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Domains of transcription factor Sp1 required for synergistic activation with sterol regulatory element binding protein 1 of low density lipoprotein receptor promoter

(synergistic transcriptional activation/cotransfection)

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ABSTRACT **Feedback regulation of transcription from the low density lipoprotein (LDL) receptor gene is fundamentally important in the maintenance of intracellular sterol balance. The region of the LDL receptor promoter responsible for normal sterol regulation contains adjacent binding sites for the ubiquitous transcription factor Sp1 and the cholesterol-sensitive sterol regulatory element-binding proteins (SREBPs). Interestingly, both are essential for normal sterol-mediated regulation of the promoter. The cooperation by Sp1 and SREBP-1 occurs at two steps in the activation process. SREBP-1 stimulates the binding of Sp1 to its adjacent recognition site in the promoter followed by enhanced stimulation of transcription after both proteins are bound to DNA. In the present report, we have defined the protein domains of Sp1 that are required for both synergistic DNA binding and transcriptional activation. The major activation domains of Sp1 that have previously been shown to be essential to activation of promoters containing multiple Sp1 sites are required for activation of the LDL receptor promoter. Additionally, the C domain is also crucial. This slightly acidic ≈120-amino acid region is not required for efficient synergistic activation by multiple Sp1 sites or in combination with other recently characterized transcriptional regulators. We also show that Sp1 domain C is essential for full, enhanced DNA binding by SREBP-1. Taken together with other recent studies on the role of Sp1 in promoter activation, the current experiments suggest a unique combinatorial mechanism for promoter activation by two distinct transcription factors that are both essential to intracellular cholesterol homeostasis.**

Intracellular sterol balance is maintained by a nutritional feedback mechanism whereby excess sterol shuts off the production of key proteins involved in its accumulation (1, 2). The protein involved in cholesterol uptake is a cell surface receptor that internalizes cholesterol-rich low density lipoprotein (LDL) particles from outside the cell. The LDL receptor gene is efficiently regulated by cholesterol, and its simple promoter is composed of three related sequence elements (repeats 1–3) with 12–16 identical base pairs. These three repeats are located upstream of a TATA box-like element (3–5).

Repeat 2 contains a special sequence, the sterol-regulatory element 1 (SRE-1), that is uniquely responsible for regulation by sterols. The SRE-1 within repeat 2 was identified by mutational studies as a 10-base-pair element that binds a family of basic helix–loop–helix (b-HLH) zipper (b-HLHzip) proteins called the SRE-binding proteins (SREBPs) (5–8). The SRE-1 functions as a conditionally positive element that activates expression only when sterol levels are low. It cannot function efficiently by itself, even when present in multiple copies (8, 9). Repeats 1 and 3 bind the universal transcription factor Sp1, and, in the native LDL receptor promoter, these two Sp1-binding sites are required in addition to the SRE-1 for efficient sterol regulation of transcription (4).

In a previous report (9), we demonstrated that inversion of the Sp1 site of repeat 3 prevents sterol regulation and that the Sp1 site could not be replaced by other common transcription factor sites. In addition, we showed that SREBP-1 increased the binding of Sp1 to repeat 3 *in vitro* and that both proteins synergistically activated the LDL receptor promoter in *Drosophila* tissue culture cells that lack endogenous Sp1. These studies documented a special role for Sp1 in sterol regulation of the LDL receptor gene.

The present studies were designed to further address the role of Sp1 in sterol regulation. By using cotransfection assays in *Drosophila* cells with wild-type and mutant forms of Sp1, we have characterized the protein domains that are required for concerted transcriptional activation and DNA binding. The results indicate that the slightly acidic domain C of Sp1 is specifically required for synergistic activation with SREBP-1. This domain of Sp1 is not essential for synergistic activation by multiple Sp1 sites (10) or in combination with NF-kB (11). Furthermore, we also show that domain C is essential for the SREBP-1-induced stimulation of DNA binding by Sp1. Therefore, synergistic activation by Sp1 and SREBP-1 occurs by a previously unrecognized mechanism.

MATERIALS AND METHODS

Plasmids. pGL2-basic (no promoter) and pGL2-promoter [containing the wild-type simian virus 40 (SV40) promoter with six Sp1 sites] were purchased from Promega and were used as the source of the luciferase gene. Standard techniques were used in all cloning procedures (12). The wild-type human LDL receptor promoter from position −225 to −116 (relative to the translation initiating ATG) containing repeats 1–3 was inserted upstream of the TATA sequence of the pSyn-TATA-luc plasmid, which contains the hamster hydroxymethylglutaryl-CoA synthase sequence from position −28 to +39 relative to the mRNA start site inserted between the *Xho* I and *Hin*dIII sites in pGL2-basic, and it has been described before (9). The pSyn-TATA-luc plasmid contains only a TATA box and is not expressed efficiently in transfection studies (9). Plasmids pPACSp1, pPACSp1ΔA, and pPACSp1ΔD have been described (10, 13, 14) and were obtained from Al Courey (University of California, Los Angeles). pPACSp1ΔC (10) was obtained from R. Tjian (University of California, Berkeley). All of the Sp1 expression plasmids contain the corresponding regions from the Sp1 coding sequence inserted downstream of

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Abbreviations: LDL, low density lipoprotein; SRE, sterol-regulatory element; SREBP, SRE-binding protein; b-HLH, basic helix–loop–helix; SV40, simian virus 40; b-HLHzip, b-HLH zipper.

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the *Drosophila* actin 5C promoter in the pPAC expression vector (13). The SREBP-1 clone used in this study represents amino acids 1–490 of the full-length protein in the pPAC expression vector and was described previously (9).

For expression in *Escherichia coli*, two different portions of the SREBP-1 protein were used in these experiments. One represents amino acids 1–490 of the full-length protein and is identical in sequence to the expression clone used in transfection studies (see above). The other smaller protein contains the sequence from amino acid residue 321 to residue 490, which contains the b-HLHzip region of SREBP-1. Both SREBP-1 derivatives were expressed as fusion proteins in *E. coli* with the histidine tag sequence at the amino terminus in the pRSET plasmid (Invitrogen). Both proteins were purified by metal-affinity chromatography. The purity and concentration of each purified protein were assessed by SDS/PAGE analysis performed with marker proteins followed by staining with Coomassie blue.

Transient DNA Transfections. *Drosophila* SL2 cells were obtained from Al Courey and cultured at 25°C in Shields and Sang *Drosophila* media (Sigma) containing 10% (vol/vol) heat-inactivated fetal bovine serum. They were seeded at 1.2×10^6 cells per 60-mm dish and were transfected by a standard calcium phosphate coprecipitation method (14).

Enzyme Assays. Luciferase activities were measured in a luminometer with a luciferin reagent from Promega. The protein concentration of all samples was determined with the Bio-Rad kit. The data presented here are average values of the ratio of luciferase to total cell protein, derived from at least three independent transfections for each plasmid.

Nuclear Extract Preparation. SL2 cells were transfected as described above, and nuclear extracts were prepared as described (15). The SL2 nuclear extracts were used in standard gel mobility-shift reactions as described below.

Gel Mobility-Shift Analyses. The proteins alone or together were incubated on ice with a ^{32}P-labeled probe consisting of a single copy each of repeats 2 and 3 of the wild-type human LDL receptor promoter. The binding conditions and electrophoresis were performed by standard methods as described (9). The region of the dried gel corresponding to the labeled DNA spots was excised and subjected to liquid scintillation counting.

RESULTS

As a first step in the identification of the protein domains of Sp1 involved in synergistic activation of the LDL receptor promoter, we performed a series of titration experiments to determine appropriate amounts of each expression plasmid to use in the transfection assays. A very small degree of activation was observed when each expression construct was transfected alone (Fig. 1 *Upper*) and the inclusion of both plasmids together resulted in a significant stimulation of luciferase expression from the LDL receptor promoter. When a constant amount of the *Drosophila* vector expressing SREBP-1 was mixed with increasing concentrations of the wild-type Sp1-expressing vector, pPACSp1, the results presented in Fig. 1 *Upper* were obtained. Maximum levels of activation occurred when 50–100 ng of pPACSp1 was used, and higher amounts resulted in a decrease in expression. A titration with pPACSp1 alone did not result in significant activation over the value shown on the graph (data not shown). A similar experiment utilizing a constant amount of pPACSp1 and increasing concentrations of the *Drosophila* vector expressing human SREBP-1 showed a dose-dependent increase in luciferase expression up to 300 ng of DNA followed by a decline (Fig. 1 *Lower*). Fig. 1 *Lower* also shows that the SREBP-1 expression plasmid was very ineffective by itself, even at very high levels of DNA.

To identify the protein domains of Sp1 involved in synergistic activation, we performed a series of cotransfection

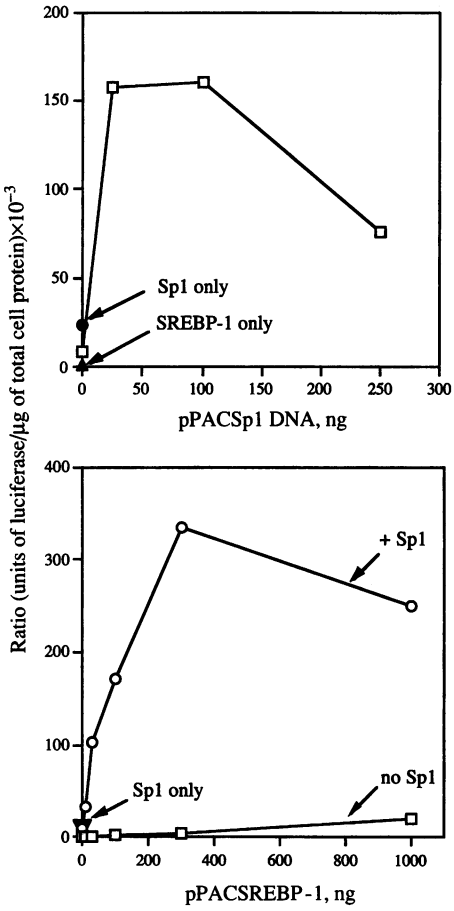

FIG. 1. Synergistic activation of the LDL receptor promoter by Sp1 and SREBP-1. (*Upper*) Titration of pPACSp1 DNA. The indicated amount of pPACSp1 expression plasmid was transfected along with 25 ng of the pPACSREBP-1 expression plasmid, and cell extracts were assayed for luciferase activity (units) as a function of the protein concentration of the extract (μg per assay) as described in *Materials and Methods* (□). The amount of activity when the Sp1 (50 ng) (●) or SREBP-1 (▲) expression plasmids were transfected alone is also noted. (*Lower*) Titration of pPACSREBP-1 DNA. Similar to *Upper* except that the concentration of pPACSREBP-1 was varied in the absence (□) or presence (○) of 50 ng of pPACSp1. The activity when Sp1 was transfected alone is also noted (▼).

experiments with mutant derivatives of Sp1. On the basis of the results shown in Fig. 1, we chose to use 25 ng of SREBP-1 and 50 ng of pPACSp1 or an appropriate mutant in our standard experiment. With these values we observed a reproducible increase of 65-fold in LDL receptor promoter activity when wild-type forms of both proteins were included in the transfection assay (Fig. 2). When mutant derivatives of Sp1 were substituted, we noted that removal of domain A, C, or D abolished the synergistic activation. Several other mutant forms of Sp1 were also analyzed, but since they all lacked at least one of the domains mentioned above, synergistic activation was not observed (data not shown).

We also analyzed the ability of the Sp1 derivatives to synergistically activate the multiple-tandem Sp1 sites of the SV40 early promoter. The results of Fig. 2 show that domains A and D were required for synergistic activation. However, removal of domain C still resulted in substantial activation of the SV40 promoter. These results are in agreement with those previously reported for synergistic activation by multiple Sp1 sites (10) and indicate that domain C of Sp1 is uniquely required for synergistic activation by the combination of Sp1 and SREBP-1 on the LDL receptor promoter. To ensure that the Sp1 mutant proteins were expressed at levels equivalent to

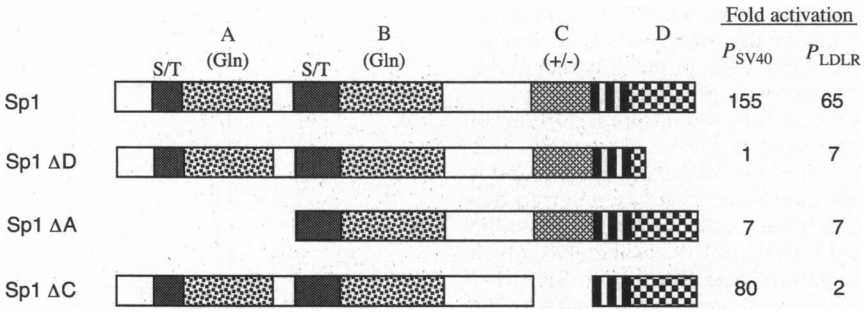

FIG. 2. Sp1 domains involved in activation of LDL receptor promoter with SREBP. (*Left*) Linear diagrams of the wild-type Sp1 protein and the indicated deletion derivatives. The basic diagram and the name given to each Sp1 derivative were taken from previous reports (10, 14). The position of the major activation domains A, B, C, and D is noted above the wild-type diagram. The location of the three zinc fingers involved in DNA binding is noted by the three vertical black boxes between domains C and D. Regions of the protein containing a high percentage of serine and threonine (S/T), glutamine (Gln), or charged (+/−) amino acids are indicated. The individual Sp1 derivatives were transfected into *Drosophila* SL2 cells along with a plasmid that expresses the first 490 amino acids of the wild-type SREBP-1 protein. (*Right*) The wild-type LDL receptor promoter (P_{LDLR}) or the six-GC-box-containing basal SV40 early promoter (P_{SV40}), each fused to the luciferase gene, was used as reporter plasmids as indicated at the right. The luciferase activity (normalized for total cell protein) obtained for each Sp1 derivative was divided by the value obtained for the reporter plasmid containing SREBP-1 alone to obtain the fold activation value. The numbers represent an average of at least four separate transfection experiments performed in duplicate for each plasmid. When the Sp1- or SREBP-1-expressing plasmids were added alone, activation was 3.5- or 1.6-fold over the LDL receptor reporter plasmid alone, respectively.

that of the wild-type protein in our transfection assays, we performed Sp1-specific gel mobility-shift assays with nuclear extracts prepared from transfected SL2 cells, since all of the Sp1 derivatives analyzed above do not remove the Sp1 DNA-binding domain. Transfection of the wild-type Sp1 protein-expressing plasmid resulted in a gel-shifted complex that was absent from nontransfected SL2 extract (Fig. 3; lanes 1–4). This band comigrated with the DNA complex containing Sp1 that was purified from HeLa cells, and it was specifically abolished by including an excess of unlabeled Sp1 site oligonucleotides in the binding reaction (data not shown). Nuclear extracts prepared from cells transfected with the mutant derivatives analyzed in Fig. 2 generated similar relative levels of Sp1 DNA binding activity (Fig. 3, lanes 5–9). The slight difference in migration rates for the Sp1–DNA complexes observed for the mutant extracts is consistent with the decrease in protein size predicted by each specific deletion. Since all of the mutant proteins are expressed at levels similar to the wild-type protein, the dramatic differences in activation are due solely to the inability of the mutant proteins to function with SREBP-1.

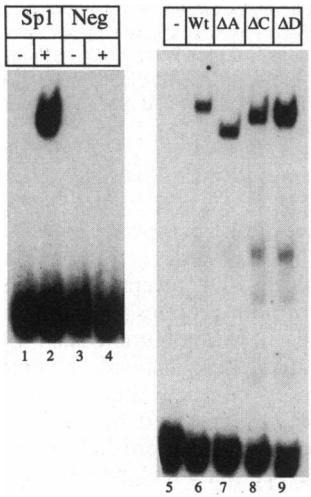

FIG. 3. Detection of Sp1 DNA binding activity in SL2 cell extracts. SL2 cells were transfected with the indicated Sp1 derivative, and nuclear extracts were prepared as described in text. Five micrograms of each extract was used in a standard gel-mobility shift assay for Sp1. "Neg" refers to an extract that was prepared from mock-transfected cells. The lanes marked with a minus sign indicate that the probe was electrophoresed without any added protein. Bands at the bottom of each lane are from the free probe, and the upper bands result from Sp1 protein–DNA complexes. Note that the protein–DNA complex from each Sp1 mutant exhibits a slightly faster migration rate due to the corresponding deletion in the protein.

In a previous report, we showed that synergistic activation by SREBP-1 and Sp1 is partially caused by an increase in Sp1 DNA-binding activity induced by SREBP-1 bound at an adjacent site (9). To determine if any of the domains of Sp1 involved in synergistic transcriptional activation were involved in synergistic DNA binding, we performed gel mobility-shift experiments with purified recombinant SREBP-1 and nuclear extracts prepared from SL2 cells expressing the wild-type or mutant derivatives of SP1 (Fig. 4). In our earlier studies, we used a cDNA clone expressing amino acids 1–490 of SREBP-1 and a DNA probe containing the wild-type sequence from the LDL receptor promoter containing the adjacent binding sites for Sp1 and SREBP-1 (9). In the present studies we used the same DNA probe and an expressed protein fragment of SREBP-1 containing the b-HLH domain and several carboxyl-terminal residues (amino acids 321–490 of SREBP-1). The data of Fig. 4 *Upper Left* show that this portion of SREBP-1 was sufficient for enhanced Sp1 DNA binding. When increasing amounts of SL2 nuclear extract expressing wild-type Sp1 were incubated alone (Fig. 4 *Upper Left*, lanes 3–5) or in the presence of SREBP-1 (lanes 6–8), a significant enhancement of Sp1 DNA binding activity was observed. This suggests that all of the information of SREBP-1 required for the enhanced DNA binding is contained in the sequence close to the DNA-binding domain. In fact, the smaller protein analyzed here was quantitatively just as active as the larger 1- to 490-amino acid protein for both simple binding to the LDL receptor SRE site as well as the synergistic binding along with Sp1 (data not shown). Consistent with our earlier studies (9), we observed an increase in both the Sp1–SREBP cocomplex with DNA as well as the Sp1-only protein–DNA complex. Based on other studies presented in the earlier manuscript, we suggested that the SREBP–DNA interaction was destabilized by Sp1 and the association is unstable to the gel electrophoresis conditions (9). Mutations of Sp1 that delete either domain A or D behaved very similarly to the full-length Sp1 protein in the gel mobility-shift assay (Fig. 4 *Upper Left* and *Lower*). However, the mutant form that lacked domain C was defective for synergistic DNA binding (Fig. 4 *Right*). We also determined that mutations that impair DNA binding by either protein (we deleted the basic domain of the SREBP-1 b-HLH or the zinc

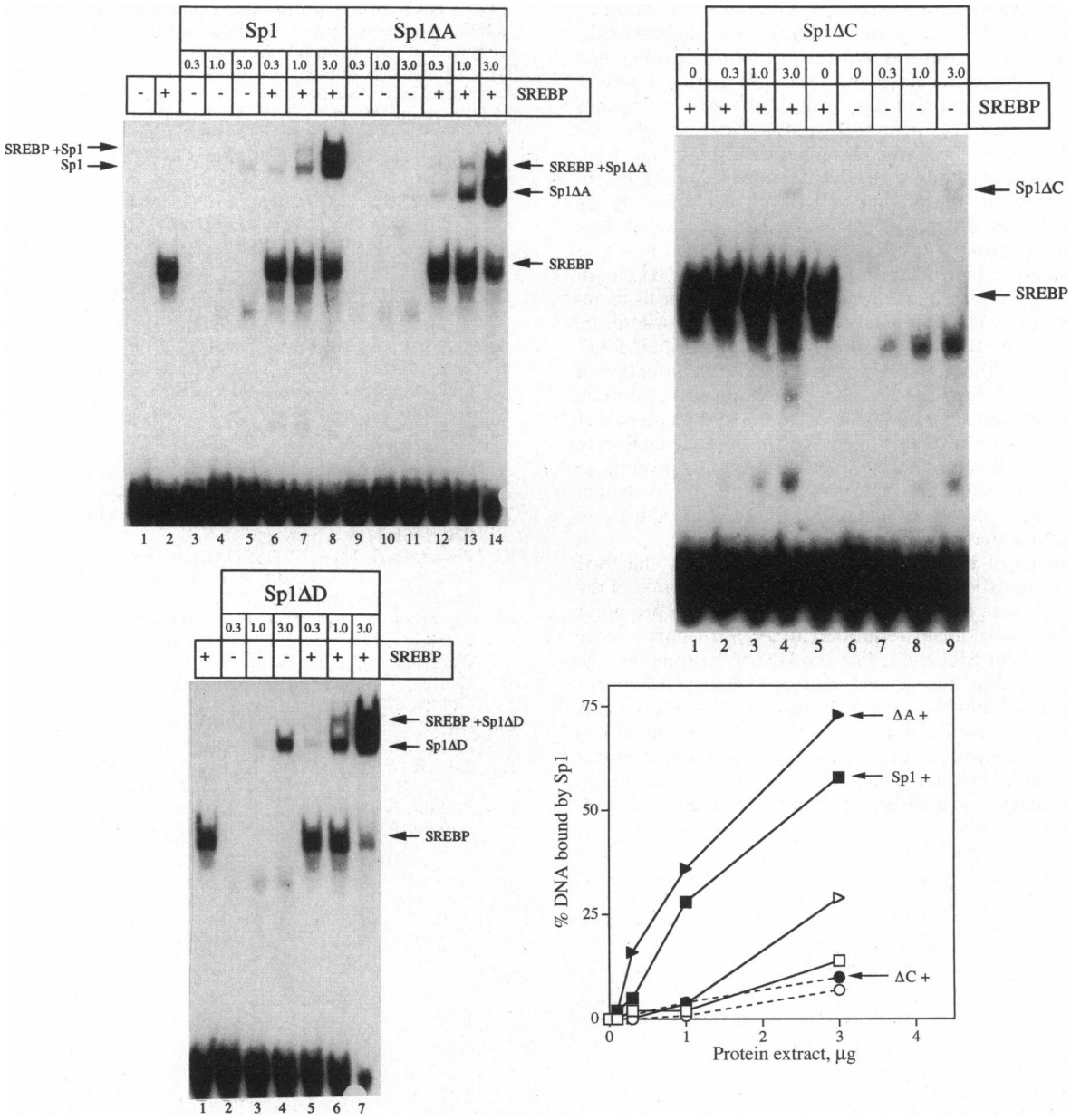

FIG. 4. Sp1 domains involved in synergistic DNA binding with SREBP. Nuclear extracts were prepared from SL2 cells transfected with Sp1 DNA or Sp1 DNA deletion derivatives as indicated and were included in a gel-mobility shift assay. The indicated amount of nuclear extract (μg) was incubated with the DNA probe alone (lanes −) or in the presence (lanes +) of 25 ng of a truncated SREBP-1 protein fragment with amino acids 320–490 containing the b-HLHzip domain. This protein was expressed in and purified from *E. coli.* Experiments in *Upper* were repeated, and after autoradiography, the bands were excised and the amount of radioactivity was determined by liquid scintillation counting. (*Lower Right*) The percentage of total DNA in the bound fraction was determined by calculating the ratio of radioactivity in the Sp1-containing bands to the total radioactivity in all bands in the specific lane. These are plotted for each sample as indicated. Open symbols denote the values obtained in the absence of SREBP-1, and closed symbols denote the values obtained in the presence of SREBP-1.

finger region of Sp1) abolish the synergistic effect *in vitro* (data not shown).

DISCUSSION

The experiments reported here were designed to define the domains of Sp1 involved in synergistic activation of transcription from the sterol-regulated LDL receptor promoter. Regions of Sp1 previously shown to be involved in synergistic activation by multiple Sp1 sites (10) were shown to be involved in synergistic activation by the combination of Sp1 and SREBP-1. Domain A of Sp1 contains functional "glutamine-rich" hydrophobic activation motifs (Fig. 2) that define an essential activation domain that is required for synergistic activation by multiple Sp1 sites (10). This domain is also essential for synergistic activation by the combination of Sp1 and SREBP (Fig. 2). The B domain of Sp1, like the A domain, contains "glutamine-rich" activation motifs (Fig. 2), and domain B is likely to be involved in synergistic activation of the LDL receptor in a manner similar to domain A. However, we did not analyze a B-domain mutation that did not also remove domain A or C (data not shown). Therefore, we were unable to unambiguously demonstrate a requirement for domain B. The D domain of Sp1 is also required for synergistic activation of multiple Sp1 sites (10) and by the combination of Sp1 and SREBP-1 as reported here. Domain D contains no obvious functional protein motif, and its role in synergistic activation is unclear. How-

ever, it appears not to be essential for simple activation by a single Sp1 site (10). Domain C of Sp1 has a slightly acidic overall character, contains clusters of both positive and negative charges, and carries out a modest-auxiliary role in activation of promoters containing multiple Sp1 binding sites (refs. 10, 13, and 14; also this study). Interestingly, this domain is crucial to synergistic activation of the LDL receptor promoter along with SREBP-1 (Fig. 2). The mechanism for this stimulation can be partially explained by the observation that domain C is also critical for synergistic DNA binding along with SREBP-1 (Fig. 4).

The amino-terminal acidic domain and the b-HLHzip motif of SREBP-1 are essential for synergistic activation in mammalian cells (16). These domains are also crucial to synergistic activation in the SL2 cotransfection assay (unpublished data). However, these domains are dispensable for the stimulation of DNA binding of Sp1 (Fig. 4). How communication between the b-HLHzip domain of SREBP-1 and the C domain of Sp1 occurs remains to be determined. We have been unable to demonstrate a direct interaction between the two proteins in solution (unpublished observations); therefore, it is likely that DNA binding by both proteins is an essential prerequisite to any interaction that may occur.

Interestingly, it has been demonstrated recently that Sp1 functions synergistically with NF-kB (p65) in stimulation of the human T-cell lymphotropic virus type 1 (HTLV-1) promoter (11, 17), but the domain requirements of Sp1 appear to be different than for SREBP-1. For the HTLV-1 promoter, the DNA binding domains of p65 and Sp1 are sufficient for synergistic DNA binding, and any mutation that retains any one of the Sp1 activation domains A, B, or D is still functional in synergistic activation. Clearly, Sp1 is a multidimensional activator, and its modular domains confer upon it the potential to participate in a diverse collection of regulated transcriptional processes.

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