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Sterol regulatory element binding protein binds to a cis element in the promoter of the farnesyl diphosphate synthase gene

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ABSTRACT **Sterol-regulated transcription of the gene for rat farnesyl diphosphate (FPP) synthase (geranyl-diphosphate:isopentenyl-diphosphate geranyl*trans*transferase, EC 2.5.1.10) is dependent in part on the binding of the ubiquitous transcription factor NF-Y to a 6-bp element within the proximal promoter. Current studies identify a second element in this promoter that is also required for sterol-regulated transcription *in vivo*. Mutation of three nucleotides (CAC) within this element blocks the 8-fold induction of FPP synthase promoter–reporter genes that normally occurs when the transfected cells are incubated in medium deprived of sterols. Gel mobility-shift assays demonstrate that the transcriptionally active 68-kDa fragment of the sterol regulatory element (SRE-1)-binding protein assays (SREBP-1) binds to an oligonucleotide containing the wild-type sequence but not to an oligonucleotide in which the CAC has been mutated. DNase I protection pattern (footprint) analysis indicates that SREBP-1 binds to nucleotides that include the CAC. Both the *in vivo* and *in vitro* assays are affected by mutagenesis of nucleotides adjacent to the CAC. Coexpression of SREBP with a wild-type FPP synthase promoter–reporter gene in CV-1 cells results in very high levels of reporter activity that is sterol-independent. In contrast, the reporter activity remained low when the promoter contained a mutation in the CAC trinucleotide. We conclude that sterol-regulated transcription of FPP synthase is controlled in part by the interaction of SREBP with a binding site that we have termed SRE-3. Identification of this element may prove useful in the identification of other genes that are both regulated by SREBP and involved in lipid biosynthesis.**

Farnesyl diphosphate (FPP) synthase (geranyl-diphosphate: isopentenyl-diphosphate geranyl*trans*transferase, EC 2.5.1.10) catalyzes the formation of FPP by the head-to-tail condensation of dimethylallyl diphosphate and two molecules of isopentenyl diphosphate (1). FPP is a key intermediate in the biosynthesis of sterols, ubiquinone, dolichol, and heme *a* (2). A distinct enzyme further elongates FPP by condensation with an additional molecule of isopentenyl diphosphate to produce geranylgeranyl diphosphate (3). The latter isoprenoid together with FPP is involved in the functionally important modification (prenylation) of an increasing number of proteins, including trimeric and monomeric GTP-binding proteins, certain kinases, and yeast mating hormones (2). Thus, FPP synthase catalyzes the synthesis of an isoprenoid that is important for many different cell functions.

The rat FPP synthase cDNA was originally cloned by differential hybridization under conditions known to regulate the expression of hepatic 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and HMG-CoA synthase 30- to 45-fold (4). Subsequent studies demonstrated that the transcription of the FPP synthase gene is regulated by the sterol status of the cell (5–7) in parallel with that of HMG-CoA reductase, HMG-CoA synthase, and the low density lipoprotein (LDL) receptor (8, 9).

Chloramphenicol acetyltransferase (CAT) reporter gene constructs containing 247 or 319 bp of the FPP synthase promoter (pFPPS-0.247 and pFPPS-0.319) are regulated 3- to 5-fold by the sterol status of the cell (6, 7, 10). Further deletion and mutational analyses of FPP synthase promoter–reporter gene constructs demonstrated that a 117-bp sequence imparted a 3- to 10-fold sterol-dependent regulation to a heterologous minimal herpes simplex virus thymidine kinase (TK) promoter–CAT gene (pTKCIII-0.117) (7). The nucleotide sequence within these 117-bp exhibits no significant homology with the sterol regulatory element 1 (SRE-1; ATCACCCCAC) characterized in detail in the promoter of the LDL receptor gene (9).

Recently, a human SRE-1-binding protein (SREBP) was purified and cloned (9, 11, 12). Further analyses demonstrated the existence of at least two major forms of SREBP—i.e., SREBP-1 and SREBP-2—encoded by two separate genes which, as a result of differential splicing of the SREBP-1 transcript, produce a total of five proteins (12, 13).

Single point mutations of 9 of the 10 bp of SRE-1 of the LDL receptor promoter block both sterol-regulated transcription *in vivo* and the binding of SREBP-1 and SREBP-2 *in vitro* (11, 13). The promoter of HMG-CoA synthase contains a sequence with 90% identity with the SRE-1 of the LDL receptor (8), and transcription of this gene is also regulated by SREBP availability (13). It is not known whether the HMG-CoA synthase SRE-1 can functionally replace the SRE-1 within the LDL receptor gene promoter.

In contrast, Osborne (14) reported that four separate point mutations within the putative SRE-1 in the promoter of HMG-CoA reductase did not affect sterol-regulated transcription. A distinct protein, termed "Red 25," was purified and shown to bind to nucleotides that overlap with the putative SRE-1, and it was hypothesized that Red 25 is involved in sterol-regulated transcription of HMG-CoA reductase (15).

Adipocyte determination- and differentiation-dependent factor 1 (ADD-1), the rat homologue of SREBP, was cloned as a result both of its ability to bind to an E-box motif *in vitro* and its increased expression during adipocyte differentiation (16, 17). Surprisingly, the E-box motif (ATCACGTGA) has limited identity with SRE-1.

The current study identifies a sequence within the FPP synthase promoter, termed SRE-3, that is required for the induced levels of transcription normally observed in cells

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Abbreviations: FPP, farnesyl diphosphate; SRE-1, sterol regulatory element 1; SREBP, SRE-1-binding protein; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; CAT, chloramphenicol acetyltransferase; CHO, Chinese hamster ovary; LDL, low density lipoprotein; ADD-1, adipocyte determination- and differentiation-dependent factor 1; UAS_{INO}, upstream-activating sequence in the promoter of the INO1 gene; CMV, cytomegalovirus; TK, thymidine kinase.

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deprived of exogenous sterols. We demonstrate that the nucleotides within SRE-3 are required both for sterol-regulated transcription *in vivo* and for binding SREBP-1 *in vitro*. Identification of this element may lead to the identification of an expanded repertoire of genes that are regulated by SREBP.

EXPERIMENTAL PROCEDURES

Materials. DNA restriction and modification enzymes were obtained from GIBCO/BRL. ^{32}P-labeled nucleotides were obtained from Amersham. Oligonucleotides were synthesized by D. Glitz (Department of Biological Chemistry, Univ. of California, Los Angeles). Purified SREBP-1a (amino acids 1–490), containing both a phage T7 and polyhistidine tag, and pCMV-CSA10, an expression vector encoding the 68-kDa transcriptionally active fragment of SREBP-1a under the control of the cytomegalovirus (CMV) promoter, were kindly provided by T. Osborne (Department of Molecular Biology and Biochemistry, Univ. of California, Irvine). Lipoprotein-deficient fetal calf serum was purchased from PerImmune (Rockville, MD). The sources of all other reagents and plasmids have been given (6, 7).

Cell Culture. CV-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum at 37°C under 92% air/8% CO_2. Chinese hamster ovary (CHO) cells were grown in Ham's F-12 medium under 95% air/5% CO_2 as described (7).

Plasmid Construction and Mutagenesis. Standard molecular biology techniques were used (18). Single-stranded pFPPS-0.319 (nucleotides −365 to −47) or pFPPS-0.247 (−293 to −47) and oligonucleotides (20–40 bp) were used to generate mutations by using the Sculptor *in vitro* mutagenesis kit (Amersham). The wild-type or mutant promoters were subcloned into the pGL2 basic luciferase vector (Promega) and sequenced as described (6) to confirm the mutations. To be consistent with earlier studies (7), the numbering of mutants 1–5 was maintained. Mutants 1–5 and p–u all contained C/A and T/G transversions across the indicated sequence (Fig. 1). Mutants A–H contained 12-bp mutations in which the indicated wild-type sequence (Fig. 1) was replaced by ACTGATGACACA. A 61-bp FPP synthase promoter fragment (nucleotides −293 to −233) was generated by PCR that used primers containing restriction sites (*Hin*dIII/*Bam*HI) compatible with pTKCIII (7). The resulting pTKCIII-0.061 construct was sequenced and found to contain the wild-type sequence. A mutant 61-bp promoter, pTKCIII-0.061q, was also generated by PCR. This promoter (nucleotides −293 to −233) contained ACA instead of the wild-type CAC at nucleotides −259 to −257.

Transient Transfection. CV-1 cells were transfected with a total of 6.0 μg of plasmid DNA per 60-mm dish by using the Stratagene MBS transfection kit. All transfections contained 2 μg of pCMV-β-galactosidase to normalize luciferase or CAT activities for transfection efficiencies (7). Where indicated, 7.5 ng of pCMV-CSA10 was included in the transfection. This latter plasmid encodes a 68-kDa transcriptionally active fragment of SREBP-1a. The cells were exposed to DNA for 3.5 hr, washed extensively, and incubated in medium supplemented with 10% (vol/vol) lipoprotein-deficient fetal calf serum (LPDS) in the absence (inducing media) or presence (repressing media) of sterols (10 μg of cholesterol and 1 μg of 25-hydroxycholesterol per ml). After incubation for 18–24 hr, cell extracts were prepared in lysis buffer (Promega) according to the manufacturer's instructions, and the activities of β-galactosidase and either luciferase or CAT were determined (7).

Gel Mobility-Shift Assay. CHO nuclear extracts were prepared as described (7). Double-stranded DNA corresponding to nucleotides −264 to −241 of the FPP synthase promoter containing either wild-type sequence or the indicated 3-bp transversion mutations was end-labeled with ^{32}P and used in gel mobility-shift assays (7). The probe (20,000 cpm, 1.5 fmol) was incubated with 2 ng of recombinant 68-kDa SREBP-1 containing a T7 tag in the presence of either nonfat milk (2.5 mg/ml) or nuclear extract (100 μg/ml) for 60 min at room temperature. Where indicated, incubations were continued on ice for 30 min following the addition of 300 ng of anti-T7-tag (Novagen).

Gel mobility-shift assays also utilized double-stranded oligonucleotides containing SRE-3 (5′-GCCCATA**CTCACAC-GAG**GTCATTC-3′), SRE-1 from the LDL receptor gene (5′-TTTGAAA**ATCACCCCAC**TGCAAAC-3′) (9), and the E-box from the S14 gene (5′-TTGCCAGTTCT**CACGTG**GT-GGCCA-3′) (19). The nucleotides in boldface letters correspond to the consensus sequence. The DNA–protein complexes were resolved on a 4% nondenaturing acrylamide gel at 4°C in 1× TBE (0.05 M Tris/0.05 M boric acid/0.001 M EDTA), vacuum-dried, and exposed to either Kodak XAR film at −70°C or a Molecular Dynamics PhosphorImager screen.

DNase I Protection Assays (Footprinting). A 117-bp fragment corresponding to nucleotides −293 to −177 of the FPP synthase promoter was ^{32}P-end-labeled, and 7.5 fmol was used in a binding reaction (7) in the presence of nonfat milk (2.5 mg/ml) and in the absence or presence of 1 μg or 3 μg of recombinant SREBP. After incubation for 60 min at room temperature, DNase I footprinting was performed by using a SureTrack footprinting kit (Pharmacia) according to the manufacturer's recommendations. Briefly, 2 μl of Ca^{2+}/Mg^{2+} solution and 0.15 unit of DNase I were added to the SREBP–DNA, and the reaction then was allowed to proceed for 1 or 4 min. The sample was extracted with chloroform/phenol, resolved on a 12% denaturing gel in 1× TBE, and analyzed as described above.

RESULTS

We recently reported that mutation of a 6-bp sequence (ATTGGC) within a 247-bp or 319-bp fragment of the FPP synthase promoter blocked the 4- to 8-fold induction of a reporter gene, which normally occurred when the transfected cells were incubated in medium deprived of sterols (7, 10). Furthermore, we demonstrated that a 117-bp fragment of the FPP synthase promoter (nucleotides −293 to −177) imparted a 3- to 10-fold sterol-dependent regulation when placed upstream of an otherwise inactive minimal herpes simplex virus TK promoter–CAT gene (7). The finding that the ubiquitous transcription factor NF-Y bound to this 6-bp regulatory element within the 117-bp sequence (10) suggested that other sequences and transcription factors might contribute to sterol regulation. Consequently, we used linker-scanning mutagenesis to alter nucleotides across these 117 bp contained within a larger 319-bp FPP synthase promoter-luciferase reporter plasmid (pFPPS-0.319L). CV-1 cells were transiently transfected with these constructs, together with a plasmid encoding β-galactosidase under the control of the CMV promoter, and incubated for 20 hr in the absence or presence of excess sterols. β-Galactosidase activity was used to correct for differences in transfection efficiencies. The nucleotide sequence of the 117 bp and the mutations that were introduced to produce mutants 1–4 and A–H are illustrated in Fig. 1*A*. The regulation of luciferase activity by sterols was reduced from 8-fold in controls (WT) to <3-fold in mutants 2 and B (Fig. 1*A*). Mutant 2 alters the ATTGGC sequence, which is known to be a binding site for the transcription factor NF-Y and to be required for sterol-regulated transcription of both FPP synthase and HMG-CoA synthase genes (10). Mutants A–C identify a second, broad, 36-bp sequence that is required for sterol-dependent regulation (Fig. 1). Seven constructs containing consecutive 3-bp transversions within this region were prepared and transfected into CV-1 cells to further localize the

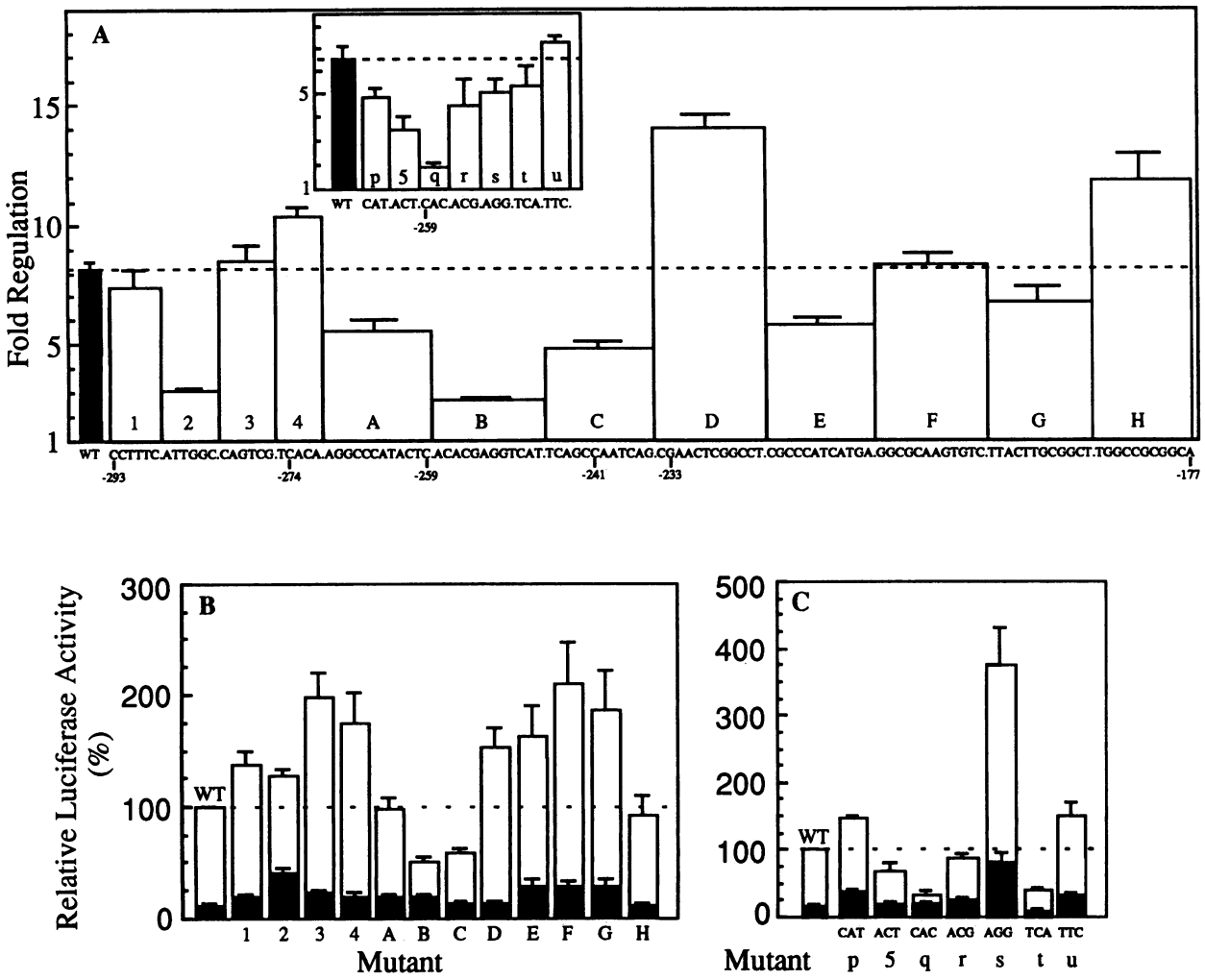

FIG. 1. Expression of FPP synthase promoter mutants in transfected CV-1 cells. Wild-type (WT) pFPPS-0.319L or mutant constructs (1–4, A–H, p, 5, and q–u) were each transfected into duplicate dishes of CV-1 cells with a β-galactosidase expression plasmid, and the cells were incubated for 20 hr in the presence of 10% lipoprotein-deficient fetal calf serum and in the absence (inducing) or presence (repressing) of sterols (10 μg of cholesterol and 1 μg of 25-hydroxycholesterol per ml). The normalized luciferase activity was determined as described in the text. Data are the means ± SEM from 3–10 experiments for each construct. (*A*) Wild-type 117-bp sequence (nucleotides −293 to −177) contained within the 319-bp promoter is shown. Nucleotides below each bar indicate those that were altered in each specific mutant construct (1–4, A–H, p, 5, and q–u) as described in the text. The sterol-dependent fold regulation for each mutant is shown and compared to the 8.3-fold regulation obtained with wild-type constructs (n = 10). (*A Inset*) Results obtained with mutants p, 5, and q–u, each of which contains a 3-bp transversion of the nucleotides shown below each bar. The wild-type sequences shown are aligned exactly above the corresponding sequence of mutants A–C. The wild-type construct (solid bar) was regulated 6.5-fold (n = 10). (*B* and *C*) Mean relative luciferase activity ± SEM is shown for wild type (WT) and the indicated mutants under inducing (open bar) or repressing (closed bar) conditions. The luciferase activity of the wild-type construct under inducing conditions was assigned a value of 100%. (*C*) Wild-type nucleotides that were mutated by transversion to produce mutants p–u are shown. Each mutant contained three non-wild-type nucleotides.

functionally important nucleotides within this 36-bp sequence (Fig. 1*A Inset*; p, 5, and q–u). Five of these constructs (p, 5, and q–s) showed a reduction in the fold regulation of reporter enzyme activity in response to the sterol status of the cells, as compared with the wild-type control (Fig. 1*A Inset*). Mutant q, containing transversions of CAC, was the most defective and was regulated only 1.9-fold as compared with 6.5-fold for the wild-type promoter (Fig. 1*A Inset*).

Fig. 1*B* illustrates the relative activities of wild-type and mutant promoters under both inducing (open bar) and repressing (solid bar) conditions. The wild-type 319-bp promoter was assigned a value of 100% under inducing conditions. The data indicate that the maximal induced or repressed activities are affected by several of the mutations. Mutants B and C are particularly defective in their ability to express high levels of luciferase activity under conditions when cells are deprived of sterols (Fig. 1*B*). Fig. 1*C* summarizes the results with mutants p, 5, and q–u, each of which contains a 3-bp transversion. Mutants q and t, and to a lesser extent mutant 5, identify nucleotides that are necessary for high reporter enzyme activity under inducing conditions (Fig. 1*C*). In the presence of sterols, the enzyme activity level of mutant t was reduced below the values seen with the wild-type construct (Fig. 1*C*). The net result was a relatively normal fold regulation of mutant t (Fig. 1*A Inset*). In contrast, the reporter enzyme activity of mutant s was significantly higher than the wild-type control under both inducing and repressing conditions (Fig. 1*C*). Taken together, the results shown in Fig. 1 are consistent with the binding of a positive transcription factor to a CAC core sequence, defined by mutant q, under inducing conditions.

A 10-bp sequence (ATCACCCCAC) termed SRE-1 has been identified in the promoters of the LDL receptor and HMG-CoA synthase genes and shown to function as a binding site for the transcription factors SREBP-1 and SREBP-2 (11, 13). There is 60% identity between the SRE-1 sequence and the sequence CTCACACGAG in the FPP synthase promoter

surrounding the CAC defined by mutant q. Single point mutations in 9 of the 10 nucleotides in the SRE-1 of the LDL receptor are known to block both sterol regulation and DNA binding of SREBP-1 and SREBP-2 (11, 13). Thus, we were surprised to observe that recombinant SREBP-1 bound to a radiolabeled probe corresponding to nucleotides −264 to −241 of the FPP synthase promoter (Fig. 2*A*, lane 3). As expected, addition of antibody to recombinant SREBP-1 resulted in a super-shifted complex (Fig. 2*A*, lane 4). Fig. 2*B* shows that binding of SREBP-1 was reduced >99% when the radiolabeled probe contained the q mutation (lane 4 vs. lane 2). SREBP-1 binding was reduced 70–94% for probes containing mutations 5, r, or s (Fig. 2*B*). Binding of SREBP-1 to the radiolabeled wild-type probe was specifically stimulated by addition of nuclear extract (unpublished data). In the presence of nuclear extract, SREBP-1 binding to mutant probes 5, r, and s was reduced only 40–80% as compared with a wild-type probe (Fig. 2*C*). In contrast, binding of SREBP-1 to the probe containing mutation q was still reduced >99%, even in the presence of nuclear extract (Fig. 2*C*). As expected, the B1 complex in Fig. 2*C* was supershifted by antibody to recombinant SREBP-1 (data not shown).

The radiolabeled wild-type probe also bound a protein(s) in the nuclear extract to form complex B2 (Fig. 2). The migration of the B2 complex was unaffected by antibody to recombinant SREBP (Fig. 2*A* and data not shown). Fig. 2*C* shows that oligonucleotides containing mutations q, r, or s do not interact with nuclear proteins to form the B2 complex. These results demonstrate that the CAC sequence, defined by mutant q, is critical for SREBP-1 binding *in vitro* and that an additional nuclear protein(s) binds to an overlapping sequence defined by mutants q–s.

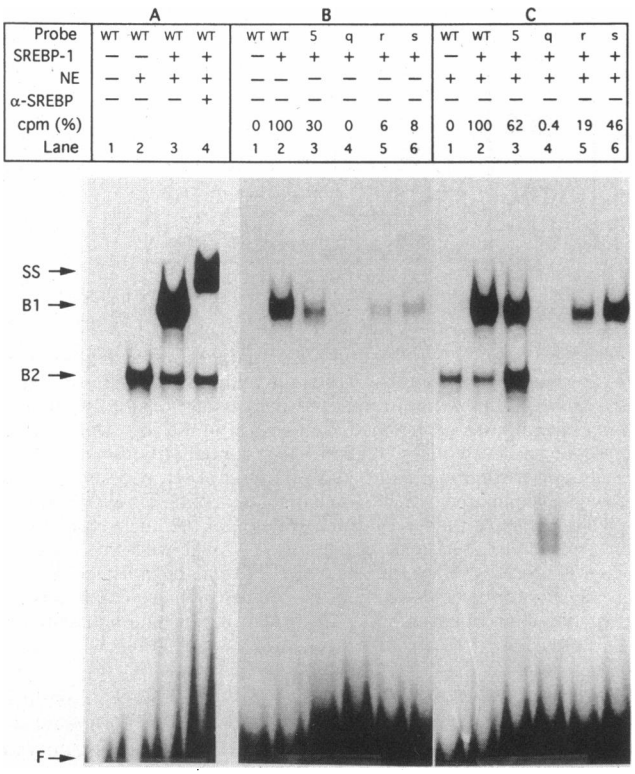

FIG. 2. The FPP synthase promoter contains a binding site for SREBP-1. A ^{32}P-labeled double-stranded oligonucleotide probe (20,000 cpm, 1.5 fmol), corresponding to nucleotides −264 to −241 of the FPP synthase promoter, was incubated with CHO nuclear extract (NE; 100 μg/ml), SREBP-1 (2 ng), and anti-T7-Tag (α-SREBP) as indicated (*A*). Radioactive wild-type or the indicated mutant probes (20,000 cpm, 1.5 fmol) were incubated with SREBP-1 in the presence of either nonfat milk (2.5 mg/ml) (*B*) or nuclear extract (100 μg/ml) (*C*) as indicated in text. After electrophoresis, the gel was dried and exposed to Kodak XAR film for 12 hr at −70°C and then to a Molecular Dynamics PhosphorImager screen. The SREBP–DNA complex is labeled B1. The supershifted complex (SS), free probe (F), and a second complex (B2) are indicated. The radioactivity present in the SREBP–DNA complexes in *B* and *C* was quantitated on a PhosphorImager and is given as a percentage of that measured in lane 2 (cpm %).

DNase I footprinting analysis showed that SREBP-1 bound to the SRE-3 sequence (Fig. 3). The footprint region extended from nucleotide −266 to −248 and included the CAC defined by mutant q (Fig. 3). A hypersensitive site was present at nucleotide −252 (Fig. 3).

The data in Figs. 1–3 demonstrate that the nucleotides necessary for NF-Y and SREBP-1 binding lie between nucleotides −293 and −233 of the FPP synthase promoter. When CV-1 cells were transiently transfected with a plasmid containing these 61 bp linked to a minimal thymidine kinase promoter–CAT reporter gene (pTKCIII-0.061) and the cells then were incubated under inducing or repressing conditions, the CAT activity was regulated 12-fold in a sterol-dependent manner (Fig. 4, Wt61). These results are consistent with the hypothesis that the 61-bp fragment contains all of the cis elements necessary for sterol-regulated transcription. Cotransfection of the plasmid encoding the 68-kDa transcriptionally active SREBP together with pTKCIII-0.061 resulted in an 18-fold increase in CAT activity in cells incubated in the presence of sterols, a 2.5-fold increase in cells deprived of sterols, and in a reduction in sterol-dependent regulation from 12- to <1.8-fold (Fig. 4, Wt61). The demonstration that CAT activity was stimulated by coexpression of the 68-kDa fragment of SREBP-1 is consistent with a physiological role for this protein in the regulation of the FPP synthase gene.

Fig. 4 also shows that mutation of CAC (nucleotides −259 to −257) within the 61-bp promoter resulted in low levels of CAT activity that were not enhanced when the cells were incubated in inducing medium (Mut61q). We conclude that this CAC sequence is critical for the high rate of transcription

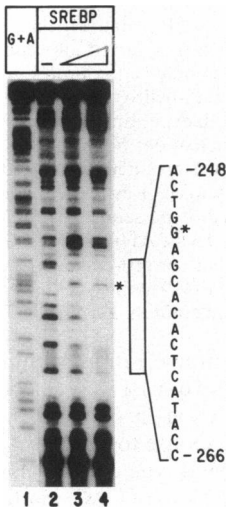

FIG. 3. DNase I footprint of the FPP synthase promoter by SREBP. A 117-bp DNA fragment of the FPP synthase promoter (nucleotides −293 to −177) was ^{32}P-end-labeled on the top strand. The gel-purified fragment was incubated for 60 min in the absence (lane 2) or presence of 1 μg (lane 3) or 3 μg (lane 4) of recombinant SREBP-1. After digestion with 0.15 unit of DNase I at room temperature for 1 min (lane 2) or 4 min (lanes 3 and 4), the reaction was subjected to electrophoresis on a 12% denaturing gel as described in the text. The end-labeled DNA was also subjected to Maxam and Gilbert sequencing reactions (lane 1; G+A) (18). The footprint (boxed area), the sequence of the protected area, and a hypersensitive site (*) are indicated. The numbers refer to the nucleotides relative to the ATG.

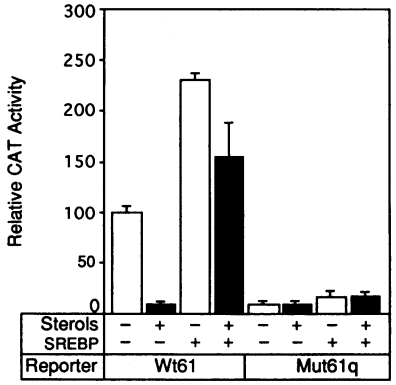

FIG. 4. Overexpression of SREBP-1 increases the activity of wild-type, but not mutant, FPP synthase–reporter genes. CV-1 cells were transfected with plasmids expressing β-galactosidase and either pTKCIII-0.061 (Wt61) or pTKCIII-0.061q (Mut61q) in the absence or presence of 7.5 ng of pCMV-CSA10 (SREBP) and incubated for 20 hr in medium containing 10% lipoprotein-deficient fetal calf serum in the absence (open bars) or presence (closed bars) of sterols (10 μg of cholesterol and 1 μg of 25-hydroxycholesterol per ml). CAT activities were determined and normalized for transfection efficiency as described (7). The CAT activity obtained from cells incubated in the absence of both sterols and SREBP was given a value of 100%. The results, obtained from two experiments, each performed in duplicate, are means ± SEM.

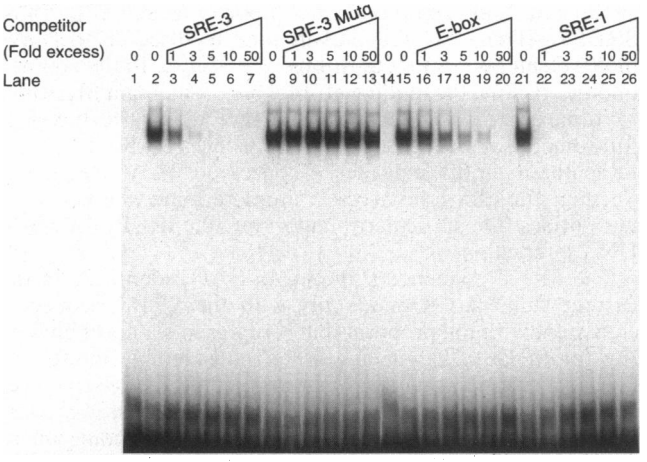

FIG. 5. SREBP binds to SRE-3, SRE-1, and the E-box. A 24-bp oligonucleotide containing the SRE-3 sequence from FPP synthase was end-labeled with ^{32}P. Equal aliquots (20,000 cpm, 0.3 pmol) were incubated with 5 ng of recombinant SREBP in the absence (lanes 2, 8, 15, and 21) or presence of the indicated molar excess of an unlabeled 24-bp competitor probe. Analysis and quantitation on a PhosphorImager were as described in the legend to Fig. 2. The radioactivity in control lanes (2, 8, 15, and 21) was given a value of 100. The relative radioactivity in lanes containing competitor DNA was; 48, 13, 8, 3.9, and 1.8 (lanes 3–7); 91, 107, 95, 98, and 105 (lanes 9–13); 69, 41, 24, 14, and 4 (lanes 16–20); and 6, 0.8, not detectable (ND), ND, and ND (lanes 22–26).

of the FPP synthase gene in cells deprived of sterols. Coexpression of SREBP together with the mutant 61-bp promoter–reporter gene stimulated CAT expression <2-fold; maximal activities were <12% of the wild-type promoter–reporter gene cotransfected with SREBP and incubated under identical conditions (Fig. 4, Mut61q). Thus, the SREBP-dependent stimulation observed with the wild-type 61-bp promoter was significantly impaired in the mutant promoter–reporter gene. This result further indicates that the high level of transcription of the FPP synthase gene that occurs in cells incubated in sterol-depleted media is dependent upon the binding of SREBP to nucleotides that include the CAC identified by mutant q.

The results in this and previous reports indicate that SREBP can bind to SRE-3, SRE-1, or an E-box (11, 13, 16, 17). To determine the relative affinities of SREBP for these different sequences, gel mobility-shift competition experiments were performed; a ^{32}P-end-labeled 24-bp oligonucleotide containing the FPP synthase SRE-3 element was incubated with recombinant SREBP in the absence (Fig. 5, lanes 2, 8, 15, and 21) or presence of increasing amounts of unlabeled 24-bp probes containing SRE-3 (Fig. 5, lanes 3–7), SRE-3 containing the q mutation (CAC to ACA) (Fig. 5, lanes 9–13), the E-box from the S14 gene (Fig. 5, lanes 16–20), or SRE-1 from the LDL receptor gene (Fig. 5, lanes 22–26). Quantitation of the radioactivity remaining in the SREBP–DNA shifted band after competition with the different oligonucleotides indicates that SREBP binds with the following affinity: SRE-1 > SRE-3 > E-box (Fig. 5).

DISCUSSION

Previous studies demonstrated that transcription of the FPP synthase gene was regulated by the sterol status of the cell (6, 7) and that these changes paralleled those for HMG-CoA synthase, HMG-CoA reductase, and the LDL receptor genes (5–8). Sterol-dependent regulation of the LDL receptor and HMG-CoA synthase genes has been shown to occur via an SREBP- and SRE-1-dependent process (9, 11–13). SREBP-1 and SREBP-2 interact with the SRE-1 sequence in a very specific manner; single point mutations within this 10-bp element in the LDL receptor–promoter disrupt both SREBP binding and sterol-regulated transcription (11, 13). Thus, it was surprising that a 117-bp (7) or a 61-bp sequence (Fig. 4) of the FPP synthase promoter contained nucleotides necessary and sufficient for sterol-regulated transcription yet did not contain sequences with high identity to SRE-1. The reason for this apparent enigma is provided in the current report.

We demonstrate that the FPP synthase promoter contains a novel binding site for SREBP. We have termed this element SRE-3 to differentiate it from the SRE-1 of the LDL receptor. Mutagenesis studies identified the trinucleotide CAC (−259 to −257) as being critical both for sterol-regulated transcription *in vivo* and for SREBP-1 binding *in vitro* (Figs. 1, 2, 4, and 5). The findings that mutagenesis of the nucleotides 5′ and 3′ of the CAC only partially interfere with these functions provide further support for the critical role of this core sequence. The identity between nucleotides −261 to −252 of the FPP synthase gene and the SRE-1 is 60%. Further studies will be necessary to define the specific nucleotides of the FPP synthase promoter which are involved in interaction with SREBP.

The data in Fig. 2*C* demonstrate that SREBP binding to SRE-3 is stimulated in the presence of crude nuclear extract. In unpublished work we have identified two separate proteins, one being NF-Y, that enhance this binding. We hypothesize that functional interaction of SREBP-1 with the SRE-3 in the FPP synthase promoter *in vivo* may be dependent on the presence of these other nuclear proteins. This hypothesis is consistent with the recent report that SREBP binds to the SRE-1 of the LDL receptor and specifically recruits Sp1 to an adjacent site and that binding of both transcription factors is necessary for high levels of transcription (20). Thus, the function of SREBP in different genes may be modulated by interaction with different transcription factors.

ADD-1, the rat homologue of SREBP, has been cloned and shown to bind to a motif (ATCACGTGA) that contains an E-box (CANNTG) (17). This motif has 60% and 50% identity with SRE-1 and SRE-3 sequences, respectively. Cotransfection of plasmids encoding ADD-1 and a reporter gene driven by a promoter that contained multiple copies of the E-box motif found in the S14 gene (CTCACGTGGT) resulted in

stimulated transcription of the reporter gene (17). Thus, SREBP/ADD-1 can functionally bind to three distinct promoter elements, SRE-1, an E-box, and SRE-3. In the absence of other factors, the binding affinity of recombinant SREBP-1 for these different sequences is: SRE-1 > SRE-3 > E-box (Fig. 5). Comparison of the nucleotide sequences contained within these three motifs indicates a conserved TCAC sequence. Further studies will be necessary to determine whether these nucleotides are particularly important for SREBP/ADD-1–DNA interaction.

The SRE-3 sequence (CTCACACGAG) identified in the current study has 60% identity with the SRE-1 sequence, consistent with the proposal that it represents a novel binding site for SREBP. It will now be of considerable interest to identify new genes that contain functional SRE-like sequences within their promoters and are transcriptionally regulated by SREBP. Potential candidates include genes encoding members of the cytochrome P450 family (21, 22). These latter genes are regulated by either sterols and/or mevinolin and contain within their promoters sequences similar to an SRE-1. In addition, the promoters of at least 13 yeast genes that encode a variety of lipid biosynthetic enzymes, including FPP synthase and fatty acid synthetase, contain a conserved sequence (UAS_{INO}) (23, 24) with a 60% identity to the mammalian SRE-3 and E-box motifs. It is not known whether all 13 yeast genes are coordinately regulated by a common transcription factor. However, based on the above observations, it is likely that SREBP/ADD-1 has a broad role as a transcription factor and is involved in the regulation of mammalian genes involved in various lipid biosynthetic pathways.

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