gene for FATP was labeled with digoxigenin dUTP and hybridized to normal metaphase chromosomes from male embryonic stem cells. Of 80 metaphase cells examined, 78 exhibited specific labeling of a medium-sized chromosome (Fig. 5A) consistent with chromosome 8 on the basis of 4',6-diamidino-2-phenylindole staining. Co-hybridization was carried out using a probe specific for the telomeric region of chromosome 8, confirming that FATP localizes to chromosome 8 (Fig. 5B). Measurement of 10 specifically hybridized chromosomes revealed that FATP is located 49% of the distance from the heterochromatic-euchromatic boundary to the telomere of chromosome 8, an area corresponding to band 8B3.3.

**Promoter Analysis**—Transient transfection assays were performed to test whether the 5'-flanking region of the FATP gene exhibits promoter activity. A 1273-bp fragment (−1189 to +84) was fused 5' to the firefly luciferase reporter gene in the forward and reverse orientations. These two constructs were then
transfected into mature 3T3-L1 adipocytes to test for their ability to drive transcription. Luciferase activities in these cells were compared with control cells which were transfected with pGL3-basic, a promoterless luciferase vector. As shown in Fig. 6, luciferase activities in cells transfected with the forward construct (−1189/+84) is 4–5-fold higher whereas the reverse construct (+84/−1189) has less luciferase activity than the control. A luciferase construct containing the proximal 7.8-kb FATP 5′-flanking sequence showed the same level of promoter activity as that of the forward construct (data not shown).

A series of luciferase constructs containing progressive 5′ deletions of the FATP 5′-flanking sequence were transfected into 3T3-L1 adipocytes. As shown in Fig. 6, deletion of sequences between −793 and −556 resulted in a 1.6-fold increase in luciferase activity whereas basal FATP promoter activity markedly decreased when sequence 3′ to −273 was deleted. Constructs with deletion beyond the transcription start sites (+53/+84) showed luciferase activity comparable to the promoterless vector. A similar pattern of expression was also observed in transfection experiments performed with 3T3-L1 preadipocytes (data not shown). These data suggested that the major determinants of the basal FATP promoter lie within 273-bp upstream of the transcription start sites, and that a potential negative regulatory element may be present between −793 and −556.

Insulin Responsive Sequences—We have previously demonstrated that insulin represses FATP transcription in 3T3-L1 adipocytes (18). To define the DNA sequences required for the down-regulation of FATP transcription, various regions of flanking sequences (−7.8 kb to +1.9 kb) were subcloned into luciferase vectors (Fig. 7A) and transiently transfected into mature 3T3-L1 adipocytes. Luciferase expression was assayed after treatment of adipocytes with or without 1 μM insulin for 18 h, conditions sufficient to maximally decrease FATP transcription as shown by nuclear runoff assay (18). As shown in Fig. 7A, insulin treatment resulted in a 50% reduction of luciferase expression in cells transfected with the construct containing the entire 7.8-kb upstream region (I). Construct IV, containing sequences between −3.7 and −1.2 kb, showed the same pattern of insulin-dependent repression. Constructs containing upstream sequences from −7.8 to −3.5 kb (II, III, and V) or downstream sequences up to +1.9 kb (VI) did not show any significant insulin repression effect. These data suggest that the insulin responsive sequence lies between −3.5 and −1.2 kb 5′ to the FATP promoter. A series of deletion constructs from this region were generated in order to further characterize the insulin responsive element. These studies showed that sequence between −3.7 kb and −1681 bp is not required to confer insulin responsiveness (Fig. 7B, constructs IV, VII, and VIII), whereas deleting sequences between −1507 and −1266 abolished the repression effect of insulin (Fig. 7B, constructs IX and X).

The nucleotide sequence between −1507 and −1266 is shown in Fig. 8A. Computer-aided analysis revealed the presence of several putative transcription binding sites: a C/EBP-binding site at −1462, an AP-1 element at −1359 and a CCAAT box at −1339. In addition, the sequence in this region also...
contains three motifs similar to the core negative insulin responsive element (T(G/A)TTTTG) of the PEPCK gene (Fig. 8B), these are referred to as phosphoenolpyruvate carboxykinase-like elements (PLEs). In order to explore whether or not the negative insulin effect on FATP expression is mediated through these motifs, two deletion constructs were made and tested for luciferase activities. 1 μM insulin was added to experimental groups 18 h prior to cell harvest. Luciferase activities of these constructs in the presence of 1 μM insulin were shown on the right as percentage of control (mean ± S.E., n = 4–6). An asterisk denotes a significant difference (p ≤ 0.05, two-tailed t test) between the control group and the insulin-treated group. Panel B, schematic representation of deletion constructs used for defining the insulin response element. Luciferase activities of these constructs in the presence of 1 μM insulin as described in Panel A are shown on the right as percentage of control (mean ± S.E., n = 4–6). An asterisk denotes a significant difference (p ≤ 0.05, two-tailed t test) between the control group and the insulin-treated group. Deletion sequences of constructs XI and XII are shown in Fig. 8A.

To determine if specific protein-DNA complexes form on the region containing PLE3, DNA electrophoretic mobility shift assays were performed using nuclear extracts prepared from day 9 3T3-L1 adipocytes treated either with or without 1 μM insulin for 18 h. As shown in Fig. 9A, incubation of radiolabeled oligonucleotide PL3 with nuclear proteins results in a gel shift complex that does not change in intensity upon insulin treatment. The protein-PL3 complex is specifically competed by unlabeled PL3 oligonucleotide (Fig. 9B). The oligonucleotide PEP (containing the PEPCK IRS) did not effectively compete for the PLE3-binding protein(s) suggesting that while the sequence shown to be functional in insulin repression is similar to that for PEPCK, different protein(s) are likely to be involved. To determine which components of the PLE3 element may be most critical for protein-DNA complex formation, gel-shift assays using oligonucleotides containing substitution mutations of the either the first or last 3 bp of the PLE3 element or the entire PLE3 element were performed. As shown in Fig. 9B, mutation of the first three bases of PLE3 decreases its ability to compete for protein binding; however, mutation of the last 3 bp enhances its ability to compete for the wild type probe. Mutation of all 7 bp of PLE3 results in an oligonucleotide which is unable to compete for protein binding to the wild type PLE3 probe. A corresponding mutation in the PLE3 of the luciferase reporter construct shows that the substitution of all seven nucleotides in the element results in a construct that is no longer responsive to insulin (Fig. 7B). Therefore, either deletion or substitution of the element yields a construct that is no longer responsive to insulin. Competition with the oligonucleotide containing AP-1, an element which overlaps PLE3 in the FATP promoter, did not effectively reduce PL3 binding, indi-
cating that while that element is present, it does not appear to be central to the formation of the protein-DNA complex. Finally, an oligonucleotide containing both the PLE1 and PLE2 sequences does not effectively compete for protein binding to PL3. This correlates with the transfection studies (Fig. 7B), which show that deletion of these elements does not affect insulin responsiveness. Further analysis of the protein-DNA complex is in progress to determine its relationship to the repressive effects of insulin on FATP gene expression.

**DISCUSSION**

To investigate the transcriptional regulation of the mouse FATP gene by insulin, we have isolated the murine gene encoding FATP, identified its chromosomal location cytogenetically, characterized its promoter, and identified the insulin response element. Analysis of genomic DNA by Southern hybridization showed that there is a single FATP gene. The gene spans approximately 16 kb and is organized into 13 exons, of which exon 1 and exon 2 do not contain any coding sequence. It should be noted that the murine FATP gene belongs to a multigene family comprised of the very long chain acyl-CoA synthetases and the fatty acid transporters (51). Members of this gene family contain two distinct motifs, one identifying a potential covalent AMP-binding site and a second domain in the carboxyl region of the protein possibly playing a role in lipid selectivity (51). While genes for all the very long chain acyl-CoA synthetases have not been reported, it will be of interest to compare the organization of those genes to the murine FATP sequences reported here.

The murine FATP gene possesses several unusual features. Exon 2 is believed to be alternatively spliced since its sequence is absent in some cDNA clones previously isolated. This point was corroborated by RT-PCR analysis of adipocyte mRNA. It is not known whether alternative splicing of FATP RNA is specific to adipose tissue or has any physiological function; cDNA clones for FATP have not been isolated from cardiac or skeletal muscle, two other cell types expressing high FATP levels. It is noteworthy that both intron 2 and intron 11 have a less common variant form of 5' splice site. The majority of eucaryotic introns have a conserved GT dinucleotide at their 5' donor site; however, in some cases, the 5' splice site is found to be GC (32, 33). Sequence comparison among this class of introns revealed that sequences flanking the donor site are highly conserved; they often have the sequence AGCAGC (34). Both the 5' intron/exon junctions of introns 2 and 11 match this conserved heptanucleotide sequence. Pre-mRNA is spliced in the nucleus by a spliceosome which is made up of small nuclear ribonucleoprotein particles and other factors. Precise base pairing of the splicing substrate with the 5' end of U1 snRNA is important in determining the 5' splice site (38). It has been shown that mismatches between the splicing substrate and U1 snRNA can be tolerated in splice sites either 5' or 3' of the cleavage site, but not both (31). The sequence of the 5' end of U1 snRNA is 5'-CUUACCU-3', which complements perfectly with the conserved flanking sequences of the GC-splice site. The lack of the usual central U:A pair from the GC sequence allows for the need of a better match from the rest of the sequence. In vitro splicing studies have shown that, besides the authentic GT site, the GC variant is the only form that will be accurately
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A nuclear protein was isolated from 3T3-L1 adipocytes which had been treated with 1 μM insulin for 0 or 18 h. The labeled oligonucleotide, PL3, was incubated with or without nuclear protein and analyzed by electrophoretic mobility gel shift assay. Panel B, nuclear protein from 3T3-L1 adipocytes was incubated with labeled oligonucleotide with or without the presence of competitor oligonucleotides in 20-, 50-, and 200-fold molar excess. The following unlabeled, double-stranded oligonucleotides were added as competitors: PL3, the insulin response element PLE3; PLM1 and PLM2, PL3 sequences with three base substitutions; PLM3, the PL3 sequence with all 7 bp of the PLE3 element substituted; PEP, the PEPCK IRS; PL12, an oligo containing both PLE1 and PLE2; and AP1, the AP-1 site overlapping PLE3. The arrows denote the positions of the protein-DNA complex (open arrow) and free probe (solid arrow), respectively.

The mouse FATP gene also contains a variant form of polyadenylation signal, ATTAAA. Among all genes, 90% have a perfect copy of ATTAAA as the polyadenylation signal. However, in the remaining 10%, this signal is absent or exists in variant forms (40). The most frequent variant form is ATTAAA, which has been shown to be efficiently processed in vitro (35). Polyadenylation sites are also determined by other elements in the proximity of the polyadenylation signal; however, the consensus for these elements is less defined. In general, they consist of a high content of G and T. The two GT-rich regions found in the 3' end of the FATP gene may play a role in determining the polyadenylation site.

Results from S1 nuclease protection analysis indicates that there are multiple transcription initiation sites; this was confirmed by both RNase protection assay and primer extension analysis. The reason for the multiple start sites remains obscure, although it is not uncommon for promoters lacking a well defined TATA box. The 5'-flanking region of the FATP gene lacks a TATA box immediately upstream to the transcriptional initiation sites and possesses several characteristics of a TATA-less promoter. The proximal sequence is G/C-rich and contains multiple binding sites for the transcription factor Sp1, which has been shown to activate the transcription of many TATA-less promoters (41–43). It is generally believed that the binding of Sp1 will recruit specific cofactors, such as TATA binding-associated factors, which in turn interact with TFIID and initiate transcription. Deletion of promoter sequence downstream of −264 greatly reduced basal transcription, suggesting the presence of a binding site(s) for a transcription factor important for basal expression. Several putative transcription binding sites are present in this region, these include: a CAAT box at −225, a C/EBP-binding site at −190, two copies of Sp1 binding sites and 2 copies of AP-2 binding sites. Construct IX, which only contains promoter sequence up to −214, had an expression level comparable with other plasmids with longer FATP promoter sequence. This suggests that the CAAT box is not crucial in basal transcription of FATP. The C/EBP-binding site at −190 is also unlikely to be a key determinant for basal transcription because FATP basal promoter activity in transfected preadipocytes is similar to that in fully differentiated adipocytes. C/EBP isomers are expressed in a differentiation-dependent manner during 3T3-L1 preadipocyte differentiation (44) and have been shown to play an important role in initiating adipose-specific gene transcription (45). It appears that Sp1 and AP-2 may be important in driving FATP basal transcription, although the involvement of other unknown factors cannot be ruled out.

FATP was localized to mouse chromosome 8 by cytogenetics. The region to which FATP localizes corresponds roughly to physical map positions between 30 and 40 centimorgans (cM) on chromosome 8. It is interesting to note that three other genes important in fatty acid homeostasis, lipoprotein lipase (33.0 cM), the mitochondrial uncoupling protein (UCP1, 37.0 cM), and sterol regulatory element-binding protein 1 (SREBP-1, 33 cM) are also localized in this region of chromosome. Like FATP, lipoprotein lipase and UCP1 expression is regulated by insulin (20). The apparent clustering of insulin-controlled genes encoding proteins involved in fatty acid metabolism is intriguing and bears further examination.

By transient transfection in 3T3-L1 adipocytes, we have shown that the sequence between −1507 and −1266 is required for mediating the transcriptional repression by insulin. Both deletion and mutation of the element at −1353 to −1347 abolished the effect of insulin, indicating that the integrity of this region is essential for insulin responsiveness. Furthermore, protein complex formation on this element in vitro can be correlated with in vivo function.

A number of negative insulin response elements have been characterized (for review, see Ref. 20); while many of them have unique sequences, the IRSs of PEPCK, IGFBP-1, and tyrosine aminotransferase genes share a common sequence, T(G/A)TTTTG. The sequence identified in the FATP promoter is very similar to this motif and has the sequence TGTTTTC. Although it is speculated that insulin may mediate its negative effect on the transcription of PEPCK, IGFBP-1, and tyrosine aminotransferase genes through HNF-3, HNF-3 itself does not directly mediate the insulin effect (25, 46). The identity of the trans-acting factor(s) that binds to the IRS remains unknown. It is postulated that an unknown insulin response protein, or proteins, may modulate the glucocorticoid response by altering the binding or trans-activation potential of HNF-3. The pattern of hormonal regulation of FATP is different from that of PEPCK, IGFBP-1, and tyrosine aminotransferase. FATP transcription is not stimulated by glucocorticoid and insulin represses FATP basal transcription rather than the glucocorticoid-stimulated transcription.
The gel mobility shift studies further demonstrate both similarities and differences between insulin regulation of FATP and that of other genes containing insulin response elements. Although the PLE3 sequence from the FATP promoter resembles the PEPCK IRS, the insulin effects mediated by these sequences appear to require the binding of distinct nuclear proteins, as demonstrated by the lack of PEPCK IRS competition for the formation of the PL3-protein complex. Neither the FATP nor the PEPCK elements show a change in the amount of protein bound in response to insulin treatment, a phenomenon which points to the complexity of insulin signaling through these elements. Although PLE3 has been shown to be important for insulin regulation of FATP, it is likely that this element functions in conjunction with other elements and protein factors.

An AP-1 motif is located just 5' of the FATP PLE3. Since insulin stimulates collagenase gene transcription through an AP-1 motif, it suggests that the presence of this element adjacent to the FATP IRS contributes to the effect of insulin on FATP transcription. Other reports also suggest that this motif may be involved in the stimulatory effect of insulin on α2microglobulin gene transcription (47) and on the inhibitory effect on albumin gene transcription (48). It is possible that insulin exerts its effect directly through the AP-1 element which is bound by the heterodimers of c-Jun and c-Fos as well as other c-Fos-related proteins. Insulin has been shown to increase the degree of phosphorylation of both c-Jun and c-Fos (49). Thus insulin can potentially mediate its effect through reversible phosphorylation of the AP-1 complex. In addition, insulin also stimulates c-fos transcription (50), resulting in an increase in the amount of AP-1 complex. It is also conceivable that the insulin effect is mediated through an unknown protein factor which modulates AP-1 activities in a fashion resembling the regulation of HNF-3 activity in PEPCK, tyrosine aminotransferase, and IGFBP-1 genes. Finally, the overlapping location of the AP-1 and PLE3 elements in the FATP promoter presents another possible regulatory mechanism. The protein(s) binding to the PLE3 sequence may sterically hinder the ability of c-Fos and c-Jun to bind to the AP-1 element, thus preventing AP-1-mediated activation of FATP transcription. However, analysis of the AP-1 site in gel-shift assays indicates that the formation of the IRS-protein complex does not require a full AP-1 site.

In summary, we have cloned the mouse FATP gene and characterized its promoter. The gene is located on chromosome 8 and is near the lipoprotein lipase gene and the UCP1 gene. We have also demonstrated that the inhibitory action of insulin on FATP transcription is mediated through a cis-acting PEPCK-like element located at the region of −1353 and −1347. These studies provide a framework to examine the functional aspects of FATP with regard to lipid transport and the possible relationship between FATP dysfunction, obesity, and type II diabetes mellitus.

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