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Identification of Glycerol-3-phosphate A cyltransferase as an Adipocyte Determination and Differentiation Factor 1- and Sterol Regulatory Element-binding Protein-responsive Gene*

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We demonstrate that the mRNA levels of glycerol-3-phosphate acyltransferase (GPAT), a mitochondrial enzyme catalyzing the initial step in glycerolipid synthesis, are induced during the differentiation of 3T3-L1 preadipocytes to adipocytes and following ectopic expression of rat adipocyte determination and differentiation factor 1 (ADD1), a protein with high homology to the human sterol regulatory element-binding protein-1 (SREBP-1). The increase in GPAT mRNA levels that occurs during differentiation is partially prevented by ectopic expression of a dominant negative form of ADD1.

Sterol regulatory element-binding proteins (SREBPs) and adipocyte determination and differentiation factor 1 (ADD1) are members of the helix-loop-helix-leucine zipper family of transcription factors (1–3). SREBP-1 was purified from HeLa cells based upon its ability to bind specifically to a 10-bp sequence, termed the sterol regulatory element-1 (SRE-1) (4). This element was shown to be involved in sterol-regulated transcription of the LDL receptor and HMG-CoA synthase genes (2, 4). Subsequently, two human SREBP genes were identified, SREBP-1a and SREBP-2, that encode at least three proteins (SREBP-1a, SREBP-1c, and SREBP-2) of approximately 125 kDa (1, 2).

ADD1 was cloned independently as the result of a screen to identify rat adipocyte proteins that bound to a DNA sequence termed an E-box (3). The E-box motif (CANNTG) is present in the promoters of a number of genes including fatty acid synthase and S14 (3, 5, 6). ADD1 mRNA levels were shown to increase significantly during the differentiation of preadipocytes to adipocytes (3, 7), consistent with a role for this transcription factor in cell differentiation. The transcription of the endogenous fatty acid synthase gene or reporter genes under the control of the fatty acid synthase promoter was induced by ADD1 and SREBP-1, consistent with a functional role for these proteins (3, 5, 6, 8). Comparison of the nucleotide/amino acid sequence derived from rat ADD1 (3) and human SREBP-1 (1) indicates that they encode the same protein.

SREBPs/ADD1 are unique transcription factors containing two transmembrane domains that anchor the proteins to the endoplasmic reticulum and the nuclear envelope (9). Low cellular sterol levels stimulate a complex set of proteolytic cleavage events of the precursor form of SREBPs to release the mature 68-kDa amino-terminal domain from the endoplasmic reticulum (10, 11). This soluble, transcriptionally active form of SREBP is targeted to the nucleus where it binds to cis-elements in the promoters of SREBP-responsive genes (2, 4). Three distinct cis-elements have been identified: SRE-1 in the promoters of genes encoding the LDL receptor and HMG-CoA synthase (12), SRE-3 in the promoter of the gene encoding FPP synthase (13), and an E-box motif in the promoters of the fatty acid synthase and S14 genes (3, 5, 8). Motifs corresponding to SRE-1 and SRE-3 have been identified in the promoter of the squalene synthase gene (14). However, the functional roles of these elements in this latter gene have not been reported.

Increased transcription of the LDL receptor, HMG-CoA synthase, and FPP synthase genes requires a second DNA-binding protein in addition to SREBP. Studies with FPP synthase and HMG-CoA synthase promoter-reporter genes identified NF-Y as the second crucial transcription factor (15). The binding of NF-Y to an inverted CCAAT box motif in the proximal promoter of the FPP synthase gene stimulated the binding of SREBP to an adjacent SRE-3 and allowed active transcription of an FPP synthase promoter-reporter gene (16). A different
ubiquitous transcription factor, Sp1, is necessary for high expression of the LDL receptor gene (17). The binding of SREBP to SRE-1 in the promoter of the latter gene stimulated the binding of Sp1 to an adjacent low affinity binding site for Sp1 (17, 18).

Based on these studies, we hypothesized that other SREBP/ADD1-responsive genes would require NF-Y or Sp1 in addition to SREBP/ADD1 for high transcription. Consequently, we searched a number of data bases to identify genes involved in lipid biosynthesis that contained potential NF-Y and SREBP/ADD1 for high transcription. Consequently, we identified a number of genes, including the one encoding FPP synthase (pSYN SRE) genes have been described (15, 17). The sources of all other reagents and plasmids have been given (13, 15, 24, 25).

GPAT catalyzes the acylation of the sn-1 hydroxyl of glycerol-3-phosphate, the initial step of glycerolipid synthesis. The product, 1-acetyl-sn-glycerol-3-phosphate is a committed intermediate in the biosynthesis of both triglycerides and phospholipids (19). There are two major mammalian forms of GPAT that are localized to either the endoplasmic reticulum or mitochondria (19). In animal studies, the activities of both GPAT isoenzymes were regulated by changes in the nutritional and hormonal status of the animal (20). However, only the protein (21), cDNA (22), and gene (23) encoding the mitochondrial GPAT have been isolated to date. Jerkins et al. (23) identified 1447 bp of the murine GPAT proximal promoter and demonstrated that the expression of GPAT promoter-reporter genes in cultured cells was regulated by glucose and insulin, presumably via a carbohydrate response element. In addition, reporter genes containing a minimum of 86 bp of the GPAT promoter were expressed at 2-fold higher levels in 3T3-L1 adipocytes as compared with preadipocytes (23). The reason for the increased expression of the reporter gene in adipocytes was not determined.

In the current study, we report the identification of distinct cis-elements in the GPAT proximal promoter that interact with either SREBP/ADD1 or NF-Y. We demonstrate that the expression of GPAT promoter-reporter genes in transiently transfected cells is increased by co-expression of mature SREBP-1a or SREBP-2. We also demonstrate that high levels of expression of GPAT promoter-reporter genes, in response to co-expressed SREBP-1a, are attenuated when the binding sites for either SREBP or NF-Y in the promoter are mutated or when cells are co-transfected with a plasmid encoding a dominant-negative form of NF-Y. Finally, we show that the endogenous GPAT mRNA levels in 3T3-L1 cells increase during differentiation of preadipocytes to adipocytes and in response to ectopic expression of ADD1.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA restriction and modification enzymes were obtained from Life Technologies, Inc. 32P-Labeled nucleotide triphosphates were obtained from Amersham Corp. pSRETB (Invitrogen) containing both a partial sequence of SREBP-1a (amino acids 1–490) and T7 plus poly-histidine tags and pCMV-CSA10, which encodes amino acids 1–490 of SREBP-1a, and pCMV-CS2, which encodes amino acids 1–485 of SREBP-2, were kindly provided by Dr. T. Osborne (Department of Molecular Biology and Biochemistry, University of California, Irvine). Lipoprotein-deficient fetal calf serum was purchased from PerImmune.

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**Transcriptional Regulation of GPAT by ADD1/SREBPs and NF-Y**

Infection of 3T3-L1 Cells with a Retrovirus Expressing either ADD1 or ADD1-DN—The P6b retroviral vector (26) containing either ADD1 or ADD1-DN (7) was used to transfect BOSC 23 cells (27). The supernatant, containing the recombinant retrovirus, was used to infect 3T3-L1 preadipocytes. After 10–14 days, puromycin-resistant cells were pooled, replated and grown to confluence (day 0 of differentiation) (7). Confluent cells were induced to differentiate by treatment with differentiation-permissive medium (7). Subsequent media changes were as described (7).

**GPAT Promoter-reporter Gene Constructs**—The GPAT promoter was obtained by a polymerase chain reaction using mouse genomic DNA as a template. The 3′-primer corresponded to nucleotides –12 to +17 of the published genomic sequence (Fig. 1) (23) and an additional 10 nucleotides that contained a BglII restriction site. The 5′-primer contained an StuI restriction site and nucleotides that corresponded to –200 to –176 of the published GPAT genomic sequence (Fig. 1) (23). The polymerase chain reaction product was subcloned directionally into pG2 basic luciferase vector (Promega) to produce pGPAT-A and sequenced to confirm the wild-type GPAT sequence. The subcloned 217-bp fragment of the GPAT promoter was used as a template in subsequent polymerase chain reactions using the same 3′-primer and 5′-primers corresponding to –176 to –160, –160 to –142, or –78 to –58 of the genomic sequence. The products were subcloned into pG2 to produce pGPAT-B, pGPAT-C, and pGPAT-D, respectively. Two additional 5′-primers were also used, 5′-TACCGAGCTCTATTGGCTGATCAAGCTCAGCC-3′ and 5′-TACCGAGCTCTATTGGCTGATCAAGCTTAAACCTAGCAGACC-3′ that contained mutations (underlined) in the putative NF-Y or SREBP binding sites, respectively. The promoter-reporter constructs containing these mutations were pGPAT-Y and pGPAT-S, respectively. All polymerase chain reaction products were sequenced after subcloning into pG2 in order to confirm the presence of wild-type or mutant sequences.

**Oligonucleotides**—Oligonucleotides were either synthesized by Life Technologies, Inc., or were obtained by polymerase chain reaction using the GPAT promoter fragment subcloned into pG2. Double-stranded DNA corresponding to GPAT cDNA was obtained by the polymerase chain reaction following the synthesis of cDNA from mouse liver mRNA using Superscript II (Life Technologies, Inc.) and an additional 10 nucleotides that contained a BglII restriction site. The 5′-primer was carried out with the same 3′-primer and 5′-primer combinations that contained mutations (underlined) in the putative NF-Y or SREBP binding sites, respectively. The polymerase chain reaction products were sequenced after subcloning into pG2 in order to confirm the presence of wild-type or mutant sequences.

Northern Blot Analysis—Total RNA was isolated from 3T3-L1 cells, either before or after the initiation of differentiation, using Trizol reagent (Life Technologies, Inc.). Ten-µg aliquots of total RNA were fractionated by electrophoresis on 1% agarose/formaldehyde gels, transferred to nylon membranes and cross-linked with UV light as described (28). [α-32P]dCTP radiolabeled DNA probes were generated by random priming (Pharmacia Biotech Inc.). Hybridization and quantitation using a PhosphorImager (Molecular Dynamics) were as described (29). Blots were also hybridized to a 36B4 probe (7), analyzed on the phosphorImager, and the values used to correct for differences in RNA loading, per lane. Gel Mobility Shift Assay—The double-stranded DNA oligonucleotides used in these studies corresponded to –192 to –171 (5′-SRE), –176 to –155 (central SRE) or –85 to –65 (NF-Y) of the GPAT promoter. The 95-bp BgIII/SstI restriction fragments of pG2-D and pG2-S contain wild-type binding sites for both SREBP-1a and NF-Y and were subcloned into Bluescript SK (Stratagene).

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NF-Y (3) with a retrovirus that expressed either ADD1 or ADD1-DN, a dominant negative form of ADD1, as described previously (13). Recombinant SREBP-1a, containing N-terminal T7 and polyhistidine (His₆) tags was purified to homogeneity from *Escherichia coli* extracts by nickel affinity chromatography as described by Sanchez et al. (17).

Complementary single-stranded DNA, corresponding to the indicated nucleotides of the GPAT promoter and containing either the wild-type sequence or the indicated mutations, were annealed, and the double-stranded probe was isolated from acrylamide gels (28). Radiolabeled probes were generated in the same manner following an initial radiolabeling of a single-stranded oligonucleotide with ³²P (28). The radiolabeled probes (20,000 cpm; 1.5 fmol) were used in gel mobility shift assays in the presence of nonfat milk (2.5 mg/ml) or in the presence of either partially purified NF-Y (5 μg) or recombinant SREBP-1a as described previously (13, 15). Where indicated, antibody (0.2 μg) to NF-YA, NF-YB, CBF-HSP, or the T7 tag (Novagen) on SREBP-1a was added to the incubations on ice for 30 min before gel electrophoresis was performed (13, 15).

**Transient Transfections and Reporter Gene Assays—**Details of the transient transfection of HepG2, CV-1, or Chinese hamster ovary cells with luciferase reporter genes, a plasmid encoding β-galactosidase, pCMV-CSA10 (encoding SREBP-1a), and pCMV-CS2 (encoding SREBP-2) appear in previous publications (13, 15, 29). Cells were then incubated for 20 h in the indicated media and lysed, and the activities of reporter genes were determined as described (13, 15). The activity of β-galactosidase was used to normalize for transfection efficiencies (13).

**RESULTS**

**GPAT Expression during Adipocyte Differentiation—**Analysis of the published nucleotide sequence for the murine GPAT promoter (23) indicated that it contained a number of potential binding sites for SREBP/ADD1 and NF-Y (Fig. 1). Consequently, we hypothesized that transcription of the endogenous GPAT gene might be enhanced under conditions in which the nuclear levels of NF-Y and/or ADD1/SREBP were increased. Previous studies have demonstrated that the differentiation of 3T3-L1 preadipocytes to adipocytes is associated with an increase in the expression of ADD1 mRNA (3, 7). Differentiated adipocytes also accumulated large amounts of triglyceride, presumably as a result of increased synthesis of this lipid.

Fig. 2 demonstrates that the mRNA levels for GPAT, an enzyme that catalyzes the initial step in triglyceride and phospholipid synthesis (19), increased 7.5-fold during the differentiation of 3T3-L1 preadipocytes to adipocytes. Maximal GPAT mRNA levels were observed 6–8 days after differentiation was initiated (Fig. 2 and data not shown).

**Effect of Ectopic Expression of either ADD1 or a Dominant Negative Form of ADD1 on GPAT mRNA Levels—**3T3-L1 cells undergo many changes in gene expression during differentiation to adipocytes (7). In order to determine whether the increased expression of GPAT mRNA was a direct consequence of the increased expression of ADD1, we infected preadipocytes with a retrovirus that expressed either ADD1 or ADD1-DN, a dominant negative form of ADD1 that has a point mutation in the DNA-binding domain and represses differentiation of 3T3-L1 cells (7).

Introduction of ADD1 into 3T3-L1 preadipocytes via retroviral infection resulted in a 6.7-fold increase in GPAT mRNA levels (Fig. 3, lane 3 versus lane 1) under conditions in which preadipocytes do not differentiate into adipocytes (7). The mRNA levels for PPARγ, adipin, and aP2 did not change under these conditions (Fig. 3). Furthermore, GPAT, PPARγ, adipin, and aP2 mRNA levels were unaffected, or decreased slightly, following the infection of the cells with the vector containing ADD1-DN (Fig. 3, lane 2 versus lane 1).

Six days after cell differentiation was initiated, GPAT mRNA levels were increased 2.5-fold in cells infected with the vector alone (Fig. 3, lane 4 versus lane 1). The increased expression of GPAT mRNA levels was attenuated in cells that ectopically expressed the dominant-negative form of ADD1 (Fig. 3, lane 5 versus lane 4). Expression of the dominant-negative form of ADD1 also resulted in reduced mRNA levels for PPARγ, adipin, and aP2 but had no effect on the expression of either ribosomal RNA or an mRNA identified by probe 36B4 (Fig. 3, data not shown).
Transcriptional Regulation of GPAT by ADD1/SREBPs and NF-Y

**Fig. 4.** Stimulation of GPAT promoter-luciferase reporter genes by co-expressed SREBP-1a. Quadruplicate dishes of HepG2 cells were transiently transfected with 2 μg of the indicated GPAT promoter-reporter gene, a plasmid encoding β-galactosidase under the control of a cytomegalovirus promoter, and pSREBP-1a (pCMV-CSA10), which encodes mature SREBP-1a. The cells were incubated for 20 h in medium containing 10% fetal bovine serum and then lysed, and enzyme activities were determined. The luciferase activities were normalized for any differences in transfection efficiency as described under “Experimental Procedures.” Cells were transfected with pGPAT-A and the indicated amount of pSREBP-1a (A) or the indicated GPAT promoter-reporter plasmid in the absence (open bars) or presence (solid bars) of 20 ng of pSREBP-1a (B). 1, 2, 3, and 4 indicate the 5′-SRE, the central SRE, the inverted CCAAT box, and the 3′-SRE, respectively (Fig. 1). M indicates a mutation in that domain, as described under “Experimental Procedures.” The luciferase values, mean ± S.E. (n = 4), are given relative to that measured in cells transfected with pGPAT-A. The -fold regulation of each reporter gene (pGPATA, -B, -C, -D, -S, and -Y) by SREBP-1a was 14.4, 13.5, 12.0, 4.4, 1.0, and 1.0, respectively. The results are representative of two separate experiments with HepG2.

eXpressed ADD1 (Fig. 3, lane 6 versus lane 1), consistent with a stimulatory role for ADD1 in the expression of GPAT mRNA. Taken together, these results demonstrate that GPAT mRNA levels are induced during adipocyte differentiation. In addition, ectopic expression of ADD1, either in preadipocytes or during adipocyte differentiation, results in increased levels of endogenous GPAT mRNA.

**Fig. 5.** Stimulation of GPAT promoter-luciferase reporter gene activity by SREBP-1a is attenuated by co-expression of dominant negative NF-YA29. Quadruplicate dishes of CV-1 cells were transiently transfected with 2 μg of pCMV-β-galactosidase, 2 μg of pGPAT-A (∆, ▲), or pLDLSRE (○, ●) in the presence of the indicated amount of pSREBP-1a (pCMV-CSA10) and in the absence (∆, ○) or presence (▲, ●) of 500 ng of pNF-YA29. The cells were incubated for 20 h in medium supplemented with 10% fetal bovine serum. The normalized luciferase activities, mean ± S.E. (n = 4), were determined as described in the legend to Fig. 4.

**Fig. 6.** Regulation of a GPAT promoter-reporter gene by cellular sterols. Quadruplicate dishes of HepG2 cells were transiently transfected with pGPAT-A or pFPPS-0.247, and the cells were incubated for 48 h in medium supplemented with 10% lipoprotein-deficient fetal calf sera and compactin (10 μg/ml) in the absence or presence of sterols (10 μg/ml cholesterol and 1.0 μg/ml 25-hydroxycholesterol). The luciferase activities were normalized for the efficiency of transfection, and the value obtained in the presence of sterols was designated as 100%. The means ± S.E. are given. The results are representative of two separate experiments.
Duplicate plates of the indicated cell type were transiently transfected with a plasmid encoding β-galactosidase and a luciferase reporter gene under the control of the indicated promoter in the absence or presence ofр SREBP-1a (pCMV-CSA10, encoding amino acids 1–490 of SREBP-1a) or pSREBP-2 (pCMV-CS2, encoding amino acids 1–485 of SREBP-2). The cells were incubated for 20 h in medium containing 10% fetal bovine serum and lysed, and enzyme assays were performed as described under “Experimental Procedures.” The luciferase activities in relative light units (RLU) are given after normalization for variations in transfection efficiency. The corrected luciferase values obtained from duplicate dishes varied by less than 10%. The numbers in parentheses indicate the -fold increase in luciferase activity relative to cells that were not transfected with plasmids encoding either SREBP-1a or SREBP-2. Similar results were obtained in duplicate experiments. The reporter genes were under the control of promoters derived from FPP synthase (pFPPS-0.247), LDL receptor (pLDL SRE) or HMG-CoA synthase (pSYN SRE) or GPAT (pGPAT-A) genes.

### Table I

| Experiment | Cell type            | Reporter gene | Luciferase activity | Relative stimulation |
|------------|----------------------|---------------|---------------------|----------------------|
|            |                      | None | pSREBP-1a | pSREBP-2 | SREBP-1a:SREBP-2 |
| A          | HepG2                | pFFPS-0.247 | 20.1 | 190 (9.5) | 438 (22) | 0.4 |
|            |                      | pLDL SRE   | 20.0 | 730 (36)  | 435 (22)  | 1.6 |
|            |                      | pSYN SRE   | 32.3 | 908 (28)  | 798 (25)  | 1.1 |
|            |                      | pGPAT-A    | 2.8  | 163 (58)  | 30 (10.6) | 5.5 |
| B          | Chinese hamster ovary| pFFPS-0.247 | 308  | 498 (1.6) | 555 (1.9) | 0.8 |
|            |                      | pLDL SRE   | 108  | 938 (8.7) | 625 (5.8) | 1.5 |
|            |                      | pSYN SRE   | 40   | 826 (20.6)| 650 (16.2)| 1.3 |
|            |                      | pGPAT-A    | 29   | 372 (12.5)| 118 (4.1) | 3.1 |
| C          | CV-1                 | pLDL SRE   | 1.3  | 6.6 (5.0) | 13.8 (10.6)| 0.5 |
|            |                      | pGPAT-A    | 0.6  | 3.4 (5.7) | 1.2 (2)   | 2.8 |

The results indicate that 1) murine GPAT promoter-luciferase genes are activated in response to increased expression of SREBP-1a, 2) a putative binding site for NF-Y is important for SREBP-1a-regulated expression of GPAT promoter-reporter genes, and 3) pGPAT-D may contain a novel binding site for SREBP-1a, since it does not contain the functionally important TCAC sequence that is found in the core of SRE-1, SRE-3, and E-box motifs. Instead, pGPAT-D contains the sequence TCAG, when mutated to TAAA impairs the induction of the reporter gene by co-expressed SREBP-1a. Nucleotide sequences surrounding the TCAG have only 40–60% identity with SRE-1, SRE-3, or E-box motifs and thus appear to represent a novel binding sequence for SREBP-1a.

**SREBP Activation of GPAT-reporter Genes Requires NF-Y**—We have previously shown that sterol-regulated transcription of FPP synthase and HMG-CoA synthase promoter-reporter genes requires both SREBP and NF-Y (13, 15, 16). In addition, electromobility shift assays were used to demonstrate that NF-Y synergistically enhanced the binding of SREBP-1a to SRE-3 in the FPP synthase proximal promoter (16). In the current study, mutations of the putative NF-Y binding site in the GPAT promoter produced a reporter gene (pGPAT-Y) that was unresponsive to co-expressed SREBP-1a (Fig. 4B). This result suggested that NF-Y might also play a functional role in the SREBP-dependent induction of GPAT promoter-reporter genes.

In order to test this hypothesis, cells were transiently transfected with pGPAT-A together with increasing amounts of pSREBP-1a in the absence or presence of a constant amount of a dominant negative form of NF-YA, pNF-YA29. The latter plasmid encodes a mutant form of NF-YA that is unable to bind DNA but can bind and sequester two separate proteins (NF-YB and NF-YC) that comprise transcriptionally active, heterotrimERIC NF-Y (30). Fig. 5 shows that co-expression of NF-YA29 attenuated by over 90% the normal increase in luciferase activity observed when SREBP-1a was co-transfected with pGPAT-A. This inhibitory effect of pNF-YA29 was not the result of a general effect on transcription, since pNF-YA29 had only a slight inhibitory effect on the luciferase activity in cells that had been transiently transfected with pSREBP-1a and a reporter gene under the control of the LDL receptor promoter (Fig. 5). This latter result is consistent with a previous study (15) and with the findings that the LDL receptor gene is transcriptionally regulated by SREBP and Sp1 (17, 18) but not NF-Y.

**Regulation of GPAT Promoter-reporter Genes by Sterols**—Transcription of a number of genes involved in either cellular cholesterol homeostasis (12–14) or fatty acid synthesis (8, 31) are known to be regulated by changes in the sterol content of the cell. The sterol-regulated transcription of these genes results from changes in the nuclear levels of SREBPs. Fig. 6 shows the results obtained when HepG2 cells were transiently transfected with pGPAT-A and then incubated for 48 h in media containing 10% lipoprotein-deficient fetal calf serum and compactin either in the absence or presence of 25-hydroxycholesterol and cholesterol. The results indicate that the luciferase activity was 4.4-fold higher in cells deprived of sterols as compared with the sterol-treated cells (Fig. 6). Under these same conditions, an FPP synthase promoter-reporter gene was regulated 4.6-fold (Fig. 6), consistent with previous studies (24).

**Differential Stimulation of Reporter Genes by SREBP-1a and SREBP-2**—The functional consequences of activation of SREBP-1 versus SREBP-2 are not known. In cell culture, SREBP-responsive promoter-reporter gene constructs are known to be stimulated by both SREBP-1 and SREBP-2. However, in vivo data indicate that hypolipidemic drugs result in increased levels of mature SREBP-2 but decreased levels of mature SREBP-1 in hepatic nuclei (32). Thus, SREBP-responsive genes may not all respond equally to different forms of this family of transcription factors.

Table I shows the results obtained after HepG2, Chinese...
hamster ovary, or CV-1 cells were transiently transfected with equivalent amounts of different promoter-luciferase reporter genes together with 20 ng of a plasmid encoding either SREBP-1a or SREBP-2. As expected, luciferase activities increased when SREBP-1a or SREBP-2 were co-expressed with reporter genes under the control of promoters derived from the LDL receptor, FPP synthase, HMG-CoA synthase, or GPAT genes (Table I). The results in Table I also demonstrate that, compared with the other reporter genes, pGPAT-A was 2–13-fold more sensitive to SREBP-1a than SREBP-2. Similar results were obtained when the amount of the co-transfected SREBP plasmids was decreased to 5 ng (data not shown).

**Electromobility Shift Assays—** Gel shift and supershift assays demonstrated that purified, recombinant SREBP-1a formed a complex with double-stranded DNA corresponding to the sequences encompassing the 5′ and central putative SREs (Fig. 1) in the proximal GPAT promoter (Fig. 7A). The addition of antibody to the epitope-tagged SREBP resulted, in each case, in a super-shifted complex (Fig. 7A). Recombinant SREBP-1a also bound to a probe that contained the 3′ putative SRE but not to a probe in which the TCAG had been mutated to TAAA (Fig. 7B). A preliminary DNase I footprinting analysis with the murine GPAT promoter (−78 to +17) and recombinant SREBP-1a indicated that this protein bound to sequences that included the TCAG at nucleotides −63 to −60 (data not shown).

Incubation of a 32P-end-labeled probe (−85 to −65), which encompassed the inverted CCAAT box (ATTGG) within the GPAT promoter, with a nuclear fraction enriched in NF-Y resulted in the formation of a major specific complex (Fig. 7C). The addition of anti-NF-YA or anti-NF-YB, but not anti-CBF-HSP, to the assay resulted in a supershifted band, consistent with the *in vitro* interaction of NF-Y with the DNA fragment (Fig. 7C).

**DISCUSSION**

The current experiments demonstrate that the gene encoding mitochondrial GPAT is regulated during the differentiation of 3T3-L1 preadipocytes to adipocytes by a process that involves ADD1/SREBP. This conclusion is based on the following observations: 1) endogenous ADD1 levels increased during this differentiation process (7), 2) the increase in GPAT mRNA levels that occurred during differentiation was enhanced significantly when ADD1 was ectopically expressed in either preadipocyte or adipocyte cells (Fig. 3), 3) the increase in GPAT mRNA levels that occurred during 3T3-L1 cell differentiation was attenuated when the cells were infected with a retrovirus that expressed a dominant negative form of ADD1 (Fig. 3), and 4) GPAT promoter-reporter genes were regulated by sterols or following the co-expression of SREBP-1a or SREBP-2 (Figs. 4–6, Table I). Thus, these studies expand the repertoire of lipid metabolic pathways that are responsive to changes in ADD1/SREBP levels; these pathways include cholesterol biosynthesis (12–14), low density lipoprotein receptor-mediated endocytosis (12), fatty acid synthesis (3, 7, 8, 31), and, in the current report, glycerolipid synthesis.

Jenkins *et al.* (23) previously reported that luciferase reporter genes that were controlled by nucleotides −86 to +102 of the murine mitochondrial GPAT promoter were expressed at

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**Fig. 7. SREBP-1a and NF-Y bind to sequences in the proximal promoter of the murine GPAT gene.** Radiolabeled oligonucleotides were used in electromobility gel shift assays exactly as described under *Experimental Procedures.* The probes contained the 5′-SRE (−192 to −171) or the central SRE (−176 to −155) (A); the 3′-SRE (−78 to +17), containing either wild type sequences or a mutation of the TCAG to TAAA (B); or wild type sequences (−85 to −65) containing the ATTGG (C). Where indicated, the probes were incubated with recombinant SREBP-1α, partially purified NF-Y (5 μg), and antibody (0.2 μg) to the T7-tagged SREBP, NF-YA, NF-YB, or CBF-HSP. The amount of SREBP-1α used was 1 ng in A and 0.2, 0.5, 1.0, and 2.0 ng in lanes 2 and 3, 8 and 9, and 5 and 10, respectively, in B. The bound (SREBP-1α or NF-Y) and supershifted (SS) probes are indicated. The free probe is not shown in B.
2-fold higher levels in adipocytes than preadipocytes. In addition, these investigators demonstrated that transient expression of luciferase reporter genes in both preadipocytes and adipocytes was decreased 9-fold when the 5' of the promoter was further deleted from nucleotide -86 to -55 (23). The current studies are in agreement with these earlier findings, since we now demonstrate that important binding sites for NF-Y and SREBP/ADD1 are located between nucleotides -78 and -55 (Fig. 1).

Previous reports demonstrated that the sterol-regulated changes in the transcription of genes encoding FPP synthase and HMG-CoA synthase were dependent on the binding of SREBP and NF-Y to the proximal promoters of these genes (13, 15, 16). More recently, we demonstrated that the binding of NF-Y to an inverted CCAAT box in the promoter of the FPP synthase promoter synergistically stimulated the binding of SREBP-1a to a cis-element, termed SRE-3, that lies 20 bp 3' of the NF-Y binding site (16). In the current studies, we investigated whether the expression of GPAT promoter-reporter genes was regulated by conditions that are known to regulate the expression of FPP synthase and HMG-CoA synthase promoter-reporter genes in order to determine whether a similar mechanism is involved for all three genes. The results (Figs. 4–6, Table I) demonstrate that GPAT promoter-reporter genes are regulated by sterols or by co-expression of SREBPs in a manner that parallels that observed with FPP synthase or HMG-CoA synthase promoter-reporter genes (2, 12, 13, 15). In addition, the SREBP-1a-induced increase in GPAT promoter-reporter gene activity was attenuated by co-expression of the dominant negative NF-YA29 (Fig. 5). We have previously demonstrated that expression of NF-YA29 attenuates the induction of FPP synthase and HMG-CoA synthase promoter-reporter genes in cells that are sterol-deprived (15). Finally, we showed that, consistent with these in vivo studies, the GPAT proximal promoter contains recognition sites for SREBP-1a and NF-Y (Fig. 7). Taken together, these results demonstrate that luciferase reporter genes under the control of promoters derived from GPAT, FPP synthase, or HMG-CoA synthase genes are all regulated by sterols, SREBP, and NF-Y. The transcription of the squalene synthase gene is also regulated by sterols (14). The finding that the proximal promoter of the squalene synthase gene also contains cis-elements that correspond to an inverted CCAAT box (the binding site for NF-Y), SRE-1 and SRE-3, and Sp1-binding sites (13) may indicate that this represents an additional gene that requires both SREBP/ADD1 and NF-Y or Sp1 for sterol-mediated regulation of transcription.

In contrast, sterol regulation of the LDL receptor, acetyl-CoA carboxylase, and fatty acid synthase genes requires SREBP/ADD1 and the ubiquitous transcription factor Sp1 (8, 17, 31). SREBP-1, SREBP-2, or ADD1 have been shown to bind to SREs in all three promoters. However, the binding of SREBP-1/ADD1 to an E-box motif (CANTNTG) in the fatty acid synthase or SRE-1 promoters is required for normal regulation (3, 6, 8). The ability of SREBP-1/ADD1 to bind to both E-box and SRE-1 motifs results, in part, from the presence of a tyrosine residue in a position that is normally occupied by an arginine in other basic-helix-loop-helix family members (6).

The most 3'-SRE in the GPAT proximal promoter (−64 to −55) is critical for induction of reporter genes by co-expressed SREBP-1a (Fig. 4A) and is bound by recombinant SREBP-1a in gel mobility shift assays (Fig. 7). This SREBP-1a binding sequence (CTCACCCCTAG) has 70% identity with SRE-3 (CTCAACGGAT) (13) and 60% identity with SRE-1 (ATCCACCCCTAC) (11) but does not contain the E-box motif (CANTNTG). Thus, mature SREBP-1a appears to be able to bind to a number of distinct nucleotide sequences that have now been functionally identified within various promoters. The GPAT proximal promoter contains two additional sequences approximately 100 bp 5' of the inverted CCAAT box (Fig. 1) that show 80% identity to the SRE-1 found in the LDL receptor promoter. Deletion of these sequences from promoter-reporter genes did not impair the induction of the reporter gene by co-expressed SREBP-1a (Fig. 4B). Thus, although both of these sequences are bound by recombinant SREBP-1a avidly in electromobility shift assays, they appear to be of little importance in the regulated expression of the promoter-reporter genes used in the current studies.

The current studies demonstrate the importance of ADD1/SREBP in the regulated expression of GPAT during adipocyte differentiation. However, other as yet unknown factors, in addition to ADD1/SREBP and NF-Y, appear to be required for the increased expression of GPAT mRNA. This latter conclusion is based on the observation that GPAT mRNA levels change less that 2-fold in the livers of rats or mice fed diets supplemented with either cholesterol or cholestyramine and mevinolin (data not shown). The mRNA levels for fatty acid synthase in these livers also change less than 2-fold (data not shown). In contrast, the mRNAs for FPP synthase and HMG-CoA synthase are regulated 10–35-fold in these same livers (33, 34).

Sheng et al. (32) demonstrated that when rodents were fed diets supplemented with cholestyramine and mevinolin, the hepatic mRNA levels for SREBP-2 increased, while the mRNA levels for SREBP-1 decreased. These investigators also demonstrated that, under conditions of hepatic cholesterol deprivation, there was an increase in the cleavage and release of mature SREBP-2 but a decrease in cleavage of SREBP-1 (32). This suggests that SREBP-2 and -1 have different regulatory functions and that SREBP-2 may be responsible for the induction of genes, such as HMG-CoA synthase, FPP synthase, and the LDL receptor, that are involved in hepatic cholesterol homeostasis.

The observation that, of four reporter genes studied, GPAT promoter-reporter constructs were more sensitive to SREBP-1a than SREBP-2 (Table I) may be important in understanding the increased expression of genes during adipocyte differentiation. The mRNA for ADD1, the rat homologue of human SREBP-1, increased during the differentiation of preadipocytes to adipocytes (3, 7), a period of increased synthesis of fatty acids and triglycerides (3). Ectopic expression of the dominant negative form of ADD1 prevents both the differentiation of preadipocytes to adipocytes and the enhanced expression of a number of genes (PPARγ, adipin, p2, and C/EBPα) that normally occurs during this process (7) (Fig. 3). Extrapolating these findings to the in vivo situation suggests that ADD1/SREBP-1 may be more important than SREBP-2 in the stimulation of GPAT expression during adipocyte differentiation. Further studies, perhaps utilizing transgenic mice that overexpress different SREBPs in adipose tissue, will be necessary to test this hypothesis.

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