Regulation of fatty acid synthase gene transcription

Sequences that confer a positive insulin effect and differentiation-dependent expression in 3T3-L1 preadipocytes are present in the 332 bp promoter

Naïma MOUSTAÏD,* Kenji SAKAMOTO,* Steven CLARKE,† R. Scott BEYER* and Hei Sook SUL*‡

*Department of Nutrition, Harvard School of Public Health, 665 Huntington Ave., Boston, MA 02115,

and †Department of Food Science and Human Nutrition, Colorado State University, Fort Collins, CO 80523, U.S.A.

We have previously reported induction of fatty acid synthase (FAS) gene expression by insulin and adipocyte differentiation in 3T3-L1 cells. In order to identify sequences responsible for insulin regulation of the FAS gene, chimaeric constructs containing serial deletions of the 5'-flanking region of the rat FAS gene ligated to the chloramphenicol acetyltransferase (CAT) reporter gene were prepared and transfected into 3T3-L1 cells. Plasmids containing 2100 (–2100CAT), 1400 (–1400CAT), 1009 (–1009CAT) and 332 (–332CAT) bp of FAS 5' flanking sequences exhibited comparable basal CAT activities in 3T3-L1 preadipocytes. This activity was 3-fold higher when these constructs were transiently transfected into 3T3-L1 adipocytes. Stably transfected 3T3-L1 cells also exhibited a 3-fold increase in CAT activity upon adipocyte differentiation, indicating that sequences required for the differentiation-dependent increase in FAS expression are located within the 332 bp promoter. Treat-

ment with 10 nM insulin increased CAT activity by 2.1 ± 0.2 -, 2.6 ± 0.1 -, 2.0 ± 0.2 - and 1.7 ± 0.2 -fold respectively in 3T3-L1 adipocytes transiently transfected with –2100CAT, –1400CAT, –1009CAT and –332CAT plasmids. CAT activity was increased by 3.0 ± 0.3 - and 3.5 ± 0.6 -fold respectively by insulin treatment in adipocytes stably transfected with –2100CAT and –1009CAT plasmids. When insulin-responsive H4IIE hepatoma cells were transiently transfected with –2100CAT, –1400CAT, –1009CAT and –332CAT plasmids and then treated with 10 nM insulin, CAT activity increased by 3.1-, 3.1 ± 0.8 -, 3.0 ± 0.7 - and 2.3 ± 0.5 -fold respectively in serum-free media, and by 2.6 ± 0.4 -, 3.3 ± 0.9 -, 3.1 ± 0.4 - and 2.9 ± 0.6 -fold respectively in the presence of 0.5% serum. These results indicate that sequences responsible for insulin regulation of FAS gene are also located within 332 bp of the transcription start site.

INTRODUCTION

Fatty acid synthase (FAS) plays a central role in *de novo* lipogenesis in mammals and birds by catalysing all of the reactions in the conversion of acetyl-CoA and malonyl-CoA to palmitate. Fatty acid synthesis is stimulated when glucose is in abundance and the flow of metabolic intermediates through glycolysis is high. FAS activity is not known to be regulated by allosteric effectors or covalent modification. However, the FAS concentration is exquisitely sensitive to nutritional, hormonal and developmental states [1,2]. The concentration of FAS in liver and adipose tissue changes drastically when animals are subjected to different nutritional and hormonal manipulations. When rats are fasted for 1–2 days, the rate of synthesis of FAS declines, and refeeding with a high carbohydrate diet increases synthesis of FAS [3]. Increased circulating insulin and decreased glucagon levels participate in the regulation of FAS synthesis. We have previously reported that FAS mRNA is not detectable in fasted mouse liver and that refeeding causes a dramatic increase in FAS mRNA [4], due to changes in the rate of transcription of the FAS gene [5]. However, the increase in transcription of the FAS gene by fasting/refeeding was not observed in diabetic mice. In addition, the mRNA level and transcription rate for FAS were very low in diabetic mice, and insulin caused a marked and rapid increase in both mRNA level and rate of transcription [5]. We have also shown that the FAS mRNA level increases about 20-fold during differentiation of 3T3-L1 cells to adipocytes due to increases in both the rate of transcription and the stability of the mRNA [6]. We have also observed that insulin increases and cyclic AMP decreases FAS mRNA levels in 3T3-L1 adipocytes

[4], indicating the existence of independent effects of insulin and cyclic AMP on FAS expression. There are, however, no reports on the molecular mechanisms underlying insulin regulation of FAS gene transcription. Insulin is known to stimulate or inhibit the expression of several genes [7–9]. However, neither the consensus *cis*-acting DNA sequences nor the *trans*-acting factors responsible for insulin regulation of gene transcription have been defined.

Although the 5' flanking regions of the rat and goose FAS genes have been isolated [10,11], the dissection of the FAS promoter has never been reported. As a first step toward understanding the positive regulation of FAS gene expression by insulin, we have tested whether the 5' flanking region of the rat FAS gene can confer insulin-responsiveness to a linked reporter gene in 3T3-L1 adipocytes and H4IIE hepatoma cells. Our results show that the promoter activity of these FAS chimaeric constructs is stimulated by insulin in both cell lines. Furthermore, the promoter activity also increases during adipose conversion of 3T3-L1 cells. We report here that the insulin-responsive sequences, as well as sequences involved in differentiation-dependent increase in FAS expression, are located within 332 bp of the transcription start site.

MATERIALS AND METHODS

Cell culture

3T3-L1 preadipocytes [12] were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Adipocyte differentiation was initiated by treating cells at confluence with 0.5 mM 1-methyl-3-isobutylxanthine and 0.25 μ M dexamethasone.

methasone for 2 days [13]. The drugs were then removed and cells were maintained for a further 2 days in serum-supplemented medium before transfection. H4IIE hepatoma cells were cultured under the same conditions as 3T3-L1 cells, except that the differentiation protocol was omitted. Cells were used for transfection at 80 % confluency.

Transient transfections

Each 60 mm dish of 3T3-L1 adipocytes or H4IIE hepatoma cells was transfected with 10 μ g of chloramphenicol acetyltransferase (CAT) construct by the calcium phosphate DNA co-precipitation method [14]. To normalize for efficiency of transfection, 2 μ g of pSV2-luciferase or pCMV- β -gal, or 7 μ g of pRSV- β -gal, was co-transfected with the test plasmids in some experiments. The precipitate was allowed to remain on the cells overnight. The transfected cells were maintained in serum-free medium before addition of insulin and were harvested after 48 h of hormone treatment. When preadipocytes were compared with adipocytes, the preadipocytes were transfected 2 days post-confluence. After transfection, both cell types were maintained for 48 h in serum-supplemented medium before harvesting.

Stable transfections

A 1 μ g portion of pSV2-Neo, whose expression confers resistance to the antibiotic geneticin, G418 (Gibco Laboratories), was co-transfected with 10 μ g samples of different CAT constructs by calcium phosphate precipitation into exponentially growing 3T3-L1 preadipocytes. Cells were incubated in serum-containing media for 2 days before G418 was added at 0.4 mg/ml. After 2–3 weeks of selection in the presence of the antibiotic, several colonies of cells resistant to G418 (of approx. 100 per 100 mm plate) were isolated and the remaining colonies were pooled. Cloned cell lines or pooled colonies were grown without G418, differentiated and assayed for CAT expression after treatment with or without insulin in serum-free media.

CAT assay

The cells were lysed by four cycles of freezing–thawing. Aliquots of the cytosolic extracts were saved for luciferase or β -galactosidase assays. The remaining extract was heated at 65 °C, then assayed for CAT activity. The reaction mixture contained 0.1 μ Ci of [¹⁴C]chloramphenicol and 70 μ g of acetyl-CoA, in a final volume of 220 μ l of 0.25 M Tris/HCl, pH 8. The reaction was carried out for between 2 h and overnight, such that CAT activity was linear with respect to the amount of extract used. The mixture was extracted with xylenes, according to the previously described method [15], and radioactivity was counted. β -Galactosidase activity was assayed spectrophotometrically as previously described [16]. Firefly luciferase activity was assayed according to previously reported methods [17], using a luminometer (Berthold). Protein concentrations of extracts were determined by the Bio-Rad (San Diego, CA, U.S.A.) Coomassie Blue dye binding assay using BSA as standard [18]. CAT activity was normalized to β -galactosidase or luciferase activity.

Plasmid constructions

FAS(–2100)CAT plasmid (henceforth designated –2100CAT) was constructed by ligating a *Bst*YI restriction fragment, containing nucleotides –2100 to +67 of the rat FAS gene, into the *Bam*HI cloning site of the pBLCAT3 vector [19]. pSV2-luciferase and pCMV- β -gal plasmids were kindly provided by Dr.

A. Brasier (MGH, Boston, MA, U.S.A.) and Dr. G. R. MacGregor (HHMI, Houston, TX, U.S.A.) respectively. Deletions of the FAS(–2100)CAT plasmid, which remove larger sequences of the 5' flanking region, were constructed using an exonuclease III/mung bean nuclease deletion kit (Stratagene, La Jolla, CA, U.S.A.). The deletions obtained were FAS(–1400)CAT, FAS(–1009)CAT and FAS(–332)CAT (designated in the remainder of the paper as –1400CAT, –1009CAT and –332CAT respectively). The sequence of these inserts was verified by dideoxy sequencing, using a Sequenase kit [20].

RESULTS AND DISCUSSION

Expression of FAS–CAT fusion genes by transient transfection of 3T3-L1 preadipocytes and adipocytes

We have previously shown that FAS gene expression increased during differentiation of 3T3-L1 fibroblasts into adipocytes and that this increase was due to both transcriptional and post-transcriptional control [4,6]. In the present studies, we used a FAS–CAT fusion gene to see if the 5' flanking sequences of the FAS gene are sufficient to confer to a reporter gene the differentiation-dependent increase in transcription that occurs during adipose conversion. Sequences between nucleotides –2100 and +67 of the FAS gene were fused to the reporter gene CAT such that transcription of the CAT gene was directed by the FAS promoter (Figure 1). In order to identify potentially important regulatory elements within the 5' flanking region of the FAS gene, the above construct was used to make a series of deletions containing smaller fragments of the 5' flanking region of FAS, i.e. –1400CAT, –1009CAT and –332CAT. All of

Figure 1 Structure of the pFAS(–2100)CAT construct

pFAS(–2100)CAT was constructed by ligating a *Bst*YI restriction fragment, containing FAS sequences from –2100 to +67, into a *Bam*HI cloning site upstream of the CAT coding sequences in plasmid pBLCAT3. SV40 intron and polyadenylation sequences are located 3' of the CAT gene. This plasmid was used to generate deletions of the 5' flanking region of FAS with 5' end points at –1400, –1009, and –332 nucleotides. All of these deletions have a common 3' end at +67.

CAT activity		(fold change)	
(% conversion)			
Preadipocytes	Adipocytes		
1.10 ± 0.15	3.92 ± 0.28	3.6 ± 0.9	—332
0.51 ± 0.04	1.59 ± 0.06	3.1 ± 0.7	—1009
1.85 ± 0.70	5.66 ± 0.55	3.1 ± 0.4	—1400
1.16 ± 0.35	3.14 ± 0.25	2.7 ± 0.3	—2100
			CAT

Figure 2 Expression of the FAS–CAT fusion genes in transient transfections of 3T3-L1 preadipocytes and adipocytes

Preadipocytes (2 days post-confluence) or adipocytes (4 days after differentiation had been induced, as indicated in the Materials and methods section) were co-transfected with 2 µg of pCMV-β-gal and 10 µg of the FAS–CAT fusion genes constructs —2100CAT, —1400CAT, —1009CAT or —332CAT. At 48 h post-transfection, cells were harvested and assayed for CAT and β-galactosidase activities. The ratio of CAT to β-galactosidase activity in each sample served as a measure of normalized CAT activity. Cytomegalovirus (CMV) promoter activity did not change appreciably during adipocyte differentiation. The basal CAT activity in preadipocytes and adipocytes is indicated as the percentage conversion of chloramphenicol to its acetylated products, and results are means ± S.E.M. of five independent experiments. Each experiment represents the average from duplicate or triplicate plates. The fold change in CAT activity in adipocytes compared with preadipocytes is indicated (means ± S.E.M. of five separate experiments).

Table 1 Expression of FAS–CAT fusion genes in preadipocytes and adipocytes of stable transfectants of 3T3-L1 cells

Growing preadipocytes were transfected with either pSV2-CAT or the fusion constructs containing 2100 and 1009 bp of the 5' flanking region of FAS gene linked to the reporter gene CAT, along with plasmid pSV2-Neo (whose expression confers resistance to the antibiotic neomycin). Some cultures were also co-transfected with 1 µg of pSV2-luciferase. After 2 weeks of selection in G418-supplemented medium, resistant clones were isolated as either individual or pooled clones. Cells were harvested as preadipocytes (2 days post-confluence) or adipocytes (5 days after differentiation was induced, as indicated in the Materials and methods section). Results are expressed as a fold change in CAT activity in adipocytes compared with preadipocytes, and are means ± S.E.M.; *n* represents the number of individual and mixed clones assayed.

Plasmid	Change in CAT activity (fold)
pSV2	0.29 ± 0.15 (<i>n</i> = 4)
—2100CAT	3.0 ± 0.5 (<i>n</i> = 3)
—1009CAT	2.6 ± 0.4 (<i>n</i> = 7)

these deletions have a common 3' end at +67. These constructs were introduced into 3T3-L1 fibroblasts or mature adipocytes to compare basal promoter activities in both cell types. The basal promoter activity was similar among the different constructs when transiently transfected into 3T3-L1 cells (Figure 2). In addition, CAT activity was 2.7 ± 0.3-, 3.1 ± 0.4-, 3.1 ± 0.7- and 3.6 ± 0.9-fold higher in differentiated than in undifferentiated cells for plasmids —2100CAT, —1400CAT, —1009CAT and —332CAT respectively (Figure 2).

Expression of FAS–CAT fusion genes in preadipocytes and adipocytes of stable transfectants

Expression from transiently transfected plasmids depends on the number of cells which take up the transfected gene, as well as on promoter sequences. We therefore isolated cell lines in which the FAS–CAT fusion gene is stably integrated, in order to confirm

the increase in CAT activity during differentiation. Several transfectants containing —2100CAT and —1009CAT have been isolated to study expression of the FAS–CAT fusion genes before and after adipocyte differentiation. When the stable transfectants containing —2100CAT and —1009CAT constructs were induced to differentiate, CAT activity increased by 3.0 ± 0.5- and 2.6 ± 0.4-fold respectively in adipocytes compared with preadipocytes (Table 1). This increase in reporter gene expression was specific to the FAS promoter, since CAT activity decreased by 70% during the differentiation process when driven by simian virus 40 (SV40) promoter sequences, which is in agreement with a previous report by Djian et al. [21]. Therefore the results obtained in stable transfections are in agreement with those obtained in transient transfections. Overall, these experiments show that the sequences required for the differentiation-dependent increase in FAS transcription are present within 332 bp upstream of the transcription start site. We have previously shown that FAS mRNA increases about 20-fold during adipocyte differentiation of 3T3-L1 cells [4]. In the present paper, FAS promoter activity increased about 3-fold during differentiation when assessed by transfection assays, compared with a 1.5-fold increase in FAS transcription in run-on assay experiments [6]. The 2-fold difference might be due to the differences in the sensitivity of CAT transfection and run-on transcription assays. Our results, however, confirm that the FAS gene is both transcriptionally and post-transcriptionally controlled during adipose conversion of these cells.

In contrast to the other adipose-specific genes such as stearyl-CoA desaturase [22] and adipose P2 (aP2) [23], whose transcription is not detectable in preadipocytes but dramatically increases during adipocyte differentiation, FAS gene transcription is high in preadipocytes, as we have shown previously by transcription run-on assays and also here by high CAT activity [6]. For the stearyl-CoA desaturase and aP2 genes, proximal promoter elements containing AP1 and C/EBP binding sites direct differentiation-dependent gene expression in cultured adipocytes, and C/EBP is a transactivator of these promoters [24]. Previous studies have identified a differentiation-dependent factor that binds to an enhancer 5 kb upstream of the aP2 gene [25–27]. Possible transactivation of FAS transcription by C/EBP or other factors during adipocyte differentiation has not been studied. The region upstream of the transcription start site of the FAS gene, extending to —332, contains an inverted CAAT sequence (ATTGGCC) between —98 and —92. The region from —290 to the CAP site is very GC-rich and contains five sequence elements similar to the Sp1 binding consensus sequence [10]. It is not known whether these sequences are involved in regulation of FAS expression during adipocyte differentiation.

Regulation of FAS–CAT fusion genes by insulin in transient and stable transfections of 3T3-L1 adipocytes

To define DNA elements which are required for regulation of the FAS gene by insulin, plasmids containing the 5' flanking region of the rat FAS gene fused to the CAT reporter gene were transfected into 3T3-L1 adipocytes. 3T3-L1 cells, when differentiated into adipocytes, show high insulin receptor numbers and increased sensitivity to insulin [12,28]. We have previously shown that FAS transcription is increased rapidly in the livers of streptozotocin-diabetic mice treated with insulin [5]. We also observed a 3-fold increase in the FAS mRNA level when 3T3-L1 adipocytes were treated with insulin, indicating an independent insulin effect [4]. Therefore these cells probably contain all the components necessary for insulin regulation of FAS gene transcription. Following transient transfection, 3T3-L1 adipocytes were maintained for 48 h in serum-free medium

Table 2 Insulin-stimulated expression of the FAS–CAT fusion genes in transient and stable transfections of 3T3-L1 adipocytes

In (a) differentiated 3T3-L1 cells were transiently co-transfected with 2 µg of pSV2–luciferase and 10 µg of –2100CAT, –1400CAT, –1009CAT or –332CAT constructs. In (b) differentiated stable transfectants containing SV40 promoter sequences or 2100 and 1009 bp sequences of the 5' flanking region of FAS were obtained as indicated in the Materials and methods section and the legend to Table 1. Adipocytes from the above transient and stable transfections were preincubated overnight in serum-free medium, then treated with or without 10 nM insulin for 48 h. Cells were then harvested and assayed for CAT and luciferase activities. The ratio of CAT to luciferase served as a measure for normalized CAT activity when both genes were co-transfected. In transient transfections, each value represents the mean ± S.E.M. of 5, 10, 11 and 12 independent experiments for the –2100CAT, –1400CAT, –1009CAT and –332CAT constructs respectively. In stable transfections, the values represent the mean ± S.E.M. of 4, 6 and 5 individual or pooled clones of 3T3-L1 cells transfected with pSV2, –2100CAT and –1009CAT constructs respectively. Each experiment represents the average from duplicate or triplicate plates of cells.

Plasmid	Increase in CAT activity (fold)
(a) Transient transfections	
–2100CAT	2.1 ± 0.2
–1400CAT	2.6 ± 0.1
–1009CAT	2.0 ± 0.2
–332CAT	1.7 ± 0.2
(b) Stable transfections	
pSV2	0.70 ± 0.08
–2100CAT	3.0 ± 0.5
–1009CAT	3.5 ± 0.6

with or without insulin. As shown in Table 2(a), sequences from the FAS gene which extend from nucleotides –2100 to +67 conferred up-regulation by insulin on the expression of the CAT gene. CAT activity increased 2.1 ± 0.2 -fold in insulin-treated cells compared with those cultured without the hormone. To further delineate the insulin-responsive sequences, 5' end deletions of the FAS gene were transfected into adipocytes and treated with insulin. Adipocytes transiently transfected with –1400CAT, –1009CAT and –332CAT constructs showed 2.6 ± 0.1 -, 2.0 ± 0.2 -, and 1.7 ± 0.2 -fold increases respectively in CAT activity upon insulin treatment. When FAS sequences were deleted to nucleotide –19, no change in reporter gene activity was observed following insulin treatment (results not shown). These results show that the *cis*-acting elements that mediate insulin responsiveness in 3T3-L1 adipocytes are located within the proximal promoter region.

We have used concentrations of insulin within the physiological range to study the regulation of FAS–CAT fusion gene expression. The maximal effect of the hormone was obtained at 10 nM (results not shown), suggesting that the insulin effect is mediated through the insulin receptor. This is in contrast to studies by Goodridge's group, reporting that the insulin effect on FAS expression was mediated by the insulin-like growth factor-1 receptor in chick embryo hepatocytes [29]. In those studies, 50% of the maximal effect of insulin on FAS expression was achieved at a concentration of 50 nM, and insulin had no effect by itself on FAS enzyme activity, mRNA content or transcription, but was only able to amplify stimulation by tri-iodothyronine (T_3) [29,30]. These results agree with our previous reports with respect to the positive transcriptional effect of T_3 on FAS expression in 3T3-L1 adipocytes [6]. However, our present studies, along with our previous report, show evidence of a transcriptional effect of insulin on FAS: we have shown that transcription of FAS is increased in the livers of diabetic mice after administration of insulin [5]. Although the studies *in vivo*

might not reflect a direct insulin effect, we also observed that FAS mRNA increased in 3T3-L1 adipocytes upon insulin treatment [4]. Our present results, therefore, indicate that the regulation of a transfected FAS-promoter–CAT fusion gene reflects that of the endogenous FAS gene. The reason for the discrepancy in insulin regulation of FAS between murine adipocytes and chick embryo hepatocytes is not known. It may be due to the fact that insulin was added to chick embryo hepatocytes at the time that the cells were put in culture [29,30], whereas in our experiments 3T3-L1 adipocytes were preincubated in serum-free, and hormone-free, media before insulin addition. Nevertheless, it is also possible that different mechanisms are involved in the regulation by insulin of FAS in chick embryo hepatocytes and murine 3T3-L1 adipocytes.

Insulin-stimulated expression of FAS–CAT fusion genes in adipocytes of stable transfectants of 3T3-L1 cells

Because in transient transfections not all cells express the transfected plasmid, we also utilized cell lines in which the FAS–CAT fusion gene is stably integrated to study the effect of insulin on FAS promoter activity. Several transfectants containing the –2100CAT and –1009CAT plasmids were generated and insulin regulation of the FAS–CAT fusion gene was studied in the same conditions as in transient transfections. The magnitude of stimulation by insulin varied between 1.5- and 7-fold depending on the transfectants when isolated clones were studied (results not shown). Therefore we have also included pooled colonies (> 500) in studying insulin responsiveness. –2100CAT and –1009CAT increased CAT activity upon insulin treatment by 3.0 ± 0.3 and 3.5 ± 0.6 -fold respectively (Table 2b). The insulin effect was specific to the 5' flanking region of FAS, since the CAT activity of stable transfectants expressing SV40–CAT fusion genes decreased by 30% upon insulin treatment. These results agree with those obtained in transient transfections, and together suggest that one or more *cis*-acting elements for insulin are located within 332 bp upstream of the transcription start site. All the culture media used in the above experiments contained a high glucose concentration. It has been previously reported that at least some of the insulin effect on some metabolic enzymes requires glucose [31–33]. Earlier studies showed carbohydrate-dependent induction of FAS in insulin-treated primary cultures of rat hepatocytes [34]. We have tested the effect of carbohydrate on FAS–CAT expression in 3T3-L1 adipocytes, and have found that no significant insulin effect was obtained in the presence of lactose; however, insulin increased CAT activity in glucose-supplemented medium, indicating that the insulin-stimulated increase in FAS transcription may be dependent on increased glycolysis (results not shown).

Insulin-stimulated expression of FAS–CAT fusion genes in transient transfections of H4IIE hepatoma cells

In order to investigate whether FAS promoter sequences mediate insulin responsiveness in other cell lines, we transiently transfected H4IIE cells with FAS–CAT fusion genes and examined the effect of insulin on CAT activity. The hepatoma cell line has been studied for both positive regulation of the glyceraldehyde-3-phosphate dehydrogenase gene [9] and negative regulation of the phosphoenolpyruvate carboxykinase gene [7,8] by insulin, and appears to be a good model system for investigating the effects of insulin on gene expression. Plasmids –2100CAT, –1400CAT, –1009CAT and –332CAT were transiently transfected into hepatoma cells. The results in Table 3 show that CAT activity was increased about 3-fold by insulin

Table 3 Insulin-stimulated expression of the FAS-CAT gene in transiently transfected H4IIE hepatoma cells

Hepatoma cells were co-transfected with pRSV- β -gal and pFAS-CAT fusion genes containing 2100, 1400, 1009 and 332 bp of the 5' flanking region of the FAS gene. Cells were preincubated in serum-free or serum-supplemented medium (0.5%) overnight, then maintained for 48 h in the same medium supplemented or not with insulin. Cells were then harvested and assayed for CAT and β -galactosidase activities. CAT activity was normalized to β -galactosidase activity when both genes were co-transfected. Results are expressed as fold changes in CAT activity in insulin-treated compared with untreated cells, and are means $\pm$ S.E.M.; *n* represents the number of independent transfections carried out in each case. In each experiment, duplicate or triplicate plates were transfected.

Plasmids	Change in CAT activity (fold)	
	Serum-free medium	0.5% serum
-2100CAT	3.1 (<i>n</i> = 2)	2.6 $\pm$ 0.4 (<i>n</i> = 5)
-1400CAT	3.1 $\pm$ 0.8 (<i>n</i> = 7)	3.3 $\pm$ 0.9 (<i>n</i> = 6)
-1009CAT	3.0 $\pm$ 0.7 (<i>n</i> = 8)	3.1 $\pm$ 0.4 (<i>n</i> = 6)
-322CAT	2.3 $\pm$ 0.5 (<i>n</i> = 7)	2.9 $\pm$ 0.6 (<i>n</i> = 5)

when any of these plasmids was transfected. The reported CAT activities were corrected for transfection efficiency using an internal reference, whose activity also increased to various extents with insulin treatment (results not shown). Thus the degrees of insulin stimulation reported here are probably at least 2-fold lower than the actual increases. Insulin responsiveness was retained when the promoter sequences were deleted to nucleotide -332. These results show that insulin-responsive sequences are located within 332 bp upstream of the transcription start site in adipocytes and hepatoma cells. Further analysis of smaller deletion mutants of this region is necessary in order to define minimal sequences required for insulin responsiveness.

Because CAT activity is significantly decreased in hepatoma cells in serum-deprived media (results not shown), some experiments were carried out at a low serum concentration (0.5%) instead of in serum-free media. Insulin induction of CAT activity was again about 3-fold after correction for the internal control, as shown in Table 3. Previous studies have shown that sequences located between -320 and -299 of the *c-fos* gene were required for serum induction [35,36]. The same SRE sequences have been shown to mediate the insulin response in CHO cells overexpressing the insulin receptor [37]. It has been reported that insulin rapidly stimulates accumulation of the proto-oncogene *c-fos* in 3T3-L1 [38] and H4IIE cells [39]. Early induction of the *c-fos* gene by insulin has therefore been postulated as one possible mechanism by which insulin regulates the expression of some genes. We have previously shown using cycloheximide that ongoing protein synthesis is required for transcriptional activation by insulin of FAS and p90, a mouse cDNA with sequence identity to *Escherichia coli* glycerol-3-phosphate acyltransferase, in streptozotocin-diabetic animals [5,40]. However, we did not detect changes in *c-fos* mRNA or transcription rate on insulin treatment in these animals.

Regulation of gene expression by insulin has been reported for a few metabolic enzymes, and an attempt has been made to define the insulin-responsive sequences in their genes. However, a consensus sequence for insulin responsiveness has not yet been established. Moreover, *trans*-acting factors mediating insulin action have not been identified or characterized. It would, therefore, be of value to study the up-regulation by insulin of FAS, an important insulin-inducible enzyme. Although insulin treatment gives an approx. 3-fold increase in FAS promoter-

reporter enzyme activity, we have clearly shown an effect of insulin by using several different systems, i.e. both stable and transient assays using two different insulin-responsive cell lines. Work is in progress to identify the minimum necessary and sufficient DNA sequences for up-regulation of the FAS gene by insulin. In addition, we are examining the 332 bp immediately upstream of the transcription start site of the FAS gene for nuclear protein binding.

This work was supported in part by National Institutes of Health grant DK 36264. N.M. was the recipient of a Juvenile Diabetes Foundation Postdoctoral Fellowship. R.S.B. was the recipient of an Individual National Research Award (F2 DK 08814).

REFERENCES

- Wakil, S. J., Stoops, J. K. and Joshi, V. C. (1983) *Annu. Rev. Biochem.* **52**, 537-579
- Volpe, J. J. and Vagelos, P. R. (1976) *Physiol. Rev.* **56**, 339-417
- Lakshmanan, M. R., Nepokroeff, C. M. and Porter, J. W. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3516-3519
- Paulauskis, J. D. and Sul, H. S. (1988) *J. Biol. Chem.* **263**, 7049-7054
- Paulauskis, J. D. and Sul, H. S. (1989) *J. Biol. Chem.* **264**, 574-577
- Moustaid, N. and Sul, H. S. (1991) *J. Biol. Chem.* **266**, 18550-18554
- O'Brien, R. M., Lucas, P. C., Forest, C., Magnuson, M. A. and Granner, D. K. (1990) *Science* **249**, 533-537
- Forest, C., O'Brien, R. M., Lucas, P. C., Magnuson, M. A. and Granner, D. K. (1990) *Mol. Endocrinol.* **4**, 1302-1310
- Nasrin, N., Ercolani, L., Denaro, M., Kong, X. F., Kang, I. and Alexander, M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5273-5277
- Amy, C. M., Williams-Ahlf, B., Naggert, J. and Smith, S. (1990) *Biochem. J.* **271**, 675-679
- Kameda, K. and Goodridge, A. G. (1991) *J. Biol. Chem.* **266**, 419-426
- Green, H. and Kehinde, O. (1974) *Cell* **1**, 113-116
- Rubin, C. S., Hirsch, A., Fung, C. and Rosen, O. M. (1978) *J. Biol. Chem.* **253**, 7570-7578
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (eds.) (1990) in *Current Protocols in Molecular Biology*, Suppl. 14, pp. 9.1.1-9.1.9, John Wiley, New York
- Seed, B. and Sheen, J. Y. (1988) *Gene* **67**, 271-277
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., pp. 16.66-16.67, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Braiser, A. R., Tate, J. E. and Habener, J. F. (1989) *Biotechniques* **7**, 1116-1121
- Sedmak, J. J. and Grossberg, S. E. (1977) *Anal. Biochem.* **79**, 544-552
- Luckow, B. and Schütz, G. (1987) *Nucleic Acids Res.* **15**, 5490
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463-5467
- Djian, P., Phillips, M. and Green, H. (1988) *Genes Dev.* **2**, 1251-1257
- Ntambi, J. M., Buhrow, S. A., Kaester, K. H., Christy, R. J., Sibley, E., Kelly, T. J. and Lane, M. D. (1988) *J. Biol. Chem.* **263**, 17297-17300
- Cook, K. S., Hunt, C. R. and Spiegelman, B. M. (1985) *J. Cell Biol.* **100**, 514-520
- Christy, R. J., Yang, V. W., Ntambi, J. N., Geiman, D. E., Landschulz, W. H., Friedman, A. D., Nakabeppu, Y., Kelly, T. J. and Lane, M. D. (1989) *Genes Dev.* **3**, 1323-1335
- Graves, R. A., Tontonoz, P., Ross, S. R. and Spiegelman, B. M. (1991) *Genes Dev.* **5**, 428-437
- Ross, S. R., Graves, R. A., Greenstein, A., Platt, K. A., Shyu, B., Mellovitz, B. and Spiegelman, B. M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9590-9594
- Graves, R. A., Tontonoz, P. and Spiegelman, B. M. (1992) *Mol. Cell. Biol.* **12**, 1202-1208
- Reed, B. C., Kaufman, S. H., Mackall, J. C., Student, A. K. and Lane, M. D. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4876-4880
- Stapleton, S. R., Mitchell, D. A., Satali, L. M. and Goodridge, A. G. (1990) *J. Biol. Chem.* **265**, 18442-18446
- Wilson, S. B., Back, D. W., Morris, S. M., Jr., Swierczynski, J. and Goodridge, A. G. (1986) *J. Biol. Chem.* **261**, 15179-15182
- Decaux, J. F., Antoine, B. and Khan, A. (1989) *J. Biol. Chem.* **264**, 11584-11590
- Mariash, C. N., Seelig, C., Schwartz, H. L. and Oppenheimer, J. H. (1986) *J. Biol. Chem.* **261**, 9583-9586
- Decaux, J. F., Marcillat, O., Pichard, A. L., Henry, J. and Khan, A. (1991) *J. Biol. Chem.* **266**, 3432-3438
- Giffhorn-Katz, S. and Katz, N. R. (1986) *Eur. J. Biochem.* **159**, 513-518
- Treisman, R. (1986) *Cell* **46**, 567-574
- Gilman, M. Z., Wilson, R. N. and Weinberg, R. A. (1986) *Mol. Cell. Biol.* **6**, 4305-4316

- 37 Stumpo, D. J., Stewart, T. N., Gilman, M. Z. and Blakshear, P. J. (1988) *J. Biol. Chem.* **263**, 1611–1614
- 38 Stumpo, D. J. and Blakshear, P. J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9453–9457
- 39 Taub, R., Roy, A., Dieter, R. and Koontz, J. (1987) *J. Biol. Chem.* **262**, 10893–10897
- 40 Shin, D. G., Paulauskis, J. D., Moustaid, N. and Sul, H. S. (1991) *J. Biol. Chem.* **266**, 23834–23839

Received 17 December 1992/22 January 1993; accepted 29 January 1993