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Promoter Selective Transcriptional Synergy Mediated by Sterol Regulatory Element Binding Protein and Sp1: a Critical Role for the Btd Domain of Sp1

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Cellular cholesterol and fatty acid levels are coordinately regulated by a family of transcriptional regulatory proteins designated sterol regulatory element binding proteins (SREBPs). SREBP-dependent transcriptional activation from all promoters examined thus far is dependent on the presence of an additional binding site for a ubiquitous coactivator. In the low-density lipoprotein (LDL) receptor, acetyl coenzyme A carboxylase (ACC), and fatty acid synthase (FAS) promoters, which are all regulated by SREBP, the coactivator is the transcription factor Sp1. In this report, we demonstrate that Sp3, another member of the Sp1 family, is capable of substituting for Sp1 in coactivating transcription from all three of these promoters. Results of an earlier study showed that efficient activation of transcription from the LDL receptor promoter required domain C of Sp1; however, this domain is not crucial for activation of the simian virus 40 promoter, where synergistic activation occurs through multiple Sp1 binding sites and does not require SREBP. Also in the present report, we further localize the critical determinant of the C domain required for activation of the LDL receptor to a small region that is highly conserved between Sp1 and Sp3. This crucial domain encompasses the buttonhead box, which is a 10-amino-acid stretch that is present in several Sp1 family members, including the *Drosophila buttonhead* gene product. Interestingly, neither the buttonhead box nor the entire C domain is required for the activation of the FAS and ACC promoters even though both SREBP and Sp1 are critical players. ACC and FAS each contain two critical SREBP sites, whereas there is only one in the LDL receptor promoter. This finding suggested that buttonhead-dependent activation by SREBP and Sp1 may be limited to promoters that naturally contain a single SREBP recognition site. Consistent with this model, a synthetic construct containing three tandem copies of the native LDL receptor SREBP site linked to a single Sp1 site was also significantly activated in a buttonhead-independent fashion. Taken together, these studies indicate that transcriptional activation through the concerted action of SREBP and Sp1 can occur by at least two different mechanisms, and promoters that are activated by each one can potentially be identified by the number of critical SREBP binding sites that they contain.

Cellular sterol balance is maintained by a feedback mechanism primarily at the level of gene transcription (3, 9, 28). When intracellular levels are low, the gene involved in cholesterol uptake, namely, the low-density lipoprotein (LDL) receptor gene, and those encoding key enzymes of the de novo synthesis pathway are turned on. Reciprocally, when sterol levels are sufficient, these genes are shut off. The core promoter region for the LDL receptor gene is composed of three related sequence elements designated repeats 1 to 3 (36). Repeat 2 contains the sterol regulatory element (SRE-1) which is the direct DNA target site for sterol regulation (7). The SRE-1 within repeat 2 was identified by mutational studies as a 10-bp binding site for a family of sterol regulatory element binding proteins (SREBPs) which activate gene expression through the SRE-1 only when new sterol accumulation is required (2, 35, 40).

The activity of the SREBPs themselves is regulated by sterol levels; a 125-kDa precursor form is anchored to intracellular membranes including the endoplasmic reticulum and nuclear envelope through two transmembrane spans (3, 12). A drop in intracellular sterol levels initiates a signaling process that results in release of membrane-bound SREBPs after two sequential proteolytic attacks (3, 31). The resulting soluble amino-terminal half of the protein which contains the transcriptional activation and DNA binding domains is targeted to the nucleus, where it activates the appropriate target genes (31, 41). The exact signaling mechanism that results in release of the SREBPs from the membrane is not well understood. There are multiple SREBP family members; SREBP-1a and SREBP-1c are alternate forms that are expressed from a single gene, and SREBP-2 is the product of a separate genetic locus (13). The physiologic roles played by the individual SREBPs are not clearly defined.

SREBPs were identified as proteins that regulate intracellular cholesterol balance (40, 44), but studies from our laboratory (1, 21) and by others (19, 37) have demonstrated they are involved in the regulation of fatty acid biosynthesis and are also activated during adipocyte differentiation. Additionally, in cultured cells (15) and in animals (34) that constitutively overproduce nuclear targeted SREBPs, the rates of synthesis for both cholesterol and fatty acids are significantly enhanced.

SREBPs control fatty acid biosynthesis through binding to variant SRE sites that are present in the promoter regions for the acetyl coenzyme A carboxylase (ACC) and fatty acid synthase (FAS) genes, which encode key enzymes of long-chain

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fatty acid biosynthesis (1, 21). SREBPs are unable to support efficient transcription activation alone, since a coregulatory protein and its associated binding site must be present to achieve optimal expression. For the LDL receptor, ACC, and FAS, this coregulator is the ubiquitous transcription factor Sp1 (1, 21, 32) which was first identified as a major transcription factor required for the expression of the simian virus 40 (SV40) early promoter (8). Subsequently, critical Sp1 sites were identified in other viral as well as cellular genes (18).

In a previous report, we showed that the major glutamine-rich activation domains and the carboxy-terminal D domain of Sp1 were required for synergistic activation of the LDL receptor promoter along with SREBP-1a (43). Moreover, we showed that domain C of Sp1 was also important (43). Interestingly, the C domain was not critical for synergistic activation of the SV40 promoter, which is activated through multiple Sp1 sites and does not respond to SREBP.

In this report, we show that a small portion of domain C which encompasses a region that is highly similar between Sp1 and a related protein called Sp3 is a critical determinant for activation of the LDL receptor promoter. This small region of Sp1-Sp3 similarity encompasses the buttonhead (Btd) box, which was first noted as a highly conserved 10-amino-acid stretch that is present in several metazoan Sp1-related genes, including a *Drosophila* Sp1 homolog called *buttonhead* (*btd*) (42). The buttonhead protein (Btd) is a gap gene product required for normal head development.

We also show that even though Sp1 and SREBP are required for activation of the ACC and FAS promoters, domain C is not. This finding indicates that the mechanism for activation of these other promoters is at least partially distinct from activation of the LDL receptor.

We also demonstrate that the Sp1-related protein called Sp3, which is another member of the Sp1 family and contains the conserved Btd box, can substitute for Sp1 in coactivation of the LDL receptor, ACC, and FAS promoters. These and similar studies will help define regions of Sp1 and perhaps SREBPs that are involved in gene-specific activation through subtly different mechanisms.

MATERIALS AND METHODS

Plasmids. Plasmid pPACSp1 was obtained from Al Courey (University of California, Los Angeles) and plasmids pPACSp1ΔC and pPACβgal were obtained from R. Tjian (University of California, Berkeley). pPACSp1ΔBtd was constructed by PCR amplification using plasmid pPACSp1 as the template. The numbering of the Sp1 amino acids is according to reference 29, where amino acid +1 corresponds to the first Sp1-specific amino acid in pPACSp1. This corresponds to amino acid +83 of full-length Sp1. Specific primers were designed to different strands of the amino-terminal portion of Sp1 to amplify the amino-terminal 500 amino acids from pPACSp1. The PCR product was cloned into *Bam*HI-digested pPACSp1; in the resulting plasmid, amino acid 500 is fused directly to amino acid 532. Thus, the plasmid contains a deletion of amino acids 501 through 531 of the Sp1 coding sequence. This mutation is referred to as btd. pPACSp3 was constructed by PCR using as a template the full-length Sp3 plasmid (20), which was obtained from A. Winoto (University of California, Berkeley). Primers were designed to amplify the entire Sp3 coding sequence with a *Bam*HI site at the 5′ end and an *Xho*I site at the 3′ end. The PCR product was digested with *Bam*HI and *Xho*I and cloned into the *Bam*HI-*Xho*I-digested pPAC vector. pPACSREBP1a contains amino acids 1 to 490 of the full-length protein in the pPAC expression vector and has been previously described (32). pPACSREBP-2 was cloned by PCR amplification using first-strand cDNA from HepG2 mRNA and primers designed to amplify amino acids 1 to 481 from the published sequence for SREBP-2. The primers contained *Bam*HI and *Xho*I sites at the 5′ and 3′ ends, respectively; after digestion with these enzymes, the fragment was cloned into the pPAC vector as described above. The DNA sequence for the amino and carboxy termini of this construct were confirmed by sequence analysis. Previous reports described the construction of the reporter plasmids for the LDL receptor (32), ACC-400 (21), and FAS-150 (1). The expression and purification of the truncated SREBP-1 protein used in DNA binding experiments was also described previously (43).

Transient DNA transfections. *Drosophila* SL2 cells were obtained from A. Courey and cultured at 25°C in Shields and Sang *Drosophila* medium (Sigma) containing 10% (vol/vol) heat-inactivated fetal bovine serum. They were plated at a density of 1.2×10^6 cell/60-mm-diameter dish and were transfected by a standard calcium phosphate coprecipitation method as described elsewhere (43). Two dishes were used for each data point; cells from both dishes were combined during harvest, and cell extracts were prepared by a standard freeze-thaw method in a total volume of 100 μl.

Enzyme assays. Luciferase activities were measured in a luminometer (Analytical Luminescence Monolight 2010), using 20 μl of cell extract and 100 μl of luciferin reagent (Promega Biotec). The β-galactosidase assays were performed with 50 μl of cell extract, using a standard colorimetric assay with 2-nitrophenyl-β-D-galactopyranoside as the substrate (25). The luciferase activities in relative light units were divided by β-galactosidase activities (optical density at 420 nm) for each sample.

Preparation of nuclear extracts and gel mobility shift analyses. SL2 cells were transfected as described above, and nuclear extracts were prepared and used for standard electrophoretic mobility shift assays as previously described (43). A ^{32}P-labeled probe containing a single copy each of repeats 2 and 3 of the wild-type human LDL receptor promoter was incubated on ice with nuclear extract in the absence or presence of purified SREBP-1. The binding and electrophoresis conditions are described elsewhere (43).

RESULTS

The SREBPs are very weak activators of transcription by themselves. In contrast, they work efficiently along with different ubiquitous coregulatory proteins. The number and identity of the coregulators and the position of their binding sites relative to those for SREBP can vary between different promoters. For example, in the LDL receptor, the singular coregulator is Sp1 (32), and for 3-hydroxy-3-methylglutaryl coenzyme A synthase and farnesyl diphosphate synthase, at least one coregulator is nuclear factor Y (16). Sp1 is also the coregulator for FAS and ACC as well, but the arrangements of the SREBP and Sp1 binding sites in these promoters are different from each other and from that of the LDL receptor (1, 21, 23).

Like the SREBPs, Sp1 is a member of a gene family, and we wished to determine if other Sp1 family members could substitute for Sp1 as a coregulator along with SREBP. Figure 1A shows that the overall domain structures of Sp1 and the related Sp3 are quite similar, although there is limited conservation at the primary amino acid sequence level outside the zinc finger DNA binding domain (11, 20). One notable exception to the lack of amino acid sequence conservation is a small region in domain C referred to as the Btd box (Fig. 1B), which was first noted by Wimmer and colleagues as a region that is highly similar in all known Sp1 family members, including a related gene from *Drosophila* called *buttonhead* (42).

Since the overall domain structures of Sp1 and Sp3 are similar, we wanted to evaluate whether Sp3 could substitute for Sp1 in synergistic activation with SREBP. To study activation by Sp3 specifically, we used a modified cotransfection assay system in *Drosophila* SL2 cells, which we have used before to study synergistic activation of Sp1 and SREBP (32). The advantage of the SL2 assay system is that the cells lack endogenous active SREBP and Sp1; therefore, SREBP- and Sp1-requiring reporter constructs are significantly activated only if we include in the transfection additional separate plasmids that express both SREBP and Sp1. Importantly, when added singly, expression vectors for SREBP or Sp1 alone do not significantly activate the LDL receptor (32).

We prepared an Sp3 *Drosophila* expression vector and compared its ability to activate the LDL receptor along with SREBP-1a to the activation resulting from cotransfection of SREBP-1a and Sp1 *Drosophila* expression vectors in SL2 cells. The results (Fig. 2A) demonstrate that when increasing amounts of an expression vector for either Sp1 or Sp3 is trans-

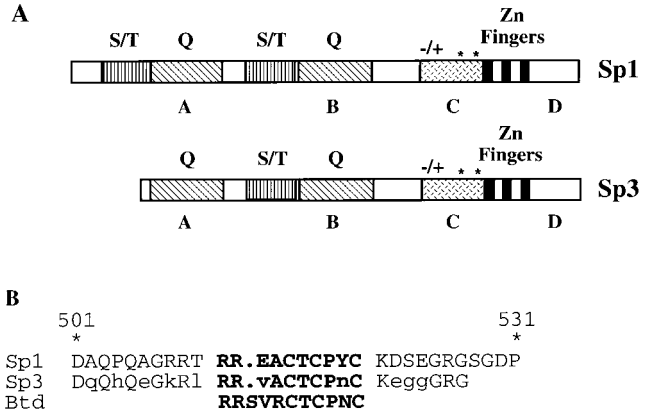

FIG. 1. Similar structural features of Sp1 and Sp3 proteins. (A) Schematic representation of the domain structures of Sp1 and Sp3 proteins. Regions rich in serine/threonine or glutamic acid and the zinc fingers motif are indicated at the top, and domains previously referred to A, B, C, and D are indicated below their positions on the map. −/+ denotes the presence of a high percentage of charged amino acids, and ** marks the Btd domain discussed in the text. (B) Sequence alignment in and around the conserved Btd box element of Sp1-related proteins. Shown is a comparison for a small conserved region in domain C which is represented in the full-length protein by the asterisks in panel A. The amino acid numbers refer to the Sp1 coding sequence in pPACSp1. The regions of 17 of 27 identical residues for Sp1 and Sp3 are shown, and mismatches in the Sp3 sequence are indicated by lowercase letters. The conserved Btd box is shown in boldface. Sequences are from human Sp1 (6, 29), Sp3 (20), and *Drosophila* buttonhead (42).

fected along with a constant amount of SREBP-1a-expressing plasmid, the LDL receptor promoter is activated in a dose-dependent fashion and Sp3 and Sp1 are essentially functionally interchangeable. When added alone, the Sp1 or Sp3 vectors failed to activate the LDL receptor (Fig. 2B and data not shown).

As mentioned above, the promoters for FAS and ACC, the two main enzymes of long-chain fatty acid biosynthesis, are also activated through the concerted action of SREBP and Sp1. To determine if Sp3 could substitute for Sp1 in activation of these other promoters, we transfected the expression vectors for Sp1 or Sp3 plus SREBP-1a along with reporter constructs for FAS and ACC into the SL2 cells (Fig. 2C and D). The results in Fig. 2C show that the ACC promoter is efficiently activated by the combination of SREBP-1a and either Sp1 or Sp3, similar to the results observed for the LDL receptor promoter (Fig. 2A and B). Comparable results were obtained for the FAS promoter (Fig. 2D). Importantly, as mentioned above, when either Sp1- or Sp3-expressing plasmids were transfected without SREBP, there was no significant activation of any of the reporter plasmids (Fig. 2B to D, lanes 3 and 4). Similarly, when the expression vector for SREBP-1a was added alone, there was no significant activation of either the LDL or ACC promoter but there was a partial activation of the FAS promoter. These results are consistent with our earlier studies (1, 21).

Since Sp3 activated these genes similarly to Sp1, a reasonable interpretation is that Sp1 and Sp3 were expressed at similar levels in the transfected SL2 cells. Additionally, experiments presented below demonstrate that equivalent amounts of Sp1 and Sp3 DNA binding activity were produced when equivalent amounts of all expression constructs were used in a slightly different transfection procedure in SL2 cells.

Synergistic activation of the LDL receptor promoter by SREBP and Sp1 occurs at two separate steps in the process of promoter activation. SREBP-1 first stimulates Sp1 to bind to its adjacent site; after both proteins are bound to DNA, they activate transcription much more effectively than either protein alone (43). Since Sp3 was able to substitute for Sp1 in activation, we reasoned that SREBP-1 should also stimulate Sp3 binding to the LDL receptor promoter. Thus, SL2 cells were transfected with either an Sp1- or Sp3-expressing plasmid, and nuclear extracts were prepared and used as a source of Sp1 or Sp3 protein in an electrophoretic mobility shift procedure similar to that used previously to evaluate stimulation of Sp1 DNA binding by SREBP-1 (43).

With nuclear extract from Sp1-transfected SL2 cells, a low level of Sp1-specific binding was observed in the absence of added SREBP-1 (Fig. 3, lanes 2 and 3). However, when purified recombinant SREBP-1 protein (containing amino acids 321 to 490, a region which includes the DNA binding basic–helix-loop-helix–leucine zipper [bHLHZip] domain) was added along with the same amounts of Sp1-expressing SL2 nuclear extract, a dramatic stimulation of Sp1 binding was observed (compare lanes 2 and 3 to lanes 7 and 8 in Fig. 3). We observed an increase in the level of both complexes that contain Sp1 protein. The fact that the complexes containing both SREBP and Sp1 as well as that containing only Sp1 were both enhanced is consistent with our previous observations (32, 43). Since the intensity of both bands representing Sp1-containing

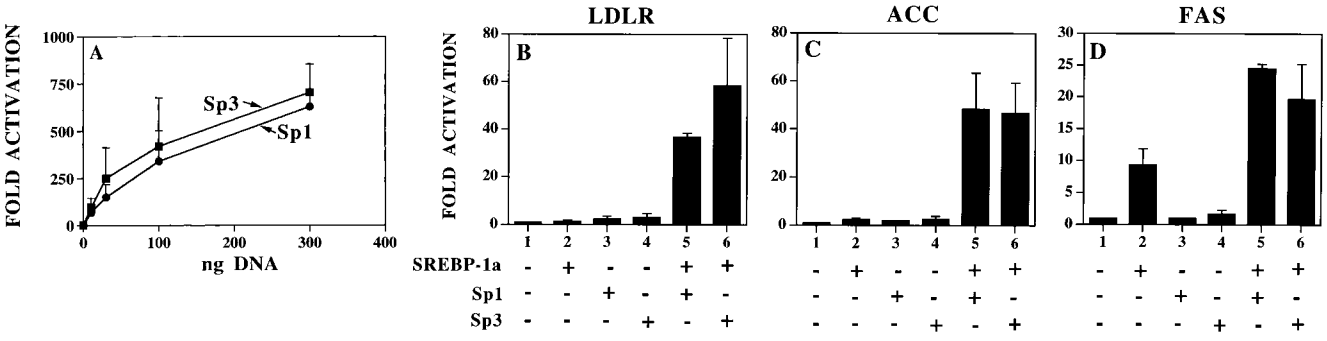

FIG. 2. Activation of the LDL receptor, ACC, and FAS promoters by SREBP and either Sp1 or Sp3. *Drosophila* SL2 cells were transfected with various amounts of pPACSp1 or pPACSp3 and a constant amount (25 ng/60-mm-diameter dish) of pPACSREBP-1a expression vectors. The wild-type LDL receptor promoter fused to the luciferase gene (2 μg/dish) was used as the reporter. Transfections, cell harvests, and enzyme assays were performed as described in Materials and Methods. Fold activation was calculated relative to cells transfected with reporter plasmid alone. (B to D) Cells were transfected with a constant amount (25 ng/60-mm-diameter dish) of Sp1, Sp3, or pPACSREBP-1a alone or in combination as indicated at the bottom. The LDL receptor promoter (LDLR), ACC, and FAS were used as luciferase reporter plasmids in panels B, C, and D, respectively. Fold activation was calculated relative to transfection with reporter alone (lane 1). An internal cytomegalovirus actin 5C promoter–β-galactosidase expression plasmid was included in all transfections, and its expression was used for normalization purposes.

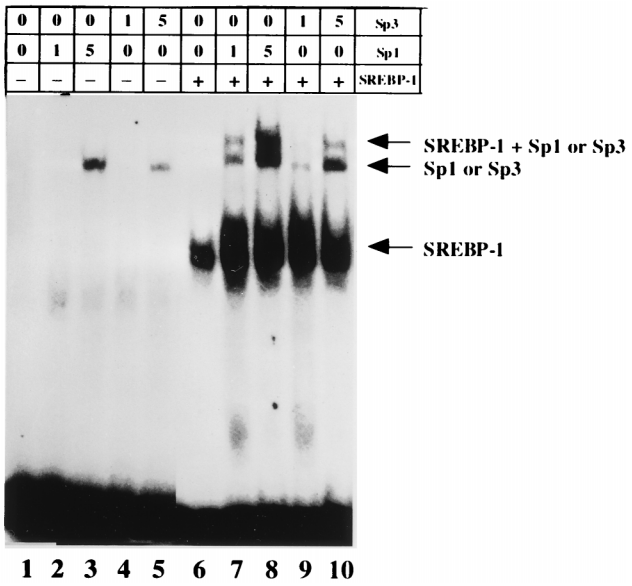

FIG. 3. DNA binding by both Sp1 and Sp3 is stimulated by SREBP-1. *Drosophila* SL2 cells were transfected with either Sp1 or Sp3 (2.5 μg/60-mm-diameter dish), and nuclear extracts were prepared and used in standard electrophoretic mobility shift assays. The indicated amounts of nuclear extract (in micrograms of total protein) were incubated with 0.25 ng of ^{32}P end-labeled DNA probe in the absence (−) or presence (+) of 50 ng of bacterially expressed SREBP-1 (amino acids 320 to 490). The proteins present in each of the three detectable complexes are indicated by arrows at the right. The DNA probe alone and probe incubated with only the recombinant SREBP-1 were loaded in lanes 1 and 6, respectively. There is a complex below the SREBP-1 species that is the result of an endogenous SL2 protein. The DNA probe is a 42-bp fragment encompassing repeats 2 and 3 of the LDL receptor and has been described before (32).

complexes are enhanced, we believe that when Sp1 binds to its recognition site adjacent to SREBP, the SREBP-DNA interaction becomes unstable, resulting in dissociation of SREBP from the DNA fragment when the sample is applied to the polyacrylamide gel and subjected to an electric field. This leaves Sp1 bound to the DNA by itself, resulting in a dramatic increase in the complex containing only Sp1. This conclusion is consistent with other studies where predicted ternary complexes appeared not to be stable to native electrophoresis (17, 27, 39).

Importantly, very similar results were obtained when nuclear extracts from Sp3-transfected SL2 cells were used in place of Sp1 (Fig. 3; compare lanes 4 and 5 with lanes 9 and 10). Thus, Sp1 and Sp3 appear to be expressed to roughly equal levels, and the DNA binding of each protein to the LDL receptor is stimulated by SREBP-1.

We previously demonstrated that synergistic activation of the LDL receptor promoter by SREBP-1a and Sp1 required the major activation domains of Sp1. Additionally, the LDL receptor required the C domain for full stimulation (43). Domain C is a ~115-amino-acid region that is not critical for synergistic activation by multiple Sp1 sites as in the SV40 promoter (29) or for synergistic activation mediated by Sp1 and NF-κB in the human immunodeficiency virus long terminal repeat promoter (30). As mentioned above, Sp1 and Sp3 have overall similar domain structures but lack extensive amino acid sequence identity outside the zinc finger DNA binding domain. However, one exception to this, as noted above, is a small stretch contained within the C domain where Sp1 and Sp3 have identical residues at 17 of 27 consecutive positions (Fig. 1B). Part of this region is also conserved between several Sp1 family members, including the *Drosophila* homolog *btd* (42). *btd* is a *Drosophila* gap gene, and loss-of-function *btd* mutants die, leaving an embryo with a button-like protrusion from the anterior end.

To determine if the conserved region encompassing the Btd domain was a determinant in domain C that was required for SREBP-Sp1 synergy, we prepared an Sp1 mutant that specifically lacked the 31 amino acids of Sp1 shown in Fig. 1B. We compared the effect of this small deletion to that of deletion of the entire 115-amino-acid C domain on synergistic activation of the LDL receptor. The results (Fig. 4A) demonstrate this Btd deletion mutant is defective for activation of the LDL receptor promoter. In fact, the results are indistinguishable from those observed when the entire C domain is deleted (Fig. 4A, lanes 1 to 3). In contrast, deletion of neither the Btd region nor the entire C domain impaired activation of the SV40 early promoter (Fig. 4A, lanes 10 to 12). These observations are consistent with our earlier study (43).

Since the promoters for ACC and FAS are also activated by the combined action of SREBP-1 and Sp1, we evaluated the effects of the C-domain deletions on activation of these key promoters of fatty acid biosynthesis. Surprisingly, neither deletion altered synergistic activation of ACC and FAS by SREBP-1a and Sp1 (Fig. 4A, lanes 4 to 9). Thus, even though the same two proteins are required, the mechanism for activation of ACC and FAS must be at least partially distinct from activation of the LDL receptor.

Like Sp1, SREBP-1 is a member of a gene family, and so far only one additional member has been identified. SREBP-1 and -2 are highly similar to each other throughout their coding regions. They are most similar (71% identity) in the bHLHZip domain (14). We analyzed the ability of SREBP-2 to function together with Sp1 for activation in SL2 cells, and the results obtained were very similar to those for SREBP-1a (compare Fig. 4A and B). Thus, both SREBP proteins are capable of activating transcription along with Sp1.

Since the Btd and C-domain mutants of Sp1 were not de-

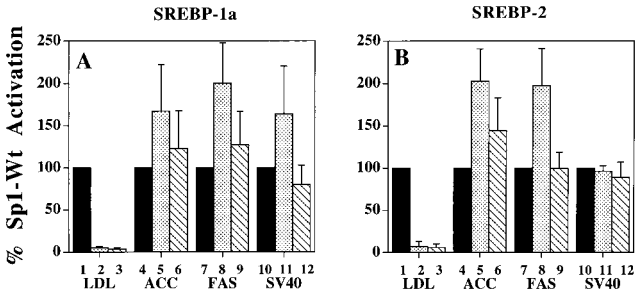

FIG. 4. Sp1 domains involved in activation of the LDL, ACC, and FAS promoters with SREBP-1a and SREBP-2. Wild-type or mutant derivatives of Sp1 (25 ng/60-mm-diameter dish) were transfected into *Drosophila* SL2 cells along with the pPACSREBP-1a (A) or pPACSREBP-2 (B) expression vector (25 ng/dish). Promoters for wild-type LDL receptor, ACC, FAS, and the six-GC-box-containing basal SV40 early gene were used as reporters. Data for wild-type Sp1 (Sp1-Wt) (filled bars), Sp1ΔC (dotted bars), and Sp1ΔBtd (cross-hatched bars) are presented as percentages of wild-type activity relative to each reporter alone. The 100% of control values for fold activation (calculated as described in the legend to Fig. 2) when pPACSp1 was cotransfected with wild-type pPACSREBP-1a (A) were as follows: LDL, 111.2; ACC, 444; FAS, 33.6; and SV40, 33.8. The 100% of control for fold activation with pPACSREBP-2 (B) were as follows: LDL, 60.5; ACC, 362; FAS, 49.25; and SV40, 28. The SV40 early promoter was evaluated in the absence of SREBP cotransfection since its multiple Sp1 sites respond to Sp1 only and are not significantly affected by SREBP (32). Data represent the averages of at least three separate transfection experiments performed in duplicate.

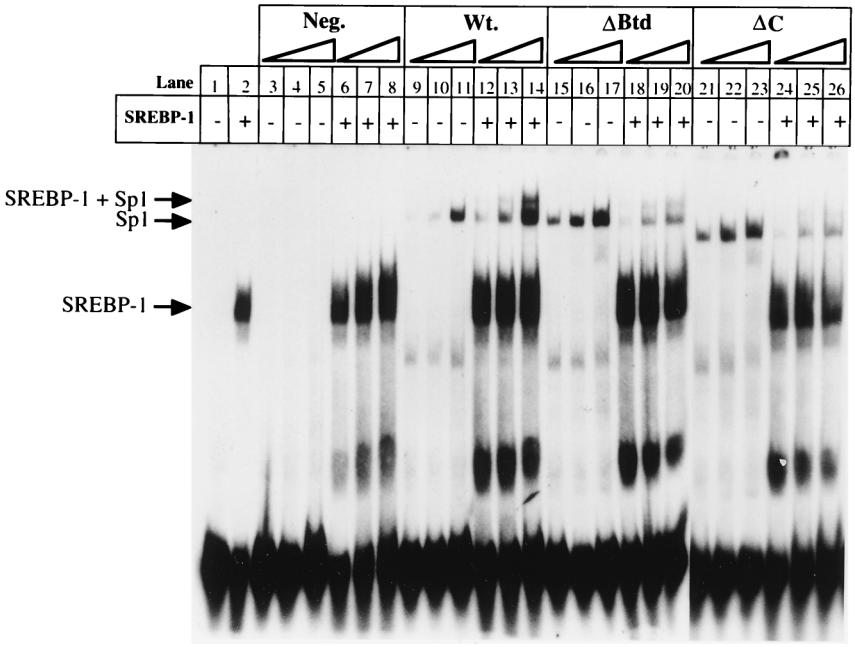

FIG. 5. SREBP-1 stimulation of Sp1 DNA binding. *Drosophila* SL2 cell nuclear extracts were prepared from normal SL2 cells (Neg.) or from cells transfected with the indicated Sp1 derivative as described in Materials and Methods. The wedges denote that increasing amounts (0.1, 0.3, and 1.0 μg) of total nuclear protein from cells transfected with the wild-type Sp1 plasmid (Wt.) or approximately equivalent amounts of extract from cells transfected with the indicated mutant Sp1 vectors or control extract were incubated with 0.25 ng of ^{32}P end-labeled DNA probe in the absence (−) or presence (+) of 50 ng of bacterially expressed SREBP-1 (amino acids 320 to 490). The proteins present in the three detectable complexes are indicated by arrows on the left.

fective for activation of the SV40, ACC, and FAS promoters, it is unlikely that the mutant proteins were not efficiently expressed in SL2 cells. In fact, in a previous study we showed that the C-domain mutant was expressed at levels similar to that of wild-type Sp1 (43). Also, in our earlier report we showed that even though the C-deletion mutant protein was expressed efficiently and bound DNA normally, it was defective for synergistic DNA binding along with SREBP-1a (43).

To determine if the Btd mutant was similarly affected, we evaluated the effects of adding recombinant SREBP-1 on the level of Sp1-specific DNA binding activity recovered from nuclear extracts of SL2 cells that had been transfected with the different Sp1 derivatives. Consistent with our earlier studies (43) and the results of Fig. 3, binding of wild-type Sp1 was efficiently stimulated and binding of the mutant lacking the entire C domain was not stimulated by SREBP-1 (Fig. 5, lanes 9 to 14 and 21 to 26). Binding of the smaller Btd deletion mutant was also not enhanced (Fig. 5; compare lanes 15 to 17 with lanes 18 to 20). Thus, deletion of the 31 amino acids including the conserved Btd domain results in the same phenotype as removal of the entire 115-amino-acid C domain. In fact, binding of the Btd and domain C deletion mutants appeared to be slightly inhibited by the inclusion of SREBP in the gel shift assay (compare, for example, lanes 15 to 17 with lanes 18 to 20 in Fig. 5). Although this was not revealed in our earlier study (43), it is possible that the binding of SREBP would sterically interfere with that of Sp1 in the absence of its stimulatory domain. This follows from the observation that the regions protected by each of SREBP and Sp1 in this region of the LDL receptor promoter significantly overlap in a DNase I footprinting experiment (32).

The results of Fig. 4 show that the Sp1 Btd domain is only required for activation of the LDL receptor even though activation of the ACC and FAS promoters also requires both SREBP and Sp1. Therefore, the mechanism for activation of ACC and FAS must be at least partially distinct and may depend on the precise arrangement of the SREBP and Sp1 sites in the different promoters. A structural comparison of the FAS and ACC promoters relative to the LDL receptor reveals a fundamental difference (Fig. 6A). In the LDL receptor promoter, there is a single SREBP site, whereas there are two closely spaced sites in the sterol regulatory regions of both the ACC and FAS promoters. Thus, the Sp1 Btd domain may be uniquely required when there is a single SREBP recognition site.

To test this hypothesis, we compared activation of the wild-type LDL receptor promoter to activation of a synthetic derivative containing three tandem copies of the LDL SREBP binding site linked to a single Sp1 site. We previously showed that this plasmid was efficiently regulated by sterols in cultured mammalian cells and that its efficient expression in SL2 cells required cotransfection of both Sp1- and SREBP-1a-expressing plasmids (32). As in our earlier studies, the wild-type LDL receptor promoter was significantly activated by the combination of SREBP-1a and wild-type Sp1, and as expected, activation was lost upon deletion of the Btd box as well as the entire C domain (Fig. 6B lanes 1, 3, and 5). Also as in our earlier studies, the synthetic construct was activated efficiently by SREBP-1a and wild-type Sp1. Additionally, this construct was still significantly activated by the Sp1ΔC and Btd domain mutants, although the degree of activation was three to fourfold lower than observed for wild-type Sp1 (Fig. 6B, lanes 2, 4, and 6). Thus, the presence of multiple SREBP sites can partially explain why activation of the ACC and FAS promoters does not require the Btd domain. To analyze this further, we attempted to evaluate activation of mutant versions of either the ACC or FAS promoter where one of the two neighboring SREBP sites in each promoter was disrupted. Unfortunately, a mutation of just one site in either promoter resulted in a dramatic loss in promoter activity (references 22 and 23 and

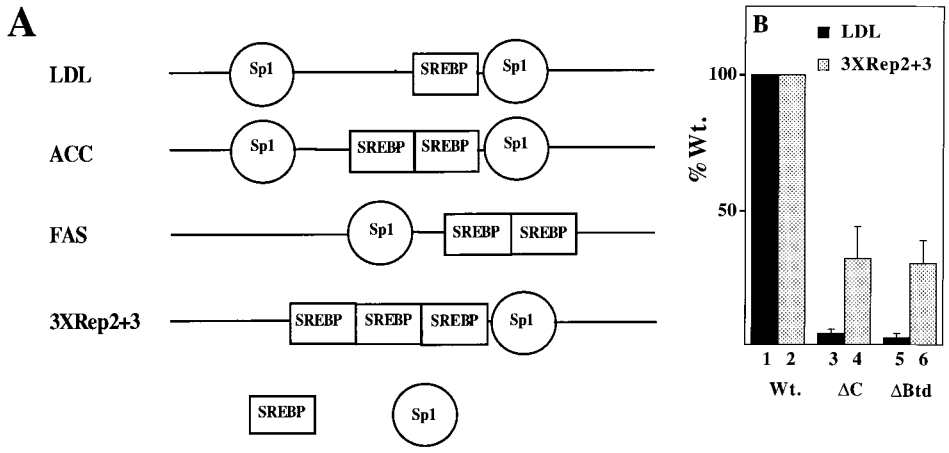

FIG. 6. Multiple SREBP sites partially overcome the requirement for the Sp1 Btd domain. (A) Schematic representations of the human wild-type LDL receptor, rat ACC, and rat FAS promoters and a synthetic promoter containing three copies of the LDL receptor SREBP site linked to a Sp1 site from the LDL receptor promoter (3XRep2+3 [32]). (B) *Drosophila* SL2 cells were transfected (25 ng/dish) with wild-type (Wt.) or mutant derivatives of Sp1 and the SREBP-1a expression vectors and analyzed as described in Materials and Methods. The wild-type LDL receptor promoter and the synthetic promoter 3XRep2+3 fused to the luciferase gene were used as reporters as indicated. Data represent the averages of three individual transfection experiments performed in duplicate.

unpublished data). This made it impossible for us to perform these comparative studies.

DISCUSSION

In our previous studies we showed that both SREBP and Sp1 were required to activate the LDL receptor promoter (32, 43). Since Sp1 is a member of a multigene family, it was important to determine if SREBPs could function together with other Sp1-related proteins to activate transcription. Plasmids expressing either Sp1 or the related Sp3 protein were transfected into SL2 cells which lack endogenous Sp1-related activity along with SREBP-expressing plasmids and reporters for the LDL receptor, ACC, and FAS. The results (Fig. 2) demonstrate that Sp3 can take the place of Sp1 for activation of all three of these promoters.

Stimulation of the LDL receptor promoter by SREBP and Sp1 occurs at two separate steps in transcriptional activation. First, SREBP stimulates Sp1 to bind to its adjacent site, and then both proteins activate transcription more efficiently than either one alone (43). The results in Fig. 3 demonstrate that SREBP stimulates Sp3 binding to the LDL receptor promoter as well. Thus, Sp1 and Sp3 appear to be functionally interchangeable for activation of the LDL receptor in combination with SREBP.

An alignment of the Sp1 and Sp3 proteins that emphasizes their similarity reveals several related structural domains throughout their coding regions. However, there are no extensive blocks of amino acid identity outside the zinc finger DNA binding region (Fig. 1A). The exception is one small area at the carboxy-terminal end of the C domain where Sp1 and Sp3 are identical at 17 of 27 consecutive residues. Ten of these amino acids are also conserved in several other Sp1-related proteins (Fig. 1B) as was first noted in a report characterizing a *Drosophila* Sp1 homolog Btd (42). Btd is a gap gene product whose expression is required in the anterior region of the developing *Drosophila* embryo for normal head development.

Initially, the importance of the C domain of Sp1 for activation was not fully appreciated since it was not critical for activation of all promoters that require Sp1 (5, 6, 29). For example, activation of the multiple-Sp1-site-containing SV40 early promoter occurs through synergistic Sp1-Sp1 interactions and requires the major glutamine activation domains A and B as well as the carboxy-terminal D domain. The C domain, which contains the Btd box, was not absolutely critical for activation of the SV40 promoter (5, 6, 29).

The significance of the C domain was fully realized in studies of the LDL receptor promoter, which contains two important Sp1 sites and a single recognition site for SREBPs. Synergistic activation required both SREBP-1a and the major activation domains A, B, and D of Sp1 (43). Additionally, deletion of the C domain of Sp1 abolished activation of the LDL receptor promoter (43). Since Sp1 and Sp3 share a region of extensive sequence identity at the carboxy end of domain C, part of which corresponds to the Btd box, we reasoned that this small conserved domain might be the critical determinant required for transcriptional activation and stimulated DNA binding along with SREBP. The results in Fig. 4 demonstrate that deletion of this conserved region from Sp1 resulted in a mutant that was just as defective for synergistic activation of the LDL receptor promoter as deletion of the entire C domain. Furthermore, stimulation of Sp1 DNA binding by SREBP-1 was also lost by deletion of just the Btd domain (Fig. 5). Thus, the region encompassing the conserved Btd box is a critical determinant from the C domain, and it is functionally conserved in Sp3.

Previous reports have suggested that Sp3 functions as a positive or negative regulator of gene expression (11, 38), and our experiments clearly show that Sp3 acts as a positive regulator. It is possible that Sp3 as well as other Sp1 family members can activate or repress specific genes, and the exact functional role could depend on the interaction of Sp1 factors with negative regulators (4, 26), as well as on the combined effect for all of the transcriptional regulatory proteins that influence the activity of the specific promoter being investigated. Indeed, a recent report indicates that Sp3 may function as an activator where there is a single recognition site and a repressor where multiple tandem recognition sites are present (24).

Sp2, another Sp1 family member, failed to activate the LDL receptor promoter along with SREBP-1a (data not shown). Sp2 also contains the conserved Btd box; however, it does not bind to the Sp1 GC box recognition site with high affinity, and it lacks the equivalent A and D domains which are required for Sp1 and Sp3 to synergistically activate transcription (reference

20 and data not shown). Sp4 is more similar to Sp1 and Sp3 since it contains the major glutamine activation domains (11). However, Sp4 failed to activate transcription synergistically at a promoter containing two adjacent Sp1 sites (10). It would be interesting to analyze the ability of Sp4 to activate the LDL, ACC, and FAS promoters along with SREBP.

Another isoform of SREBP, SREBP-2, is encoded by a distinct gene, and its mRNA does not appear to be subjected to alternative processing (14). The results in Fig. 4B demonstrate that SREBP-2 can substitute for SREBP-1a in activation of the LDL receptor, ACC, and FAS promoters. Thus, SREBP-2 can efficiently activate transcription along with Sp1 and Sp3. Although this observation may suggest at least a partially redundant function for these two proteins, SREBP-2 is the isoform that was specifically activated in livers of animals fed a diet designed to induce cholesterol uptake and synthesis (33). Further studies are required to clearly define unique roles for SREBP-1 and -2.

As discussed above, the critical determinant of domain C is the region encompassing the Btd box, since the Sp1 mutant lacking this region was just as defective as a mutant with a deletion of the entire ~115-amino-acid C domain for activation of LDL receptor promoter along with both SREBP-1a and SREBP-2 (Fig. 4). Importantly, DNA binding by the Btd protein was also not stimulated by SREBP-1 (Fig. 5). Additionally and consistent with our earlier studies (43), deletion of the Btd domain as well as the entire C domain did not alter activation of the SV40 promoter (Fig. 4). These results indicated that synergistic activation by Sp1 can occur by at least two separate mechanisms: one that requires only multiple Sp1 binding sites and does not require the Btd domain, and one where Sp1 works together with another protein such as SREBP that requires the Btd domain.

Another mode of activation by Sp1 is suggested by other results reported here. Activation of the ACC and FAS promoters also occurs through the concerted action of SREBP and Sp1 (references 1 and 21 and Fig. 2). However, deletion of the Btd domain, which abolished activation of the LDL receptor promoter, surprisingly did not decrease activation of the ACC or FAS promoter (Fig. 4). Thus, even though activation of the LDL receptor, ACC, and FAS promoters requires SREBP and Sp1, the domains required for the response are distinct. This observation implies there is a fundamental difference in the mechanism for activation of the ACC and FAS promoters relative to the activation of the LDL receptor.

A close examination of the LDL receptor, ACC, and FAS promoters revealed a basic difference in their architecture (Fig. 6A); the LDL receptor promoter has only a single SREBP binding site, whereas the ACC and FAS promoters each have two tandem sites (references 21 and 23 and Fig. 6A). We postulated that the Btd domain might be critical for activation of promoters containing a single binding site for SREBP. To test this hypothesis, we analyzed the activation of a synthetic construct containing three tandem SREBP binding sites linked to a single Sp1 site. We previously demonstrated this synthetic promoter was efficiently regulated by sterols in cultured cells (32).

The results (Fig. 6B) demonstrate that this promoter was still significantly activated by the Sp1 mutants which lack either the Btd box or the entire C domain. However, the activity is still three- to fourfold below that observed for activation by wild-type Sp1.

We attempted to evaluate the role of the Btd domain in another natural promoter setting where only one SREBP site was present through the analysis of mutant versions of the ACC and FAS promoters, which normally contain two neighboring SREBP sites. Unfortunately, a mutation of a single site in either promoter resulted in a dramatic loss in promoter activity that prevented the analysis (references 22 and 23 and unpublished data).

Thus, the presence of multiple SREBP binding sites in the ACC and FAS promoters may partially explain why the Btd box region is not required for efficient stimulation of their expression. In addition to the multiple SREBP sites, the ACC and FAS promoters differ from the LDL receptor in the spacing and the arrangement of the SREBP and Sp1 sites, which probably also has an influence on the mechanism of activation.

Of the three genes discussed here which require SREBP and Sp1 for activation, one is involved in cholesterol uptake (LDL receptor) and two are involved in fatty acid biosynthesis (ACC and FAS). Although these two different pathways must be linked during times of rapid cell growth for accumulation of new membrane lipid and possibly for the ordered assembly of very low density lipoprotein particles in the liver, which requires both cholesterol and fatty acids, it is also important that the two pathways be controlled separately in different tissues and in response to fluctuations in the availability of specific nutrients. The separation of cholesterol uptake from fatty acid biosynthesis may be achieved in part through a mechanism targeting the Btd domain of Sp1.

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