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Regulation of the Rat SREBP-1c Promoter in Primary Rat Hepatocytes

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We have cloned 5 kb of genomic DNA encompassing 1.72 kb of 5′-regulatory sequence and exons 1-c and 2 of the rat SREBP-1c gene. A 1.5-kb segment upstream from the transcription start site was ligated ahead of the luciferase reporter gene and tested for promoter activity by transient transfection assays in primary rat hepatocytes. We discovered that insulin strongly activated the full-length promoter, regardless of whether 5 or 20 mM glucose was in the culture medium during treatment. Stimulation by insulin was blocked by dibutyryl-cAMP and by polyunsaturated fatty acids, such as α-linolenic acid, γ-linolenic acid, or eicosapentaenoic acid; palmitic or oleic acids, however, had no inhibitory effect. A truncated promoter containing 149 bp of 5′ flanking DNA, including proximal NF-Y, E-box, SRE, and Sp1 sites, retained most of the response. This is the first report that insulin, cAMP, and polyunsaturated fatty acids modulate the proximal SREBP-1c promoter in rat hepatocytes mirroring physiological regulation of SREBP-1c *in vivo*.

Insulin increases transcription of fatty acid synthase (FAS) and acetyl CoA carboxylase-1 (ACC-1) in primary cultures of rat hepatocytes (1). This effect of insulin is opposed by exogenous cAMP, or by agents that elevate intracellular cAMP concentrations (2), and by unsaturated fatty acids (reviewed in 3). At present a consensus insulin response element (IRE) remains elusive (4). The promoters of the FAS and other genes involved in hepatic lipogenesis share a common sequence that can bind transcription factors of the basic helix-loop helix-leucine zipper family (reviewed in 5). Several lines of evidence point to SREBP-1c, also known as ADD1 (6), as the transcription factor mediating the positive effects of insulin through this element (reviewed in 7).

The gene for SREBP-1 uses alternative promoters to generate SREBP-1a or SREBP-1c, which differ only in their first exon. SREBP-1c is the predominant product in liver and adipose and has stronger effects on transcription of lipogenic enzymes than on those involved in cholesterol metabolism (8). Hepatic content of SREBP-1c mRNA and protein is reduced by fasting and increased by refeeding, while expression of SREBP-1a and SREBP-2 are only minimally affected (9). Levels of hepatic SREBP-1c mRNA also fall in streptozotocin induced diabetes and rebound after insulin treatment (10). Expression of SREBP-1c in cultured rat hepatocytes is stimulated by insulin and inhibited by cAMP and by unsaturated fatty acids (11–13). Insulin induces, and glucagon and dibutyryl c-AMP (db-cAMP) suppress, SREBP-1c gene transcription as determined by nuclear run-on measurements (11). The underlying mechanisms by which SREBP-1c gene transcription is modulated by these factors have not been elucidated.

The human (14) and mouse (15) SREBP-1 genes have been cloned. The proximal promoter of the mouse SREBP-1c gene contains potential binding sites for SREBP-1c, NF-Y, and Sp1 (15), as well as LXR (16, 17). In transient transfection experiments, transcription driven by the proximal mouse SREBP-1c promoter in HEK 293 cells was stimulated by cotransfection of the active form of SREBP-1c (15) or of LXRα or LXRβ (16) or by treatment with synthetic LXR agonists (16, 17). Responsiveness of SREBP-1c promoter to insulin and cAMP, as judged by experiments using transfection, have not yet been reported.

To facilitate studies of rat models of chronic hyperinsulinemia, we have cloned the rat SREBP-1c gene promoter. We now report that 149 bp of this promoter is sufficient to drive its expression in primary cultures

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FIG. 1. Sequence of the proximal 1239 base pairs of the rat SREBP-1c promoter. For comparison, the published sequence of the mouse SREBP-1c promoter is included (15–17). Functional response elements that have been identified in the mouse promoter (two LXRE sites and a cluster of response elements comprising adjacent NF-Y, E-box, SRE, and Sp1 sites) are highlighted in bold type. The putative transcription start site is indicated with an enlarged letter. Sequences or individual nucleotides within the rat or mouse promoter that have no counterpart are indicated by a series of dots in the opposite sequence. The 5′ ends of the 412 and 149 bp promoter constructs used in this study are indicated by arrow; the 5′ end of the 1546 bp construct lies upstream from the displayed sequence. All three promoter segments terminate at the 3′ end of the displayed sequence.

of rat hepatocytes and to elicit responsiveness to insulin, cAMP, and unsaturated fatty acids.

MATERIALS AND METHODS

Primary hepatocyte culture. Hepatocytes were obtained from livers of male Sprague–Dawley rats (approximately 300 g; Harlan Laboratories, Indianapolis, IN) by collagenase perfusion as previously described (18). Cells were suspended in Williams E medium (Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum (Sigma, St. Louis, MO), 10 mM glucose, 1 μM dexamethasone and 100 nM insulin. Each sixty mm culture dish, coated with rat tail collagen (Collaborative Biochemical Products, Bedford, MA), was seeded with 3×10^6 cells; after 4 h, nonadherent cells were removed, and adherent cells were incubated overnight in Williams E medium without serum or hormones. Incubation was continued for a further 24 h in fresh Williams E medium supplemented with 0.75% delipidated bovine serum albumin (BSA), 20 mM glucose, and 100 nM dexamethasone.

Measurement of steady-state levels of SREBP-1 mRNA. Total RNA was extracted with RNA Stat-60 (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions and quantified by absorbance at 260 nm. Fifteen μg of total RNA was loaded per lane of a formaldehyde/0.8% agarose gel, electrophoresed in 1× Mops buffer, blotted onto Nytran membranes (Schleicher and Schuell, Keane, NH), and UV-cross linked. Ribosomal RNA bands were visualized by staining with ethidium bromide prior to transfer. Blots were prehybridized for 3 h at 42°C in 50% formamide, 5× SSPE, 5× Denhardt's solution (5Prime-3Prime, Boulder, CO), 7.5% dextran sulfate, 1.5% sodium dodecyl sulfate (SDS), and 100 μg/ml sheared salmon sperm DNA (Ambion, Inc., Austin, TX).

Plasmids used to prepare cDNA probes for measurement of SREBP-1 and FAS mRNAs were generously provided by Dr. Bruce M. Spiegelman (Dana Farber Cancer Institute. Boston, MAc) and Dr. Stuart Smith (Children's Hospital Research Institute, Oakland, CA). β-actin mRNA was measured using mouse β-actin DECAprobe (Ambion, Inc., Austin, TX). SREBP-1, FAS, and β-actin mRNA were detected by overnight hybridization at 42°C with the cDNA probes, ^{32}P-labeled by the random primer method using a commercial kit (Invitrogen, Carlsbad, CA). Unbound probe was removed by washing twice with 2× SSC + 0.1% SDS at room temperature and then twice with 0.1× SSC + 0.1% SDS at 65°C for 30 min each. Membranes were exposed to Bio-Max MS film (Eastman Kodak, Rochester, NY); a digital image of the developed film was created and RNA bands quantitated by densitometry (Alpha Innotech Corp., San Leandro, CA).

Preparation of SREBP-1c promoter luciferase constructs. Genomic DNA fragments were amplified by PCR using primers incorporating *Kpn*I (upstream; GGTACC) or *Bgl*II (downstream; AGATCT) sequence at their 5′ ends; the downstream primer was complementary to a sequence within the 5′ untranslated region of exon 1c. Amplified DNA fragments were ligated into pGL3 basic luciferase (Promega, Madison, WI) that had been linearized with *Kpn*I and *Bgl*II, 5′ phosphates removed with calf-intestinal phosphatase (Promega, Madison, WI), and gel-purified (Geneclean III; Bio 101, Carlsbad, CA). Orientation of the cloned insert was confirmed by sequencing. PCR primers utilized were: 5′-GCAAGATCT**TAGGGCGTGCAGACGCTACCCCTA** (downstream); 5′-AGTGGTACC**GCTGGACACGGCCTGTACA** (upstream, to amplify a 1546 bp segment), 5′-AGTGGTACC**TGGGTGTGTGCGAACCAGCGGTAG** (upstream, to amplify a 412 bp segment), and 5′-AGTGGTACC**TTATTGGGGCGCGGCGCGGCTGCT** (upstream, to amplify a 149 bp segment). Sequencing was performed at St. Jude Children's Research Hospital Molecular Biotechnology Center (Memphis, TN).

Transient transfection of primary cultures of rat hepatocytes. Freshly isolated rat hepatocytes were incubated overnight with 2μg of SREBP–1c promoter vector (pSREBP (−1516/+40) luc, pSREBP (−372/+40) luc, or pSREBP (−109/+40)) luc expressing Photinus luciferase plus 1 μg of control vector expressing *Renilla* luciferase (p-RL-TK; Promega, Madison, WI) dispersed with 8 μl Lipofectin reagent (Invitrogen, Carlsbad, CA) in Williams E medium. Medium was then removed, and cells incubated for 24 h in Williams E medium containing 0.75% delipidated BSA, 20 mM glucose, and 100 nM dexamethasone alone, or with the treatments detailed. Cells were lysed and luciferase activity was quantified fluorimetrically (TD-20/20 Luminometer; Turner Designs, Sunnyvale, CA) using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). To normalize for variation in transfection efficiency, data are expressed as the ratio of Photinus to Renilla luciferase activity.

RESULTS AND DISCUSSION

Cloning and sequence of the rat SREBP-1c promoter. A BAC clone was obtained from a screen of a rat genomic DNA library (Incyte Genomics, Palo Alto, CA) utilizing a portion of rat SREBP-1 exon 2 cDNA as probe (4). By Southern analysis and sequencing a 5-kb *Sac*I fragment derived from this clone was deduced to contain exon 2, exon 1c and 1.72 kb of 5′ flanking sequence from the rat SREBP-1 gene. The proximal 1200 base pairs this sequence, terminating within the 5′ untranslated region, and the corresponding sequence from mouse (from references 15,17) are displayed in Fig. 1. There is an overall 82% homology between the rat and mouse promoters. The homology over the first 270 base pairs proximal to the start site of transcription is 97%. The 5′ flanking region adjacent to exon 1c of SREBP-1c of the rat SREBP-1c promoter contains a cluster of potential response elements, consisting of an Sp1 site (−52 to −60), SRE (−61 to −68), E-box (−73 to −78), and NF-Y site (−80 to −85), as well as two potential LXREs at −168 to −184 and −218 to −234 that are homologous to corresponding elements in the mouse promoter (15–17). Consensus sequence search of 1.72 kb of the 5′ flanking region of the rat SREBP-1c promoter (TFSEARCH, MatInspector) indicates the presence of numerous additional potential regulatory elements in the upstream sequence, whose significance remains to be determined. The extraordinary conservation of sequences in the proximal promoter and the location of many common *cis*-acting elements suggests the functional importance of these elements.

Insulin stimulates transcription driven by the SREBP-1c promoter in rat hepatocytes. To determine whether the cloned portion of the rat SREBP-1c promoter can drive efficient expression and respond to insulin in rat hepatocytes, cells were transfected for 18 h with pSREBP-(−1516/+40)-luc. Transfected hepatocytes were incubated for a further 24 h in medium containing 0.75% delipidated BSA, 20 mM glucose, and 100 nM dexamethasone with or without addition of bovine insulin (100 nM), db-cAMP (100 μM),

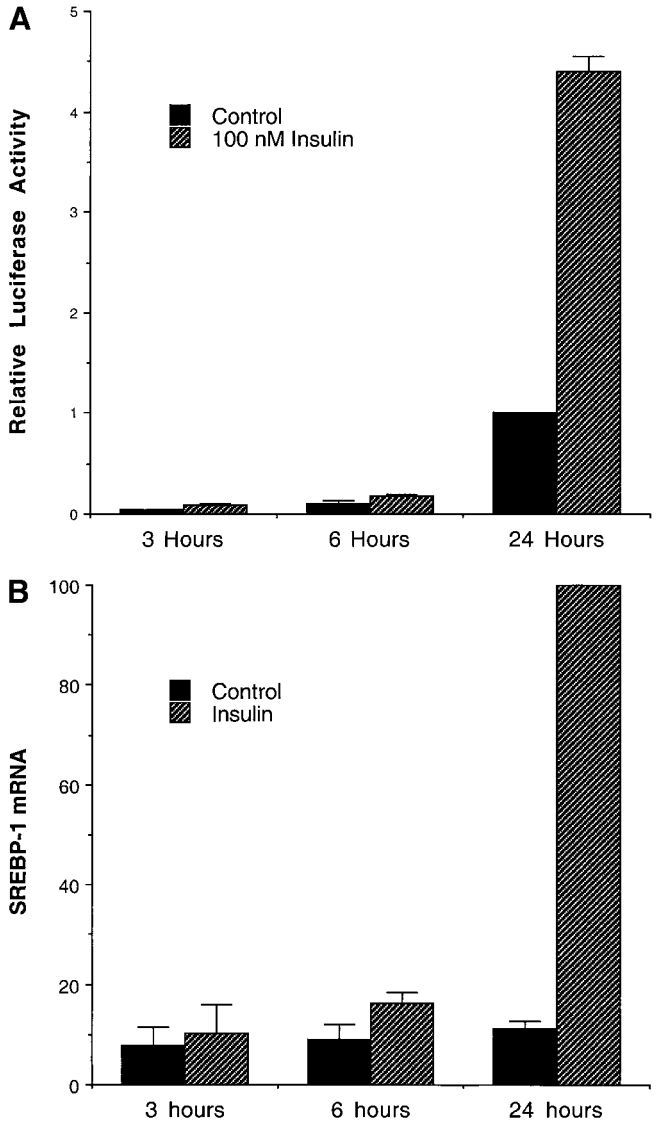

FIG. 2. (A) Time course of the increased transcriptional activity of the SREBP-1c promoter produced by insulin. Freshly isolated hepatocytes were transfected overnight with 2 μg of pSREBP (−1516/+40) luc containing 1.5 kb of rat SREBP-1c 5′-flanking DNA ligated upstream of *Photinus* luciferase reporter gene. Concurrent transfections with 1 μg of pRL-TK expressing *Renilla* luciferase were performed as a control for transfection efficiency. Cells were then incubated in medium with no additions or supplemented with 100 nM insulin. Samples were collected after 3, 6, or 24 h. Data, expressed as the ratio of *Photinus* to *Renilla* luciferase activity, from two hepatocyte preparations, with three plates in each preparation, are shown. (B) Time course of induction of SREBP-1 mRNA, determined by Northern analysis, by insulin in primary rat hepatocyte cultures. Hepatocytes were maintained for up to 24 h in medium containing glucose (20 mM) and dexamethasone (100 nM) with or without bovine insulin (100 nM). Data from 3 hepatocyte preparations are shown.

or eicosapentaenoic acid (0.5 mM). Insulin stimulated expression of luciferase activity driven by the "full-length" SREBP-1c promoter (Fig. 2A) and steady-state levels of SREBP-1 mRNA (Fig. 2B) as early as 6 h. Maximum effects of insulin on SREBP-1c promoter were seen 24 h posttransfection. Interestingly, insulin did not stimulate SREBP-1c promoter activity in H4IIE hepatoma cells (data not shown). The stimulatory effect of insulin on the SREBP-1c promoter of 24 h of exposure to insulin was inhibited completely by db-cAMP and by 62% by eicosapentaenoic acid (Fig. 3). These data indicate that this portion of the rat SREBP-1c promoter contains sequences needed for hormonal regulation of the gene.

Effect of glucose on transcriptional activation of the SREBP-1c promoter. Elevated glucose concentrations are required for maximal effect of insulin on expression of lipogenic enzymes in rat hepatocytes (1). Evidence has been provided that, while the insulin acts through regulatory elements in the promoters of these genes that bind SREBP-1c, glucose acts through a distinct site, the carbohydrate response element (ChoRE), that binds to an as yet incompletely characterized carbohydrate responsive factor (ChoRF) (19, 20). Homologous ChoREs have been identified in the promoters of several glucose-responsive genes, including FAS (19), ACC-1 (21), L type pyruvate kinase (22), and the lipogenic factor S14 (23).

The effect of glucose on SREBP-1c gene transcription is controversial (11, 24). We found no difference in the

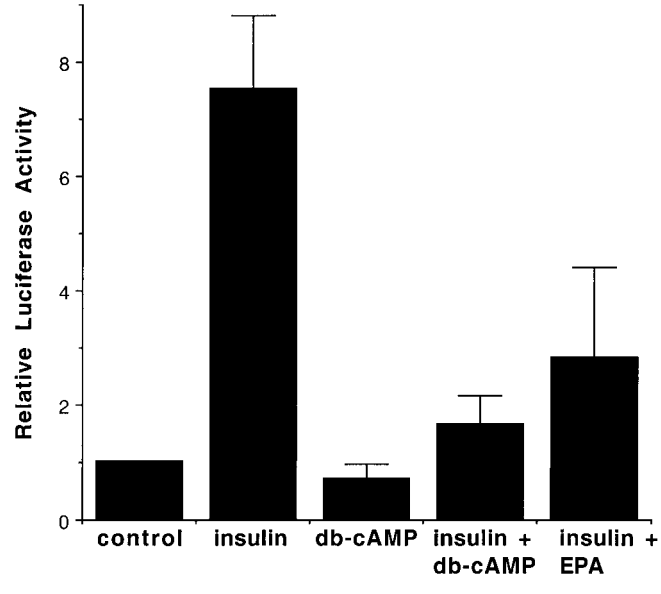

FIG. 3. Regulation of rat SREBP-1c promoter activity by insulin, cAMP, and eicosapentaenoic acid. Freshly isolated hepatocytes were transfected overnight with 2 μg of pSREBP (−1516/+40) luc and 1 μg of pRL-TK-expressing *Renilla* luciferase and then incubated in medium containing 0.75% delipidated BSA, 20 mM glucose, 100 nM dexamethasone, and the indicated additions. After 24 h, SREBP-1c promoter activity was increased by insulin (100 nM). db-cAMP (100 μM) reduced both basal promoter activity and reversed insulin-induced promoter activation. Similarly, eicosapentaenoic acid (20:5; 0.5 mM) reversed insulin-induced promoter activity. Data from 3 hepatocyte preparations (with three plates in each group) are displayed.

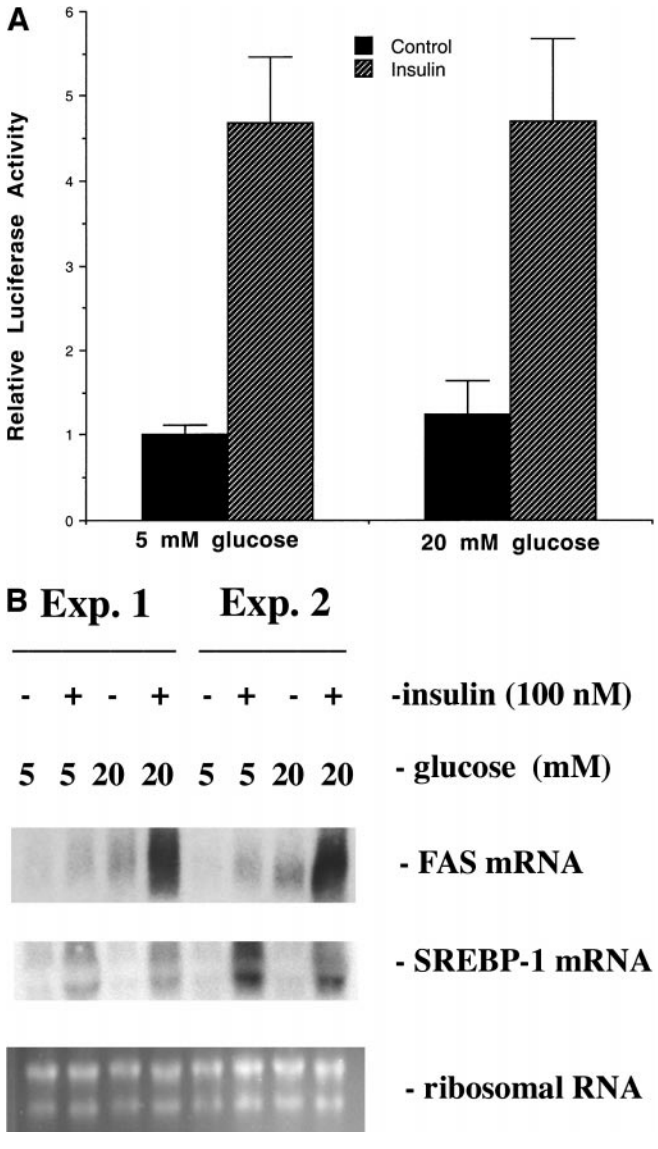

FIG. 4. (A) Effect of glucose concentration on transcription through the SREBP-1c promoter. Hepatocytes were transfected with pSREBP(−1516/+40)luc as described in the legend to Fig. 2 and subsequently incubated for 24 h in medium containing 5 or 20 mM glucose with or without addition of insulin (100 nM). Increasing glucose concentration did not increase transcription driven by the SREBP-1c proximal promoter either in the presence or absence of insulin. Data from 4 hepatocyte preparations with three plates in each group are shown. (B) Effect of glucose concentration on levels of FAS and SREBP-1 mRNA. Northern blots of RNA extracted from hepatocytes incubated for 24 h in the presence or absence of insulin (100 nM) in medium containing 5 or 20 mM glucose are displayed. Data from two hepatocyte preparations are shown.

basal or insulin stimulated activity of the 1.5 kb rat SREBP-1c 5′ flanking DNA whether incubations occurred in medium containing 5 or 20 mM glucose (Fig. 4A). In agreement with the findings of Foretz *et al.* (11), glucose concentration also had no effect on levels of SREBP-1 mRNA (Fig. 4B). In contrast, levels of FAS mRNA were elevated by insulin only at the higher glucose concentration (Fig. 4B). Based on these data, we surmise that the ChoRE is not present within the rat SREBP-1c promoter.

Regulation of the SREBP-1c promoter by fatty acids. To determine whether the capacity of fatty acids to suppress transcription of SREBP-1c depends on the degree of unsaturation, we transfected rat hepatocytes with pSREBP(−372/+40)luc, and incubated these cells for 24 hr in a medium containing insulin (100 nM) and 0.5 mM palmitic acid (16:0); oleic acid (18:1 ω9); γ-linolenic acid (18:3 ω6); linolenic acid (18:3 ω3); or eicosapentaenoic acid (20:5 ω3) complexed to 0.75% delipidated BSA or with BSA alone. Neither palmitic acid nor oleic acid caused significant inhibition of insulin induced luciferase expression, but transcriptional activity of the SREBP-1c promoter was inhibited by fatty acids bearing multiple double bonds (Fig. 5). These results are consistent with an earlier report that the efficacy of fatty acids in reducing levels of SREBP-1a and SREBP-1c mRNA in HEK-293 cells is in proportion to the degree of unsaturation (24).

Defining the minimal sequence of the rat SREBP-1c promoter that responds to insulin, db-cAMP, and eicosapentaenoic acid. To determine the minimal sequence in the SREBP-1c promoter required for basal expression and for responsiveness to insulin, db-cAMP, and eicosapentaenoic acid, rat hepatocytes were transfected with pSREBP(−1516/+40)luc, pSREBP(−372/+40)luc, or pSREBP(−109/+40) and incubated for 24 h

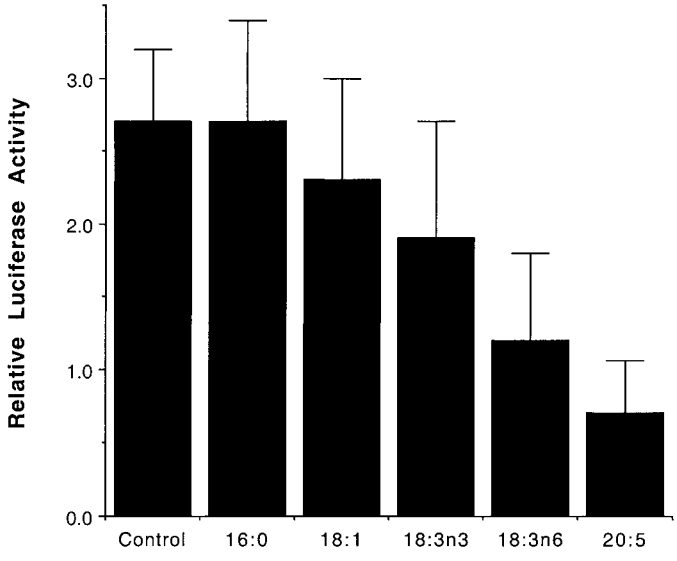

FIG. 5. Effect of fatty acids on transcription through the rat SREBP-1c promoter. Hepatocytes were transfected overnight with pSREBP (−372/+40)luc as described in the legend to Fig. 2 and subsequently incubated in medium containing 100 nM insulin plus 0.75% delipidated BSA and 0.5 mM palmitic acid, oleic acid, linolenic acid, γ-linolenic acid, eicosapentaenoic acid, or no fatty acid. Data from 4 hepatocyte preparations (with three plates in each group) are shown.

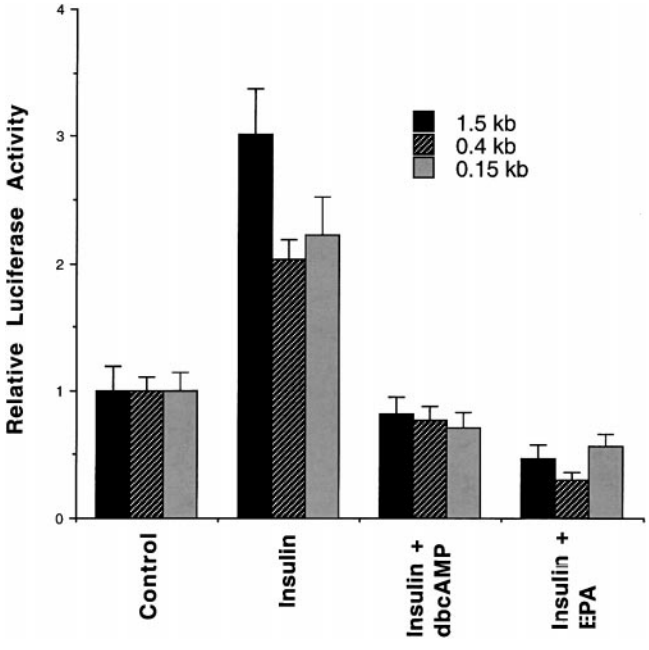

FIG. 6. Response of truncation of the rat SREBP-1c promoter to insulin (100 nM), insulin (100 nM) + dbcAMP (100 μM), or insulin (100 nM) + eicosapentaenoic acid (0.5 mM). Rat hepatocytes were transfected with pSREBP (−1516/+40)luc, pSREBP(−372/+40)luc, or pSREBP(−109/+40)luc as described in the legend to Fig. 2 and then incubated 24 h in medium containing the indicated additions. Data from two hepatocyte preparations (with three plates in each group) are shown.

in medium containing no additions or 100 nM insulin; 100 nM insulin + 100μM db-cAMP; or 100 nM insulin + eicosapentaenoic acid. The promoter construct containing 1.5 kb of 5′ flanking sequence showed greater transcriptional stimulation by insulin than the shorter constructs. Surprisingly, however, the truncated 149 bp rat SREBP-1c promoter, containing the SRE and adjacent elements between −52 and −85, retained 75% of the responsiveness of the "full-length" promoter to insulin and inhibition of that response by db-cAMP and eicosapentaenoic acid (Fig. 6).

The nucleotide sequences of the complex of elements located in the −109/+40 bp segment of the rat SREBP-1c promoter are identical to the homologous sequence in the mouse SREBP-1c gene (Fig. 1; 15). A segment of mouse SREBP-1c proximal promoter containing these elements responds positively to overexpression of SREBP-1c in HEK 293 cells (15). Amemiya-Kudo *et al.* have speculated that the SREBP responsive element in the SREBP-1c promoter may amplify the response to insulin (15), although this has not yet been demonstrated and the precise mechanisms of auto-activation remain unknown. It is possible that the effects of insulin, db-cAMP and eicosapentaenoic on pSREBP-1c-luc in transiently transfected primary hepatocytes are partly due to induction of endogenous SREBP-1c.

On the other hand, in mice homozygous for a functionally disabling mutation in the SREBP-1c gene that would not be susceptible to auto-activation of SREBP-1c gene transcription, insulin retains most of its capacity to elevate hepatic levels of SREBP-1c mRNA (29). This suggests that a substantial portion of the total stimulus provided by insulin is independent of amplification by expressed SREBP-1c protein.

It is highly likely that regulation of the SREBP-1c promoter by insulin, db-cAMP, and eicosapentaenoic acid involves participation of additional trans-activating proteins that may be affected independently. Trans-activation by SREBP-1c in other gene promoters, such as that of FAS, ATP dependent citrate lyase, and S14) requires simultaneous binding of Sp1 or NF-Y to adjacent sites (25–27). Rapid and enhanced expression of Sp1 by insulin has been documented in rat liver and in rat hepatoma cells (28).

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