Co-stimulation of Promoter for Low Density Lipoprotein Receptor Gene by Sterol Regulatory Element-binding Protein and Sp1 Is Specifically Disrupted by the Yin Yang 1 Protein*

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Sterol regulation of gene expression in mammalian cells is mediated by an interaction between the cholesterol-sensitive sterol regulatory element-binding proteins (SREBPs) and promoter-specific but generic co-regulatory transcription factors such as Sp1 and NF-Y/CFB. Thus, sterol-regulated promoters that require different co-regulatory factors could be regulated independently through targeting the specific interaction between the SREBPs and the individual co-regulatory proteins. In the present studies we demonstrate that transiently expressed yin yang 1 protein (YY1) inhibits the SREBP-mediated activation of the low density lipoprotein (LDL) receptor in a sensitive and dose-dependent manner. The inhibition is independent of YY1 binding directly to the LDL receptor promoter, and we show that the same region of YY1 that interacts in solution with Sp1 also interacts with SREBP. Furthermore, other SREBP-regulated genes that are not co-regulated by Sp1 are either not affected at all or are not as sensitive to the repression. Thus, the specific interaction that occurs between SREBPs and Sp1 to stimulate the LDL receptor promoter is a specific target for inhibition by the YY1 protein, and we provide evidence that the mechanism can be at least partially explained by the ability of YY1 to inhibit the interaction between SREBP and Sp1 in solution in vitro. The LDL receptor is the key gene of cholesterol uptake, and the rate-controlling genes of cholesterol synthesis are stimulated by the concerted action of SREBPs along with coregulators that are distinct from Sp1. Therefore, repression of gene expression through specifically targeting the interaction between SREBP and Sp1 would provide a molecular mechanism to explain how cholesterol uptake can be regulated independently from cholesterol biosynthesis in mammalian cells.

Cholesterol homeostasis in mammals is achieved through a sensitive regulatory circuit that responds to changes in both the need and availability of cellular cholesterol (1). High levels trigger a classic negative feedback loop that prevents its over accumulation. This negative regulatory process is governed by a conditionally positive mechanism (2). Suboptimal cholesterol levels result in the proteolytic release of the membrane-bound precursor forms of the sterol regulatory element-binding transcriptional activator proteins or SREBPs. Once liberated from their membrane anchor, the mature transcription factors accumulate in the nucleus where they activate genes required for cholesterol uptake and biosynthesis (2, 3).

The SREBP proteins also activate genes of fatty acid metabolism (2, 3) and thus provide a molecular link to coordinate the activation of the two major lipid pathways in mammals. Although there are situations in metabolism where coordinate regulation of fatty acids and cholesterol is essential such as for new cellular membrane synthesis and possibly for the assembly of lipoprotein particles, there are other times where separate regulation is required, for example when excess dietary calories are converted into triglycerides for storage or when cholesterol is needed for the biosynthesis of steroid hormones or bile acids. Recent work has suggested that this may be achieved at least in part through the preferential activation of genes of either fatty acid or cholesterol metabolism by different isoforms of the SREBPs (4, 5).

It is at least equally important for cells to have a mechanism to selectively activate cholesterol uptake from its endogenous biosynthesis to maintain homeostasis in response to wide fluctuations in the availability of cholesterol in the diet. The SREBP proteins are inefficient transcriptional regulatory proteins by themselves, and they require a coregulatory factor and its associated DNA-binding site to efficiently activate all of the carefully studied SREBP target genes analyzed to date (2, 3). The identity and binding site location of the coregulatory factor relative to the binding site(s) for SREBP in individual promoters is likely to provide at least part of the mechanistic basis for the independent regulation for individual genes of cholesterol synthetic enzymes versus the LDL receptor.

In our search for regulatory proteins that selectively affect either cholesterol uptake or cholesterol synthesis, we report in the current studies that the LDL receptor gene promoter is selectively down-regulated by the yin yang 1 (YY1) protein. YY1 has been characterized as both an activator and repressor of specific gene expression in other systems (6). We specifically demonstrate that the interaction between SREBP and Sp1 that is necessary for the two proteins to synergistically activate the

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1 The abbreviations used are: SREBP, sterol regulatory element-binding protein; HMG, 3-hydroxy-3-methylglutaryl; LDL, low density lipoprotein; YY1, yin yang 1 protein; CMV, cytomegalovirus; GST, glutathione S-transferase; btd, buttonhead; aa, amino acid(s); HSV, herpes simplex virus; PAGE, polyacrylamide gel electrophoresis; bHLH, basic helix-loop-helix.
Transcriptional Repression of LDL Receptor Promoter by YY1

LDL receptor promoter is inhibited by YY1. Along with other recent data these studies provide a regulatory mechanism that could preferentially alter cholesterol uptake with minimal or no effect on cholesterol biosynthesis.

MATERIALS AND METHODS

Plasmids—CMV-SREBP-1a and CMV-SREBP-2 contain aa 1–490 or aa 1–481 of the full-length human proteins in the CMV-5 expression vector and have been previously described (9). A prokaryotic expression vector for production of full-length human YY1 as a poly-histidine fusion protein was a gift of Tom Shenk (Princeton University). CMV-YY1 was prepared by excising the YY1 coding sequence and inserting it into the CMV-5 expression vector. YY1 GAL4 fusion proteins were prepared by designing hybridization primers that were subsequently used to amplify specific regions of the YY1 cDNA using CMV YY1 as a template. The YY1 coding fragments were then fused in frame at the carboxy end of the GAL4 aa 1–147 fragment in the pSG424 expression vector (10). Amino acids 1–147 of GAL4 contains its DNA-binding and nuclear targeting domains.

The CMV expression clones containing the HSV glycoprotein D epitope tag attached to the NH2 terminus followed by the YY1 full-length protein or similarly constructed mutants with deletions in the central B region were prepared by polymerase chain reaction, and a small fragment encoding a methionine residue followed by the HSV epitope sequence was attached at the amino terminus of all of these constructs. GST fusion proteins were constructed by inserting the corresponding coding sequence of YY1 or SREBP into the pGEX2T vector (Amersham Pharmacia Biotech). All other plasmids have been previously described (11–13).

Cells and Media—HepG2 cells were cultured in minimal essential medium (Life Technologies, Inc.) with 10% fetal bovine serum (v/v) at 37 °C and 5% CO2. CV-1 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% (v/v) fetal bovine serum at 37 °C and 8% CO2. Lipoprotein-deficient serum was prepared by ultracentrifugation (14), and stock solutions of cholesterol and 25-hydroxycholesterol (Steraloids, Inc.) were prepared in ethanol.

Transient DNA Transfections—Transfections were performed by a standard calcium phosphate co-precipitation method as described (13). All reporter plasmids and the CMV-β-galactosidase control plasmid were added at 5 μg/60-mm dish of cells.

HepG2 cells were plated at 1.75 × 105 cells/60-mm dish and transfectioned the following day. 4–6 h after transfection, dishes were subjected to a glycerol shock and rinsed three times with phosphate-buffered saline and normal medium, and cells were harvested an additional 24 h prior to harvest. CV-1 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% (v/v) fetal bovine serum at 37 °C and 8% CO2. Lipoprotein-deficient serum was prepared by ultracentrifugation (14), and stock solutions of cholesterol and 25-hydroxycholesterol (Steraloids, Inc.) were prepared in ethanol.

Transcriptional Repression of LDL Receptor Promoter by YY1

Expression from the LDL receptor promoter is dependent on the combined action of the SREBP and Sp1 transcription factors. Normally, SREBPs are nuclear only when cells are sterol depleted. However, transfection of a plasmid designed to encode a constitutively expressed and nuclear-targeted version of the SREBP’s results in a dose-dependent activation of the LDL receptor without regard for the sterol status of the cell (13, 16). In Fig. 1A we show that when a plasmid expressing the mature forms of either SREBP-1a or -2 is added in transient transfections along with the LDL receptor promoter reporter plasmid, that including an YY1-expressing plasmid results in a dose-dependent inhibition of the SREBP-activated expression. Over the same concentration range YY1 had a modest repressive effect on SREBP activation of the HMG CoA synthase promoter, and activation of the SV40 early promoter was essentially unaffected. Fig. 1B shows results for a similar experiment where activation of the LDL receptor promoter was directly compared with the HMG CoA reductase promoter. Activation of the HMG CoA reductase promoter by exogenously added SREBP’s is not as sensitive as the LDL receptor and HMG CoA synthase promoters, and to observe significant activation we had to provide higher levels of added SREBP plasmid (17). Even at this level of added SREBP plasmid the LDL receptor promoter was efficiently repressed by YY1, and there was no inhibitory effect at all on the HMG CoA reductase promoter. We have failed to see any effect on the reductase promoter even at 10-fold higher levels of YY1 as that used in this figure (data not shown).

As mentioned above, endogenously produced SREBPs only accumulate in the nucleus when cultured cells are depleted of sterols. Under these conditions SREBP-activated promoters are induced. The experiment in Fig. 1C demonstrates that addition of the YY1-expressing plasmid also resulted in a dose-dependent decrease in this induced level of expression from the LDL receptor promoter. Fig. 1C: YY1 had no appreciable effect on the basal level of LDL receptor promoter activity in the absence of co-transfected SREBPs or in sterol replete cells (data not shown).

Unlike the LDL receptor promoter that requires both Sp1 and SREBP, the SV40 promoter is activated by Sp1 and not SREBP, and the HMG CoA synthase and reductase promoters are activated by SREBP but not Sp1. Thus, it is likely that the sensitivity of the LDL receptor promoter to inhibition by the co-expression of YY1 is a result of YY1 interfering with the
synergistic activation mediated by the combined action of SREBP and Sp1.

In an initial attempt to analyze the specificity and potential mechanism for this inhibition, we first evaluated whether an increase in the amount of added SREBP would reverse the inhibitory effect of YY1 (Fig. 1D). Similar to the experiment in Fig. 1A, YY1 inhibited the activation by a small amount of added SREBP-1a (Fig. 1D, bars 1–3). When additional SREBP-expressing plasmid was added on top of the YY1, a dose-dependent reactivation of the LDL receptor promoter was observed (Fig. 1D, bars 4–6).

To further analyze the inhibitory effect, we introduced mutations into the YY1 coding sequence to characterize the protein domain(s) that were required. Initially, we prepared three mutant versions as shown in Fig. 2A. In one mutant (ΔA) we deleted the first 82 amino acids, in another (ΔC) we deleted the last 100 amino acids removing the last two zinc finger regions, and in a third mutant we combined the NH2 and COOH truncations to delete both termini. Because the carboxyl-terminal domain contains both the DNA-binding and nuclear targeting functions of YY1 (18), we fused these mutants (as well as the full-length YY1) to the DNA-binding and nuclear targeting domains of the yeast GAL4 protein in an expression vector designed to express GAL4 fusion proteins in mammalian cells from the SV40 promoter (10). The resulting GAL4-YY1 fusion proteins would be targeted to the nucleus independent of the nuclear targeting elements of YY1.

When these GAL4 fusion proteins were transfected into HepG2 cells the construct containing the entire YY1 coding sequence inhibited the SREBP-Sp1-dependent activation of the LDL receptor promoter in a dose-dependent manner, and the control vector that expressed only the GAL4 portion of the protein did not. All three of the YY1 deletion constructs inhibited activation of the LDL receptor, indicating that the region required for inhibition was contained within the central B region. YY1 deletions that lack the carboxy-terminal two zinc fingers destroy the ability of the resulting protein to bind DNA (28) and there are no GAL4 DNA-binding sites in the LDL

**Fig. 1.** YY1 inhibition of LDL receptor promoter activation by SREBP. A, the LDL receptor and HMG CoA synthase luciferase reporter plasmids were transfected into HepG2 cells with or without the CMV-SREBP-1a or SREBP-2 expression vector (10 ng/60-mm dish), and the degree of activation mediated by SREBPs relative to the reporter alone was set at 100% of control. LDL(1a) represents the values for SREBP-1a activation, whereas LDL(2) represents data for SREBP-2 activation. The SV40 promoter was transfected without CMV-SREBP-1a. Increasing amounts of a plasmid that expresses the full-length wild type YY1 protein from the CMV promoter were included in the transfection as indicated on the abscissa, and the degree of repression is plotted as a percentage of the control. The mean fold activation (relative to reporter alone) for SREBP-1a was 38.14 and 57.09 for the LDL receptor and HMG CoA synthase promoters, respectively. The mean fold activation for SREBP-2 on the LDL receptor was 40.7. B, the activation of the LDL receptor (LDL, ■) and HMG CoA reductase (Red, ○) promoters were directly compared in this experiment that was essentially identical to the experiment shown in A except that 100 ng of SREBP-expressing plasmid was added. The results shown are from a typical experiment and are characteristic of over 10 individual experiments. The fold activation for HMG CoA reductase and LDL receptor promoters were 13.0 and 27.4, respectively. C, CV-1 cells were transfected with the LDL receptor promoter luciferase construct, and in some dishes the CMV YY1 expression plasmid was included in amounts as indicated on the abscissa. The transfected cells were cultured in the absence of serum lipoproteins as detailed under “Materials and Methods.” The open square denotes the suppressed value for the promoter activity obtained from cells cultured in the presence of sterols and not transfected with the YY1 expression plasmid, YY1 addition to the suppressed cells had no effect on promoter activity (data not shown). D, HepG2 cells were transfected with the LDL receptor promoter as in A. SREBP-1a- or YY1-expressing plasmids were included as indicated at the bottom of the figure. In bars 4–6 an additional 30, 100, or 300 ng of SREBP-1a-expressing plasmid were included. In all transfections, an internal control plasmid expressing β-galactosidase from the CMV promoter was included, and its activity was used for normalization purposes as detailed under “Materials and Methods.” Fold activation was calculated as the ratio of corrected luciferase activity relative to that from cells transfected with reporter alone. In A, B, and C the data were plotted as the percentages of activation of SREBP-1a or SREBP-2, and in D the values were plotted directly as fold activation. The data for A, C, and D represent the mean values for three individual experiments, and positive error bars are shown.
receptor promoter; this suggests that YY1 inhibition of the LDL receptor occurs by a novel DNA-independent mechanism.

We constructed another set of YY1 deletion constructs to further localize the determinants within the B region that are required for inhibiting the LDL receptor promoter. Because deletion of neither the NH2-terminal A region nor the COOH-terminal C region affected repression, these new deletions left these regions intact and we focused on the central B region. Because the YY1 nuclear targeting region is contained within the C region, we did not have to fuse these new constructs to a heterologous nuclear targeting element. The first new mutant deleted amino acids 84–255, and the second one deleted only amino acids 256–354. These were called B2 (lacking aa 84–255) and B1 (lacking aa 256–354), respectively, and their positions within the YY1 coding sequence are schematically represented in Fig. 3A. Because these mutants were analyzed in a different expression vector that has an HSV antibody epitope added to the fusion proteins, we also reanalyzed the deletion of the entire B region and a version containing the full-length YY1 protein as well. The wild type construct inhibited the SREBP-stimulated activity of the LDL receptor promoter. The data presented thus far indicate that YY1 inhibits the SREBP-stimulated activity of the LDL receptor promoter through a mechanism that does not require YY1 to be bound to the promoter. Additionally, the repression is dependent on either two separate domains of YY1 or a single domain that overlaps the junction defined by the two B region deletions.

**Fig. 2.** YY1 inhibition of LDL receptor promoter does not require its DNA-binding domain. A, shown is a schematic diagram of the wild type YY1 protein and the three regions DA, B, and DC that were evaluated by mutagenesis. Both wild type and mutant derivatives were fused to the yeast Gal4 protein as described under “Materials and Methods.” The A, C, or B mutants are lacking the indicated regions from the wild type YY1 protein. The B region was further subdivided for the studies in Fig. 3 into B1 and B2 as indicated. The putative roles for some domains of the YY1 protein that have been identified are discussed in the text, their positions are indicated above the wild type sequence, and the four zinc fingers that are crucial to binding DNA are indicated (Zn). B, transfection experiments with the indicated YY1 proteins fused to GAL4 were performed, and the data are plotted as described in the legend to Fig. 1 and under “Materials and Methods.” The GAL4 fusion vector used for these experiments is driven by an SV40-based promoter expression vector instead of CMV as in Fig. 1. The unmodified Gal4 vector (Neg) was included as a negative control.

**Fig. 3.** Two regions in YY1 domain B are necessary for inhibition of LDL receptor promoter. A, expression plasmids for HSV-YY1 fusion protein or mutant versions lacking the entire B region or just the B1 or B2 subregions are diagrammed, and the HSV epitope tag located at the amino terminus is also noted. B, the YY1 expression constructs described in A were cotransfected along with the LDL receptor reporter plasmid as indicated. Transfections were performed, and the data were analyzed as described in the legend to Fig. 1 and under “Materials and Methods.” The GAL4 fusion vector used for these experiments is driven by an SV40-based promoter expression vector instead of CMV as in Fig. 1. The unmodified Gal4 vector (Neg) was included as a negative control.
Materials and Methods.

Protein-protein interaction assays were performed as described under lanes between YY1 and SREBP-1a. A recombinant version of proteins were also used to evaluate the potential interaction 298–331 (7, 8). A more extensive panel of YY1-GST fusion localized the Sp1-interacting region of YY1 to amino acids studies as B2. This is consistent with previous reports that Sp1 interacts with YY1 through the region defined in our regions of the YY1 protein. The data in Fig. 4C show that a recombinant version of SREBP that extends from aa 321–490 and contains the bHLH region but deletes the activation domain and entire amino-terminal domain of SREBP-1a still interacts with the B region of YY1. Thus, the bHLH domain of SREBP-1a and Sp1 interact with the same 100-amino acid B2 region of YY1, and importantly, this region of the YY1 protein is required to inhibit expression of the LDL receptor promoter as well (Figs. 2 and 3).

We previously demonstrated that SREBP-1a and Sp1 synergistically activate the LDL receptor promoter through a novel mechanism where SREBP-1a stimulates Sp1 to bind to an adjacent DNA-binding site on the promoter and it requires the buttonhead (btd) domain of Sp1 (11, 13, 19). We used a GST interaction assay to evaluate whether SREBP and Sp1 were able to interact in solution in the absence of DNA (Fig. 5A). When glutathione-bound beads were premixed with a GST-SREBP-1a fusion construct and subsequently incubated with nuclear extracts from control SL2 cells (lane 4) or SL2 cells that were transfected with a wild type Sp1 expression construct (lane 5) or a mutant Sp1 expression construct with a deletion of the btd domain (lanes 6), only extracts from cells transfected with the full-length protein generated an Sp1 immunoreactive band after electrophoresis of specifically bound proteins. The wild type and Δbtd protein were expressed at similar levels in transfected SL2 cells (compare lanes 1–3). Thus, SREBP-1a and Sp1 interact in the absence of DNA, and this interaction is dependent on the btd domain of Sp1, which is uniquely required for SREBP-Sp1 synergy at the LDL receptor promoter (11).

Because SREBP and Sp1 interact with each other and with a common domain of YY1, we sought to determine whether the bimolecular interactions were mutually exclusive. We first preincubated purified Sp1 with increasing amounts of a purified recombinant version of YY1 (shown in Fig. 5D) prior to the GST assay for SREBP-Sp1 interaction. The results of the immunoblotting protocol demonstrated that YY1 inhibited the interaction between Sp1 and SREBP-1a in a dose-dependent fashion (Fig. 5B). We also evaluated the ability of recombinant SREBP-1a (shown in Fig. 5D) to inhibit the interaction between YY1 and Sp1. In the experiment shown in Fig. 5C, SL2 nuclear extract from SL2 cells transfected with the Sp1 expression vector was incubated with increasing amounts of recombinant SREBP-1a prior to evaluation of Sp1 interaction with GST-bound YY1 protein. The results demonstrated that in a reciprocal fashion, SREBP-1a inhibited the interaction of Sp1 with the GST-bound YY1 protein (Fig. 5C). The results in Fig. 5C also show that the Sp1 Δbtd mutant protein does not efficiently interact with GST-bound YY1 (lanes 7 and 8). Thus, it is likely that the unique mechanism for YY1-mediated inhibition of the LDL receptor promoter is a result of the ability of YY1 to inhibit the protein-protein interaction between SREBP and Sp1, which is essential for the two proteins to effectively activate the promoter.

Discussion

YY1 is a multifunctional protein that can stimulate mRNA expression through binding to RNA polymerase II initiator elements (20), or it can either activate or repress transcription through interaction with upstream promoter elements (6). Different mechanisms have been proposed for YY1 inhibition in-
Neither SREBP nor Sp1 can simultaneously bind each other and YY1. Protein-protein interaction assays were performed as described under “Materials and Methods.” A, nuclear extract (5 μg) from SL2 cells (lane 1) or SL2 cells transfected with full-length Sp1 (lane 2) or the Δbtd mutant of Sp1 (lane 3) were analyzed by 7.5% SDS-PAGE and immunoblotting with an Sp1 antibody. In lanes 4–6 the same nuclear extracts as in lanes 1–3 were incubated with a GST fusion protein encoding amino acids 321–490 of SREBP-1a (called GST-bHLH) prior to binding to glutathione-agarose beads prior to gel analysis. The positions for the migration of molecular mass markers (in kDa) that were electrophoresed in a companion lane on the same gel are indicated at the right. B, purified Sp1 was incubated on ice alone (lane 2) or with increasing amounts of purified recombinant YY1 (lanes 3–7) corresponding to 0.09, 0.3, 0.9, 3.0, and 9.0 μg of protein, respectively, in a total volume of 100 μl of 0.1 buffer for 1 h. Then the samples were allowed to interact with glutathione-agarose-bound SREBP-1a (aa 1–490), and specifically bound proteins were eluted and analyzed by SDS-PAGE and immunoblotting for Sp1. Untreated Sp1 protein was loaded without any preincubation in lane 1. The positions for the migration of molecular mass standards (in kDa) from a companion lane are indicated at the left. C, nuclear extract (5 μg) prepared from SL2 cells that were transfected with an Sp1 expression plasmid was used as a source of Sp1. Nuclear extract was incubated on ice alone (lane 2) or with increasing amounts of purified recombinant SREBP-1a (lanes 3–6) corresponding to 0.2, 0.6, 2.0, or 6.0 μg of purified protein, respectively, in a total volume of 100 μl of 0.1 buffer for 1 h. Then the samples were allowed to interact with glutathione-agarose-bound YY1, and specifically bound proteins were eluted and analyzed by SDS-PAGE and immunoblotting for Sp1. Untreated Sp1 expressing SL2 extract was loaded in lane 1 as a control. Nuclear extract (5 μg) from Δbtd transfected SL2 cells was loaded in lane 7, and in lane 8 the Δbtd SL2 nuclear extract was allowed to interact with glutathione-agarose-bound GST YY1. Material specifically eluted from the beads was electrophoresed prior to SDS-PAGE and immunoblotting for Δbtd Sp1 retention. D, purified recombinant SREBP-1 (aa 1–490) or full-length YY1 (1 or 5 μl as indicated; SREBP-1a is at 0.2 μg/μl, and YY1 is at 0.3 μg/μl) that were used in the experiments of B and C were analyzed by SDS-PAGE followed by staining with Coomassie Blue.

SREBPs and Sp1 synergistically activate the LDL receptor promoter through binding to adjacent sites in the promoter (13). We have previously demonstrated that this occurs through a mechanism where SREBPs bind and enhance the initial on-rate of Sp1 binding to the adjacent site (11, 13, 19). This absolutely requires the conserved btd domain of Sp1, which is in large part dispensable for efficient activation of other Sp1-regulated genes (11, 25). We hypothesized that YY1 inhibition may target the interactions between SREBP and Sp1 that would provide an explanation for the sensitivity of the inhibition.

We show that YY1 and SREBP interact in solution through a domain that is also required for YY1 to interact with Sp1 (Figs. 4 and 5). The data in Fig. 5 further demonstrate that YY1 can directly interfere with the SREBP-Sp1 interaction and, in a complementary fashion, increasing amounts of SREBP-1a inhibit Sp1 from interacting with YY1. Thus, these experiments support our hypothesis and provide a molecular mechanism for the cell based transcriptional repression studies of Figs. 1–3. Sp1 was previously shown to interact with YY1, but the significance of this interaction was not clearly established (7, 8). The experiments reported here provide evidence for a functional significance to this interaction.

It is noteworthy that btd is uniquely required for SREBP-Sp1 synergy on the LDL receptor promoter and that this domain of Sp1 is required for efficient interaction with both SREBP and YY1. Because the btd domain is critical for SREBP-Sp1 stimulation of the LDL receptor promoter but not for Sp1-Sp1
synergy on the SV40 promoter, this provides a specific target for inhibition that would selectively affect activation of the LDL receptor promoter. Consistent with this notion, we show that activation of the SV40 promoter is not affected by YY1 expression (Fig. 1).

The promoters for enzymes of cholesterol biosynthesis that have been studied in detail so far require SREBP but not Sp1 for activation following sterol depletion (12, 26, 27). In the current studies we show that the promoter for HMG CoA reductase was not affected at all (Fig. 1B) and the HMG CoA synthase promoter was partially repressed but not to the same degree as the LDL receptor (Fig. 1A).

The region of the YY1 coding sequence that was crucial for inhibition of the LDL receptor promoter maps to the central B region. We further separated the B region into two parts, B1 and B2, and the data in Fig. 3 show that both are required for repression. B2 contains two zinc fingers that were shown by others to harbor a repression element. In these previous studies YY1 derivatives were expressed as GAL4 fusion proteins, and repression was mediated through binding to multiple GAL4 DNA sites in a synthetic promoter (28). The B1 region contains another separately defined repression domain that interacts with the histone deacetylase mRPD3/HDAC2 (22).

Because the B2 region of YY1 is capable of interacting with both SREBP and Sp1 but B1 is also required for inhibition of the LDL receptor promoter, a simple competitive interaction between YY1 and each activator protein is not sufficient to explain the entire repression phenomenon. We have shown that synergistic activation of the LDL receptor promoter occurs at two stages in promoter activation (25). The initial DNA binding event is one target, and stimulation at this level is dependent only on the bHLH domain of SREBP and the btd DNA-binding domains from Sp1. However, this is not enough because the activation domain of SREBP together with domains A, B, and D of Sp1 are all additionally required to achieve high levels of promoter activity in transfected cells. It is possible that the B2 region of YY1 interferes with the initial DNA binding event but the B1 region inhibits a subsequent step in promoter activation. This is consistent with the fact that the B1 domain interacts with a histone deacetylase (22) that could augment an inhibitory effect through local alterations in histone acetylation. However, further experiments are required to address the role of B1 in repression of the LDL receptor promoter.

Specific DNA binding by YY1 requires the presence of 5′-ACAT-3′ or 5′-CCAT-3′ as the core DNA recognition element. However, specific additional flanking bases significantly influence binding specificity, but the exact combination of flanking residues can vary significantly between individual sites (29, 30). Thus, there are many potential YY1 sites in the genome, and because the 4-base pair 5′-CCAT-3′ element is also one alternative half-site for SREBP recognition (17), a significant fraction of SREBP sites would also be predicted to bind YY1 in vitro.

In fact, YY1 was purified as a protein that bound to an element that overlaps a critical binding site for SREBP in the HMG CoA reductase promoter (31). However, as mentioned above, YY1 has no effect on expression of the HMG CoA reductase promoter in transient assays. Thus, its role if any in expression of HMG CoA reductase remains uncertain.

There is also a binding site for YY1 in the promoter for HMG CoA synthase. Here, the YY1 site does not overlap a site for SREBP, but it partially overlaps the binding site for the NF-Y/ICBF factor, which is required for sterol-regulated promoter activity along with two SREBP-binding sites (12). However, a single-base mutation that abolishes YY1 binding but not NF-Y/ICBF binding to the overlapping site has no effect on HMG CoA synthase promoter activity or sterol regulation. Additionally, using techniques similar to those described here we have been unable to demonstrate that YY1 interacts with the heterotrimeric NF-Y/ICBF protein or any combination of its isolated subunits.2

There are copies of the 4-base core YY1 recognition elements in the LDL receptor promoter as well. However, mutations that disrupt these elements had no effect on sterol regulation (32). Coupled with the observation that mutant versions of YY1 that lack its DNA-binding domain still repress the LDL receptor promoter (Fig. 2), all of the available data indicate that YY1 has a unique role in cholesterol regulation that is independent of its binding to DNA.

Our in vitro interaction studies (Figs. 4 and 5) coupled with the dose-dependent inhibition of the LDL receptor promoter by YY1 (Fig. 1, A and C) and its reactivation by additional SREBP (Fig. 1D) indicate that the relative concentrations of SREBPs, YY1, and Sp1 in an individual cell govern the ultimate level of LDL receptor promoter expression. This is emphasized by the model shown in Fig. 6. In this model, a high ratio of SREBP to YY1 would favor the activation pathway on the left, whereas a high level of YY1 relative to SREBP would favor the pathway for inhibition on the right.

This mechanism could have a profound impact on nutrient homeostasis and metabolic regulation because it suggests that high levels of YY1 would inhibit LDL uptake even if nuclear levels of SREBP were elevated due to sterol depletion. This would selectively activate the cholesterol synthetic pathway whose genes are dependent on SREBP but not Sp1. Alternatively, in tissues where there are relatively low levels of YY1 relative to Sp1, cholesterol uptake and biosynthesis would be coordinately activated by sterol depletion.

Different levels of YY1 or changes in the relative levels of all three of the key LDL receptor transcription factors could explain, at least in part, the whole animal experiments of Ditesch and colleagues (33–36). These studies demonstrate that cholesterol uptake and biosynthesis can be regulated independently in the liver and that cholesterol synthesis occurs at a relatively high rate relative to its receptor-mediated uptake in nonhepatic tissues in the body (33–36). Additionally, the selective repressive effect of YY1 on the LDL receptor promoter may explain why LDL receptor mRNA expression is only mildly increased in animals fed diets to mimic cholesterol depletion where at least some cholesterol biosynthetic genes are dramat-

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2 M. K. Bennett, T. T. Ngo, J. N. Athanikar, J. M. Rosenfeld, and T. F. Osborne, unpublished observations.
Y1 mRNA is widely expressed in animals (6), and the protein may be subject to down-regulation during the differentiation of specific cultured cell lines (38, 39). One report suggests that Y1 may be degraded by a calcium-activated protease (40), which may account for the activation of LDL receptor mRNA expression that occurs after treatment of cultured cells with a calcium ionophore (41).

Thus, our studies have uncovered a novel molecular mechanism for how Y1 selectively modulates expression of the LDL receptor gene that may provide a specific target for therapeutic strategies to selectively alter cholesterol uptake. Future studies are required to fully explore this possibility and to realize the significance of these observations in the physiological regulation of cholesterol metabolism.

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