SREBP-1 integrates the actions of thyroid hormone, insulin, cAMP, and medium-chain fatty acids on ACCα transcription in hepatocytes

Yanqiao Zhang, Liya Yin, and F. Bradley Hillgartner[1]

Department of Biochemistry and Molecular Pharmacology, School of Medicine, P.O. Box 9142, West Virginia University, Morgantown, WV

Abstract **In chick embryo hepatocytes, activation of acetyl-CoA carboxylase-α (ACCα) transcription by 3,5,3′-triiodothyronine (T3) is mediated by a *cis*-acting regulatory unit (−101 to −71 bp) that binds the nuclear T3 receptor (TR) and sterol regulatory element-binding protein-1 (SREBP-1). SREBP-1 directly interacts with TR on the ACCα gene to enhance T3-induced transcription. Here, we show that treating hepatocytes with T3 or insulin stimulates a 4-fold increase in the concentration of the mature, active form of SREBP-1. When T3 and insulin are added together, a 7-fold increase in the mature SREBP-1 concentration is observed. Time course studies indicate that the T3-induced increase in mature SREBP-1 abundance is closely associated with changes in ACCα transcription and that the mechanism mediating the effect of T3 on mature SREBP-1 is distinct from that mediating the effect of insulin. Transfection analyses indicate that inhibition of ACCα transcription by cAMP or hexanoate is mediated by ACCα sequences between −101 and −71 bp. Treatment with cAMP or hexanoate suppresses the increase in mature SREBP-1 abundance caused by T3 and insulin. These results establish a new interaction between the SREBP-1 and TR signaling pathways and provide evidence that SREBP-1 plays an active role in mediating the effects of T3, insulin, cAMP, and hexanoate on ACCα transcription.**—Zhang, Y., L. Yin, and F. B. Hillgartner. **SREBP-1 integrates the actions of thyroid hormone, insulin, cAMP, and medium-chain fatty acids on ACCα transcription in hepatocytes.** ***J. Lipid Res.*** **2003.** 44: **356–368.**

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In livers of avians and mammals, consumption of a high-carbohydrate, low-fat diet coordinately stimulates the transcription of genes involved in the conversion of carbohydrate to triacylglycerols (1). These genes include glucokinase, L-pyruvate kinase, ATP-citrate lyase, acetyl-CoA carboxylase-α (ACCα), fatty acid synthase, malic enzyme, and glycerol-3-phosphate acyltransferase. To date, three signaling pathways have been identified that mediate the effects of dietary carbohydrate on lipogenic gene transcription. One pathway is activated by increased glucose metabolism, and its end target is carbohydrate response factor or carbohydrate response element binding protein (ChREBP) (2, 3). Increased glucose metabolism enhances the ability of ChREBP to bind the L-pyruvate kinase gene and activate L-pyruvate kinase transcription (2). A second pathway that signals changes in carbohydrate consumption to lipogenic genes is activated by 3,5,3′-triiodothyronine (T3), the active form of thyroid hormone (1). T3 activates transcription through its interactions with nuclear T3 receptors (TRs) bound to T3 response elements (T3REs) of target genes (4). Functional T3REs have been identified in the genes for ACCα (5), fatty acid synthase (6), and malic enzyme (7). A third pathway that signals alterations in dietary carbohydrate status to lipogenic genes is activated by insulin, and its end target is sterol regulatory element binding protein (SREBP)-1 (8, 9). SREBP-1 is synthesized as a 125 kDa precursor protein that is anchored to the endoplasmic reticulum. To become transcriptionally active, precursor SREBP-1 is translocated to Golgi, where it is cleaved by two proteases, resulting in the release of the N-terminal segment of SREBP-1, referred to as mature SREBP-1. Mature SREBP-1 is transported into the nucleus, where it binds the promoter/regulatory regions of several lipogenic genes, including ACCα (10) and fatty acid synthase (11, 12). In rat hepatocytes, insulin increases the concentration of mature

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Abbreviations: ACCα, acetyl-CoA carboxylase-α; CAT, chloramphenicol acetyltransferase; CEH, chick embryo hepatocyte; ChREBP, carbohydrate response element binding protein; HNF-4, hepatocyte nuclear factor-4; LXR, liver X receptor; PUFA, polyunsaturated fatty acid; RXR, retinoid X receptor; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein; T3, 3,5,3′-triiodothyronine; T3RE, T3 response element; TK, thymidine kinase; TR, nuclear T3 receptor.

[1] To whom correspondence should be addressed.
e-mail: fbhillgartner@hsc.wvu.edu

SREBP-1, resulting in an activation lipogenic gene transcription (13–15).

The ChREBP, TR, and SREBP-1 signaling pathways also mediate the effects of nutrients and hormones that inhibit lipogenic gene transcription. For example, long-chain fatty acids suppress the activation of ChREBP, TR, and SREBP-1 by glucose, T3, and insulin, respectively (16–20). Glucagon, a hormone that signals the starved state in animals, also inhibits the activation of ChREBP by glucose (2). Whether glucagon inhibits lipogenic gene transcription by suppressing positive signaling through the TR or SREBP-1 pathways is presently not known.

ACC catalyzes the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA, which is the donor of all but two of the carbon atoms for the synthesis of long-chain fatty acids. This reaction is the pace-setting step of the fatty acid synthesis pathway (1). There are two ACC isoforms that are encoded by distinct genes. ACCβ is the principal isoform expressed in heart and skeletal muscle, where it is thought to function primarily in the regulation of β-oxidation of fatty acids (21). ACCα is the major isoform expressed in tissues such as liver and adipose tissue that exhibit high rates of fatty acid synthesis. Transcription of the ACCα gene is subject to nutritional regulation. For example, in livers of starved chickens, the rate of ACCα transcription is low; consumption of a high-carbohydrate, low-fat diet stimulates an 11-fold increase in ACCα transcription (22). The induction of ACCα transcription caused by dietary carbohydrate is preceded or paralleled by increases in the molar ratio of insulin/glucagon and the level of T3 in the blood (23). In primary cultures of chick embryo hepatocytes (CEHs), addition of T3 to the culture medium stimulates a 7-fold increase in ACCα transcription (24). Insulin has no effect by itself but amplifies the increase in ACCα transcription caused by T3. Glucagon acting through cAMP suppresses the induction of ACCα transcription caused by T3 and insulin.

Fatty acids containing six to eight carbons also inhibit ACCα transcription in the presence of T3 and insulin and do so within 2 h of addition of the fatty acid (25, 26). Hexanoate and octanoate per se are not likely to be physiological regulators of ACCα transcription, because the levels of these fatty acids in the blood are not high enough to inhibit transcription. However, the potent, rapid, and selective effects of hexanoate and octanoate on ACCα transcription suggest that the intracellular intermediates and signaling pathways mediating the effects of these fatty acids on transcription are physiologically relevant (25). For example, during starvation, a condition in which ACCα transcription is inhibited, increased rates of fatty acid oxidation may cause a change in the level of a metabolic intermediate that, in turn, inhibits ACCα transcription. In hepatocytes in culture, addition of hexanoate and octanoate to the culture medium may mimic the effects of starvation on the level of this intermediate. The identity of the active intermediate(s) mediating the effects of hexanoate and octanoate on ACCα transcription is of interest because it may aid in the development of new therapies to prevent and treat obesity and cardiovascular disease.

The ACCα gene is transcribed from two promoters, generating mRNAs with heterogeneity in their 5′-untranslated regions (27). Alterations in the activity of the more downstream promoter (promoter 2) account for the majority of the changes in ACCα mRNA abundance caused by starvation and refeeding a high-carbohydrate diet in intact chickens and by T3, insulin, cAMP, and hexanoate in CEH (26). The stimulatory effect of T3 on ACCα promoter 2 activity is mediated by a T3RE (−101 to −86 bp) that enhances ACCα transcription in both the absence and the presence of T3, with a greater stimulation observed in the presence of T3 (5). The enhancer activity in the absence of T3 is mediated by the binding of protein complexes containing liver X receptor (LXR)·retinoid X receptor (RXR) heterodimers. The increase in enhancer activity caused by T3 treatment is mediated by the binding of a different set of protein complexes. One of these complexes contains TR·RXR heterodimers, and another contains LXR·RXR heterodimers. Immediately downstream of the ACCα T3RE is a sterol regulatory element (SRE)-1 (−80 to −71 bp) that augments the ability of the ACCα T3RE to stimulate ACCα transcription in the presence of T3 (10). Results from transfection, protein binding, and DNA binding assays suggest that the stimulatory effect of the SRE-1 on ACCα transcription is mediated by a direct and T3-inducible interaction between SREBP-1 and TR and that this interaction facilitates the formation of a SREBP-1·SREBP-1/TR·RXR tetrameric complex on the ACCα gene. Complex formation between TR·RXR and SREBP-1·SREBP-1 stabilizes the binding of SREBP-1 to the SRE-1. Thus, optimal induction of ACCα transcription by T3 is dependent on an interaction between TR and SREBP-1 on the ACCα gene. Both of these signaling pathways represent potential targets mediating the actions of insulin, cAMP, and medium-chain fatty acids on T3-induced ACCα transcription. The mechanisms by which insulin, cAMP, and medium-chain fatty acids control ACCα transcription remain to be determined.

In the present study, we have identified a new interaction between the SREBP-1 and TR signaling pathways. T3 increases the concentration of the mature, active form of SREBP-1 in CEH. In addition, we provide evidence that insulin, cAMP, and medium-chain fatty acids regulate ACCα transcription by modulating the abundance of mature SREBP-1.

EXPERIMENTAL PROCEDURES

Plasmids

Reporter plasmids are named by designating the 5′- and 3′- ends of the ACCα DNA fragment relative to the transcription start site of promoter 2. A series of 5′-deletions and 3′-deletions of ACCα promoter 2 gene in the context of p[ACC−4900/+274] chloramphenicol acetyltransferase (CAT) have been previously described (5). ACCα promoter constructs containing mutations of the SRE-1 between −79 and −72 bp have been described previously (10). pBLCAT2 (pTKCAT) was obtained from B. Luckow and G. Schutz (German Cancer Research Center) (28). p[ACC−108/−82]TK-

CAT, p[ACC−108/−66]TKCAT, p[ACC−84/−66]TKCAT, and pTKCAT constructs containing mutations in the −108 to −66 bp ACCα fragment are described in (10).

A full-length cDNA for chicken SREBP-1 was obtained by screening a chicken liver cDNA library (Stratagene) using a human SREBP-1 cDNA probe (nucleotides 721 to 1,103 relative to the start site of translation) and by 5′-rapid amplification of cDNA ends (RACE) (Y. Zhang and F. B. Hillgartner, unpublished observations). The N-terminal amino acid sequence of this chicken SREBP-1 (GenBank accession number: AY029224) more closely resembles the 1a isoform than the 1c isoform described in mammalian species (29). Data from 5′-RACE and RNase protection analyses indicate that other forms of SREBP-1 containing variations in the N-terminus are not expressed in chicken cells. An expression plasmid encoding the mature form of chicken SREBP-1 was developed by subcloning an SREBP-1 cDNA fragment encoding amino acids 1 to 464 into pSV-SPORT1 (Invitrogen) to form pSV-SPORT1-SREBP-1 (1-464).

Cell culture and transient transfection

Primary cultures of CEH were prepared as previously described (30) and maintained in serum-free Waymouth's medium MD705/1 containing 1 μM corticosterone, 50 nM insulin (gift from Eli Lilly Corp.), and 25 mM glucose. CEHs were incubated at 40°C in a humidified atmosphere of 5% CO_2 and 95% air. CEHs were transfected using a modification of the method of Baillie et al. (31). Briefly, CEHs were isolated as described above and incubated on 60 mm petri dishes (Fisher Scientific). At 6 h of incubation, the medium was replaced with one containing 20 μg of lipofectin (Invitrogen), 2.5 μg of p[ACC−4900/+274]CAT or an equimolar amount of another reporter plasmid and pBluescript KS(+) to bring the total amount of transfected DNA to 3.0 μg per plate. At 18 h of incubation, the transfection medium was replaced with fresh medium containing corticosterone, insulin, 25 mM glucose, and T3 (1.5 μM). On some cells, the medium was supplemented with dibutyryl cAMP (50 μM) or hexanoate (2.5 mM). At 66 h of incubation, CEHs were harvested and cell extracts were prepared (31). CAT activity (32) and protein (33) were assayed by the indicated methods. All DNAs used in transfection experiments were purified using the Qiagen endotoxin-free kit.

Preparation of membrane and nuclear extracts

All procedures were carried out at 4°C. To prevent proteolysis, a mixture of protease inhibitors (Complete, Roche Molecular Biochemicals) was included in all the buffers. Nuclear extracts were prepared from CEHs by a modification of the method described by Dignam et al. (34). Briefly, CEHs from four 100 mm plates were pooled and centrifuged at 1,000 *g* for 5 min at 4°C. The resulting cell pellet was homogenized in Buffer 1 [10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol] using 20 strokes in a Dounce homogenizer. The homogenate was centrifuged at 1,100 *g* for 10 min, and the resulting nuclear pellet was washed once in buffer 1. The nuclear pellet was resuspended in Buffer 2 [20 mM Hepes (pH 7.9), 420 mM NaCl, 25% (v/v) glycerol, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol]. This suspension was rotated for 30 min and then centrifuged 15,000 *g* for 30 min. The resulting supernatant is designated as the nuclear extract fraction. The membrane extract fraction was prepared by centrifuging the supernatant of the original 1,100 *g* spin for 1 h at 100,000 *g*. The resulting membrane pellet was dissolved in Buffer 3 [10 mM Tris (pH 6.8), 100 mM NaCl, 1% (w/v) SDS, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol]. The protein content of the nuclear and membrane extracts was determined as described (33).

Gel mobility shift analysis

Double-stranded oligonucleotides were prepared by combining equal amounts of the complementary single-stranded DNA in a solution containing 10 mM Tris (pH 8.0) and 1 mM EDTA followed by heating to 90°C for 2 min, and then cooling to room temperature. The annealed oligonucleotides were labeled by filling in overhanging 5′-ends using the Klenow fragment of *Escherichia coli* DNA polymerase in the presence of [α-^{32}P]dCTP and/or [α-^{32}P]dGTP. Binding reactions were carried out in 20 μl of 20 mM Tris (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol (v/v), 0.3 mg/ml BSA, and 2 μg of poly[d(I·C)]. A typical reaction contained 20,000 cpm of labeled DNA and 20 μg of nuclear extract. The reactions were performed at 20°C for 20 min (ACCα SRE-1 probe) (Figs. 1A, B, 7B) or on ice for 60 min (ACCα T3RE probe, Fig. 6). DNA and DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gels at 4°C in 0.5 × TBE (45 mM Tris (pH 8.3), 45 mM boric acid, 1 mM EDTA). Following electrophoresis, the gels were dried and subjected to storage phosphor autoradiography. For competition experiments, unlabeled competitor DNA was mixed with radiolabeled oligomer prior to addition of nuclear extract. For antibody supershift experiments, nuclear extracts were incubated with antibodies for 30 min prior to addition of the oligonucleotide probe. Mouse monoclonal antibodies against SREBP-1 (IgG-2A4) and SREBP-2 (IgG-1D2) were obtained from American Type Tissue Collection (Manassas, VA). The sequence of ACCα SRE-1 probe and ACCα T3RE probe was 5′-TCGCATCACACCACCGCGG-3′ and 5′-AGGTGGTTGACCCGAGGTTAACCCCTCG-3′, respectively.

Western blot analysis

Proteins in nuclear extract and membrane fractions were subjected to electrophoresis in 8% SDS-polyacrylamide gels and then transferred to PDVF membranes (Amersham Pharmacia) using an electroblotting apparatus (Owl Scientific). Immunoblot analyses were performed as described in the Western blotting protocol from Santa Cruz Biotechnology. Briefly, the blots were blocked in Blotto [5% nonfat dry milk, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl] at 4°C overnight, and then incubated with monoclonal antibody against SREBP-1 (IgG-2A4) diluted to 1 μg/ml in Blotto containing 0.01% (v/v) Tween-20. After incubation with primary antibody for 1 h at room temperature, the blots were washed in TBS [10 mM Tris-HCl (pH 8.0), 150 mM NaCl] containing 0.01% Tween-20. Next, the blots were incubated with a donkey anti-mouse IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch) diluted 1:10,000 in Blotto, 0.01% Tween-20 for 1 h at room temperature. After washing with TBS, 0.01% Tween-20, antibody/protein complexes on blots were detected using enhanced chemiluminescence (Amersham Pharmacia). Chemiluminescence on the blots was visualized using a FluorChem 8000 imager (Alpha Innotech Corporation), and signals for the precursor and mature forms of SREBP-1 were quantified using FluorChem V200 software.

RNase protection assay

A chicken SREBP-1 cDNA fragment (300 bp) containing sequences between nucleotides 829 and 1,128 relative to the start site of translation was subcloned into the *Hind*III and *Bam*HI sites of pBluescript KS+ (Stratagene). The subclone was linearized with *Bam*HI, and antisense RNA was transcribed with α-[^{32}P]CTP (specific activity 3,000 Ci/mmol) using bacteriophage T3 RNA polymerase (Promega). An 18 S rRNA probe, which was used as a control for RNA loading, was made from pRTI 18 S template (Ambion, Austin, TX). Labeled RNAs were purified by polyacrylamide gel electrophoresis. RNA was extracted from CEH by the guanidinium thiocyanate-phenol-chloroform method (35).

RNase protection assays were performed using the RPA II kit (Ambion). Total RNA (20 μg) was hybridized to 4×10^4 cpm of ^{32}P-labeled RNA at 45°C for 16 h. The sample was then digested with a mixture of RNase A and RNase T. Protected fragments were separated on 8 M urea/5% polyacrylamide gels. Gels were dried and subjected to storage phosphor autoradiography. Images were quantified using ImageQuaNT software by Molecular Dynamics.

RESULTS

T3 and insulin increase the concentration of mature SREBP-1 in CEH

In a previous report, we showed that the mature form of SREBP-1 interacted with TR on the ACCα gene to enhance the stimulatory effect of T3 on ACCα transcription in CEH (10). This observation plus other work demonstrating a role of T3 in signaling changes in dietary carbohydrate status to lipogenic genes (1) have led us to hypothesize that T3 modulates SREBP levels in CEH. As insulin enhances the ability of T3 to activate ACCα transcription in CEH (24), we have further hypothesized that insulin controls SREBP levels. As a first step in investigating these questions, gel mobility shift experiments were performed to assess the binding of SREBP to the ACCα SRE-1 in CEH. Incubation of a ^{32}P-labeled oligonucleotide probe containing the ACCα SRE-1 (−84 to −66 bp) with nuclear extracts from CEH resulted in the formation of three protein-DNA complexes (**Fig. 1A**, left panel). The binding activity of the upper two complexes was specific, insofar as it was competed by a 100-fold molar excess of unlabeled ACCα SRE-1 probe but not by a 100-fold molar excess of an oligonucleotide containing an unrelated sequence. These complexes were designated *a* and *b* in the order of increasing mobility. Preincubation of nuclear extract with an antibody against SREBP-1 disrupted the formation of complex *a* but had no effect on the formation of complex *b*. Preincubation of nuclear extract with an antibody against SREBP-2 had no effect on the formation of complex *a* and complex *b*. Incubation of the ACCα SRE-1 probe with in vitro synthesized mature SREBP-1 (amino acids 1 to 464) resulted in the formation of a SREBP-1 homodimeric complex whose migration was identical to that of complex *a* (Fig. 1A, right panel). Collectively, these data suggest that complex *a* is comprised of SREBP-1 homodimers and that SREBP-1 is the predominant isoform of SREBP that binds ACCα promoter 2 in CEH. The identity of the proteins in complex *b* is presently unclear. Previous work has shown that the SRE-1 binds multiple protein complexes in crude nuclear extracts and that the transcriptional activity of the SRE-1 is correlated only with the binding of nuclear complexes containing SREBP (36, 37). Thus, complex *a* containing SREBP-1 homodimers likely accounts for the activity of the ACCα SRE-1 in CEH.

In CEH incubated in the absence of hormones, the binding of complex *a* to the ACCα SRE-1 was barely detectable (Fig. 1B). Addition of T3 or insulin to the culture medium for 24 h stimulated an increase in the binding of complex *a*. A greater stimulation of complex *a* binding activity was observed when T3 and insulin were added together. Thus, T3 and insulin enhance the binding of SREBP-1 to the ACCα SRE-1 in CEH. To determine whether the increase in SREBP-1 binding activity caused by T3 and insulin was associated with an elevation in the amount of mature SREBP-1, Western analyses were performed using nuclear extracts from CEH. In CEH incubated in medium containing high glucose (25 mM), treatment with T3, insulin, or T3 plus insulin for 24 h increased the concentration of mature SREBP-1 (Fig. 1C, E). The extent of the increase in mature SREBP-1 concentration caused by T3, insulin, and T3 plus insulin was 4.5-, 4.4-, and 7.0-fold, respectively. Similar effects of T3, insulin, and T3 plus insulin on mature SREBP-1 levels were observed when CEHs were incubated in medium containing low glucose (5 mM). Treatment of CEH with T3, insulin, or T3 plus insulin in the presence of 5 mM glucose or 25 mM glucose also increased the level of precursor SREBP-1 in membranes, but the extent of the effect of these treatments (1.5- to 2.0-fold) was substantially less than that observed for mature SREBP-1 (Fig. 1C, E). This observation suggests that alterations in the synthesis of precursor SREBP-1 account for part of the effects of T3 and insulin on mature SREBP-1 levels. T3 and insulin may also modulate the proteolytic processing of precursor SREBP-1 to mature SREBP-1 or the turnover of mature SREBP-1.

The abundance of SREBP-1 mRNA was also measured in CEH during different hormonal conditions. In CEH incubated in medium containing 25 mM glucose, addition of T3, insulin, or T3 plus insulin for 24 h increased the abundance of SREBP-1 mRNA (Fig. 1D, E). The magnitude of the increase in SREBP-1 mRNA abundance caused by T3, insulin, and T3 plus insulin was 2.0-, 1.6-, and 2.4-fold, respectively. Similar effects of T3, insulin, and T3 plus insulin on SREBP-1 mRNA abundance were observed in CEH incubated in medium containing 5 mM glucose. These results provide further evidence that alterations in the synthesis of precursor SREBP-1 account for part of the increase in mature SREBP-1 concentration caused by T3 and insulin.

To further analyze the mechanism mediating the effects of T3 and insulin on SREBP-1 levels in CEH, the time course of the effects of T3, insulin, and T3 plus insulin on the abundance of mature SREBP-1 protein, precursor SREBP-1 protein, and SREBP-1 mRNA was determined in CEH incubated in medium containing 25 mM glucose. Treatment of CEH with T3 for 2 h had no effect on the concentration of mature SREBP-1 (**Fig. 2A, C**). A small increase in mature SREBP-1 concentration (1.5-fold) was observed at 6 h of T3 treatment. A substantially larger increase in mature SREBP-1 concentration (4.2-fold) was observed at 24 h of T3 treatment. Longer incubations with T3 did not result in a further stimulation of mature SREBP-1 concentration (data not shown). The increase in precursor SREBP-1 protein and SREBP-1 mRNA levels caused by T3 was also maximal at 24 h of treatment. Incubating CEH with T3 for 24 h increased the amount of pre-

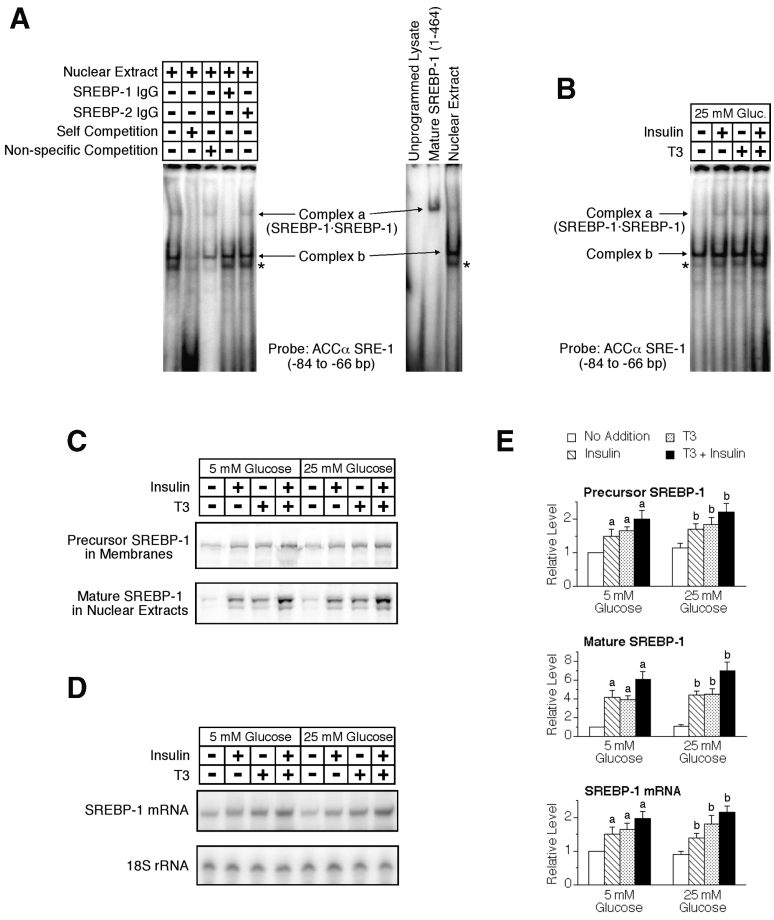

Fig. 1. 3,5,3′-Triiodothyronine (T3) and insulin increase the concentration of mature sterol regulatory element binding protein (SREBP)-1 in chick embryo hepatocytes (CEHs). Hepatocytes were isolated from livers of 18-day-old chick embryos and incubated in serum-free Waymouth's medium containing corticosterone and 5 mM glucose. At 18 h of incubation, the medium was changed to one of the same composition or to one containing corticosterone and 25 mM glucose. T3, insulin, or T3 plus insulin was added at this time. At 42 h of incubation, cellular extracts or total RNA were prepared as described under Experimental Procedures. A: Gel mobility shift assays were performed using nuclear extracts from hepatocytes or in vitro synthesized mature SREBP-1 (amino acids 1 to 464) and an oligonucleotide probe containing the acetyl-CoA carboxylase-α (ACCα) sterol regulatory element (SRE)-1 (−84 to −66 bp). Positions of specific protein-DNA complexes (arrows) and nonspecific complexes (asterisk) are indicated. Left panel: The ACCα SRE-1 probe was incubated with nuclear extract from hepatocytes treated with T3 plus insulin in the presence of 25 mM glucose. In some incubations, nuclear extract was incubated with antibodies against SREBP-1 or SREBP-2 prior to addition of the probe. Competition analysis was performed by mixing the labeled probe with a 100-fold molar excess of unlabeled probe (self competition) or unrelated sequence (nonspecific competition) prior to the addition of nuclear extract. Right panel: Comparison of the migration of complex *a* with that of SREBP-1 dimers bound to the ACCα SRE-1 probe. The ACCα SRE-1 probe was incubated with in vitro synthesized mature SREBP-1 or nuclear extract from hepatocytes treated with T3 plus insulin in the presence of 25 mM glucose. B: Gel mobility shift assays were performed using the ACCα SRE-1 probe and nuclear extracts from hepatocytes treated with T3, insulin, or T3 plus insulin in the presence of 25 mM glucose. Positions of specific protein-DNA complexes (arrows) and nonspecific complexes (asterisk) are indicated. C: The abundance of precursor SREBP-1 in membrane fractions and mature SREBP-1 in nuclear extracts was measured by Western analyses. These data are from a representative experiment. D: The abundance of SREBP-1 mRNA and 18S rRNA was measured using an RNase protection assay. These data are from a representative experiment. E: Signals for precursor SREBP-1 protein and mature SREBP-1 protein from Western analyses and SREBP-1 mRNA from RNase protection analyses were quantitated. Levels of precursor SREBP-1 protein, mature SREBP-1 protein, and SREBP-1 mRNA in hepatocytes treated with 5 mM glucose and no additional hormones were set at 1. Values are the means ± SEM of four experiments. a: Mean is significantly ($P < 0.05$) different from that of cells treated with 5 mM glucose and no additional hormones. b: Mean is significantly ($P < 0.05$) different from that of cells treated with 25 mM glucose and no additional hormones. Experimental details for Western and RNase protection analyses are described in Experimental Procedures.

cursor SREBP-1 protein and SREBP-1 mRNA by 1.6- and 1.9-fold, respectively (Fig. 2).

In contrast to the delayed effect of T3 on the abundance of mature SREBP-1, insulin caused a rapid increase in the abundance of mature SREBP-1 in CEH. Treatment with insulin for 2 h stimulated a 3.3-fold increase in the concentration of mature SREBP-1. Insulin also increased the concentration of mature SREBP-1 at 6 h (3.4-fold) and 24 h (4.0-fold) of treatment. The difference between T3 and insulin in the time course of induction of mature SREBP-1 levels suggests that these hormones act through different mechanisms to enhance the abundance of mature SREBP-1 in CEH. Whereas insulin caused a substantial increase in the concentration of mature SREBP-1 at 2 and 6 h of treatment, there was no effect

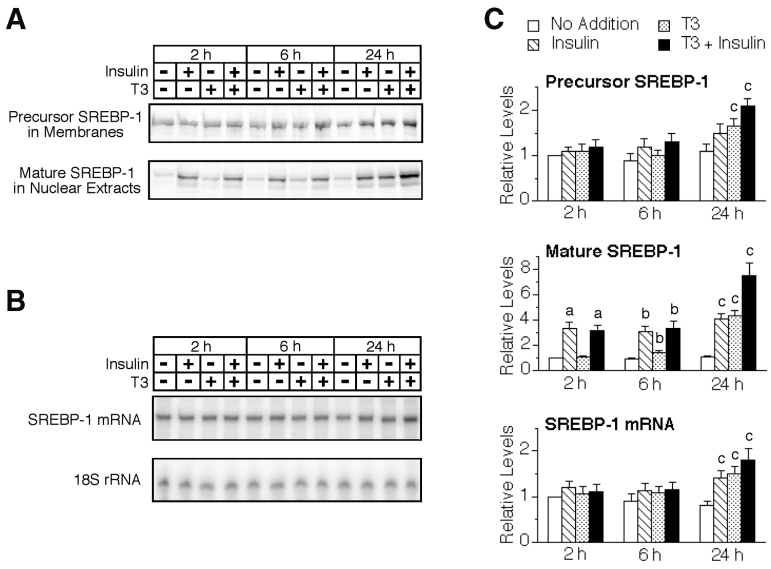

Fig. 2. Time course of the effects of T3 and insulin on levels of precursor SREBP-1 protein, mature SREBP-1 protein, and SREBP-1 mRNA in CEH. Hepatocytes were isolated and incubated in serum-free Waymouth's medium containing corticosterone and 25 mM glucose. At 18 h of incubation, the medium was changed to one of the same composition. T3, insulin, or T3 plus insulin was added at this time. After 2, 6, and 24 h of hormone treatment, cellular extracts or total RNA were prepared as described under Experimental Procedures. A: The abundance of precursor SREBP-1 in membrane fractions and mature SREBP-1 in nuclear extracts was measured by Western analyses. These data are from a representative experiment. B: The abundance of SREBP-1 mRNA and 18S rRNA was measured using an RNase protection assay. These data are from a representative experiment. C: Signals for precursor SREBP-1 protein and mature SREBP-1 protein from Western analyses and SREBP-1 mRNA from RNase protection analyses were quantitated. Levels of precursor SREBP-1 protein, mature SREBP-1 protein, and SREBP-1 mRNA in hepatocytes treated with no additional hormones for 2 h were set at 1. Values are the means ± SEM of four experiments. a: Mean is significantly ($P < 0.05$) different from that of cells incubated with no additional hormones for 2 h. b: Mean is significantly ($P < 0.05$) different from that of cells incubated with no additional hormones for 6 h. c: Mean is significantly ($P < 0.05$) different from that of cells incubated with no additional hormones for 24 h.

of this hormone on the concentration of SREBP-1 precursor protein and SREBP-1 mRNA at these time points. This finding suggests that most if not all of the increase in mature SREBP-1 concentration caused by 2 and 6 h of insulin treatment is mediated by changes in the processing of precursor SREBP-1 to mature SREBP-1 or turnover of mature SREBP-1. Incubating CEH with insulin for 24 h resulted in a small increase (1.7-fold) in SREBP-1 mRNA abundance. This effect may account for the slightly greater increase in mature SREBP-1 levels at 24 h of insulin treatment relative to 2 and 6 h of insulin treatment. The time course of the effects of T3 plus insulin on the abundance of mature SREBP-1 protein, precursor SREBP-1 protein, and SREBP-1 mRNA was similar to that of insulin alone except that the magnitude of the effect of T3 plus insulin on mature SREBP-1 levels at 24 h of treatment was greater than that of insulin alone at 24 h of treatment. The latter observation is consistent with the proposal that T3 increases the abundance of mature SREBP-1 via a mechanism that is distinct from that mediating the increase of mature SREBP-1 levels by insulin.

The ACCα SRE-1 and T3RE constitute a response unit that mediates the inhibitory effects of cAMP and medium-chain fatty acids on ACCα transcription

Treatment of CEH with cAMP or medium-chain fatty acids inhibits the activation of ACCα transcription caused by T3 and insulin (24–26). Previous work demonstrating that SREBP-1 interacts with TR to augment T3-induced ACCα transcription (10) plus results of the present study demonstrating that T3 and insulin increases the concentration of mature SREBP-1 (Figs. 1, 2) have led us to hypothesize that SREBP-1 plays a role in mediating the inhibition of ACCα transcription by cAMP and medium-chain fatty acids. To investigate this possibility, transient transfection experiments were performed to identify the *cis*-acting sequences that conferred the inhibitory effects of cAMP and hexanoate on ACCα transcription in the presence of T3 and insulin. In our initial experiments, CEHs were transfected with a series of constructs containing 5′-deletions of ACCα promoter 2 linked to the CAT gene. In CEH transfected with a construct containing 4,900 bp of 5′-flanking DNA (p[ACC−4900/+274]CAT), cAMP and hexanoate decreased promoter activity by 72% and 51%, respectively (**Fig. 3**). Deletion of ACCα sequences to −2,054, −854, −391, and −212, −136, and −108 bp had no effect on cAMP and hexanoate responsiveness. Deletion of sequences containing one of the half-sites of the ACCα T3RE (−108 to −94 bp) abolished the hexanoate-mediated inhibition of ACCα transcription and diminished cAMP-mediated inhibition of ACCα transcription by 32%. This deletion also decreased T3-induced ACCα promoter activity by 95%. Further deletion of ACCα sequences to 5′-end points of −84, −59, and −41 bp had no effect on residual cAMP responsiveness. These data suggest that the ACCα T3RE between −101 and −86 bp is required for optimal suppression of ACCα transcription by cAMP and hex-

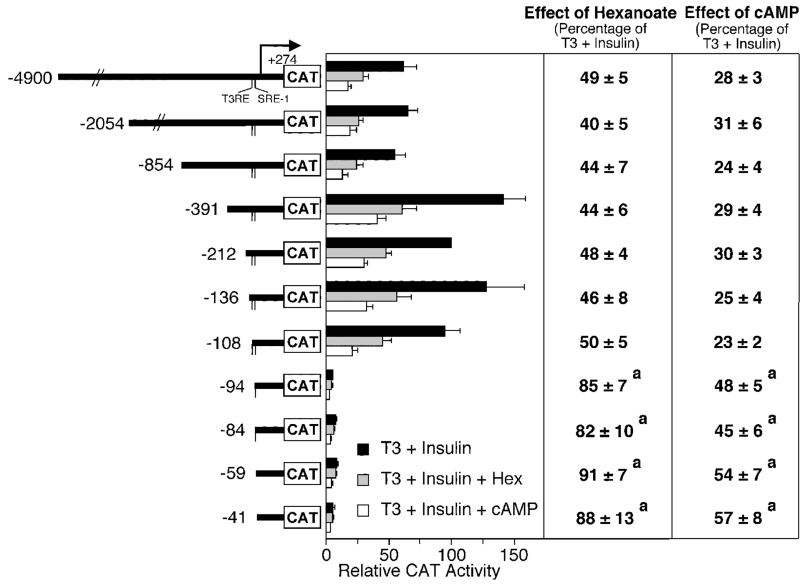

Fig. 3. Effects of deletions of the 5′-flanking region of ACCα promoter 2 on transcriptional activity in the absence and presence of cAMP or hexanoate. CEHs were transiently transfected with p[ACC−4900/+274] chloramphenicol acetyltransferase (CAT) or equimolar amounts of other plasmids as described under Experimental Procedures. After transfection, cells were treated with T3 and insulin in the absence or presence of dibutyryl cAMP or hexanoate for 48 h. Cells were then harvested, extracts prepared, and CAT assays performed. Left: The constructs used in these experiments. The number at the left of each construct is the 5′-end of ACCα DNA in nucleotides relative to the transcription initiation site of promoter 2. The 3′-end of each construct was +274 bp. The location of the T3 response element (T3RE) (−101 to −86 bp) and SRE-1 (−80 to −71 bp) is indicated by the vertical lines. Right: CAT activity of cells transfected with p[ACC−212/+274]CAT and treated with T3 and insulin was set at 100, and the other activities were adjusted proportionately. The effect of cAMP is the CAT activity of cells treated with T3, insulin, and cAMP expressed as a percentage of that in cells treated with T3 and insulin. The effect of hexanoate was calculated in a similar manner. The effects of cAMP and hexanoate were calculated for individual experiments and then averaged. The results are the means ± SEM of six experiments. Significant differences between means within a column ($P < 0.05$) are as follows: [a] versus p[ACC−108/+274]CAT.

anoate. In addition, ACCα sequences downstream of −41 bp are also involved in conferring cAMP regulation on ACCα transcription.

To further analyze the role of the ACCα T3RE in mediating the actions of cAMP and hexanoate on ACCα transcription, CEHs were transfected with constructs containing fragments of the ACCα gene linked to the minimal promoter of the herpes simplex virus thymidine kinase (TK) gene. The TK promoter alone was unresponsive to cAMP and hexanoate (**Fig. 4**). When a DNA fragment containing the ACCα T3RE (−108 to−82 bp) was linked to TKCAT, cAMP had no effect on promoter activity and hexanoate caused a small decrease (20%) in promoter activity. These results suggest that sequences in addition to the ACCα T3RE are required for optimal inhibition of transcription by cAMP and hexanoate. When a DNA fragment containing both the ACCα T3RE and SRE-1 (−108 to −66 bp) was linked to TKCAT, cAMP and hexanoate inhibited transcription by 52% and 60%, respectively. The increase in cAMP and hexanoate responsiveness caused by the inclusion of the ACCα SRE-1 was not due to changes in the spacing between the T3RE and the TK promoter, because the effects of cAMP and hexanoate on a construct containing a mutation of the ACCα SRE-1 (SRE-1 mut) in the context of p[ACC−108/−66]TKCAT were similar to those observed for p[ACC−108/−82]TKCAT. In CEH transfected with a construct containing the SRE-1 alone (−84 to −66 bp) linked to TKCAT, cAMP and hexanoate had no effect on promoter activity. Thus, the ACCα SRE-1 requires the presence of the ACCα T3RE in order to confer regulation of transcription by cAMP and hexanoate. Collectively, the data in Figs. 3 and 4 suggest that the ACCα SRE-1 and T3RE constitute a response unit that mediates the inhibitory effects of cAMP and hexanoate on gene transcription.

We next investigated the effects of the ACCα SRE-1 on the regulation of ACCα promoter 2 by cAMP and hexanoate. Mutation of the ACCα SRE-1 (−79 to −72 bp) in the context of the ACCα promoter region extending from −108 to +274 bp decreased cAMP and hexanoate responsiveness by 43% and 61%, respectively (**Fig. 5**). A similar result was obtained when the ACCα SRE-1 was mutated in the context of the ACCα promoter region extending from −108 to +31. Thus, the presence of the ACCα SRE-1 augments the inhibitory actions of cAMP and hexanoate on ACCα transcription. The observation that mutation of the ACCα SRE-1 does not completely inhibit cAMP responsiveness of ACCα promoter 2 supports the 5′-deletion data (Fig. 3), suggesting the presence of an additional cAMP response element(s) located downstream of −41 bp. The

3′ boundary of this cAMP response element is probably located upstream of +31 bp, because the activity of p[ACC−108/+31]CAT containing the SRE-1 mutation is regulated by cAMP.

Effects of cAMP and hexanoate on the binding of nuclear proteins to the ACCα T3RE and SRE-1

On the basis of the observation that the ACCα T3RE is required for optimal inhibition of ACCα transcription by cAMP and hexanoate (Figs. 3 and 4), we have hypothesized that the effects of cAMP and hexanoate on ACCα transcription are mediated by alterations in the binding of nuclear proteins to the ACCα T3RE. Such changes in protein binding to the ACCα T3RE would decrease the activity of the ACCα T3RE per se and/or inhibit the ability of the ACCα T3RE to interact with the ACCα SRE-1. To investigate this possibility, gel mobility shift analyses were conducted using nuclear extracts from CEH incubated with or without cAMP or hexanoate. We previously reported that a probe containing the ACCα T3RE (−108 to −82 bp) bound to four complexes designated 1 to 4 in the order of increasing mobility (5). Complexes 1, 2, and 3 contained LXR·RXR heterodimers, whereas complex 4 contained TR·RXR heterodimers. T3 treatment for 24 h caused a marked increase in the binding of complex 3 and complex 4 and a decrease in the binding of complex 1 and complex 2. On the basis of these data, we have proposed that T3-induced ACCα transcription is mediated by the binding of protein complexes containing TR·RXR (complex 4) and LXR·RXR (complex 3). In **Fig. 6** of the present report, we show that addition of cAMP or hexanoate at the beginning or during the last 2 h of a 24 h incubation with T3 and insulin has no effect on the binding of complexes 1–4. These results suggest that the inhibitory effects of cAMP and hexanoate on ACCα transcription are not mediated by alterations in protein binding to the ACCα T3RE.

Data in Figs. 4 and 5 indicate that cAMP and hexanoate suppress the ability of the ACCα SRE-1 to activate ACCα transcription in the presence of T3 and insulin. cAMP and hexanoate may decrease the activity of the ACCα SRE-1 by suppressing the induction of mature SREBP-1 levels caused by T3 and insulin. To investigate this possibility, Western analyses were conducted using nuclear extracts from CEH incubated in the absence or presence of cAMP or hexanoate. Treatment of CEH with cAMP or hexanoate during the last 2 h of a 24 h incubation with T3 and insulin decreased the concentration of mature SREBP-1 (**Fig. 7A, D**). The extent of reduction in mature SREBP-1 concentration caused by cAMP and hexanoate was 71% and 52%, respectively. Similar results were obtained when cAMP and hexanoate were added at the beginning of a 24 h incubation with T3 and insulin. Further evidence that cAMP and hexanoate decreased the concentration of mature SREBP-1 was obtained from gel mobility shift experiments using the ACCα SRE-1 as a probe. In CEH incubated in the presence of T3 and insulin, addition of cAMP or hexanoate decreased the binding activity of complex *a* (Fig. 7B). These data suggest that cAMP and hexanoate

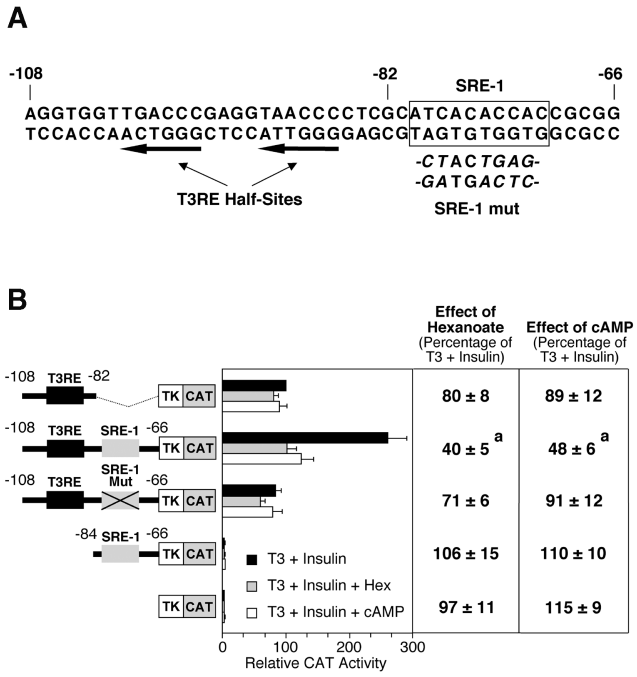

Fig. 4. The ACCα T3RE and SRE-1 confer cAMP and hexanoate responsiveness on a heterologous promoter. A: Sequence of the chicken ACCα gene between −108 and −66 bp. The hexameric half-sites comprising the T3RE are indicated by arrows. The SRE-1 is boxed. The sequence of a block mutation of the SRE-1 (SRE-1 mut) is shown underneath the SRE-1. B: Fragments of the ACCα gene containing the T3RE and/or SRE-1 were linked to the minimal thymidine kinase (TK) promoter in TKCAT. CEHs were transiently transfected with these constructs and treated with T3 and insulin in the absence or presence of dibutyryl cAMP or hexanoate as described in the legend to Fig. 3 and under Experimental Procedures. Left: Constructs used in these experiments. Numbers indicate the 5′ and 3′ boundaries of ACCα DNA relative to the transcription initiation site of promoter 2. Right: CAT activity in CEH transfected with p[ACC−108/−82]TKCAT and treated with T3 and insulin was set at 100, and the other activities were adjusted proportionately. The effects of cAMP and hexanoate on promoter activity were calculated as described in the legend to Fig. 3. The results are the means ± SEM of six experiments. Significant differences between means within a column ($P < 0.05$) are as follows: [a] versus any other construct.

inhibit ACCα SRE-1 activity by decreasing the concentration of mature SREBP-1.

To investigate the mechanism for the reduction in mature SREBP-1 levels caused by cAMP and hexanoate, the abundance of precursor SREBP-1 protein and SREBP-1 mRNA was measured in CEH incubated in the absence or presence of cAMP or hexanoate. Addition of cAMP or hexanoate during the last 2 h of a 24 h incubation with T3 and insulin had no effect on the concentration of precursor SREBP-1, whereas addition of cAMP or hexanoate at the beginning of a 24 h incubation with T3 and insulin increased the concentration of precursor SREBP-1 by 1.5- to 1.7-fold (Fig. 7A, D). Thus, the decrease in the concentration of mature SREBP-1 caused by cAMP and hexanoate is not associated with a reduction in the concentration of precursor SREBP-1, suggesting that a posttranslational process is involved in mediating the effects of cAMP and

hexanoate on mature SREBP-1 levels in CEH. We next investigated whether cAMP and hexanoate modulated the abundance of SREBP-1 mRNA. Addition of cAMP between 1 and 24 h or between 22 and 24 h of a 24 h incubation with T3 and insulin had no effect on SREBP-1 mRNA abundance (Fig. 7C, D). In contrast, addition of hexanoate during the last 2 h of a 24 h incubation with T3 and insulin decreased SREBP-1 mRNA abundance by 51%. Similar results were obtained when hexanoate was added at the beginning of a 24 h incubation with T3 and insulin. These data suggest that hexanoate also regulates the concentration of mature SREBP-1 at a pretranslational step.

DISCUSSION

Previous work from our laboratory has shown that SREBP-1 interacts with TR on the ACCα gene to enhance T3-induced ACCα transcription (10). In the present study, we have identified a new interaction between the SREBP-1 and TR signaling pathways. T3 stimulates an increase in the concentration of the mature, active form of SREBP-1 in CEH. These data are the first to show that T3 modulates the activity of an accessory transcription factor that controls TR activity.

An interesting feature of the regulation of the ACCα gene in CEH is that maximal activation of transcription by T3 requires a relatively long time (≥24 h), with most of the increase in transcription occurring between 5 h and 24 h of hormone treatment (24). Results of previous studies analyzing the binding of nuclear proteins to the ACCα T3RE have suggested that the increase in transcription between 5 and 24 h of T3 treatment is mediated by a decrease in the binding of protein complexes containing LXR·RXR (complexes 1 and 2) and an increase in the binding of protein complexes containing TR·RXR (complex 4) and LXR·RXR (complex 3) (5). Results of time course analyses in the present study suggest that alterations in SREBP-1 binding to the ACCα SRE-1 also play a role in mediating the increase in ACCα transcription caused by T3. Addition of T3 increases the concentration of mature SREBP-1 between 5 and 24 h of hormone treatment (Fig. 2). The stimulatory effects of T3 on transcription of fatty acid synthase (6), ATP-citrate lyase (38), stearoyl-CoA desaturase (39), and spot 14 (40) may also be mediated, at least in part, by the increase in mature SREBP-1 abundance caused by T3, because these genes contain functional SREs (11, 12, 18, 41, 42).

Previous studies have shown that the ability of T3 to activate ACCα transcription in CEH is enhanced by the presence of insulin in the culture medium (24). Data from the present study indicate that insulin increases the concentration of mature SREBP-1 in CEH and that this effect is additive with that of T3 (Figs. 1, 2). This observation provides support for a role of SREBP-1 in mediating the effects of insulin on T3-induced ACCα transcription. Other mechanisms may be involved in mediating the actions of insulin on ACCα transcription, because most of the increase in mature SREBP-1 levels caused by insulin is observed within the first 2 h of insulin treatment (Fig. 2), whereas most of the increase in ACCα transcription caused by T3 is observed between 5 and 24 h of T3 treatment (24).

In contrast to the chicken, two isoforms of SREBP-1, designated as SREBP-1a and SREBP-1c, are expressed in mammals (29). These different SREBP-1 isoforms vary in structure at their N-termini and are derived from different promoters on the SREBP-1 gene. SREBP-1c is the predominant isoform of SREBP-1 expressed in liver; its activity is subject to nutritional and hormonal regulation (15, 43, 44). In rat hepatocyte cultures, insulin increases the concentration of mature SREBP-1c (13–15). Thus, insulin has

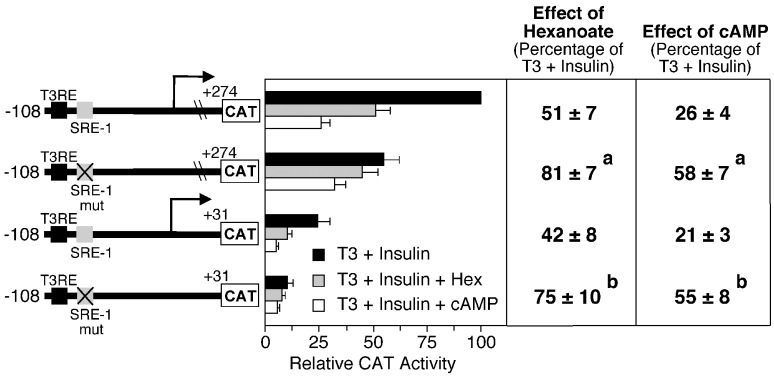

Fig. 5. The ACCα SRE-1 is required for optimal regulation of ACCα promoter 2 activity by cAMP and hexanoate. p[ACC−108/+274]CAT, p[ACC−108/+31]CAT, and constructs containing block mutations of the SRE-1 (SRE-1 mut) in the context of p[ACC−108/+274]CAT or p[ACC−108/+31]CAT were transiently transfected into CEH as described under Experimental Procedures. After transfection, cells were treated with T3 and insulin in the absence or presence of dibutyryl cAMP or hexanoate as described in the legend to Fig. 3 and under Experimental Procedures. Left: The constructs used in these experiments. Numbers indicate the 5′ and 3′ boundaries of ACCα DNA in nucleotides relative to the transcription initiation site of promoter 2. Right: CAT activity of cells transfected with p[ACC−108/+274]CAT and treated with T3 and insulin was set at 100, and the other activities were adjusted proportionately. The effects of cAMP and hexanoate were calculated as described in the legend to Fig. 3. The results are the means ± SEM of five experiments. Significant differences between means within a column ($P < 0.05$) are as follows: [a] versus p[ACC−108/+274]CAT; [b] versus p[ACC−108/+31]CAT.

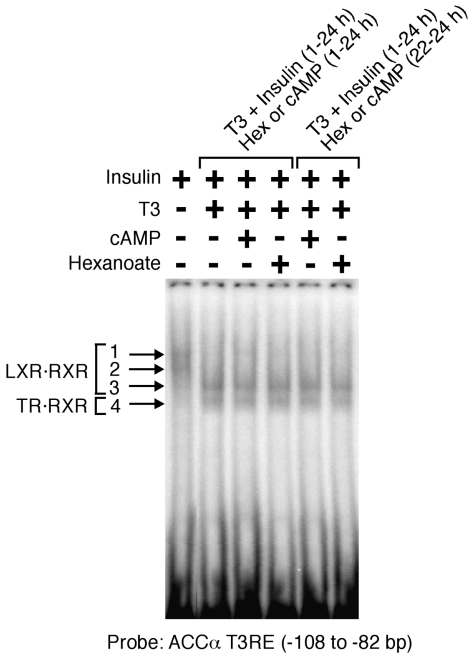

Fig. 6. Effect of cAMP and hexanoate on the binding of hepatic nuclear proteins to the ACCα T3RE. Eighteen hours after being placed in culture, CEHs were incubated in Waymouth's medium containing corticosterone, insulin, and 25 mM glucose with or without T3, T3 plus dibutyryl cAMP, or T3 plus hexanoate for the indicated times. Cells were harvested and nuclear extracts were prepared as described in Experimental Procedures. Nuclear extracts were subjected to gel mobility shift analyses using an oligonucleotide probe containing the ACCα T3RE (−108 to −82 bp). Specific protein-DNA complexes are indicated by arrows. Previous studies have shown that complexes 1, 2, and 3 contain liver X receptor (LXR)·retinoid X receptor (RXR) heterodimers, whereas complex 4 contains nuclear T3 receptor (TR)·RXR heterodimers (5). These data are representative of five experiments employing independent preparations of nuclear extract.

similar effects on the concentration of mature SREBP-1c in rat hepatocytes and the concentration of mature SREBP-1 in CEH. Further analyses have shown that insulin-induced increase in mature SREBP-1c abundance in rat hepatocytes is mediated primarily by changes in SREBP-1c mRNA abundance (13–15). These findings contrast with those of the present study, suggesting that post-translational processes play a prominent role in mediating the effects of insulin on mature SREBP-1 levels in CEH. The reason for the differences between avians and mammals in the diversity of SREBP-1 isoforms and the mechanism mediating the effects of insulin on mature SREBP-1 levels is not clear. They may reflect subtle class-dependent differences in the role of SREBP-1 in the control of lipogenesis and/or other metabolic processes in liver.

In addition to participating in the activation of ACCα transcription by T3 and insulin, SREBP-1 plays a role in mediating the inhibition of ACCα transcription caused by cAMP. Results from transfection, DNA binding, and Western analyses indicate that cAMP inhibits ACCα transcription in part by repressing the ability of T3 and insulin to increase mature SREBP-1 levels (Figs. 3–5, 7). This effect, in turn, disrupts the positive interaction between SREBP-1 and T3-bound TR on the ACCα gene. cAMP causes a rapid decrease (≤2 h) in mature SREBP-1 concentration that precedes or parallels the reduction in ACCα transcription caused by cAMP (24). To our knowledge, these observations are the first to establish a role for SREBP-1 in mediating the inhibitory effects of cAMP on lipogenic gene transcription.

cAMP inhibits the transcription of other lipogenic genes, such as glucokinase, L-pyruvate kinase, fatty acid synthase, malic enzyme, glycerol-3-phosphate acyltransferase, and spot 14 (1). For some of these genes, the mechanism by which cAMP inhibits transcription has been analyzed. For example, in rat hepatoma cells, the inhibitory actions of cAMP on fatty acid synthase transcription are mediated by an inverted CCAAT box in the proximal promoter region of the fatty acid synthase gene (45). This element constitutively binds the transcription factor, nuclear factor-Y (46). How cAMP modulates nuclear factor-Y activity is presently not known. In CEH, inhibition of malic enzyme transcription by cAMP is mediated by at least four *cis*-acting elements in the malic enzyme 5′-flanking DNA (47). One of these elements binds c-Fos and activating transcription factor-2 in CEH incubated in the presence of cAMP. The mechanism by which c-Fos/activating transcription factor-2 inhibits malic enzyme transcription is presently not known. In rat hepatocytes, inhibition of L-pyruvate kinase transcription by cAMP is mediated by two contiguous *cis*-acting elements referred to as L3 and L4 (48). These elements also mediate the stimulatory effect of glucose on L-pyruvate kinase transcription. L3 binds the hepatocyte nuclear factor-4 (HNF-4) and L4 binds ChREBP (2, 49). Both HNF-4 and ChREBP activate L-pyruvate kinase transcription. Recent work has shown that protein kinase A phosphorylates ChREBP at Ser^{196} and Thr^{666}, causing a block in the transport of ChREBP from the cytoplasm to the nucleus and a decrease in the binding of ChREBP to the L4 element (2). Protein kinase A also phosphorylates HNF-4, causing a decrease in the binding of HNF-4 to the L3 element (49). Thus, cAMP inhibits L-pyruvate kinase transcription by repressing the stimulatory effects of HNF-4 and ChREBP on transcription. These findings, in combination with those of the present study, indicate that a wide variety of mechanisms are involved in mediating the inhibitory actions of cAMP on lipogenic gene transcription.

Results of the present study indicate that SREBP-1 also plays a role in mediating the inhibitory effects of hexanoate on ACCα transcription. As observed with cAMP, hexanoate rapidly decreases (≤2 h) the concentration of mature SREBP-1 in CEH incubated in the presence of T3 and insulin (Fig. 7). Results from Western and Northern analyses suggest that the reduction in mature SREBP-1 levels caused by hexanoate is mediated by both pretranslational and posttranslational processes. In contrast, the inhibitory effect of cAMP on mature SREBP-1 levels appears to be mediated solely by a posttranslational mechanism. These observations suggest that hexanoate and cAMP act through common as well as separate signaling pathways to suppress mature SREBP-1 levels in CEH.

Recently, Kawaguchi et al. (16) have shown that acetate,

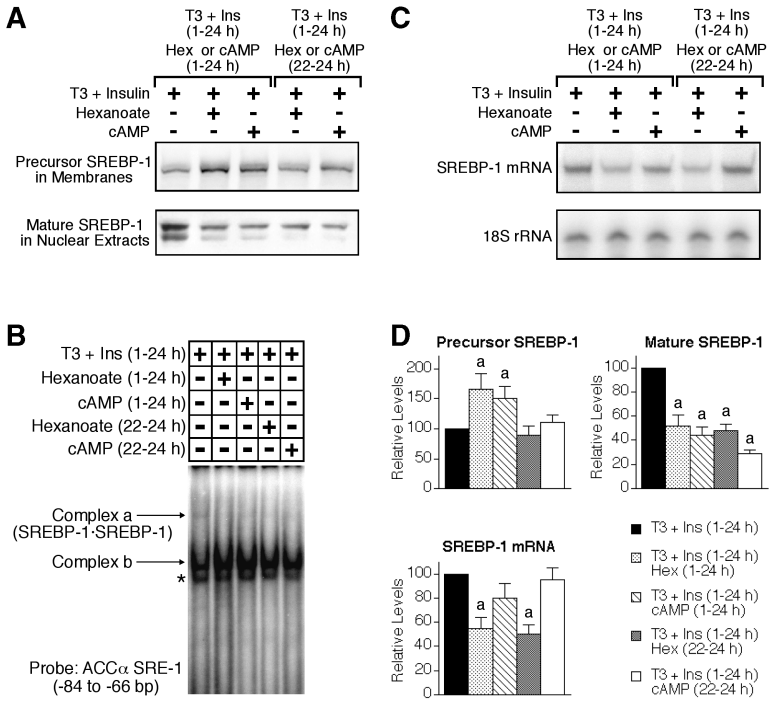

Fig. 7. cAMP and hexanoate suppress the stimulatory effects of T3 and insulin on the concentration of mature SREBP-1 in CEH. Eighteen hours after being placed in culture, CEHs were incubated in Waymouth's medium containing corticosterone, insulin, 25 mM glucose, and T3 with or without dibutyryl cAMP or hexanoate for the indicated times. Cell extracts or total RNA were prepared as described in Experimental Procedures. A: The abundance of precursor SREBP-1 in membrane fractions and mature SREBP-1 in nuclear extracts was measured by Western analyses. These data are from a representative experiment. B: Gel mobility shift assays were performed using nuclear extracts from hepatocytes and an oligonucleotide probe containing the ACCα SRE-1 (−84 to −66 bp). Positions of specific protein-DNA complexes (arrows) and nonspecific complexes (asterisk) are indicated. C: The abundance of SREBP-1 mRNA and 18S rRNA was measured using an RNase protection assay. These data are from a representative experiment. D: Signals for precursor SREBP-1 protein and mature SREBP-1 protein from Western analyses and SREBP-1 mRNA from RNase protection analyses were quantitated. Levels of precursor SREBP-1 protein, mature SREBP-1 protein, and SREBP-1 mRNA in hepatocytes treated with T3 and insulin without cAMP and hexanoate were set at 1. Values are the means ± SEM of four experiments. a: mean is significantly ($P < 0.05$) different from that of cells treated with T3 and insulin without cAMP and hexanoate.

octanoate, and palmitate inhibit L-pyruvate kinase transcription in rat hepatocytes by abolishing the binding of ChREBP to the L4 element. This effect is mediated by an increase in the phosphorylation of ChREBP at Ser^{568} by AMP-activated protein kinase. Recent work by Zhou et al. (50) suggests that AMP-activated protein kinase also controls SREBP-1 activity. Treatment of rat hepatocytes with agents that stimulate AMP-activated protein kinase activity (5-amino-imidazole carboxamide riboside and the antidiabetic drug, metformin) results in a reduction SREBP-1 protein levels and SREBP-1 mRNA abundance. These agents also inhibit expression of ACCα and fatty acid synthase in rat hepatocytes (50, 51). We postulate that hexanoate decreases mature SREBP-1 levels in CEH by stimulating the activity of AMP-activated protein kinase. Alterations in AMP-activated protein kinase activity may directly or indirectly regulate the activity of factors that control the abundance of SREBP-1 mRNA and/or the processing of precursor SREBP-1 to mature SREBP-1. Experiments are underway to explore this hypothesis.

In addition to hexanoate and octanoate, long-chain polyunsaturated fatty acids (PUFAs) inhibit lipogenic gene transcription in hepatocytes incubated in the presence of insulin and T3 (52). In the case of the genes for fatty acid synthase, stearoyl-CoA desaturase, and spot 14, inhibition of transcription by PUFA is mediated, at least in part, by a reduction in the concentration of mature SREBP-1 (18, 41, 53). Further analyses have shown that the decrease in mature SREBP-1 concentration caused by PUFA is associated with a reduction in SREBP-1 mRNA abundance (18, 20). The decrease in mature SREBP-1 concentration caused by hexanoate is also associated with a reduction in SREBP-1 mRNA abundance (Fig. 7). On the basis of these observations, we postulate that the active metabolite(s) mediating the effect of PUFAs on SREBP-1 expression is similar to the metabolite mediating the effect of hexanoate on SREBP-1 expression. Further analysis of the mechanisms by which medium-chain fatty acids and PUFAs control SREBP-1 activity should aid in identifying this metabolite.

In summary, we have shown that SREBP-1 is an accessory transcription factor that plays an active and central role in the hormonal and nutritional regulation of ACCα transcription in hepatocytes. Through interactions with the TR, SREBP-1 functions as an integrator of multiple stimulatory and inhibitory signals that influence rates of lipogenesis. We propose that, in intact animals, this regulatory system allows for the rapid and fine control of

ACCα transcription in response to alterations in dietary carbohydrate intake.

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