Sterol regulation of acetyl coenzyme A carboxylase promoter requires two interdependent binding sites for sterol regulatory element binding proteins

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Abstract The sterol regulatory element binding proteins (SREBPs) are central regulators of lipid homeostasis in mammalian cells. Their activity is controlled by a sterol-regulated two-step proteolytic process that releases the nuclear targeted amino-terminal domain from the membrane anchored carboxyl-terminal remnant. This ensures that transcriptional stimulation of the appropriate genes occurs only when increased intracellular sterol accumulation is required. Gene targets for SREBP encode key proteins of cholesterol metabolism as well as essential proteins of fatty acid biosynthesis, providing a mechanism for coordinate control of these two major lipid pathways when sterols and fatty acids need to accumulate together. However, the regulatory mechanism must provide a way to uncouple these two pathways to allow separate regulation when sterol or fat levels need to increase independently of each other. We compared the similarities and differences for how SREBP activates the promoter for the low density lipoprotein (LDL) receptor, which is the key protein involved in cholesterol uptake, relative to how it activates promoters for acetyl coenzyme A carboxylase (ACC) and fatty acid synthase (FAS), which are both key enzymes of fatty acid biosynthesis. In the current studies we show there are two distinct sites for SREBP binding that control activation of the ACC PII promoter whereas previous work has shown there is only a single SREBP site in the LDL receptor. Additionally, disruption of either ACC site results in a total loss in promoter function and a severe decrease in SREBP binding even to the neighboring unaltered site. Thus, the two sites are equally important and dependent on one another for optimal function. This is in contrast to the FAS promoter where SREBP binds to two adjacent sites independently and the one located closer to the binding site for the Sp1 co-regulator is more critical for sterol regulation and activation by SREBP over-expression.—**Magaña, M. M., S. S. Lin, K. A. Dooley, and T. F. Osborne.** Sterol regulation of acetyl coenzyme A carboxylase promoter requires two interdependent binding sites for sterol regulatory element binding proteins. *J. Lipid Res.* 1997. **38:** 1630–1638.

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The sterol regulatory element binding proteins (SREBPs) are basic-helix-loop-helix leucine zipper(bHLHZip) containing transcription factors that control the expression of genes involved in lipid homeostasis (1). They were first identified as proteins that activated expression from the LDL receptor promoter through binding to its sterol regulatory element (SRE) only when sterol levels were low (2–5). Subsequently, SREBPs have been shown to activate expression of several genes of cholesterol and fatty acid metabolism through *cis*-acting sites that resemble the direct repeat SRE-1 element of the LDL receptor promoter (6–10).

SREBPs are very weak activators in isolation (2, 11) and they function efficiently only with a co-regulatory protein that binds to a neighboring site (12, 13). Thus, the single SREBP binding site in the LDL receptor promoter is not sufficient for activation as both SREBP and the generic co-regulator Sp1 are required for normal promoter function (11) and mutations that disrupt either DNA binding site result in the same constitutively suppressed phenotype (12, 13).

Although Sp1 is the co-regulatory protein for the LDL receptor promoter, other SREBP requiring promoters use different generic factors as co-regulators. For example, the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase and farnesyl diphosphate synthase (FPDS) promoters require the heterotrimeric nuclear factor-Y as a co-regulator (7, 14).

Acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS) are the two main enzymes of saturated long chain fatty acid biosynthesis and the promoters for their respective genes are regulated by the addition of exoge-

Abbreviations: SREBP, sterol regulatory element binding protein; ACC, acetyl coenzyme A carboxylase; LDL, low density lipoprotein; SRE, sterol regulatory element; LPDS, lipoprotein-depleted serum; CMV, cytomegalovirus; FAS, fatty acid synthase; bHLHZip, basic-helix-loop-helix-zipper.

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nous sterols to cultured cells and by over-expression of SREBPs in both cell culture and transgenic animals (6, 8, 15). Additionally, over-expression of transcriptionally active SREBPs results in the over-accumulation of both cholesterol and fatty acids in CHO cells and livers of transgenic mice as well (15, 16). Similar to the LDL receptor, the promoters for FAS and the housekeeping PII promoter for ACC each contain binding sites for both SREBP and Sp1 that are required for efficient expression (6, 8).

As SREBPs and Sp1 activate expression of important proteins of both cholesterol and fatty acid biosynthesis, coordinate regulation of these major lipid pathways by the concerted action of SREBP and Sp1 provides a mechanism whereby cholesterol and fatty acid levels can rise together for cellular membrane biogenesis and perhaps for the assembly of VLDL particles in the liver as well.

However, there must be differences in the activation mechanism to allow cholesterol and fatty acid metabolism to be uncoupled when coordinate accumulation is not required. Independent regulation of SREBP requiring promoters that utilize different co-regulatory proteins can be achieved simply through direct regulation of the co-regulator or by targeting a unique step involved in SREBP interaction with each specific co-regulator. Independent regulation of promoters that require SREBP and utilize the same co-regulatory factor presents a more fundamental problem. Thus, it is crucial to characterize the similarities and differences in how the LDL receptor is activated by the concerted action of SREBP and Sp1 relative to how the ACC and FAS promoters are activated by the same two regulators.

In our initial attempts to explore this mechanistically, we have demonstrated there are two tandem binding sites for SREBP that are required for SREBP-Sp1 activation of the FAS promoter (10). Additionally, we suggested that there were two critical SREBP sites in the ACC PII promoter as well, but the potential individual sites were not analyzed separately so the functional contribution of each was not determined (8). The identification of multiple SREBP binding sites required in both ACC and FAS is significant as there is only a single SREBP site in the LDL receptor sterol regulatory region (3).

In the current studies we have introduced targeted mutations into each potential separate SREBP site of the ACC PII promoter and have analyzed the consequences on sterol regulation and activation by exogenously supplied SREBPs in cultured cells as well as the effects on the binding of purified SREBPs in vitro. The data show there are indeed two crucial adjacent SREBP sites and both must be intact for a high level of activation by SREBP and for efficient regulation by the addition of exogenous sterols. As both ACC sites are absolutely crucial for promoter activity and appear to be functionally identical, there are distinct differences with the FAS promoter where there are also two adjacent SREBP sites. Both FAS sites were required for maximal activity, but mutations that disrupt one of the sites were only partially defective for sterol regulation and activation by SREBP over-expression. Additionally, mutations in each FAS site did not affect binding to the adjacent-unaltered site whereas SREBP binding to the individual site mutants of the ACC promoter showed reduced protection of the neighboring unaltered site. This indicates that binding to the two sites is not independent.

METHODS

Cells and media

CV-1 cells were obtained from Dr. K. Cho (University of California, Irvine). HepG2 cells were obtained from the ATCC. All cell culture materials were purchased from Life Technologies Inc. Lipoprotein-deficient serum was prepared by ultracentrifugation from newborn bovine serum as described (17). Cholesterol and 25-OH cholesterol were obtained from Steraloids Inc., and stock solutions were dissolved in absolute ethanol.

Plasmids

The rat ACC PII promoter fragment from −400 to −238 (here called wild type or WT) and the ACC fragment from −400 to the Sac I site at −286 (here called negative or NEG), were inserted upstream of a generic TATA box which was linked to the luciferase coding sequence. These plasmids were described previously (8). Mutant derivatives were made by inserting synthetic DNAs containing the ACC sequence from −286 to −238, with the targeted tri base substitutions as indicated in the text and figure legends, into the Sac I site of the ACC NEG plasmid by standard cloning procedures. This reconstituted the normal ACC promoter from −400 to −238 with the desired substitution mutation as shown at the bottom of Fig. 1. The DNA sequence of each mutant clone was confirmed by direct DNA sequencing of the region before the transfection studies were performed.

Cell culture and transient transfection assay

CV-1 cells were grown in DMEM containing 10% fetal bovine serum and were plated at 125,000 cells/60-mm dish on day 0. On day 1 cells were transfected by the calcium phosphate co-precipitation method and refed the same media essentially as described before (11).

Precipitates contained 20 μg each of the test plasmid and the non-sterol regulated CMV2 β-galactosidase plasmid, which contains the cytomegalovirus (CMV) promoter fused to the *E. coli* β-galactosidase gene as an internal control for transfection efficiency, in addition to 10 μg of salmon sperm DNA in a final volume of 2 ml. An equal volume (0.5 ml) of precipitate was added to each of four dishes which were incubated at 37°C and 7% CO_2. Twelve to 16 h later the cells were washed three times with phosphate-buffered saline and refed either induced (DMEM and 10% lipid-deficient serum) or suppressed media (same as induced media but also containing 12 μg/ml cholesterol and 1 μg/ml 25-OH cholesterol) and the dishes were returned to the incubator. Cells were harvested on day 3 by scraping and duplicate dishes were pooled prior to preparation of soluble protein extracts by three freeze–thaw cycles. Luciferase and β-galactosidase activities were measured on aliquots of the extract as described under Enzyme assays below.

HepG2 cells were cultured in MEM containing 10% fetal bovine serum and seeded for experiments at 175,000 cells/60-mm dish on day 0. The cells were transfected the following day by the calcium phosphate co-precipitation method as above. Precipitates contained 10 μg of each test plasmid and the control plasmid CMV2 β-gal and 5 μg of salmon sperm DNA. In addition, half of the precipitates received 10 or 30 ng of a CMV promoter expression clone encoding amino acids 1–490 of SREBP-1a (11) or a similar expression clone encoding amino acids 1–481 of SREBP-2 as described previously (10). Five hours after transfection, 2 ml of 10% glycerol in phosphate-buffered saline was added and allowed to incubate with the cells for 2 min. The dishes were washed three times with phosphate-buffered saline and refed MEM containing 10% fetal bovine serum and cultured for 24 h before they were harvested and assayed as described for CV-1 cells above.

Enzyme assays

The luciferase activities were measured in an Analytical Luminescence monolight model 2010 luminometer with a luciferin reagent from Promega Biotec. β-Galactosidase assays were performed by a standard colorimetric procedure with 2-nitrophenyl-β-D-galactopyrannoside as substrate (18). The normalized luciferase values were determined by dividing the luciferase activity in relative light units (RLU) by the β-galactosidase activity (activity/h). The data presented here are from several experiments performed in duplicate for each plasmid (see figure legends for exact number of individual experiments).

Protein purification and DNase I footprinting

Recombinant SREBP-1a and SREBP-2 proteins were purified by nickel chelation chromatography from a 6XHis-containing fusion protein as described (11). The concentration and purity of these proteins was determined by SDS-PAGE analysis performed with marker proteins followed by staining with Coomassie blue. ^{32}P end-labeled DNA probes were prepared from the indicated plasmids and incubated with SREBP-1a or SREBP-2 and digested with DNase I as described (10, 19, 20).

RESULTS

We previously identified a region between −238 and −283 upstream from the mRNA start site for the rat ACC PII promoter as containing an element required for sterol regulation of transcription (8). This interval contains an Sp1 binding site as well as a region that binds SREBP. Using DNase I footprinting and gel mobility shift assays, we suggested that there were two closely spaced SREBP binding sites in this region. With a synthetic double-stranded probe we showed there were two protein–DNA complexes that appeared as the amount of SREBP was increased in the binding reaction of a gel mobility shift assay. Over the same concentration range of SREBP we only observed one complex with a DNA probe containing the single LDL receptor SREBP site. Additionally, the size of the DNase I protected region was larger than expected for a single site. Thus, we assumed there were two distinct sites for SREBP binding in this region. However, the DNA sequence of the interval does not closely resemble that of the known direct repeat SREBP binding site from the LDL receptor nor the palindromic E-box that is the classic recognition site for bHLH proteins such as SREBP (8).

Based on our recent studies of the DNA recognition properties for SREBP-1a in the HMG CoA reductase promoter (9) we noted there were two potential SREBP "half-sites" each corresponding to one-half of the LDL receptor direct repeat. These are underlined at the bottom of **Fig. 1.** To determine whether these two half-sites were core recognition elements for SREBP and to define the sterol regulatory element within this region of the ACC promoter more precisely, we introduced two tri-base substitution mutations that disrupt either half-site separately and we combined the mutations together to generate the double mutant. The exact tri-base substitutions are referred to as A and B and are shown in bold at the bottom of Fig. 1.

The effects of the mutations on sterol regulation were evaluated by the experiments described in Fig. 1. The wild type plasmid contained the native sequence from −400 through −238 fused to a TATA element upstream of the luciferase reporter gene. This plasmid contains

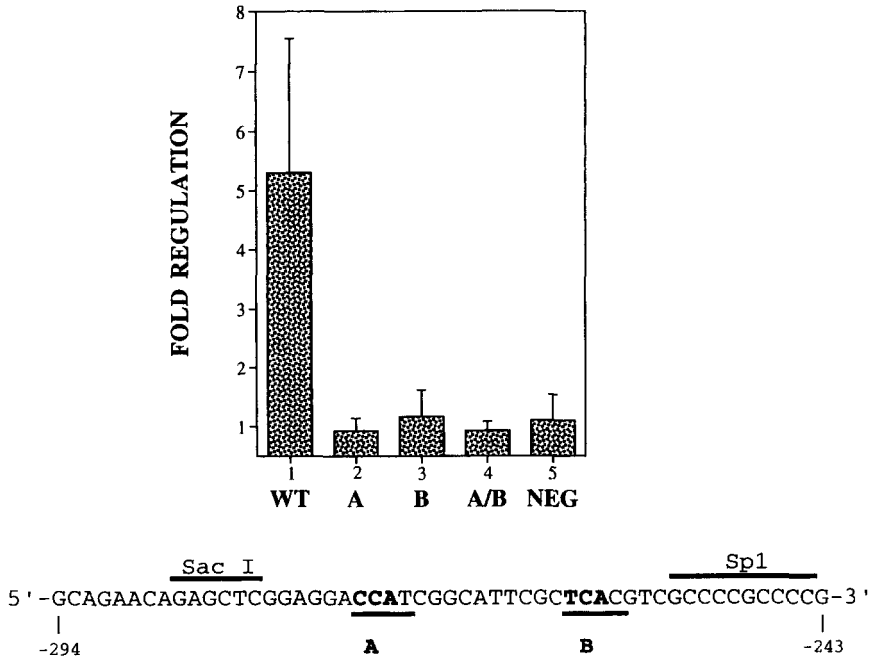

Fig. 1. Sterol regulation of the ACC promoter in CV-1 cells. The DNA sequence corresponding to the top strand of the rat ACC PII promoter from −294 to −243 is shown. The position of each tri-base mutation is shown by the boldface letters within the sequence. The letters beneath the bold regions designate the site or sites mutated in each of the various mutant derivatives. The wild-type (WT) plasmid corresponds to the normal ACC promoter from −400 to −238 (note only a portion of this sequence is shown here) fused to the generic TATA element. The negative (NEG) corresponds to a mutant plasmid in which the region 3′ of the Sac I site was deleted. These plasmids were described previously (8). Plasmids were analyzed for sterol regulation by a transient DNA transfection assay as described in Methods. Fold regulation by sterols refers to the normalized luciferase values for cells cultured in the absence of regulatory sterols divided by the normalized luciferase values obtained from cells cultured in the presence of regulatory sterols. The mean fold regulation and standard error values for three individual experiments performed in duplicate are shown. With the induced level (cells cultured in LPDS) for the wild-type construct set at 100% the values for the suppressed levels (cells cultured in LPDS plus sterols) for the wild-type, mutant A, mutant B, the double mutant A/B, and NEG were 19%, 47%, 23%, 35% and 2.1%, respectively. The two underlined regions in the DNA sequence correspond to each of two core SREBP recognition sites as described in the text. The Sac I site at −286 used to construct the negative plasmid is labeled and overlined. The overlined Sp1 consensus site is also shown; this Sp1 site along with SREBP is critical for maximal sterol regulation of the ACC promoter. In the "A" site mutant the CCA sequence was changed to AAC and in the "B" site mutant the TCA sequence was changed to GAC. The two were combined to generate the A/B double site mutant.

all of the *cis*-acting information required for efficient sterol regulation and activation by SREBP-1 including the SREBP binding region discussed above (8). The wild type and mutant promoter constructs were analyzed by a transient DNA transfection assay for sterol regulation by our standard assay procedure in cultured CV-1 cells (11). As shown in Fig. 1, the normal promoter (WT) was expressed and efficiently suppressed when sterols were added to the culture dish (lane 1). A mutation that removes the sequence from −283 to −238 was severely compromised for sterol regulation (NEG). This mutation deletes the Sp1 site and the two SREBP core half-sites and was included as a negative control. These current results are consistent with our previous observations (8). The CCA and TCA tri-base elements shown in bold were changed to AAC and GAC, respectively. Both single mutants (A and B) and the double mutant (A/B) were totally defective for sterol regulation. Thus, each of these two elements is critical for sterol regulation of transcription.

The experiments of Fig. 1 demonstrate there are two closely spaced *cis*-acting sites that are both required for sterol regulation. Next, we compared the mutant promoters to wild type in a mammalian transfection assay designed to directly evaluate activation by exogenously supplied SREBP proteins (**Fig. 2**). A plasmid that expresses the mature form of the SREBP-1a protein (amino acids 1–490) from the strong cytomegalovirus promoter (CMV) was transfected at two different levels along with either the wild-type or mutant ACC promoter-luciferase reporter constructs into HepG2 cells and extracts were prepared and assayed for activation of luciferase enzyme expression (Fig. 2A). Expression from the wild type ACC promoter was dramatically stim-

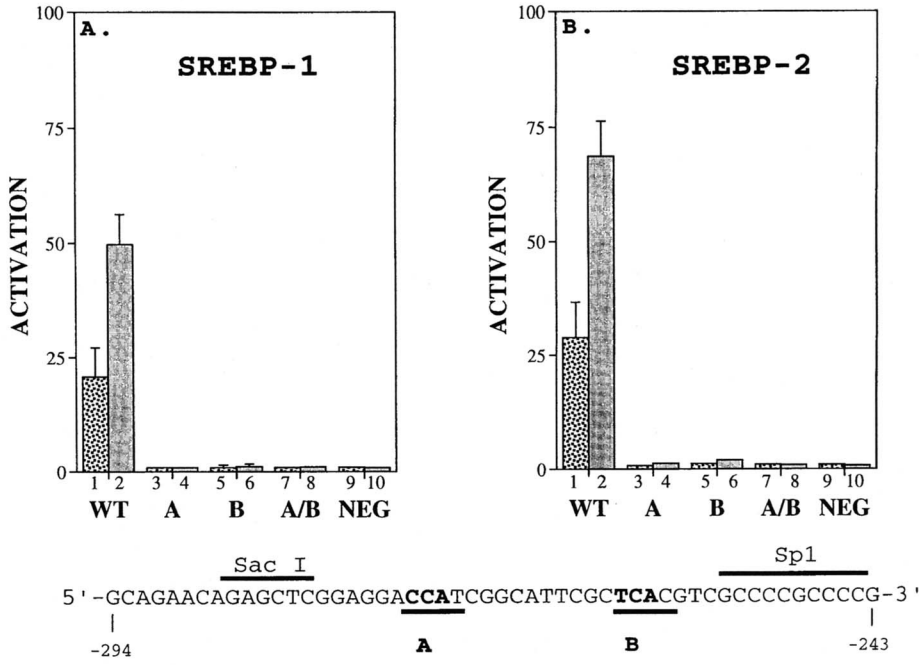

Fig. 2. Activation of the ACC promoter by overexpression of SREBP-1a or SREBP-2 in HepG2 cells. HepG2 cells were transfected with the wild type or mutant ACC constructs as described in Methods. In addition to the ACC promoter plasmid duplicate dishes received either 10 or 30 ng of a CMV promoter-based construct expressing amino acids 1–490 of the human SREBP-1a protein. Fold-activation was determined by the ratio of normalized luciferase activity in dishes with co-transfection of SREBP-1a divided by the normalized luciferase activity in the dishes without the SREBP-1a protein expression vector. For Fig. 2B a similar expression construct encoding amino acids 1–481 for the human SREBP-2 protein was co-transfected. The checkered bars on each graph correspond to the mean fold-activation value when 10 ng of SREBP-1a (A) or SREBP-2 (B) was co-transfected. The shaded bar represents the mean fold-activation when 30 ng of SREBP-1a (A) or SREBP-2 (B) was included in the transfection. The mean-fold activation for two independent experiments performed in duplicate is shown for each figure, along with the standard error. All other symbols and notations are the same as described in Fig. 1.

ulated (WT, lanes 1 and 2) and, similar to our earlier observations (8), the same negative control mutant used in Fig. 1, which removes the sequence from −283 to −238, was unresponsive (NEG, lanes 9 and 10). Consistent with the sterol regulation studies of Fig. 1, both single mutants (lanes 3–6) and the double mutant (lanes 7–8) were totally unresponsive to ectopic expression of SREBP-1a.

When a plasmid that encodes an equivalent version of the mature form of SREBP-2 protein (amino acids 1–481) was substituted for SREBP-1a, very similar results were obtained (compare lanes from Fig. 2A with the corresponding lanes from Fig. 2B). Thus, both SREBP core half-sites are required for sterol regulation and activation by ectopically expressed SREBP proteins. This is consistent with both sites being direct targets for SREBP action.

To directly determine how the above mutations affect SREBP binding to this region of the ACC promoter, we performed DNase I footprinting experiments with recombinant SREBP-1a or -2 proteins and end-labeled DNA probes prepared from the wild-type and the A and B mutant promoters from Fig. 1. The results for a representative experiment using SREBP-1a are shown in **Fig. 3A** and for SREBP-2 in Fig. 3B. The binding of SREBP-1a to the wild type promoter (Fig. 3A lanes 1–6) is identical to what we reported in our earlier study (8). There is a broad region bounded by the two sets of brackets at the left of Fig. 3A. When binding of SREBP-1a to mutants A and B was evaluated, dramatically reduced protection was observed (lanes 7–12 and 13–18, respectively). The A mutant resulted in a loss of binding over the area where the mutation was introduced (labeled site I) but it retained weak binding over the remaining region (labeled site II). The B mutant abolished binding in the region corresponding to its mutation (site II) and also retained weak binding over the other half of the sequence protected on the wild type probe (site I).

The results for SREBP-2 binding were almost identical to those observed for SREBP-1a (compare identical lanes in Figs. 3A and B). Thus, there appears to be two binding sites for both SREBP-1a and -2 within this re-

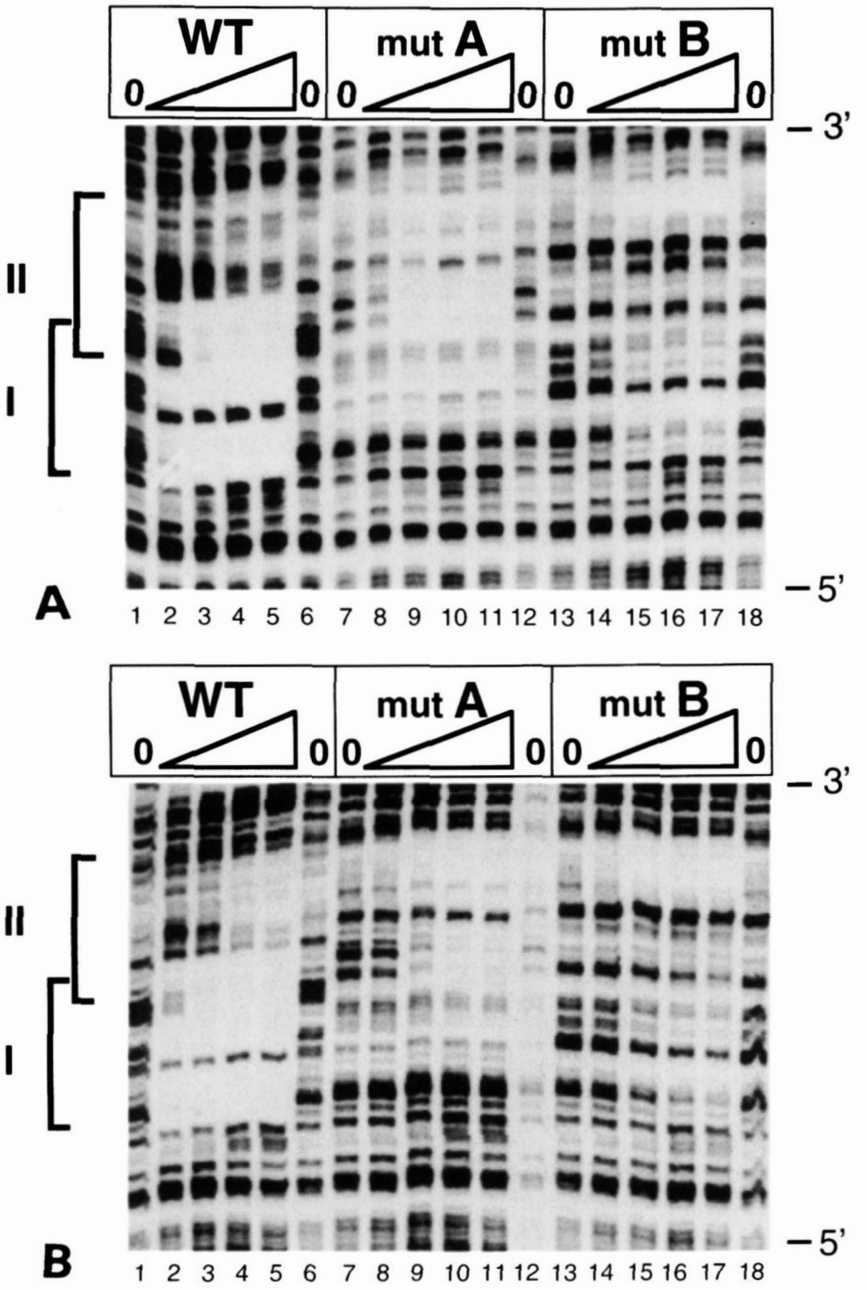

Fig. 3. Identification of two SREBP binding sites in the ACC promoter: DNase I analysis with SREBP-1 and SREBP-2. The recombinant human SREBP-1a (amino acids 1–490) and SREBP-2 (amino acids 1–481) proteins were purified as described (11). The top strand of DNA for the ACC promoter (or mutants) was labeled at −400 and the double-stranded probe was used for the DNase I footprint studies. The probes were incubated alone, indicated by a zero or with increasing amounts of SREBP-1a in A (60, 180, 480, and 780 ng of SREBP-1a) (lanes 2–5, 8–11 and 14–17) or SREBP-2 in B (36, 120, 300, and 480 ng of SREBP-2) (lanes 2–5, 8–11 and 14–17) prior to DNase I digestion. The exact conditions for the footprint studies are further described in (8, 11). The bottom of the gel corresponds to the 5′ end and the top of the gel corresponds to the 3′ end of the probe, as shown at the right of the figure. The overlapping brackets on the left indicate the two SREBP binding sites (I and II) seen in the wild type ACC promoter and are discussed in the text.

gion and the simplest interpretation is that each mutation disrupts one recognition site. In addition, when one site was mutated, the binding to the neighboring unaltered site appeared to be weaker. This suggests that SREBPs may bind to these two sites in a cooperative manner and further studies are required to investigate this more thoroughly.

DISCUSSION

The current experiments demonstrate that the region of the rat acetyl coenzyme A carboxylase PII promoter required for sterol regulation contains two separate binding sites for sterol regulatory element binding proteins -1 and -2. We predicted this from the results of our earlier report because we observed two gel shift complexes with a DNA binding site from this region and the area of protection from DNase I digestion was larger than expected for a single binding site (8).

An alignment of the ACC sites relative to SREBP sites from known sterol regulatory elements of other promoters reveals a limited degree of sequence similarity between the ACC elements and other direct repeat SREs (**Fig. 4**). Thus, it was impossible to identify these sites by visually scanning the DNA sequence. Each site contains a half-site homology relative to the direct repeat element of the LDL receptor. The core of the half site sequence is 5′-PyCAPy-3′ (9, 10). Because not every 5′-PyCAPy-3′ element binds SREBP, this core probably forms the central recognition component and flanking bases contribute significantly to specificity and allow multiple separate elements to be recognized efficiently (9, 10).

Additionally, the ACC SREBP sites identified here are weak recognition elements that are entirely nonfunctional when they are present alone. In all three assay systems used in the current studies which include: in vitro binding by purified SREBPs (Fig. 3), activation by ectopically expressed SREBPs (Fig. 2), and transient regulation sterols (Fig. 1), the two sites only functioned efficiently when present together. This suggests SREBP binding at the two sites is not independent and may be cooperative. Our initial experiments suggest there is a small degree of cooperativity but the two sites are so close together that we have been unable to measure the quantitative contribution of cooperativity to the overall free energy of DNA binding.

In a recent report we demonstrated that there are also two closely spaced SREBP binding sites required for activation and sterol regulation of the promoter for fatty acid synthase (10). However, upon inspection of the DNase I footprinting gels, SREBP binding to each

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     ---> --->
5'-ATCACACCAC-3'   Ham/Rat/Frog LDLR
   ATCACCCCAC      HumLDLR/RatFAS -150
   ATCACCCCAT      Mouse LDLR
   CTCACCCCAC      HamSyn 1
   GCCACCCTAC      HamSyn 2
   ACCGCACCAT      HamRed -150
   CTCTCACCAC      HamRed -165
   CTCACACGAG      RatFPPS
   GTCAGCCCAT      RatFAS -72
   GCCACGCCAC      RatFAS -62

   GGAGGACCAT      RatACC I
   CTCACGTCGC      RatACC II
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Fig. 4. Alignment of SREBP sites from known sterol responsive regions to the ACC sites. The sequence of the LDL receptor (LDLR) SREBP sites from hamster (Ham) (22), rat (1), Xenopus (Frog) (23), Human (Hum) (24) and mouse (1), are shown as well as the two sites from the hamster HMG-CoA synthase promoter (HamSyn 1 and HamSyn 2) (25), two sites and their locations from the hamster HMG-CoA reductase (HamRed -150 and HamRed -165) (9), the farnesyl diphosphate synthase element identified by Ericsson et al. (7), and the two adjacent rat FAS sites (10) are shown. The direction for each is 5′ to 3′. The sequence for the RatFAS −150 element is identical to the human LDL receptor. The sequence surrounding the two ACC core sites (I and II) identified here are also shown. These are aligned for maximal similarity to the LDL receptor site. The tri-nucleotide elements that were mutated in the present studies are shown in bold. The arrows at the top are positioned over each copy of the direct repeat element 5′-PyCAPy-3′, and the underlined residue corresponds to a base that separates the two direct repeats and is naturally an A or a C, and through mutagenesis it was shown that a G can be present without a loss of sterol regulation (2).

site in the FAS promoter appeared to be independent. Additionally, the results for sterol regulation and activation by SREBP supported this observation. Mutations that disrupted the FAS site labeled −62 in Fig. 4 resulted in a decrease, but not a total loss, in sterol regulation and activation by SREBP co-transfection. In fact, these FAS −62 site mutants were activated by SREBP-2 co-transfection nearly as efficiently as the wild type promoter. In contrast, mutations in the −72 FAS site were totally defective (10).

These results are clearly different form those reported here. Disruption of either SREBP site of the ACC promoter resulted in a total loss of sterol regulation and completely eliminated activation by cotransfection of either SREBP-1a or SREBP-2 expression vectors (Figs. 1 and 2).

The arrangement of the SREBP sites in the ACC promoter and the results for sterol regulation of the single site mutants are reminiscent of the HMG-CoA reductase promoter. Within the region of the HMG-CoA reductase promoter that is required for sterol regulation there are two closely spaced SREBP half sites (shown in Fig. 4) approximately the same distance apart as the

ACC sites are and both reductase sites must be intact for normal sterol regulation (9, 21). However, the difference is that Sp1 is not a co-regulator for HMG-CoA reductase.

The studies reported here, along with the results from other recent reports, suggest that the mechanism for gene activation by the SREBPs depends on the arrangement of the SREBP binding sites relative to each other (when there are more than one) and relative to the binding site for the neighboring obligatory co-regulatory protein. In fact, in experiments to be reported elsewhere (J. Athanikar and T. F. Osborne, unpublished results) we have demonstrated that even though SREBP and the ubiquitous co-regulator Sp1 activate both the LDL receptor and ACC promoters, the domains of Sp1 required for activation of each promoter are not equivalent. Thus, even in two promoters where the same co-regulatory protein is recruited by SREBP the mechanism for synergistic activation must be at least partially distinct.

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REFERENCES

1. Osborne, T. F. 1995. Transcriptional control mechanisms in the regulation of cholesterol balance *Crit. Rev. Euk. Gene Exp.* **5:** 317–335.
2. Briggs, M. R., C. Yokoyama, X. Wang, M. S. Brown, and J. L. Goldstein. 1993. Nuclear protein that binds sterol regulatory element of low density receptor promoter I: identification of the protein and delineation of its target nucleotide sequence. *J. Biol. Chem.* **268:** 14490–14496.
3. Wang, X., M. R. Briggs, X. Hua, C. Yokoyama, J. L. Goldstein, and M. S. Brown. 1993. Nuclear protein that binds sterol regulatory element of low density receptor promoter II: purification and characterization. *J. Biol. Chem.* **268:** 14497–14504.
4. Yokoyama, C., X. Wang, M. R. Briggs, A. Admon, J. Wu, X. Hua, J. L. Goldstein, and M. S. Brown. 1993. SREBP-1, a basic-helix-loop-helix-zipper protein that controls transcription of the low density receptor gene. *Cell.* **75:** 185–197.
5. Hua, X., C. Yokoyama, J. Wu, M. R. Briggs, M. S. Brown, J. L. Goldstein, and X. Wang. 1993. SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element. *Proc. Natl. Acad. Sci. USA.* **90:** 11603–11607.
6. Bennett, M. K., J. M. Lopez, H. B. Sanchez, and T. F. Osborne. 1995. Sterol regulation of fatty acid synthase promoter: coordinate feedback regulation of two major lipid pathways. *J. Biol. Chem.* **270:** 25578–25583.
7. Ericsson, J., S. M. Jackson, B. C. Lee, and P. A. Edwards. 1996. Sterol regulatory element binding protein binds to a novel *cis* element in the promoter of the farnesyl diphosphate synthase gene. *Proc. Natl. Acad. Sci. USA.* **93:** 945–950.
8. Lopez, J. M., M. K. Bennett, H. B. Sanchez, J. M. Rosenfeld, and T. F. Osborne. 1996. Sterol regulation of acetyl CoA carboxylase: a mechanism for coordinate control of cellular lipid. *Proc. Natl. Acad. Sci. USA.* **93:** 1049–1053.
9. Millinder Vallett, S., H. B. Sanchez, J. M. Rosenfeld, and T. F. Osborne. 1996. A direct role for sterol regulatory element binding protein in activation of 3-hydroxy-3-methylglutaryl coenzyme A reductase gene. *J. Biol. Chem.* **271:** 12247–12253.
10. Magaña, M. M., and T. F. Osborne. 1996. Two tandem binding sites for sterol regulatory element binding proteins are required for sterol regulation of fatty acid synthase promoter. *J. Biol. Chem.* **271:** 32689–32694.
11. Sanchez, H. B., L. Yieh, and T. F. Osborne. 1995. Cooperation by SREBP and Sp1 in sterol regulation of low density lipoprotein receptor gene. *J. Biol. Chem.* **270:** 1161–1169.
12. Sudhof, T., D. Van der Westhuyzen, J. Goldstein, M. Brown, and D. Russell. 1987. Sterol dependent repression of low density lipoprotein receptor promoter mediated by 16 base-pair sequence adjacent to binding site for transcription factor Sp1. *J. Biol. Chem.* **262:** 10773–10779.
13. Smith, J. R., T. F. Osborne, J. L. Goldstein, and M. S. Brown. 1990. Identification of nucleotides responsible for enhancer activity of sterol regulatory element in low density lipoprotein receptor gene. *J. Biol. Chem.* **265:** 2306–2310.
14. Jackson, S. M., J. Ericsson, T. F. Osborne, and P. A. Edwards. 1995. NF-Y has a novel role in sterol-dependent transcription of two cholesterogenic genes. *J. Biol. Chem.* **270:** 21445–21448.
15. Shimano, H., J. D. Horton, R. E. Hammer, I. Shimomura, M. S. Brown, and J. L. Goldstein. 1996. Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *J. Clin. Invest.* **98:** 1575–1584.
16. Jackson, S. M., J. Ericsson, J. E. Metherall, and P. A. Edwards. 1996. A role for sterol regulatory element binding protein in the regulation of farnesyl diphosphate synthase and in the control of cellular levels of cholesterol and triglyceride: evidence from sterol regulation-defective cells. *J. Lipid Res.* **37:** 1712–1721.
17. Goldstein, J., S. Basu, and M. Brown. 1983. Receptor-mediated endocytosis of LDL in cultured cells. *Methods Enzymol.* **98:** 241–260.
18. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Press, New York.
19. Dynan, W. S., and R. T. Tjian. 1983. The promoter specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. *Cell.* **35:** 79–87.
20. Osborne, T. F., G. Gil, M. S. Brown, R. C. Kowal, and J. L. Goldstein. 1987. Identification of promoter elements

required for in vitro transcription of hamster 3-hydroxy-3-methylglutaryl coenzyme A reductase gene. *Proc. Natl. Acad. Sci. USA.* **84:** 3614–3618.
21. Osborne, T. F. 1991. Single nucleotide resolution of sterol regulatory region in promoter for 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Biol. Chem.* **266:** 13947–13951.
22. Bishop, R. W. 1992. Structure of the hamster low density lipoprotein receptor gene. *J. Lipid Res.* **33:** 549–557.
23. Mehta, K. D., M. S. Brown, D. W. Bilheimer, and J. L. Goldstein. 1991. The low density receptor in *Xenopus laevis*. *J. Biol. Chem.* **266:** 10415–10419.
24. Sudhof, T., D. Russell, M. Brown, and J. Goldstein. 1987. 42 bp element from LDL receptor gene confers end-product repression by sterols when inserted into viral TK promoter. *Cell.* **48:** 1061–1069.
25. Smith, J. R., T. F. Osborne, M. S. Brown, J. L. Goldstein, and G. Gil. 1988. Multiple sterol regulatory elements in promoter for hamster 3-hydroxy-3-methylglutaryl coenzyme A synthase. *J. Biol. Chem.* **263:** 18480–18487.