Two Tandem Binding Sites for Sterol Regulatory Element Binding Proteins Are Required for Sterol Regulation of Fatty-acid Synthase Promoter*

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We previously reported that sterol regulation of the rat fatty-acid synthase was lost when the DNA sequence between −73 and −43 of the promoter was deleted from a luciferase reporter construct (Bennett, M. K., Lopez, J. M., Sanchez, H. B., and Osborne, T. F. (1995) J. Biol. Chem. 270, 25578–25583). We also showed that there was a binding site for sterol regulatory element binding protein-1 (SREBP-1) in this region that contains a palindromic E-box motif (5'-CANNTG-3'). This is the consensus recognition element for basic-helix-loop-helix leucine zipper containing proteins such as the SREBPs. However, the SREBPs are unique basic-helix-loop-helix leucine zipper proteins that not only bind to a subset of E-boxes but also to the direct repeat SRE-1 element of the low density lipoprotein receptor promoter as well as to variant sites present in the promoters for key enzymes of both cholesterol and fatty acid biosynthesis. Based on the sequence of the variant SREBP recognition protein; SRE, sterol regulatory element; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CMV, cytomegalovirus, FAS, fatty-acid synthase; SRE, sterol regulatory element; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CMV, cytomegalovirus, FAS, fatty-acid synthase; bHLHZip, basic-helix-loop-helix zipper; LDL, low density lipoprotein.

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§ The abbreviations used are: SREBP, sterol regulatory element binding protein; SRE, sterol regulatory element; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CMV, cytomegalovirus, FAS, fatty-acid synthase; bHLHZip, basic-helix-loop-helix zipper; LDL, low density lipoprotein.
ble overexpression of the mature form of the wild type SREBP1c/ADD1 protein activated a subset of genes characteristic of the adipocyte program including FAS in normal fibroblasts (11).

We showed that the 30-base pair sterol regulatory region in the FAS promoter bound SREBP-1 and contained a classic 5′-CANNTG-3′ E-box motif that is the hallmark binding motif for bHLH proteins (15, 16), and we presumed it was the target for SREBP-1 in this region (12).

However, another unique feature of the SREBP1s is that they contain an atypical tyrosine residue in the basic domain at a key position that is occupied by an arginine in almost all other bHLHZip proteins. This single amino acid plays a critical role in allowing SREBP to not only recognize the consensus E-box which contains two half-sites in an inverted orientation but also the SRE-1 element in the LDL receptor which contains a direct repeat of two half-sites with one intervening base (3, 10). Additionally, SREBPs also bind to variant SRE-1 sites present in the promoters for HMGC-oA reductase, farnesyl-diphosphate synthase, squaleine synthase, and acetyl-CoA carboxylase (12, 13, 17–19). In the FAS sterol regulatory region we noted that there was a potential half-site element in addition to the two halves of the E-box (Fig. 1).

To determine if SREBP-1 was acting through the E-box site or the half-site variant elements in the FAS promoter, we introduced a series of mutations targeted at the potential recognition motifs and evaluated the effects on sterol regulation, activation by ectopic expression of SREBP-1a and 2, and binding by purified SREBP-1a and -2 proteins.

Our results indicate that the E-box contained in this sequence interval is not the target for SREBP binding or sterol regulation. Rather, there are two separate SREBP binding sites, and each one encompasses a distinct half of the E-box along with associated flanking residues. Both SREBP sites are required for maximal sterol regulation and activation by SREBPs. However, one site is more crucial than the other. We show that SREBP1a and -2 each bind to both FAS sites in a similar but slightly different manner and that both SREBPs activate the FAS promoter.

**EXPERIMENTAL PROCEDURES**

**Cells and Media—**CV-1 cells were obtained from Dr. K. Cho at University of California, Irvine. HepG2 cells were obtained from the ATCC. All cell culture materials were obtained from Life Technologies, Inc. Lipoprotein-deficient serum was prepared by ultracentrifugation starting with newborn bovine serum as described (20). Cholesterol and 25-OH cholesterol were purchased from Steraloids Inc., and stock solutions were dissolved in absolute ethanol.

**FAS Promoter Plasmids and Transient Transfection Assay—**The rat FAS promoter FAS (-1594) CAT (21) was a kind gift of Dr. Steve Clarke (University of Texas at Austin). The FAS promoter fragment from −150 to −43 (here called wild type) or −150 to −73 (here called negative or NEG), respectively, were linked to a generic TATA box sequence positioned upstream of the luciferase coding sequence. These plasmids were described previously (12).

The mutations described in the figures were generated by PCR using the wild type FAS promoter DNA as template with Pfu polymerase and mutagenic oligonucleotides designed to introduce each specific multi-base point mutation. A specific mutagenic oligonucleotide of 20–40 bases was combined with a common 5′ primer that hybridized to the wild type FAS sequence beginning at −150. This was the same primer used to generate the original FAS −150 promoter plasmid that was efficiently regulated by sterols (12). Each mutagenic primer was designed to hybridize to the wild type FAS sequence beginning at −43 and extend upstream with the desired mutations positioned accordingly. The PCR reaction product was digested with restriction enzymes and inserted upstream from the generic TATA box positioned in front of the luciferase coding sequence as mentioned above and described previously (12). The DNA sequence of each mutant clone was confirmed before initiating the transfection studies.

**Cell Culture and Transient DNA Transfections—**CV-1 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and plated on day 0 in 60-mm dishes at 125,000 cells/dish. On day 1 the cells were refed the same media and transfected by the calcium phosphate co-precipitation method. Precipitates contained 20 μg each of test plasmid and internal control CMV ß-galactosidase plasmid that contains the cytomegalovirus (CMV) promoter fused to the Echerichia coli ß-galactosidase gene plus 10 μg of salmon sperm DNA in 2 ml of solution. 0.5 ml were added to each of four dishes that were incubated for 12–16 h at 37 °C and 7% CO2. The dishes were washed three times with phosphate-buffered saline, and duplicate dishes were refed either induced (Dulbecco’s modified Eagle’s medium containing 10% lipoprotein-depleted serum) or suppressed (same but containing 12 μg/ml cholesterol and 1 μg/ml 25-OH cholesterol) media. Following a 24 h incubation the cells from duplicate dishes were harvested by scraping and were pooled. Soluble protein extracts were prepared by three freeze-thaw cycles.

For activation studies with over-expressed SREBP-1a, a CMV promoter expression clone encoding amino acids 1–490 of SREBP-1a was used, and it was described previously (22). A similar expression clone encoding amino acids 1–481 of SREBP-2 was used to evaluate activation by SREBP-2. HepG2 cells were plated at 175,000 cells per dish and transfected the following day with the individual FAS-luciferase reporter construct and the CMV2 ß-galactosidase as described above. Half...
of the dishes received 30 ng per dish of the SREBP-1a or -2 expression plasmid. The transfected cells were subjected to a glyceral shock 5 h after the transfection and cultured in modified Eagle’s medium containing 10% fetal bovine serum for an additional 24 h prior to harvesting as described above.

**Enzyme Assays**—Luciferase activities were measured in a luminometer with a luciferin reagent from Promega Biotec. β-Galactosidase assays were performed by a standard colorimetric procedure with 2-nitrophenyl-β-D-galactopyranoside as substrate (23). The ratio of luciferase activity in relative light units was divided by the β-galactosidase activity (activity/h) for each extract to give a normalized luciferase value. The data presented are from several independent transfections performed in duplicate for each plasmid (see figure legends for the number of individual experiments).

**Protein Purification**—Recombinant SREBP-1a and SREBP-2 proteins were purified by nickel chelation chromatography from a 6XHis containing fusion protein as described (22). The purity and concentration of the purified protein were assessed by SDS-polyacrylamide gel electrophoresis analysis performed with marker proteins followed by staining with Coomassie Blue.

**DNase I Footprinting**—32P DNA probes were prepared from the indicated plasmids incubated with SREBP and digested with DNase I as described (24, 25).

**RESULTS**

We previously identified a region between −73 and −43 upstream from the mRNA start site for the rat FAS gene as harboring an SREBP binding site that was critical for regulation of the promoter by sterol levels in cultured cells (12). Within this interval is a classic E-box element (see Fig. 1), and we postulated that the bHLHZip domain of SREBP probably recognized the E-box. However, based on our recent studies of the DNA recognition properties for SREBP-1 in the HMG-CoA reductase promoter (19), we noted that there was the potential for more than one SREBP binding site within this region of the FAS promoter (Fig. 1). To define the sterol regulatory element and the binding site or sites for SREBP within this interval with more precision, we introduced several tri-base substitution mutations separately or in combination and evaluated the effects on sterol regulation, activation by ectopic expression of SREBPs, and binding by purified SREBP protein.

The parent plasmid contained the wild type sequence from −150 through −43 fused to a TATA element upstream of the luciferase reporter gene. This plasmid contains all of the cis-acting information required for efficient sterol regulation and activation by SREBP-1 (12). Within this parent construct, four separate mutations were introduced that alter each of four contiguous three base regions (referred to as A, B, C, and D in Fig. 1). We also mixed the separate mutations together in different combinations. Each mutant promoter construct was evaluated by a transient DNA transfection assay for sterol regulation in cultured CV-1 cells. As shown in Fig. 1, the parent plasmid (wild type) was expressed and efficiently suppressed when sterols were added to the culture dish. A mutation that deletes the sequence from −73 to −43 was severely compromised for sterol regulation (NEG). These results are consistent with our earlier study (12).

The single mutants A and B were moderately defective for regulation. However, mutants C and D were severely defective

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Identification of two non-E-box SREBP binding sites in the sterol regulatory region of the FAS promoter: DNase I footprint analysis with SREBP-1 and SREBP-2. A, DNA probe from the wild type FAS promoter was end-labeled on the top strand at −150 and used in a standard DNase I footprint experiment as described before (12, 22). The probe was incubated alone with DNase I (lanes 1, 6, 7, and 12) or with increasing amounts (5, 10, 30, and 50 ng) of purified recombinant SREBP-1 (lanes 2–5) or SREBP-2 (lanes 8–11) as indicated by the arrows above the lanes. The arrows at the left denote the dramatic hyper-cleavage sites induced upon binding of SREBP-1. The bottom of the gel corresponds to the 5′ end of the promoter and the top to the 3′ end as noted at the left. The DNase I protected region corresponding to the two tandem SREBP-1 sites in the wild type FAS promoter are marked by the brackets at the right and are discussed in the text. All other symbols and notations are the same as described in Fig. 1. B, probes from the wild type or mutant FAS promoters were labeled on the top strand and used in standard DNase I footprint reactions. Samples are shown for each probe in sets of three lanes; the outside lanes denoted by a 0 at the top denote that probe DNA alone was digested with DNase I. The middle lanes denoted by a + are from samples where saturating amounts of SREBP-1a (50 ng) were incubated with probe DNA prior to digestion with DNase I. All other symbols and notations are as described above and in the legend to Fig. 1. C, the indicated plasmid DNAs were subjected to DNase I footprint analysis as in B except 50 ng of recombinant SREBP-2 was used.
for sterol regulation. When the A and B mutations were combined the resultant double mutant lost all sterol regulation. If the E-box was the sterol regulatory site then the effects of mutations B and C, which change each half of the E-box element, should be identical and both should be defective for regulation. Additionally, the effects of mutations A and D should be minimal or silent. Thus, the results in Fig. 1 are inconsistent with the E-box being the sterol regulatory site.

To evaluate the effects of the above mutations on SREBP binding, we performed DNase I footprinting with recombinant SREBP-1a or -2 protein and end-labeled DNA probes prepared from the wild type promoter and several of the mutants analyzed in Fig. 1. The results of representative experiments are shown in Fig. 2. The DNase I-protected region corresponding to the wild type promoter when SREBP-1 was evaluated (Fig. 2A, lanes 1–6) is identical to what we reported previously (12). SREBP-1 binding induces two prominent DNase I hyper-cleavage sites at the 3′ end of the footprint (see arrows on the left of Fig. 2A). A similar hyper-cleavage site is induced upon the binding of SREBP-1 to the SRE-1 of the LDL receptor promoter (22) and the HMG-CoA synthase promoter.2 The protection pattern for SREBP-2 is similar, but the prominent SREBP-1 induced hyper-cleavage sites are not produced (lanes 7–12).

The binding of SREBP-1 and -2 was evaluated on a subset of the mutant FAS constructs to correlate the results of the regulation studies of Fig. 1 with the binding of SREBP. The results for SREBP-1 binding are shown in Fig. 2B. When mutant C was used as a probe the footprint was reduced in size and was localized to the 3′ side of the wild type protected region (lanes 4–6). This result is consistent with mutant C destroying one recognition site for SREBP-1 but leaving one site completely unaltered.

When either mutant A or B was analyzed (lanes 7–12), the region of DNase I protection was again reduced. Protection was maintained on the 5′ side of the footprint region seen with the wild type probe, but the protection on the 3′ side was lost and was replaced by a prominent hyper-cleavage site. The B/C and A/C double mutations resulted in a total loss of all SREBP binding (lanes 13–18), and the A/B double mutant abolished binding at the 3′ side but maintained binding at the 5′ side of the site (lanes 19–21). This pattern is identical to the results for either the A or B single mutant (compare to lanes 7–12). The A/B/C triple mutation abolished all SREBP-1 binding to the probe (lanes 22–24). Taken together the DNase I protection data are consistent with two independent recognition sites for SREBP-1 within this region; one disrupted by mutants A and B and the other disrupted by mutant C.

SREBP-2 binding to the mutant FAS promoters was also evaluated, and the results were consistent with the results with SREBP-1a. The most significant results are presented in Fig. 2C. Similar to SREBP-1 (Fig. 2B), SREBP-2 protection over the 3′-half was largely abolished by the A/B double mutant but protection over the 5′-half was not altered (compare lanes 1–3 and 7–9). Similar results were obtained for each of the single mutants as well (data not shown). SREBP-2 binding to the mutant C resulted in the reciprocal result (lanes 4–6). Protection over the 5′-half was abolished, and the protection over the 3′-half was maintained.

To further evaluate the possibility of two important SREBP recognition sites in this region, we assessed the activation of the mutant promoters by ectopically expressed SREBP-1 and -2 in a mammalian cell transfection assay (Fig. 3). The results for activation by an expression vector encoding the mature form of SREBP-1a (amino acids 1–490) are presented in Fig. 3A. The wild type FAS promoter was efficiently activated and consistent with our earlier studies (12); a mutant that deletes the sequence from −73 to −43 which removes the sterol regulatory region was unresponsive (NEG). The results for activation studies performed with the new mutants are consistent with the sterol regulation experiments of Fig. 1 (see Fig. 3A). Mutants A and B are partially and mutants C and D are severely defective for activation by SREBP-1a. Once again, the A/B double mutation is more defective than either single mutant alone, and the A/B/C triple mutant was unresponsive. The results for activation by an expression vector encoding the mature form of SREBP-2 (amino acids 1–481) are presented in Fig. 3B. The results for mutants A and B and the A/B double mutant are not statistically significant from the wild type. This is different than for SREBP-1 where the single mutants were partially defective for activation, and the A/B double mutant was severely crippled. The single mutants C and D are each severely defective for activation which is similar to the results for SREBP-1.

SREBPs are weak activators of transcription by themselves and have been shown to synergistically activate promoters along with a ubiquitous transcription factor that binds to an
adjacent site (1, 22). In the FAS promoter this co-regulator is likely to be Sp1 since there is an Sp1 site beginning at −91, and we have shown that SREBP and Sp1 synergistically activate the FAS promoter (12).

To study this further we analyzed the effect of changing the distance between the SREBP binding region between −73 and −43 and the Sp1 site at −91. Two insertion mutations were constructed that add either 4 or 10 bases between the two elements, and the effects on both sterol regulation and activation by ectopic SREBP-1 expression were evaluated (Fig. 4). Both insertion mutations were totally defective for both sterol regulation (Fig. 4A) and activation by ectopically expressed SREBP-1a (Fig. 4B). This provides further evidence that both Sp1 and SREBP are required for efficient sterol regulation of the FAS promoter and that the distance and not helical phasing of the two sites is critical for optimal activity.

**DISCUSSION**

In the present studies we demonstrate that the region of the rat fatty-acid synthase promoter required for sterol regulation contains two independent binding sites for sterol regulatory element binding proteins −1 and −2. The surprising find was that the classic bHLH DNA recognition element, the E-box, which is present in this region is not the binding site for the bHLH containing SREBP protein. Instead, each half of the E-box is contained in a different SREBP site (Figs. 1 and 2).

An alignment of the FAS sites identified here relative to known SREBP sites present in sterol regulatory elements contained in other promoters is presented in Fig. 5. This lineup reveals a high degree of sequence similarity between these FAS elements and other direct repeat SRE elements. Thus, SREBP probably recognizes all these sites in a similar manner.

SREBPs are inherently weak activators by themselves, and they function synergistically with ubiquitous factors that bind close by to achieve a high level of promoter activation (1, 22). There is a single SREBP site in the LDL receptor promoter that is required for efficient sterol regulation. Why are there two sites required in the FAS promoter? At least two SREBP sites are also required for sterol regulation in both the HMG-CoA synthase (26) and reductase promoters (19).

The difference with the LDL receptor may partially be due to the position of the SREBP site relative to the essential neighboring co-regulatory factor. In the LDL receptor promoter the Sp1 site is very close to the SREBP site. In fact, the footprints for the two individual proteins partially overlap. In the FAS promoter the co-regulatory Sp1 site is located 10 bases away, and in the synthase promoter the co-regulator is probably nuclear factor-Y and its recognition site is about 20 bases away from the nearest SREBP site. However, it is unlikely that the distance between the SREBP and co-regulatory sites alone can explain the entire effect since a single SREBP site is located about 20 bases away from the co-regulatory nuclear factor-Y site in the farnesyl-diphosphate synthase promoter (18).

Interestingly, although both of these FAS SREBP sites are required for maximal activation by SREBP-1 and sterol regulation, the site disrupted by mutations C and D which is closer to the upstream Sp1 site seems to be more critical (see Figs. 1 and 3). In fact, the Sp1 site which is disrupted by mutants A and B is even less crucial for activation by SREBP-2 than by SREBP-1 (Fig. 3, A and B).

Insertion of 4 or 10 bases between the Sp1 and SREBP sites

**Fig. 4. Sterol regulation and SREBP activation of insertion mutations between the SREBP and Sp1 sites in the FAS promoter.** The DNA sequence of the rat FAS promoter is shown at the bottom with an arrow indicating the position of the insertion mutations. The sequence and number of bases for each insertion are shown below the arrow. A, wild type and mutant FAS promoters were analyzed by a transient DNA transfection in CV-1 cells as described under “Experimental Procedures” and in the legend to Fig. 1. Mean -fold regulation values for each plasmid and the standard error for two independent experiments performed in duplicate are presented. Fold regulation is calculated as discussed in the legend to Fig. 1.

**Fig. 5. Alignment of SREBP sites present in known sterol responsive regions.** The sequence of the LDL receptor SREBP sites from hamster (Ham), rat, Xenopus (Frog), human (Hum), and mouse are shown as well as the two sites from the hamster HMG-CoA synthase promoter (HamSyn 1 and HamSyn 2); two sites and their locations from the hamster HMG-CoA reductase (hamRed −150 and hamRed −165) are shown. The direction for each is 5’ to 3’. References for these elements are given in Ref. 5. The sequence for the RatFAS −150 SREBP binding site is identical to the human LDL receptor SREBP site, and the two RatFAS elements referred to in the text are shown at the bottom. The four tri-base elements (A, B, C, D) that were mutated and analyzed in the present study are in bold. The A/B site begins at nucleotide 62, and the bottom strand of the DNA is presented since it is in the opposite orientation relative to the C/D site which begins at position −72 as indicated. The arrows at the top are positioned over each copy of the direct repeat element 5’-PyCpAp-3’ (where Py = pyrimidine), 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 (1).
resulted in a loss of sterol regulation (Fig. 4) indicating that the spacing, but not helical positioning, of the sites is important for normal FAS promoter function. This observation coupled with the regulation and activation studies for the substitution mutants discussed above are consistent with the fact that SREBP and Sp1 must interact together for optimal promoter activity. A cis-acting site required for insulin regulation of the FAS promoter was mapped to a region that overlaps the two SREBP sites studied here (27). The same laboratory provided evidence that the ubiquitous bHLHZip protein upstream stimulatory factor binds to this region, presumably recognizing the E-box motif, and it was suggested that upstream stimulating factor might play a role in insulin regulation of FAS expression (28). It will be interesting to see how the mutations analyzed here affect insulin regulation to determine if insulin and sterol signaling are mediated through the same or different sites in the promoter.

Through the use of an in vitro PCR-assisted DNA binding site selection assay, SREBP-1c/ADD1 was shown to prefer the palindromic E-box containing sequence 5′-ATCACGTG-3′ and, presumably to a lesser extent, the direct repeat SRE-1 sequence 5′-CACGTG-3′ (10). The preference for the CG dinucleotide in the center of the E-box is identical to that for Myc/Max, upstream stimulatory factor, and other Myc-related bHLHZip proteins although preferred outside flanking bases can differ slightly for these other proteins (29–32). Surprisingly, the E-box palindrome is not contained within any natural sterol response regions that are known to bind the SREBP (5, 12, 17–19, 26, 33, 34).

The reason why a high affinity E-box has not been identified in a known SREBP sterol response element is unclear. However, since several SREBP-related bHLHZip proteins such as upstream stimulatory factor and Myc/Max would also bind to a high affinity 5′-CACGTG-3′ E-box if it was present, the direct repeat SRE element would ensure more precise regulation since it would be highly specific for the SREBP proteins.

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