Synergistic Binding of Sterol Regulatory Element-binding Protein and NF-Y to the Farnesyl Diphosphate Synthase Promoter Is Critical for Sterol-regulated Expression of the Gene*

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Sterol-regulated transcription of the farnesyl diphosphate (FPP) synthase gene is dependent on two cis elements in the proximal promoter. These elements, an inverted CCAAT box and sterol regulatory element 3 (SRE-3), bind NF-Y and sterol regulatory element-binding protein 1 (SREBP-1), respectively. We now demonstrate that the binding of recombinant SREBP-1 to its cognate site (SRE-3) within the FPP synthase promoter in vitro is enhanced by binding of NF-Y to the upstream inverted CCAAT box. Using an FPP synthase promoter fragment containing the binding sites for both NF-Y and SREBP-1 in gel mobility shift assays, we demonstrate that the addition of NF-Y increases the binding of SREBP-1 to SRE-3 over 20-fold. In contrast, NF-Y does not stimulate the binding of SREBP-1 to SRE-3 when the inverted CCAAT box is either mutated or 4 base pairs (bp) are inserted between the inverted CCAAT box and SRE-3.

Promoter-reporter genes, containing either the wild-type FPP synthase promoter sequence or containing the 4-bp insertion between the inverted CCAAT box and SRE-3, were transiently transfected into cells. The activity of the wild-type promoter-reporter gene increased when the cells were either incubated in sterol-depleted medium or were co-transfected with an expression vector encoding transcriptionally active SREBP-1. This increase in activity was attenuated when the promoter contained the 4-bp insert, consistent with defective binding of SREBP to the promoter in vivo. These studies suggest that the binding of SREBP-1 to SRE-3 in the FPP synthase promoter, and subsequent stimulation of transcription, is dependent on synergistic binding and a functional interaction between SREBP-1 and NF-Y.

The 15-carbon isoprenoid farnesyl diphosphate (FPP)† is a required intermediate in the biosynthesis of sterols, ubiquitome, heme a, dolichols and in the prenylation of proteins (1, 2). In plants, FPP is a precursor of a large number of diverse compounds, including carotenoids, rubber, and hormones (3). Farnesol, the dephosphorylated form of FPP, has recently been proposed to have regulatory roles in mammalian cells, either by enhancing the degradation of HMG-CoA reductase (4) or by stimulating the activity of the farnesoid receptor and 9-cis-retinoic acid receptor heterodimer (5). Thus, changes in the rate of FPP and farnesol biosynthesis and/or levels is expected to affect diverse cellular functions.

FPP synthase, a peroxisomal enzyme (6), was originally cloned as a result of its differential expression in the livers of rats fed diets supplemented with either hypolipidemic drugs or cholesterol (7). The transcription of a number of genes involved in controlling cellular cholesterol homeostasis, including FPP synthase (8–10), HMG-CoA reductase (11), HMG-CoA synthase (12), the LDL receptor (11, 12), and squalene synthase (13) is high in cells deprived of sterols and low in cells exposed to high levels of exogenous sterols.

Recent studies have identified two cis elements, an inverted CCAAT box (ATTGG) and SRE-3, in the proximal promoter of the rat FPP synthase gene that are required for sterol-regulated transcription (10, 14, 15). Mutation of either of these elements, which bind NF-Y and SREBP, respectively, impaired sterol-regulated expression of FPP synthase promoter-reporter genes (10, 14, 15). In addition, co-transfection of a plasmid expressing a dominant-negative form of NF-Y resulted in the impaired regulation of reporter genes driven by promoters derived from either FPP synthase or HMG-CoA synthase (14). Consequently, we proposed that NF-Y and SREBP played similar roles in the sterol-regulated transcription of both the HMG-CoA synthase and FPP synthase genes (14).

NF-Y is a ubiquitous transcription factor that binds as a heterotrimer (NF-YA/NF-YB/NF-YC) to a CCAAT or inverted CCAAT (ATTGG) box (16). NF-Y is one of many transcription factors reported to bind to this or closely related sequences (17). SREBP is also ubiquitously expressed (18). It was purified and cloned based on its ability to bind to SRE-1, a 10-base pair sequence, present in the promoters of the LDL receptor and HMG-CoA synthase genes (12, 19, 20). The increased transcription of the LDL receptor and HMG-CoA synthase genes that occurs in cells deprived of sterols was shown to be dependent, in part, on the interaction of the 68-kDa mature SREBP with SRE-1 (18, 19). The proteolytic release of the mature, transcriptionally active form of SREBP from the 125-kDa precursor SREBP, which is localized to the endoplasmic reticulum, is regulated by cellular sterols (21). However, enhanced transcription of the LDL receptor gene required the additional

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‡ These abbreviations used are: FPP, farnesyl diphosphate; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein; NF-Y, nuclear factor Y; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CAT, chloramphenicol acetyltransferase; LDL, low density lipoprotein; bp, base pair(s); ADD-1, adipocyte determination- and differentiation-dependent factor 1.
binding of the constitutive transcription factor Sp1 to a DNA sequence adjacent to the SRE-1 (22).

ADD-1 (adipocyte determination- and differentiation-dependent factor 1), the rat homologue of human SREBP, was cloned as a result both of its increased expression during adipocyte differentiation and its ability to recognize an E-box motif (23, 24). This motif has 60% or 50% identity with SRE-1 or SRE-3, respectively (15). Taken together, these reports demonstrate that SREBP/ADD-1 recognizes a number of diverse but related motifs including SRE-1, SRE-3, and an E-box.

The current investigations were designed to address the roles of NF-Y and SREBP in sterol-regulated transcription of the FPP synthase gene. We show that the binding of SREBP to SRE-3 in vitro is enhanced significantly when NF-Y is bound to the inverted CCAAT box 20 bp 5′ of the SRE-3 site. In contrast, neither unbound NF-Y nor NF-Y bound to a separate oligonucleotide stimulate the binding of SREBP to SRE-3. These results form the basis for a model to explain how SREBP and NF-Y synergetically enhance the binding of both proteins to the FPP synthase promoter resulting in enhanced transcription of the gene.

EXPERIMENTAL PROCEDURES

Materials—DNA restriction and modification enzymes were obtained from Life Technologies, Inc. 32P-Labeled nucleotide triphosphates were obtained from Amersham Corp. pRSETB (Invitrogen) containing both a partial sequence of SREBP-1a (amino acids 1–490) and T7 and polyhistidine tags and pCMV-CSA10, which encodes amino acids 1–490 of SREBP-1a, were kindly provided by Dr. T. Osborne (Department of Molecular Biology and Biochemistry, University of California, Irvine). Lipoprotein-deficient fetal calf serum was purchased from PerImmune. Antibodies to human NF-YA and rat CBF-A (homologous to human NF-YB) were generous gifts from Dr. R. Mantovani and Dr. B. de Crombrugghe, respectively. The T7 antibody was from Invitrogen. The sources of all other reagents and plasmids have been given elsewhere (9, 10, 14, 15).

Cell Culture—CV-1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 8% CO2 and 37 °C. Chinese hamster ovary cells were grown at 5% CO2 as described previously (10).

Oligonucleotides—Oligonucleotides were synthesized by either Dr. D. Gold (Department of Biological Chemistry, UCLA) or Life Technologies, Inc. or were obtained by polymerase chain reaction using the FPP synthase promoter-reporter plasmid (pFPPS-0.319 in Ref. 9) as a template. The double-stranded DNA oligonucleotides used in these studies were as follows: a 58-bp probe of the FPP synthase promoter (nucleotides –298 to –241); GCCACCTTTCATGTCAGCTGTCATCAAG; GCCCATACCTCAGCTCGTAGTCCAGC; containing the inverted CCAAT box (underlined TATTGG) and SRE-3 (double underlined); a similar 58-bp mutant probe, which contained an A → C transversion at nucleotide –287 (*); a 27-bp probe (–298 to –272); and a similar 27-bp probe (–298 to –272), which contained a transversion (A → C) at nucleotide –287 (*). A 62-bp probe, containing nucleotides –298 to –241 and an additional 4-bp insert (AAAA) between –268 and –267, was generated by polymerase chain reaction of a mutant promoter containing these additional 4 nucleotides (see below).

Gel Mobility Shift Assay—Nuclear extracts were prepared as described (10). Where indicated, crude HeLa nuclear extracts, provided by Dr. M. Carey (UCLA), were separated by size exclusion chromatography on a Superose 12 (Pharmacia) FPLC column using buffer A (50 mM Hepes-KOH, pH 7.5, 100 mM KCl, 20% glycerol, 1 mM dithiothreitol, 10 μg/ml leupeptin, and 10 μg/ml phenylmethylsulfonyl fluoride). Thirty 0.5 ml fractions were collected and stored at –20 °C. Recombinant SREBP-1a, containing N-terminal T7 and polyhistidine (His8) tags, was purified to homogeneity from Escherichia coli extracts by nickel affinity chromatography as described by Sanchez et al. (22).

Complementary single-stranded DNA, corresponding to the indicated nucleotides of the FPP synthase promoter and containing either the wild-type sequence or the indicated mutations, were annealed, and the double-stranded probe isolated from acrylamide gels (25). Radiolabeled probes were generated in the same manner following an initial radiolabeling of a single-stranded oligonucleotide with 32P (25). The radiolabeled probes (20,000 cpm; 1.5 fmol) were used in gel mobility shift assays either in the presence of nonfat milk (2.5 mg/ml) as described previously (14, 15) or in the presence of partially purified NF-Y (5 μg) obtained from HeLa cell nuclei.

Immunodepletion—An NF-Y-enriched nuclear fraction was preincubated for 1 h at 4 °C with a preformed Protein A-Sepharose-anti-NF-Y antibody complex. The immunodepleted supernatant, obtained after removal of the Protein A-Sepharose complex, was stored at –70 °C. Mock-depleted samples were incubated with Protein A-Sepharose under otherwise identical conditions.

Western Blots—The Amersham ECL assay was used to detect NF-Y as described by the supplier. Antibodies to human NF-YA and rat CBF-A were generously supplied by Drs. R. Mantovani and B. de Crombrugghe, respectively.

Transient Transfections and Reporter Gene Assays—The Sculptor mutagenesis kit (Amersham) was used with single-stranded pFPSS-0.247 (10) to generate a mutant promoter containing a 4-bp insert (AAAA) between nucleotides –268 and –267, as described previously (10). The wild-type and mutant promoters were subcloned into the pG2L basic vector (Promega) and sequenced to confirm the mutations (9, 25). A 53-bp FPP synthase promoter fragment (nucleotides –293 to –241) and a mutant promoter fragment (nucleotides –293 to –241), containing the additional 4-bp insert between –268 and –267, were generated by polymerase chain reaction using wild-type or mutant pFPSS-0.247 as templates. Primers contained restriction sites (HindIII/BamHI) compatible with pTKIII-0.053 and pTKIII-0.053In (15)). The resultant plasmids (pTKIII-0.053 and pTKIII-0.053In) were sequenced to confirm the mutation. Details of the transient transfection of CV-1 cells with luciferase or chloramphenicol acetyltransferase reporter genes, β-galactosidase, and pCMV-CSA10 (encoding SREBP-1a); incubation of the cells in medium supplemented with 10% lipoprotein-deficient fetal calf serum in the absence (inducing medium) or presence (repressing medium) of sterols (10 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol); and subsequent assays of reporter gene activities have been described (14, 15). The activity of β-galactosidase was used to normalize for transfection efficiencies (15).

RESULTS

Enrichment of NF-Y from HeLa Nuclear Extracts—Sterol-regulated transcription of the FPP synthase gene is dependent on the binding of two transcription factors, NF-Y and SREBP-1, to the proximal promoter (14, 15). We hypothesized that NF-Y might stimulate the binding of SREBP-1 to SRE-3, its cognate binding site. In order to test this hypothesis, we initially used gel mobility shift assays and immunoblots to identify HeLa nuclear fractions, eluted from a size-exclusion column, that were enriched in NF-Y.

Polyclonal antibodies raised against human NF-YA (26) and rat CBF-A (homologous to human NF-YB) (27) reacted with NF-YB (30 kDa), but not NF-YA (40 kDa) in unfractonated HeLa nuclear extract (Fig. 1, right lane). These same antibodies reacted with both subunits of NF-Y in four fractions eluted from the column (Fig. 1, fractions 8–11). Presumably NF-YA was sufficiently enriched in fractions 8–11, as compared to the unfractonated extract, to allow immunodetection. Gel mobility shift assays demonstrated that fractions 8–11 contained a protein that bound to a 32P-end-labeled probe corresponding to nucleotides –298 to –272 of the FPP synthase promoter but not to a probe containing any mutation in the NF-Y binding site (ATTGG) (data not shown). These NF-Y-enriched fractions (fractions 8–11) were used in subsequent studies.

Fig. 1. Identification of HeLa nuclear fractions enriched in NF-Y. HeLa cell nuclear extract was fractionated as described under "Experimental Procedures." Protein (5 μg) from the indicated column fractions obtained after size exclusion chromatography or from unfractonated HeLa cell nuclear extract (NE; 25 μg) was subjected to ECL Western blot analysis using anti-NF-YA and anti-NF-YB antibody. The locations of the NF-YA and NF-YB subunits are indicated. Fractions 17–30 did not contain detectable NF-Y (data not shown).
Binding of Recombinant SREBP-1 to SRE-3 in Vitro Is Stimulated by NF-Y—We have used an oligonucleotide, which corresponds to 58 bp of the FPP synthase promoter and contains binding sites for both NF-Y and SREBP, in gel mobility shift assays. Incubation of this \( ^{32} \)P end-labeled probe with an NF-Y-enriched fraction resulted in the formation of one major specific complex, consistent with binding of NF-Y to the oligonucleotide (Fig. 2, lanes 2 and 6). A second, faster migrating, nonspecific complex, which did not contain NF-Y, was also formed in the presence of the NF-Y-enriched fraction (Fig. 2; data not shown). In contrast, recombinant SREBP bound poorly, if at all, to the probe under these gel mobility shift assay conditions (Fig. 2, lane 1). However, addition of SREBP-1, together with the NF-Y-enriched fraction, resulted in the formation of a slower migrating SREBP-NF-Y complex (Fig. 2, lane 7) with a concomitant decrease in the NF-Y complex (Fig. 2, lane 7 versus lane 6). Similar results were obtained when the NF-Y fraction had been mock-depleted of NF-Y (Fig. 2, lanes 2 and 3 versus lanes 6 and 7). In contrast, the NF-Y and SREBP-NF-Y complexes were reduced by greater than 95% when the NF-Y-enriched fraction had been immunodepleted of NF-Y by pretreatment with antibodies to NF-Y (Fig. 2, lanes 4 and 5 versus lanes 6 and 7). The formation of a small amount of the SREBP-NF-Y-DNA complex under these latter conditions (Fig. 2, lane 5) is consistent with synergistic binding and increased affinity of NF-Y and SREBP for the DNA. The SREBP-NF-Y complex observed in lane 7 was supershifted when antibodies to either NF-Y or the T7 tagged SREBP were included in the assay (data not shown), consistent with the presence of both NF-Y and SREBP in the complex. Taken together, these results demonstrate that the \( >20 \)-fold stimulation of SREBP-1 binding to the SRE-3 sequence within the 58-bp oligonucleotide (Fig. 2, compare lane 1 with the SREBP-NF-Y complex in lane 7) is dependent on NF-Y.

We next determined whether the NF-Y-stimulated binding of SREBP to DNA requires both proteins to bind to the same oligonucleotide. Fig. 3 shows that the formation of the SREBP-NF-Y complex was reduced significantly when the gel mobility shift assay was carried out in the presence of an unlabeled 27-bp oligonucleotide containing a single binding site for NF-Y (ATTGG) (Fig. 3, lane 4 versus lane 3). As expected, this competitor oligonucleotide prevented the binding of NF-Y to the radiolabeled probe (compare lanes 4 and 3). In contrast, addition of a 27-bp competitor oligonucleotide containing a point mutation in the NF-Y binding site (ATTGG, lane 4) or a mutant (CTTGG, lane 5) NF-Y binding site was added where indicated. The migration of the DNA-protein complexes containing NF-Y, SREBP, and SREBP-NF-Y are indicated. The free probe is not shown.

In the presence of a constant amount of NF-Y, addition of increasing amounts of recombinant SREBP-1a to the 58-bp radiolabeled probe resulted in increased levels of the SREBP-NF-Y complex (Fig. 4). Under these conditions, there was an increase in the level of the SREBP-DNA complex and a decrease in the NF-Y-DNA complex (Fig. 4, lanes 1–4 and 5–7). In contrast, neither the NF-Y nor SREBP-NF-Y complexes were observed when the radiolabeled probe contained a single A \( \rightarrow \) C point mutation within the inverted CCAAT box, the binding site for NF-Y (Fig. 4, lanes 8–11). These studies demonstrated that increased binding of SREBP to the SRE-3 motif did not occur when the NF-Y was present, but not bound to the same oligonucleotide (Fig. 4, lanes 9–11 versus lanes 2–4).

Fig. 4 also shows that a 2-fold increase in the NF-Y-enriched fraction, in the presence of a constant amount of SREBP, resulted in a synergistic increase in the formation of the SREBP-NF-Y complex. The relative levels of radioactivity in the SREBP-NF-Y complex in lanes 5 versus lane 2, lane 6 versus lane 3, and lane 7 versus lane 4 in Fig. 4 were 5.8, 3.2, and 2.9, respectively. In the absence of NF-Y there was no detectable SREBP-DNA complex (Figs. 2 and 5 and data not shown). Thus, we are unable to determine the absolute stimulation of SREBP binding to DNA by NF-Y.

The inverted CCAAT box and SRE-3 motif in the FPP synthase promoter are separated by two turns of the DNA helix (20 bp). A direct interaction of NF-Y and SREBP, bound to these motifs on the same oligonucleotide fragment, might be expected to depend on the relative spacing of the two proteins on the DNA. In order to test this hypothesis, we repeated the gel mobility shift assay with an oligonucleotide that contained a 4-bp insert between the inverted CCAAT box and the SRE-3.
The formation of the SREBP-NF-Y complex observed in the presence of NF-Y and an increasing amount of SREBP was attenuated when the probe contained the 4-bp insert (Fig. 5, lanes 6 and 7 versus lanes 3 and 4), consistent with a requirement for specific spacing between the two motifs.

Transcriptional Activity of FPP Synthase Promoter-Reporter Genes Is Dependent on the Interaction of SREBP and NF-Y—In order to determine whether the 4-bp insertion had physiological consequences, FPP synthase promoter-reporter constructs containing either a 247-bp wild-type promoter sequence or the same promoter containing the 4-bp insert between the inverted CCAAT box and SRE-3 were transiently transfected into CV-1 cells. The cells were then incubated in medium depleted of sterols (inducing medium) or in the same medium supplemented with cholesterol and 25-hydroxycholesterol (repressing medium). As expected, expression of the luciferase reporter gene under the control of the wild-type FPP synthase promoter was regulated 4.5-fold under these incubation conditions (Table I). In contrast, expression of the luciferase reporter gene was regulated only 2.0-fold when the FPP synthase promoter contained the 4-bp insert (Table I). This defective regulation of the mutant promoter-luciferase gene resulted largely from a lack of induction when cells were incubated in the inducing medium (Table I). This latter result is consistent with the proposal that the interaction between SREBP and NF-Y is dependent on the exact spacing of the binding sites for the two transcription factors. This proposal was further supported by the data shown in Fig. 6; co-expression of the mature form of SREBP-1a increased CAT activity 5.5-fold when the reporter gene was under the control of the wild-type 53-bp FPP synthase promoter but only 1.4-fold when the promoter contained the 4-bp insert.

**DISCUSSION**

Mutation of the binding sites for either SREBP or NF-Y in the promoters of FPP synthase (10, 14, 15) or HMG-CoA synthase\(^2\) (11) reporter genes impairs the transcriptional induction of these genes normally observed when cells are incubated in medium deprived of sterols (11, 14, 15). In addition, the increased expression of both reporter genes, which normally occurs when cells are incubated under such inducing conditions, is attenuated when cells are co-transfected with a dominant negative form of NF-YA (14). Together, these studies indicate that NF-Y is required for sterol-regulated transcription of both the FPP synthase and HMG-CoA synthase genes.

*Insights into the mechanism by which the ubiquitous transcription factor NF-Y stimulates the transcription of the FPP synthase gene in cells deprived of sterols are provided in the current report.*

We now demonstrate that binding of the mature form of SREBP to SRE-3 within the FPP synthase promoter is enhanced significantly when NF-Y binds to an inverted CCAAT box located 20 bp upstream of SRE-3. Under these conditions, a major shifted complex comprising both SREBP and NF-Y was observed (Figs. 2–5). In contrast, when NF-Y was not bound to the same DNA fragment, SREBP bound poorly, if at all, to SRE-3 (Figs. 2–5). We conclude that NF-Y, bound to this inverted CCAAT box, plays an important role in the recruitment of mature SREBP to SRE-3 (Figs. 2–5). These results are consistent with the requirement for both NF-Y and mature SREBP for the high levels of transcription of the FPP synthase gene in cells deprived of sterols. Indeed, FPP synthase promoter-reporter constructs that contained mutations in either the inverted CCAAT box or the SRE-3 motif showed attenuated sterol-regulated expression (14, 15). It is possible that binding

\(^2\) T. Osborne, personal communication.
of NF-Y and SREBP to DNA results in an altered conformation of the protein(s), increased affinity of the two proteins for each other or a conformational change in the DNA. However, since we have been unable to demonstrate a direct interaction of NF-Y and SREBP in solution, we cannot rule out the possibility that another, as yet unidentified, protein is involved in the formation of the SREBP-NF-Y-DNA complex.

The two cis-elements that bind NF-Y and SREBP in the FPP synthase promoter are separated by two turns of the DNA helix (20 bp). Insertion of a 4 bp between the inverted CCAAT box and SRE-3 resulted in a decrease both in the binding of SREBP to SRE-3 in vitro (Fig. 5) and the sterol- or SREBP-regulated expression of promoter-reporter genes in vivo (Table I; Fig. 6). These results are consistent with the proposal that the relative spacing of SREBP and NF-Y, bound to the FPP synthase promoter, is important for activation of transcription.

A somewhat analogous situation has been described in studies with the LDL receptor gene. The binding of SREBP to SRE-1 in the LDL receptor promoter is not sufficient to stimulate transcription (12, 22). High levels of transcription of the LDL receptor gene require the binding of both SREBP and the ubiquitous transcription factor Sp1 (22, 28, 29). Sanchez et al. have reported that the binding of SREBP to SRE-1 within the proximal LDL receptor promoter stimulates the binding of Sp1 to an adjacent 3' motif (22, 29). The latter motif is a non-consensus Sp1 binding site and represents a relatively weak binding site for this transcription factor. Sanchez et al. also reported that a number of other cis elements are unable to fully functionally substitute for the Sp1 binding site in the LDL receptor promoter (22). However, in these latter studies, the effects of either inserting extra base pairs between the Sp1 and SREBP binding sites or replacing the Sp1 binding site with an NF-Y binding site were not determined. Further investigations will be required to ascertain whether NF-Y and Sp1 function interchangeably in different promoters. In this regard, it is noteworthy that NF-YA (30), like Sp1 (31) contains a glutamine-rich activation domain. In contrast, SREBP contains an acidic N terminus that may function as a transactivation domain (32). Thus, the synergistic binding of NF-Y and SREBP to the FPP synthase promoter is likely to provide the appropriate activation domains that result in the transcriptional activation of the gene. These same two transcription factors may have important roles in the sterol-regulated transcription of HMG-CoA synthase (11, 15) and squelene synthase (13). The promoter of the latter gene contains an inverted CCAAT box, an SRE-1 motif, and a motif that has 11/12 identity with the nucleotides of SRE-3.

The importance of both the relative orientation and spacing of transcription factor-binding sites in other promoters has recently been investigated; activation of a reporter gene under the control of the T cell receptor α enhancer, which contains binding sites for three distinct transcription factors, was impaired when the binding sites were either switched or separated by extra nucleotides that would be expected to alter the helical phasing of the promoters (33). One of these factors, lymphoid enhancer factor 1 (LEF-1), is known to bind to and induce a sharp bend in the DNA (34). Such bending of the DNA may be necessary for the subsequent interaction of transcription factors that are not bound adjacently on the DNA and for the interaction of these proteins with the basal transcriptional machinery (35, 36). We are currently investigating whether SREBP causes bending of the DNA, analogous to that observed with LEF-1 and other DNA-binding proteins.

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