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NUTRITIONAL AND HORMONAL REGULATION OF ENZYMES IN FAT SYNTHESIS: Studies of Fatty Acid Synthase and Mitochondrial Glycerol-3-Phosphate Acyltransferase Gene Transcription

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ABSTRACT

The activities of critical enzymes in fatty acid and triacylglycerol biosynthesis are tightly controlled by different nutritional, hormonal, and developmental conditions. Feeding previously fasted animals high-carbohydrate, low-fat diets causes a dramatic induction of enzymes—such as fatty acid synthase (FAS) and mitochondrial glycerol-3-phosphate acyltransferase (GPAT)—involved in fatty acid and triacylglycerol synthesis. During fasting and refeeding, transcription of these two enzymes is coordinately regulated by nutrients and hormones, such as glucose, insulin, glucagon, glucocorticoids, and thyroid hormone. Insulin stimulates transcription of the FAS and mitochondrial GPAT genes, and glucagon antagonizes the insulin effect through the *cis*-acting elements within the promoters and their bound *trans*-acting factors. This review discusses advances made in the understanding of the transcriptional regulation of FAS and mitochondrial GPAT genes, with emphasis on elucidation of the mechanisms by which multiple nutrients and hormones achieve their effects.

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INTRODUCTION

When total energy intake is in excess of energy expenditure, fatty acids and triacylglycerol are synthesized and triacylglycerol is deposited in adipose tissue. Reactions of de novo fatty acid synthesis are catalyzed by acetyl-coenzyme A carboxylase (ACC) (EC 6.4.1.2) and fatty acid synthase (FAS) (EC 2.3.1.85) in the cytoplasm (Figure 1). Under lipogenic conditions, excess glucose in the cell is first converted to pyruvate via glycolysis. Pyruvate is converted to acetyl-coenzyme A (CoA) and transported as citrate from mitochondria into cytoplasm. ATP citrate lyase (EC 4.1.3.8) then converts citrate back to acetyl-CoA. ACC catalyzes the carboxylation of acetyl-CoA to malonyl-CoA in an ATP-dependent manner. Acetyl-CoA and malonyl-CoA are then used as the substrates for the production of palmitate by the seven enzymatic reactions catalyzed by FAS. FAS uses NADPH as the reducing equivalent to synthesize palmitate. NADPH is generated by the malic enzyme and the pentose phosphate shunt. The fatty acids thus produced, along with those transported into the cell, are then used for the synthesis of triacylglycerol. Esterification of glycerol-3-phosphate with fatty acyl-CoA at the sn-1 position to form 1-acylglycerol-3-phosphate (lysophosphatidic acid), the first committed and presumably rate-limiting step of the triacylglycerol synthesis, is catalyzed by glycerol-3-phosphate acyltransferase (GPAT) (EC 2.3.1.15) in mitochondria and endoplasmic reticulum. Lysophosphatidic acid is further esterified to form triacylglycerol. Because of variations in the composition and quantity of the diet, rates of de novo fatty acid and triacylglycerol synthesis are tightly regulated by nutritional and hormonal status to meet the body's requirement for lipid and energy storage.

As demonstrated in the regulation of ACC, short-term changes in the enzymatic activity of ACC are through allosteric regulation and phosphorylation/dephosphorylation events, and do not involve new protein synthesis. On the other hand, long-term regulation of ACC and other lipogenic enzymes involves changes in concentrations of the regulated enzymes, for example, those observed by subjecting animals to fasting and then feeding them. Fasting

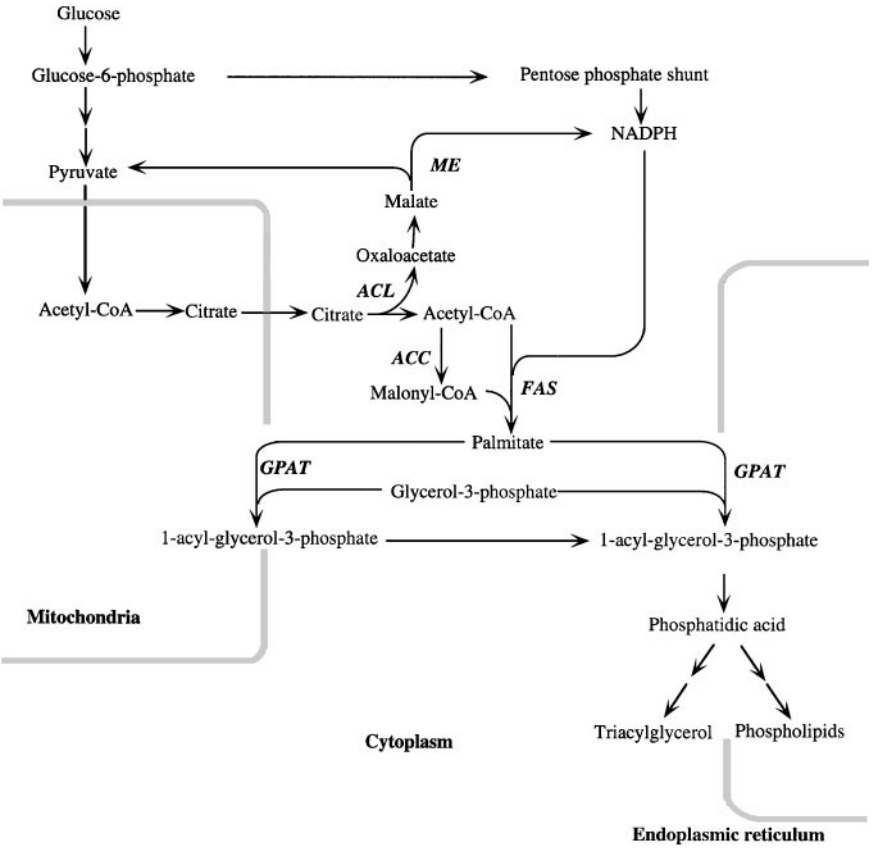

Figure 1 Pathways for fatty acid and triacylgycerol biosynthesis. (*bold, italic letters*) Enzymes. ACC, Acetyl-coenzyme A carboxylase; ACL, ATP citrate lyase; CoA, coenzyme A; FAS, fatty acid synthase; GPAT, glycerol-3-phosphate acyltransferase; ME, malic enzyme.

rodents for a few days decreases fatty acid and triaclyglycerol biosynthesis. When fasted animals are subsequently fed a diet high in carbohydrate and low in fat, there is a prompt rise in the production of fatty acids and triacylglycerol to levels above those observed in animals fed normally (26, 35, 41, 124, 138). During this induction process, concentrations of enzymes involved in fatty acid and triacylglycerol biosynthesis—such as the malic enzyme (117), ATP citrate lyase (116), ACC (58), FAS (84), and the mitochondrial GPAT (96, 135)—are increased dramatically. Based on studies of the mRNA levels and transcriptional rates of these enzymes, these long-term effects are mainly accounted for by increases in the rates of transcription of genes encoding these enzymes. Regulation of lipogenic enzymes is well coordinated with the regulation of

glucose transport, glycolysis, and gluconeogenesis. Increases in the activities of lipogenic enzymes in refed animals are accompanied by increases in glucose uptake via glucose transporters and the activities of glycolytic enzymes, such as L-pyruvate kinase (L-PK). On the other hand, the activity of PEPCK, the rate-limiting step in gluconeogenesis, is decreased.

FAS and mitochondrial GPAT have critical roles in fatty acid and triacylglycerol biosynthesis. Thus, elucidating molecular mechanisms of the regulation of these two enzymes is important for understanding the normal regulation of triacylglycerol biosynthesis and its pathophysiology during some human diseases. In this review, we discuss recent advances in the understanding of the regulation of FAS and GPAT, with emphasis on transcriptional regulation. Several reviews provide excellent coverage of the regulation of other lipogenic enzymes and related topics (39, 41, 58, 120).

REGULATION OF THE FAS AND MITOCHONDRIAL GPAT TRANSCRIPTION

FAS and Mitochondrial GPAT as Critical Enzymes in Fatty Acid and Triacylglycerol Biosynthesis

FAS catalyzes the synthesis of long-chain fatty acids, primarily palmitate, using acetyl-CoA and malonyl-CoA as substrates and NADPH as the reducing equivalent (Figure 1). The most important feature of animal FAS is its multifunctional character. The functional enzyme is a homodimer of the 250-kDa subunits. From the N-terminus of FAS to its C-terminus, amino acids encoding β-ketoacyl synthatase, acetyl-CoA transacylase, malonyl-CoA transacylase, dehydratase, enoyl reductase, ketoacyl reductase, acyl carrier protein, and thioesterase activities are organized into discrete domains in the order indicated (126). This is in contrast to bacterial and plant FASs, which are multienzyme complexes consisting of discrete monofunctional enzymes (10), and fungal FAS, which has functional components that are distributed between two nonidentical polypeptides (100). The multifunctional organization of FAS in vertebrates was probably selected in the course of evolution because it is more efficient and because the partial activities are more easily regulated in a coordinate fashion. Substrates for each of the reactions can be channeled to the active center of the subsequent enzymatic step within the same polypeptide, thus providing high efficiency in long-chain fatty acid synthesis. Moreover, regulating production of the single polypeptide affects all seven catalytic steps in a concerted manner. Excellent reviews exist for a more thorough description of the structure and function of FAS (112, 125).

FAS is encoded by a single-copy gene that produces a single mRNA species in mice (84) and two mRNAs in rats and chickens. The two mRNAs are generated

by usage of alternative polyadenylation sites (3, 21, 55, 137) and are coordinately expressed (101). The rat FAS gene contains 43 exons and 42 introns, whose junctions follow the universal GT/AG rule for splice sites (2). Because the locations of the introns generally coincide with the boundaries between the constituent enzymatic activities of the multifunctional FAS polypeptide (2), and because sequences encoding the seven partial activities are homologous to those of the discrete monofunctional enzymes in bacteria and plants (131), it is hypothesized that the multifunctional FAS is generated by gene fusion (2).

GPAT catalyzes the first committed and presumably rate-limiting step in triacylglycerol and phospholipid biosynthesis. There are two isoforms of GPAT, one in the mitochondrial outer membrane and the other on the endoplasmic reticulum (6, 109). Besides the difference in their locations, these two isoforms also differ in their substrate preference and sensitivity to heat, protease, and sulfhydryl group reagents such as N-ethylmaleimide (NEM). Although the microsomal GPAT uses saturated and unsaturated fatty acyl-CoAs equally well and is sensitive to NEM, the mitochondrial GPAT is resistant to NEM and prefers saturated fatty acyl-CoA (6, 136); this may explain the prevalence of saturated fatty acid in the sn-1 position of the naturally occurring phospholipids and triacylglycerol. Moreover, only mitochondrial GPAT is regulated by nutritional and hormonal status (84, 107). The highly inducible mitochondrial GPAT may provide a substrate cycle for fatty acyl-CoA in lipogenic tissues by converting it to lysophosphatidic acid and preventing fatty acyl-CoA from undergoing β-oxidation in mitochondria, thereby directing fatty acids to triacylglycerol synthesis (12). GPAT genes from *Escherichia coli* (68) and from plants (8, 13, 14, 34, 37, 47, 78, 129) have been cloned and characterized, but only murine mitochondrial GPAT has been cloned from a mammalian source (84, 107). By taking advantage of the fact that mitochondrial GPAT mRNA is decreased during fasting and induced after refeeding with a high-carbohydrate, fat-free diet, a 6.8-kb mouse GPAT cDNA was isolated by differential hybridization (84). An approximately 300–amino acid stretch of mitochondrial GPAT shares a 30% sequence identity and an additional 42% similarity to the *E. coli* GPAT (107, 135, 136). Recombinant mitochondrial GPAT was prepared and purified, and GPAT activity was reconstituted using phospholipids (133). The 5′-flanking region of the murine mitochondrial GPAT gene also was cloned (50). Microsomal GPAT gene has yet to be isolated, and its regulatory properties remain unknown. Later in this review, GPAT and mitochondrial GPAT are used interchangeably.

Nutritional and Hormonal Regulation of the Transcription of FAS and Mitochondrial GPAT

Under normal conditions, FAS and mitochondrial GPAT are expressed mostly in the lipogenic tissues, i.e. liver and adipose tissue; other tissues contain

low activities of these enzymes. Changes in nutrient intake cause changes in circulating glucose, which in turn signal the secretion of hormones. It is generally accepted that insulin in the circulation is elevated during ingestion of a high-carbohydrate diet and induces enzymes involved in fatty acid and triacylglycerol synthesis. Glucagon, on the other hand, is elevated during starvation and suppresses activities of enzymes in fatty acid and triacylglycerol biosynthesis by increasing the intracellular cAMP level. During fasting and refeeding, inhibition and induction of the FAS and GPAT expression are large in magnitude in liver and adipose tissue (63, 85, 113). They may follow different time courses in these organs (45, 46). Unlike ACC, FAS and mitochondrial GPAT are not subject to allosteric regulation and posttranslational modification. Rather, the FAS and mitochondrial GPAT genes are regulated mainly by controlling their rates of transcription. Actinomycin D inhibits induction of FAS mRNA by refeeding. More significantly, transcription run-on assays with isolated nuclei showed that the transcription rate of the mouse FAS gene increases by up to 39-fold at 6 h after refeeding, and the maximal transcription is maintained up to 16 h (85).

The effects of insulin and glucagon on transcription of the FAS and GPAT genes have been examined by administering insulin to diabetic animals and glucagon or dibutyryl cAMP to normal animals. FAS (85) and GPAT (107) mRNA levels were low in streptozotocin-diabetic mice and increased by two- and fourfold, respectively, within 1 h after insulin injection. Within 6 h, insulin caused a maximum 19-fold stimulation, comparable to increases in the FAS and GPAT mRNAs caused by feeding fasted mice a high-carbohydrate, fat-free diet (63, 107). The FAS transcription rate increased 3.5-fold after 30 min after insulin injection, reached a maximum of 7-fold after 2 h of insulin administration, and was maintained at the maximum level up to 6 h in diabetic mice (85). Cycloheximide abolished the insulin effect (85, 107) suggesting that transcriptional regulation of the FAS and GPAT genes by insulin requires ongoing protein synthesis.

Administration of glucagon or dibutyryl cAMP during refeeding of animals subjected to fasting completely blocked elevation of the FAS and GPAT transcription rates, which suggests that glucagon, via cAMP, antagonizes the insulin effect and inhibits transcription of the FAS and mitochondrial GPAT genes (85). The antagonism of the effect of cAMP on insulin-stimulated expression of FAS in liver was mimicked ex vivo in H4IIE hepatoma cells in culture (88). Growth hormone also antagonized the effects of insulin on FAS. In growing rats as well as pig adipose tissue in culture, porcine growth hormone decreased FAS mRNA level by inhibiting transcription of the FAS gene by 80% (30, 31).

A high level of glucose is not only required for the insulin stimulation of FAS transcription (74); it is also involved in the stabilization of FAS mRNA in HepG2

cells (104). Stabilization of FAS mRNA also was observed during adipocyte differentiation (75). Signaling molecules that mediate stimulation of FAS and mitochondrial GPAT by glucose and insulin need to be elucidated. Glucose-6-phosphate has been postulated to be the signaling molecule for the glucose/insulin stimulation of transcription of L-PK, S14, and FAS genes (39, 120). More recently, it has been reported that xylulose-5-phosphate, which is derived from glucose-6-phosphate via the pentose phosphate pathway, mediated glucose stimulation of L-PK transcription (29). Further studies are necessary to confirm the identity of the proximal metabolite that mediates glucose effect.

In addition to changes in the levels of circulating insulin and glucagon, the level of thyroid hormone also is elevated during refeeding of animals. Thyroid hormone stimulates FAS expression and probably utilizes a mechanism that is independent of insulin stimulation, because the effect of thyroid hormone was synergistic with a high-carbohydrate diet in the regulation of FAS activity (71). Injecting thyroid hormone into rats for seven days increased FAS activity two- to threefold in liver (27, 70), and hypothyroidism reduced the liver FAS activity (123). The effects of thyroid hormone on FAS expression also are seen in cultured cells. Treating primary rat and chick embryo hepatocytes with T3 produced a two- to threefold increase in FAS activity (114) and a fivefold increase in FAS mRNA (130), due to an increase in FAS transcription (115). When mature 3T3-L1 adipocytes were treated with 10 nM T3, the relative rate of FAS synthesis, the steady-state mRNA level, and the transcriptional rate all increased within hours and were maintained at the stimulated level for as long as 24 h (75).

Although molecular mechanisms underlying the suppression of FAS transcription by fatty acids are unclear, addition of 6- to 12-carbon fatty acids in cultured chick embryo hepatocytes inhibited T3-stimulated FAS gene transcription (94). Furthermore, dietary polyunsaturated fatty acids (PUFA) can suppress the expression of several genes involved in hepatic lipid metabolism (52), including transcription of the FAS and S14 genes (9, 23). On the other hand, transcription of the PEPCK gene in liver is unaffected by dietary PUFA (9). In adipose tissue, PUFA has only a slight suppressive effect on FAS mRNA levels, although both saturated and unsaturated fatty acids depress de novo fatty acid synthesis (4, 24, 76). Inhibition of T3-stimulated FAS transcription by fatty acids could be explained by the fact that long-chain fatty acids were shown to decrease T3 binding to its receptor (44, 94). FAS transcription also may be affected via the well-documented activation of peroxisome proliferator-activated receptor (PPAR) by fatty acids (11). However, experimental evidence supporting either of these possibilities needs to be provided.

Glucocorticoids also regulate FAS gene expression. In cultured explants of fetal rat lung, dexamethasone increased FAS activity and mRNA level, due to

stimulation of transcription and stabilization of the mRNA. These results are consistent with the finding that actinomycin D only partially inhibited the stimulatory effect of glucocorticoids on FAS mRNA levels (133, 134). As measured by transcription run-on assays, an approximate threefold maximum stimulatory effect of dexamethasone was observed 1–2 h after addition of the hormone and was still apparent for up to 48 h (134). The half-life of FAS mRNA, which is 4 h in the explants of fetal rat lung under normal conditions, was increased by 40% at 5 h and by 84% at 44 h after dexamethasone was added (133). Retinoic acid antagonizes the glucocorticoid effect on FAS mRNA levels (134). Whether the mechanisms involved in insulin stimulation are related to those for glucocorticoid stimulation of the FAS expression is unknown.

Nutritional and hormonal stimuli control transcription of the FAS and GPAT genes through interactions of *cis*-acting elements and *trans*-acting factors. Based on studies of transgenic mice carrying 2.1 kb of 5′-flanking region (-2100 to $+67$) of the rat FAS gene fused to a chloramphenicol acetyltransferase (CAT) reporter gene, *cis*-acting elements necessary for tissue-specific and nutritional and hormonal regulation of the FAS expression are mostly contained in the 2.1 kb of 5′-flanking DNA (113). There is strong positive correlation between the mRNA levels and the tissue-specific gene expression patterns of the reporter and the endogenous FAS in these transgenic mice (113). Fasting and refeeding, insulin, dibutyryl cAMP, and glucocorticoid regulated expression of the reporter gene and the endogenous FAS gene in a similar manner. In contrast, PUFA (menhaden oil enriched in long-chain n-3 fatty acids) dramatically suppressed the endogenous FAS and mRNA in both liver and adipose tissue of transgenic mice vis-á-vis those fed oleic acid, but CAT activities in these tissues of PUFA-treated mice were only 50% lower than those of mice on the oleic acid diet (113). Thus, the stabilization of FAS mRNA that is observed during 3T3-L1 adipocyte differentiation (75) and high-glucose treatment in HepG2 cells (104) also may contribute to regulation of FAS mRNA levels by PUFA. Alternatively, elements not contained in the first 2.1 kb of the FAS 5′-flanking DNA may be necessary for the full suppression of FAS transcription by PUFA. Identification of the *cis*-acting nucleotide sequences and *trans*-acting factors involved in the nutritional and hormonal regulation of FAS and GPAT genes will greatly facilitate understanding of the underlying molecular mechanisms.

Molecular Mechanisms of the Transcriptional Regulation of FAS and GPAT Genes

As discussed above, long-term regulation of FAS and GPAT expression is mostly through regulation of transcription. The cloning of the FAS and GPAT genes has facilitated analysis of the molecular mechanisms underlying nutritional and hormonal regulation of transcription. *Cis*-acting elements within the 5′-flanking

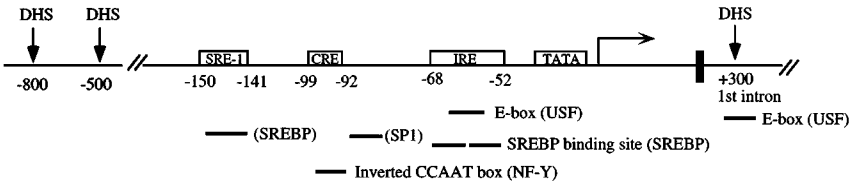

Figure 2 The *cis*-acting elements and *trans*-acting factors involved in the regulation of transcription of the FAS gene. *Numbers* indicate the positions of each element relative to the transcription start site. CRE, cAMP response element; DHS, DNase I hypersensitive site; IRE, insulin response element; SRE, sterol response element; SREBP, SRE binding proteins; TATA, TATA box; USF, upstream stimulatory factor. Transcription factors that bind to the elements are in *parentheses*.

DNA of the rat FAS and mouse GPAT genes and *trans*-acting factors that bind to the *cis*-elements have been characterized in Figure 2. DNA elements that confer the hormonal regulation on transcription of the FAS gene are located at the proximal region of the 5′-flanking DNA. Sequences of these DNA elements are well conserved among the known FAS genes (1, 5, 42, 54). Because the proximal promoter regions of the FAS genes from different species share greater sequence similarity than does the distal region, it is likely that the underlying mechanisms for the nutritional and hormonal control of the FAS gene are well conserved among these species.

INSULIN REGULATION OF THE FAS AND MITOCHONDRIAL GPAT GENES 3T3-L1 cells represent an excellent model to study regulation of genes involved in fatty acid and triacylglycerol synthesis. Upon treatment with dexamethasone and methylisobutylxanthine, 3T3-L1 cells normally growing as fibroblast cells undergo adipocyte differentiation over a period of about 7 days (40, 95). The cells acquire adipocyte morphology, accumulate large lipid droplets due to increased de novo long-chain fatty acid biosynthesis and esterification to triacylglycerol, and become responsive to lipolytic and lipogenic hormones (95). Treatment of mature 3T3-L1 adipocytes with insulin elicited a threefold increase in both the rate of synthesis and the mRNA content of FAS, whereas treatment with dibutyryl cAMP caused a 60% and 80% decrease in FAS mRNA and the rate of enzyme synthesis, respectively (84). When constructs containing 5′-deletions of the FAS 5′-flanking DNA linked to the CAT reporter gene were transiently transfected into fully differentiated 3T3-L1 adipocytes, plasmids containing 5′-deletions at −2100, −1400, −1009, and −332 to +67 (relative to the transcription start site) positions all exhibited a two- to threefold increase in CAT activity when the cells were treated with physiological concentrations (10 nM) of insulin in the presence of glucose (74). Similarly, stable 3T3-L1 transfectants of all four constructs and transient transfection in H4IIE hepatoma cells showed a threefold increase in CAT activity when the adipocytes were treated

with insulin (74). These experiments localized the insulin response sequence (IRS) to the first 332 bp of FAS 5′-flanking DNA.

Additional constructs progressively deleted the FAS promoter region from −318 to −67; each one retained the stimulation of the luciferase reporter activity by 10 nM insulin in transiently transfected 3T3-L1 adipocytes. However, no insulin effect was found when FAS 5′-flanking DNA was deleted up to −25, which suggests that the IRS is located between −67 and −25. Further localization of the FAS IRS to the −68/−52 region was based on the facts that (*a*) the −68/−52 region is protected in a DNase I footprinting analysis, (*b*) it interacts with specific nuclear factors from mouse liver and 3T3-L1 adipocyte nuclear extracts on gel mobility shift assays, and (*c*) three tandem repeats of the −68/−52 region linked to the heterologous SV40 promoter confer responsiveness to insulin (73). Although the identified FAS IRS itself is sufficient to elicit an insulin response when linked to a heterologous promoter, additional DNA elements within the FAS 5′-flanking DNA could be involved in insulin stimulation that occurs during fasting and refeeding.

Studies of the chromatin structure of liver nuclei during fasting and refeeding of rats demonstrated DNase I hypersensitive sites in the FAS 5′-flanking DNA and in the first intron (92). Hypersensitive sites at approximately −500 and −800 bp were specific to nuclei from refed rats. A tripartite structure—consisting of 5′-GCCT, a 6-bp spacer, and a 3′-palindrome sequence at −518 to −495—was postulated to represent the insulin response element of the FAS gene (92, 132). Direct evidence that this tripartite sequence confers an insulin response to a heterologous promoter or mutations of this region that affect the insulin response of the FAS promoter have not been reported. The role of this sequence, if any, is not clear. There also is a DNase I hypersensitive site within the first intron of the FAS gene in liver but not in spleen (36). An upstream stimulatory factor (USF) binding site at +292/+297 bp in the first intron was reported to be a positive regulatory element (81) involved in glucose responsiveness of the FAS gene (36). In transgenic mice expressing the CAT reporter driven by the first 2.1-kb FAS 5′-flanking DNA, however, sequences in the first intron were not required for regulation of FAS by fasting/refeeding. Thus, the intron element may not be essential or redundant elements may be involved (113).

Within the FAS IRS, an E-box DNA binding motif (5′-CATGTG-3′) is located at −65/−60 bp. The E-box motif appears to be important because in vitro binding of nuclear factors is abolished when the E-box motif is mutated (73). In transient as well as stable transfection assays, mutations of the E-box sequence within the −2.1-kb FAS 5′-flanking DNA context greatly reduce the insulin response (128). USF1 and USF2, members of the basic-helix-loop-helix (bHLH) family of transcription factors that recognize the E-box motif, bind to the FAS −65/−60 E-box in vitro (127). USF binding to the E-box is functionally

required for insulin regulation because mutations within the E-box abolish both the insulin response and the interaction with USF. Dominant negative USFs, when cotransfected into 3T3- L1 adipocytes, decreased insulin-stimulated FAS promoter activity (128). In addition to its involvement in insulin regulation of the FAS gene, the −65/−60 E-box is also important for basal activity of the FAS promoter. Mutation of the E-box in the context of −2.1-kb 5′-flanking DNA and deletion of the −73/−43 bp in context of 150 bp of 5′-flanking DNA decreased basal promoter activity by as much as 75% (7, 128).

Sterol response element–binding proteins (SREBPs) (reviewed in reference 15), members of the bHLH family of transcription factors, also were reported to bind the FAS 5′-flanking DNA (7, 69). Transgenic mice expressing high levels of active SREBP1a developed massive enlargement of their livers and of hepatocytes because of cholesterol and triglyceride accumulation, and FAS mRNA levels were elevated markedly (106). However, feeding mice with sterol-depleted or -enriched diets did not cause a consistent change in FAS mRNA level (33; M Soncini, Y Moon, HS Sul, unpublished data). In addition, dietary sterol does not significantly change GPAT mRNA level in livers of rats and mice (33). In HepG2 cells, FAS mRNA level was decreased by sterols (7). Over-expression of active SREBP stimulated FAS and GPAT promoter activities (7, 33). In vitro, SREBP binds to two regions of the FAS 5′-flanking DNA. One resides at −150/−140 bp and matches the SRE-1 sequence found in the LDL receptor 5′-flanking DNA, but it is dispensable for the sterol regulation of FAS mRNA (7). The other is within the FAS IRS and includes two tandem copies of SREBP binding sites that split the −65/−60 E-box in the middle (69). Binding of USF to the −65/−60 E-box and SREBP to the flanking sites seem to be independent processes (128). Because the USF and SREBP binding sites overlap, distinct transcription factors may be utilized to regulate FAS gene transcription under different regulatory conditions.

Based on sequence comparisons, three putative SREBP binding sites (−186/−177 bp, −170/−161 bp, and −64/−55 bp) may be present in the GPAT gene (33). Only the 3′-SRE bound strongly to recombinant SREBP in vitro; this SRE also was required for stimulation by over-expression of SREBP. Sp1 may cooperate with SREBP to stimulate the FAS promoter (69); similarly, NF-Y bound to the inverted CCAAT box at −78/−74 bp and was required for the activation of GPAT proximal promoter by SREBP (33).

Whether USF interaction with the E-box is a common mechanism in the transcriptional regulation of the genes involved in fatty acid and triacylglycerol biosynthesis needs further investigation. E-box sequences are functionally important in the regulation of lipogenic genes even in lower eukaryotes such as yeast. In *Saccharomyces cerevisiae*, activation sites that are 5′ to each FAS gene (FAS1 and FAS2) have the consensus sequence, TYTTCACATGY,

that contains the cognate 5′-CANNTG-3′ E-box sequence (99). Moreover, the same sequence motif is found in the upstream regions of all characterized yeast genes encoding enzymes of phospholipid biosynthesis (99). Originally identified by their ability to bind to the adenovirus major late promoter (17, 72, 98), both USF1 and USF2 bind to the cognate E-box sequence as homo- and heterodimers and are expressed ubiquitously among various types of mammalian tissues and cells (110, 111). Specific USF binding sites are found in a number of genes (16, 18, 20, 22, 65, 66, 77, 82, 86, 87, 90, 91, 97, 103, 105, 139); among them, the L-PK (105) and S14 (65) genes also are regulated by fasting and refeeding in animals and by glucose in cells in culture (53, 64, 67). Similar to the findings for insulin-regulated transcription of the FAS gene, over-expression of dominant negative USFs blocked the glucose response of the L-PK gene (65). As demonstrated with USF2 −/− knock-out mice, USF2 is required for the normal transcriptional response of the L-PK and S14 genes to glucose in liver under in vivo conditions (121). Evidence against the direct involvement of USF in the regulation of L-PK and S14 genes also has been reported (56).

The −328-bp region of the 5′-flanking DNA of the mitochondrial GPAT gene confers the insulin regulation of the GPAT promoter (50) and contains an E-box sequence at −320 bp that perfectly matches the FAS E-box. Based on the fact that FAS and GPAT are regulated coordinately by fasting and refeeding cycles and by insulin administration, it is possible that the *cis-trans* system utilized in insulin-regulated expression of the GPAT gene is similar to that employed by the FAS gene.

The signaling pathway leading to the transcriptional activation of the lipogenic genes by insulin and its coordination with other insulin-regulated enzymes needs to be elucidated. The best studied gene with known physiological relevance is the PEPCK gene; it is negatively regulated by insulin and catalyzes the rate-limiting step in hepatic gluconeogenesis (79). Although signaling molecules and molecular mechanisms have yet to be elucidated fully, phosphatidylinositol 3-kinase (PI-3 kinase), but not Ras-MAP kinase or p70 S6 kinase, is required for PEPCK gene repression by insulin (118). Involvement of insulin-dependent phosphorylation of transcription factors at the end of the signaling pathways in insulin-regulated gene expression needs to be investigated. c-Fos, an AP-1 transcription factor of the immediate early gene family, is transcriptionally activated and phosphorylated by insulin treatment (59, 119). Phosphorylation of Stat3, a component in IL-6 action, is stimulated by insulin (19). Because of the diversity of genes that are regulated by insulin, a consensus molecular mechanism, such as a consensus insulin response element or transcription factor common to insulin regulation of several genes has yet to be found. Different signal transduction pathways, in combination with distinct transcription factors that bind to different cognate response elements,

may mediate insulin regulation of various genes in diverse biological settings. For insulin regulation of genes involved in intermediary metabolism, exemplified by the PEPCK gene, the PI-3 kinase pathway may play an important role. When constitutively active P110 catalytic subunit of the PI-3 kinase (60) was cotransfected with the FAS −2.1-kb reporter gene construct, the promoter activity was elevated to a level comparable to that resulting from insulin treatment. In addition, the FAS promoter was no longer stimulated by insulin (D Wang, HS Sul, unpublished data). This is consistent with an involvement of the PI-3 kinase pathway in insulin regulation of the FAS promoter.

GLUCAGON (cAMP) REGULATION OF THE FAS GENE Glucagon antagonizes the stimulatory effect of insulin on FAS transcription by increasing the intracellular concentration of cAMP. In H4IIE hepatoma cells, cAMP inhibited the insulin-stimulated CAT activity driven by the FAS promoter (88). 5′-Deletion analysis of FAS 5′-flanking DNA revealed that the cAMP response element is located between −149 and +68 bp and that the FAS IRS is required for the cAMP effect (88). In addition to the FAS IRS (73), an inverted CCAAT box at −99/−92 (Figure 2) was protected in a DNase I footprinting analysis. Mutation of the inverted CCAAT box abolished responsiveness to cAMP. Consistent with localization of the FAS IRS to the −68/−52-bp region, insulin responsiveness of 5′-flanking DNA containing the mutated inverted CCAAT box was not affected (88). However, other elements within the 5′-flanking DNA of the FAS gene also may be critical for the cAMP effect because the −124 to −30 region of the FAS gene (containing both the cAMP response sequence and the IRS) linked to the heterologous thymidine kinase promoter was not responsive to the antagonistic effect of cAMP (88). Identification of the cAMP response element of the FAS gene as an inverted CCAAT box put the FAS gene into a small group of cAMP-regulated genes that do not use the more common CREB or ATF-1 binding sites for transcriptional regulation. In fact, the basal transcription factor NF-Y or a related protein binds to the inverted CCAAT box of the FAS promoter in vitro (93). Consistent with the fact that NF-Y is constitutively expressed, there was no difference in protein-DNA complex formation when nuclear extracts prepared from control or cAMP-treated H4IIe cells were used. If NF-Y is involved in the cAMP response, modifications or other associated factors may be involved in the cAMP antagonism.

Although the nutritional and hormonal regulations of genes involved in fatty acid and triacylglycerol synthesis are mediated by distinct *cis*- and *trans*-components, the *cis-trans* systems for different hormonal stimuli are likely to interact with each other to create concerted regulation. For cAMP inhibition of FAS transcription, both the FAS IRS and the cAMP response element were required (88). Similarly, in the promoter of the L-PK gene that is also

stimulated by glucose/insulin and inhibited by glucagon (cAMP) when fasted animals are refed, the glucose response element (L4 site, to which USFs bind) functions in close cooperation with the contiguous L3 site to which the hepatocyte nuclear factor 4 (HNF-4) binds (28) for both glucose/insulin and cAMP effects. Although it is not known whether HNF-4 directly interacts with USF during glucose/insulin or cAMP regulation of the L-PK gene, and though it was suggested that USF binding was not dependent on nutritional and hormonal status (28), DNA binding activity of HNF-4 is modulated by cAMP-dependent protein kinase A (122). Phosphorylation on serine residues within the DNA binding domain strongly suppresses the DNA binding ability of HNF-4. However, HNF-4 binding to the FAS promoter and possible involvement of HNF-4 modulation in the glucagon (cAMP) inhibition of the FAS gene have not been reported.

In the nutritional and hormonal regulation of the PEPCK gene, glucocorticoid stimulation and insulin inhibition of PEPCK transcription involves a complex array of *cis*-acting elements. In addition to the two glucocorticoid receptor binding sites (GR1 and GR2), maximal glucocorticoid stimulation requires three accessory factor binding sites, including AF1, which binds the orphan receptors COUP-TF and HNF-4; AF2, which binds HNF-3; and AF3, which binds COUP-TF (43, 102). Insulin inhibition of PEPCK requires the distal IRS with a core sequence of T(G/A)TTTTG, which overlaps the AF2 site and a yet-to-be-identified proximal IRS (80). Although the transcription factors required for insulin's inhibition of the PEPCK gene remain to be identified, overlap of the IRS with the AF2 site makes it plausible that execution of one hormone effect is accompanied by the inhibition of the other antagonistic hormonal effect at the molecular level.

SUMMARY AND FUTURE DIRECTIONS

The importance of de novo fatty acid and triacylglycerol biosynthesis may explain the lack of mutations of the key enzymes in the lipogenic pathway. Loss of control of the synthesis of lipogenic enzymes, on the other hand, may be involved in human diseases such as diabetes, obesity, hyperlipidemia, and cancer (25, 32, 38, 49, 61, 62, 108). Long-term regulation of the critical enzymes in fatty acid and triacylglycerol synthesis by nutritional and hormonal factors is carried out mainly at the transcriptional level. During recent years, studies of the molecular mechanisms underlying the transcriptional regulation have been facilitated by molecular biological techniques. Significant progress has been made toward identifying the *cis*-acting elements within the promoters as well as toward characterization of the *trans*-acting factors that bind to the DNA elements. As demonstrated by subjecting animals to fasting and refeeding,

multiple nutritional and hormonal factors are involved in the concerted regulation of transcription of the FAS and mitochondrial GPAT genes. Recent studies have provided new understanding of the molecular events underlying the FAS regulation by insulin and glucagon during fasting and refeeding. Stimulation of the FAS gene by insulin requires the FAS IRS and its interaction with USF. For the antagonistic effect of cAMP, an inverted CCAAT box that binds NF-Y was identified as the cAMP response element, and both the FAS IRS and the inverted CCAAT box are required to mediate suppression of FAS transcription by cAMP.

Based on current knowledge of the transcriptional regulation of the FAS gene, all the identified hormone-specific response elements—including the IRS, the inverted CCAAT box, and the SREBP binding sites—are important for basal promoter activity. Regulation of basal FAS expression and hormone stimulated/inhibited transcription may share some regulatory factors. In order to provide specific responses to hormones, additional components may be needed so that regulation by hormonal stimuli can be differentiated from maintenance of basal activity. This additional control could be mediated through modifications to transcription factors (or their interacting proteins) that bind to the DNA elements. Protein components that act upstream of the transcription factors remain to be identified. The signaling pathways that transduce nutritional and hormonal stimuli and finally lead to the nuclear events to activate/inhibit transcription need to be elucidated. The array of multiple *cis*-acting elements and binding factors may be more complicated than those currently known. Moreover, protein-protein interactions among the transcription factors and with the basal transcriptional machinery also may contribute to coordination of transcriptional regulation induced by multiple stimuli. Recruitment of transcription factors to a certain position on the promoter by one physiological stimulus may cause conformational and even covalent changes of the interacting proteins and, thus, affect the DNA binding and/or transactivation activities involved in the regulation by other stimuli. Changes of the higher-order chromatin structure also may contribute to concerted transcriptional regulation. Further investigation of these aspects will provide more insights into the molecular mechanisms underlying the nutritional and hormonal regulation of the lipogenic genes.

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