A Direct Role for Sterol Regulatory Element Binding Protein in Activation of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Gene*

(Received for publication, January 30, 1996, and in revised form, March 8, 1996)

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In earlier studies the DNA site required for sterol regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase was shown to be distinct from the classic sterol regulatory element (SRE-1) of the low density lipoprotein receptor gene (Osborne, T. F. (1991) J. Biol. Chem. 266, 13947-13951). However, oxysterol-resistant cells that continuously overproduce one of the sterol regulatory element binding proteins in the nucleus result in high unregulated expression of both genes (Yang, J., Brown, M. S., Ho, Y. K., and Goldstein, J. L. (1995) J. Biol. Chem. 270, 12152-12161) suggesting a direct role for the SREBP binding sites in the activation of the reductase gene. In the present studies we demonstrate that SREBP-1 binds to two adjacent sites within the previously identified sterol regulatory element of the reductase gene even though there is only limited homology with the SRE-1 of the receptor. We also show that SREBP-1 specifically activates the reductase promoter in transient DNA transfection studies in HepG2 cells and that mutations which eliminate sterol regulation and SREBP-1 binding also abolish transient activation by SREBP-1. Although specific, the magnitude of the activation observed is considerably lower than for the low density lipoprotein (LDL) receptor analyzed in parallel, suggesting there is an additional protein required for activation of the reductase promoter that is limiting in the transient assay. SREBP also binds to two additional sites in the reductase promoter which probably play an auxiliary role in expression. When the DNA sequence within the sites are aligned with each other and with the LDL receptor SRE-1, a consensus half-site is revealed 5'-PyCAPy-3'. The LDL receptor element contains two half-sites oriented as a direct repeat spaced by one nucleotide.

The SREBP proteins are special members of the basic-helix-loop-helix-zipper (bHLHZip) family of DNA binding proteins since they bind the classic palindromic E-box site as well as the direct repeat SRE-1 element. The SREBP binding sites in both the reductase and those recently identified in other sterol regulated promoters appear to contain a half-site with considerable divergence in the flanking residues. Here we also show that a 22-amino acid domain located immediately adjacent to the basic domain of the bHLHZip region is required for SREBP to efficiently recognize divergent sites in the reductase and 3-hydroxy-3-methylglutaryl-CoA synthase promoters but, interestingly, this domain is not required for efficient binding to the LDL direct repeat SRE-1 or to a palindromic high-affinity E-box element.

Balanced cholesterol metabolism in mammalian cells is maintained through the feedback regulation of key proteins involved in its cellular uptake and biosynthesis. A major control point is at the level of transcription for genes that encode important proteins of both processes (1, 2). DNA sequences required for sterol regulation of important enzymes of cholesterol biosynthesis and the low density lipoprotein (LDL) receptor, the unique protein required for cholesterol uptake, have been identified by DNA transfection studies in cultured cells (1, 2). The original studies identified a sequence similarity in the target site for cholesterol regulation (called the SRE-1) in the promoter for the LDL receptor and HMG-CoA synthase (3-5). Further studies demonstrated that the SRE-1 of the LDL receptor is a 10-base pair site that binds a specific subfamily of basic-helix-loop-helix-zipper (bHLHZip) DNA binding proteins called the sterol regulatory element binding proteins or SREBP's (6-8).

When mammalian cells are starved for cholesterol the SREBP proteins are cleaved from the membrane of the endoplasmic reticulum and nuclear envelope by proteolysis and the soluble amino-terminal fragment containing the DNA binding and transcriptional activation functions is translocated to the nucleus where it activates expression of appropriate target genes. The first evidence for this maturation process came from classic pulse-chase and gel electrophoresis studies on cells that were cultured in the presence and absence of sterols (9). SREBP-1 and -2 proteins were regulated in an identical fashion in tissue culture cells (9). However, a recent study in animals has suggested that the two SREBP's are regulated independently by cholesterol deprivation in the liver (10). Nonetheless, the two proteins have a similar domain structure, bind to the same DNA target sites in vitro, and appear to activate the same target genes when overexpressed in transfection studies (8, 11, 12).

A fundamental role for SREBP processing in cholesterol reg-

*This work was supported in part by grants from the National Institutes of Health (HL48044), the American Heart Association (93001330), and the Lucile P. Markey Charitable Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ Supported by a postdoctoral fellowship from the National Institutes of Health.

§ Supported by an undergraduate fellowship from the National Institutes of Health.

‖ Supported by a predoctoral fellowship from the American Heart Association, California Affiliate.

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1 The abbreviations used are: LDL, low density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; bp, base pair(s); SRE-1, sterol regulatory element-1; SREBP, sterol regulatory element binding protein; bHLHZip, basic-helix-loop-helix-zipper.
ulation was uncovered when SREBP expression was analyzed in mutant cell lines that were selected for resistance to the toxic effects of oxysterols (11, 13). Several independently derived oxysterol-resistant cell lines all resulted from genomic rearrangements that lead to production of an altered form of the SREBP-2 protein. All variant proteins lack the membrane spanning domains which are responsible for localizing the precursor to the endoplasmic reticulum in a hairpin configuration (12, 14). Thus, SREBP-2 is constitutively targeted to the nucleus resulting in high expression of the LDL receptor and cholesterol synthetic enzymes independent of the cholesterol content of the culture medium.

We have shown previously that the DNA target site for cholesterol regulation of HMG-CoA reductase appeared to be distinct from the direct repeat SRE-1 recognition site for SREBP in the LDL receptor (15) and the palindromic E-box that is the characteristic recognition site of bHLH proteins and was identified for SREBP-1 in a previous study (16). In addition, we have purified a protein that binds to the reductase regulatory site that is unrelated to SREBP (17). The involvement of a unique protein in regulation of HMG-CoA reductase would allow the cell to independently regulate cholesterol uptake from its biosynthesis. However, since the oxysterol-resistant mutant cell lines constitutively overproduce HMG-CoA reductase is directly activated by SREBP as well.

In the present studies we demonstrate that SREBP-1 directly activates the promoter for HMG-CoA reductase through non-canonical recognition sites in the previously characterized sterol regulatory region of the reductase promoter.

**EXPERIMENTAL PROCEDURES**

Cells and Media—HepG2 cells were obtained from the ATCC and maintained in modified essential medium containing 10% (v/v) fetal bovine serum. All cell culture reagents were obtained from Life Technologies.

Plasmids—The wild type hamster HMG-CoA reductase promoter from −277 to +20 was fused to the luciferase coding sequence of the plasmid pGL2 basic (Promega, Inc.). The mutation containing a scrambled substitution of the bases from −161 to −152 in the reductase sterol regulatory DNA site of the other wild type promoter was described before as the chloramphenicol acetyltransferase fusion construct plasmid (5). The −277 to +20 region from this plasmid was fused to the luciferase coding sequence of pGL2 basic as well and referred to as “mut” in the present studies.

The plasmid with a generic TATA element from the hamster HMG-CoA synthase promoter fused to the luciferase gene has been described previously (18). The plasmid "3X WT" and "3Xmut" were constructed in two steps. First, the simple Sp1 site of repeat 3 from the human LDL receptor promoter was inserted upstream of the generic TATA element in the above plasmid. Then oligonucleotides containing three tandem copies of the sequence from the wild type reductase promoter from −161 to −136 or a mutant derivative with two base changes at −156 and −155 in all three otherwise normal copies were inserted upstream of the Sp1 site. Plasmids containing the desired inserts in the wild type orientation were identified and confirmed by restriction enzyme digestion and DNA sequencing before further analyses. Methods for the oligonucleotide insertion procedures have been described previously (18). Amino-terminal truncation derivatives of SREBP-1 were constructed by polymerase chain reaction with the wild type plasmid as template and appropriately designed oligonucleotides with a 10 to 1 mixture of Taq and Pfu polymerases. The polymerase chain reaction fragments were subcloned into the pRSET expression vector from Invitrogen.

Transient Transfections—HepG2 cells were plated on day 0 in 60-mm dishes at 175,000 cells/dish. On day 1 the dishes were refed and transfected by the calcium phosphate co-precipitation method. Precipitates contained 20 μg of each reductase-luciferase plasmid and CMVβ-Gal plus 10 μg of salmon sperm DNA in 2 ml of solution. 0.5 ml were added to each of four dishes which were incubated for 4–6 h at 37 °C and 5% CO2. The dishes were treated with 1 ml of glycerol (20% (v/v)) in 1 x phosphate-buffered saline and then washed three times with phosphate-buffered saline and refed the same medium as above. Following a 36-h incubation the cells were harvested by scraping, cells from duplicate dishes were pooled, and extracts were prepared by 3 freeze-thaw cycles. A cytomegalovirus promoter based expression plasmid was used to express a transcriptionally active fragment of the human SREBP-1 protein (amino acids 1–490) and this plasmid had been described before (18). This expression plasmid was included in the DNA precipitate such that the amount indicated in the figure was added to each 60-mm dish.

Enzyme Assays—Luciferase activities were measured in a luminometer with a luciferin reagent from Promega Biotech. β-Galactosidase assays were performed by a standard colorimetric procedure with 2-nitrophenyl-β-D-galactopyranoside as substrate (19). The ratio of luciferase activity in relative light units was divided by the β-galactosidase activity (activity/h) for each extract. The experiments presented here represent data from three independent transfections performed in duplicate for each plasmid. There was less than 10% variation between individual experiments. Fold activation was calculated as the ratio of corrected luciferase activity (ratio of luciferase to β-gal) obtained for a specific plasmid measured when cells were transfected with the indicated amount of SREBP-1 expression plasmid relative to the corrected luciferase activity measured for the reporter plasmid when it was transfected alone.

Protein Purification—Wild type and mutant forms of recombinant SREBP-1 protein were purified by metal chelate affinity chromatography from extracts of Escherichia coli as described (8, 18). The quality of the purified proteins was assessed by SDS-polyacrylamide gel electrophoresis analysis performed with marker proteins following by staining with Coomassie Blue. The concentration of active protein was determined by performing a gel mobility shift titration with varying amounts of protein extract in the presence of a vast excess of 32P-labeled SREBP binding site from the LDL receptor. The concentration of DNA-protein complex at the saturation point was used to estimate the active concentration (μm) of SREBP protein assuming a 1:1 molar ratio of protein to DNA in the complex.

Oligonucleotide Probes—The LDL receptor SRE-1 element oligonucleotide was described before (18). The sequence of the reductase wild type oligonucleotide is shown in Fig. 1C. Single nucleotide mutant derivatives of this element were made at positions indicated in Fig. 1A. The top DNA strand of the other double stranded oligonucleotides used in the binding studies were as follows (important homology elements are underlined). The 3′ SRE-1 element of the HMG-CoA synthase promoter was 5′-GATCCAGTAACTAGTCTACCCGCTTCC-3′, the 5′ SRE-1 from the synthase was 5′-GATCTGGTTCTGCCCACCTACGTCAG-3′. These were taken from the study of Smith et al. (4). The high affinity E-box site was 5′-GATCCGTACGCTAGGAGGAG-3′ and was taken from the binding site selection studies performed by Kim et al. (16). The complementary oligonucleotides were designed to hybridize such that there was a four-base sticky end at each side. DGATC for ease of labeling with polynucleotide kinase and [γ32P]ATP.

Gel Mobility Shift Experiments—Analytical experiments were performed with 0.02 pmol of DNA probe and 10 ng of recombinant SREBP-1 protein (or an equivalent molar amount of a truncated version). Gel conditions were exactly as described before (18). For the competition experiments of Fig. 1A the competitor DNAs (200-fold molar excess) were preincubated with protein for 15 min on ice prior to the addition of the 32P-labeled wild type reductase DNA probe. For the quantitative experiments of Fig. 4 and Table 1, protein extract was serially diluted in buffer containing 200 μM of cytochrome c stabilize the diluted SREBP-1 protein. DNA binding and gel electrophoresis were identical to the standard analytical experiments. The gels were stained with a mixture of acetic acid (0.2%) and methanol (10% (v/v)) prior to drying and analysis with the aid of a Bio-Rad model GS250 phosphoimaging system. Regions corresponding to the singly liganded complex and the free DNA were identified in each lane and integrated using a constant spot volume (pixel density units) which was then analyzed using the Sigmaplot software package. The probe DNA/Luciferase activity (Fig. 5) was plotted as the fraction of DNA bound (ordinate) versus active protein (μm, abscissa). The equilibrium association constants (Kd, μm−1) were determined by fitting the fraction of bound DNA to the Langmuir expression (20).

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\text{Fraction bound} = \frac{[\text{SREBP}]K_d}{[\text{SREBP}]K_d + [\text{SREBP}]K_w}
\]  

(Eq. 1)

The equilibrium dissociation constant (Kd) is equal to 1/K. DNA Footprinting and Methylation Interference—[32P]DNA probes were prepared from the indicated plasmids, incubated with
RESULTS

The strong genetic evidence discussed above suggested the HMG-CoA reductase promoter may be a direct target for activation by SREBP-1 and -2 have a similar domain structure and seem to bind and activate the same promoters, all of the present studies have been performed with SREBP-1. A DNA fragment corresponding to the well characterized sterol regulatory site from −161 to −136 of the hamster reductase promoter was used as a probe in a gel mobility shift experiment with a recombinant form of SREBP-1 protein corresponding to amino acids 1–490 (Fig. 1A). This includes all of the protein information required for DNA binding and transcriptional activation (9, 12, 18). SREBP-1 bound with high affinity and specificity to the reductase probe. The specificity was evaluated by using a series of competitor oligonucleotides corresponding to the exact same DNA fragment except for a single base change as indicated above each lane on the gel in Fig. 1A. Competitor DNAs containing a single mutation at each contiguous base from −156 to −152 corresponding to ATGGT (lanes 5–9) did not compete for SREBP binding and a change from a guanosine to a thymidine (lane 12) also competed less effectively than the other DNA probes. All other single point mutant derivatives competed effectively indicating that alterations at these other positions did not perturb specific recognition by SREBP-1. In addition, binding was also abolished by including a competitor DNA containing the classic SRE-1 element from the LDL receptor (lane 16).

To further characterize this reductase SREBP-1 binding site we performed a methylation interference experiment (Fig. 1B). Methylation of the N-7 position of the guanosine residues at −151, −153, −154, and −157 on the top strand decreased binding by SREBP-1. The data of Fig. 1, A and B, are summarized on the DNA sequence of this region shown in Fig. 1C. The SREBP binding region corresponds to a focused area with critical residues that span 9 base pairs from −156 to −149. Importantly, the nucleotide mutations that diminish SREBP-1 binding also decreased regulation by sterols in a previous study (Ref. 15 and see Fig. 2B). It should be noted that this site corresponds to the previously identified sterol regulatory site and not the overlapping SRE-1 homology (underlined in Fig. 1C) that was originally proposed (4).

We also evaluated the binding of SREBP-1 to the reductase promoter by the DNase I footprinting procedure. A radioactive probe labeled on the top strand was incubated with recombinant SREBP-1 before treatment with DNase I and electrophoresis through a denaturing polyacrylamide gel. The results demonstrate that SREBP-1 binds specifically to three separated regions on the HMG-CoA reductase promoter fragment which are labeled footprints 1–3 in Fig. 2A. Footprint 2 corresponds to the DNA region used in the gel mobility shift studies of Fig. 1 and to the cis-acting site required for sterol regulation defined by our earlier studies. Footprints 1 and 3 define other sites of SREBP binding in the reductase promoter. A mutation of footprint 1 was not directly evaluated in previous studies and its role in sterol regulation is unclear (Ref. 5 and see "Discussion").
The sterol regulatory regions of the LDL receptor promoter (footprint 2) were prepared for DNase I footprinting studies from mutant promoters with a single base mutation at each clustered sterol-sensitive region. When a probe with a sterol compromising mutation at −155 was used in the DNase I protection experiment, SREBP bound normally to footprints 1 and 3 but the degree of protection over the footprint 2 region was confined to a smaller area on one side of the wild type footprint and away from the position of the point mutation (Fig. 2A, compare lanes 4 and 7). A similar result was obtained when a probe with a single base mutation at −166 was used, however, the narrowed area of nuclease protection was confined to the opposite side of the wild type footprint which again is in the area away from the location of the single point mutation (compare lanes 4, 7, and 10).

These DNase I footprinting results indicate there are three regions where SREBP-1 binds to the promoter for HMG-CoA reductase and one of them (footprint 2) contains two recognition sites. When the sequence of all three footprint elements are compared with each other and the two sterol sensitive sites of protection in footprint 2 are considered separately there is a limited degree of sequence similarity. Interestingly, this similarity (underlined in Fig. 2B) corresponds to an SREBP-1 “half-site” as proposed by Smith (21) and Yokoyama et al. (8).

The experiments presented so far document that SREBP-1 binds specifically to the HMG-CoA reductase promoter in a manner consistent with it being involved in sterol regulation. Next, we performed transient DNA transfection studies to evaluate activation by SREBP-1. The wild type reductase promoter was fused to the luciferase reporter gene and the resulting plasmid was transfected into HepG2 cells along with a plasmid that expressed the mature transcriptionally active form of SREBP-1 (amino acids 1–490) constitutively from the cytomegalovirus promoter. We reproducibly observed a 10–20-fold activation of the reductase promoter by SREBP-1 which was not observed when a mutant containing a sterol defective block substitution mutation (altering all bases between −161 to −152; mutant D1 of (5)) was analyzed in parallel (mut. in Fig. 3). This 20-fold specific activation is much less than that observed for the LDL receptor promoter analyzed in parallel (Fig. 3B).

To determine if SREBP-1 could activate gene expression directly through binding to one of the non-canonical sites present in the sterol regulatory region of footprint 2 we prepared a synthetic promoter containing three tandem copies of one of the reductase SREBP binding site from footprint 2 (the one shown in Fig. 1C) linked to the generic binding site for Sp1 contained in repeat 3 of the LDL receptor promoter (see the bottom of Fig. 3). The inclusion of the generic Sp1 site was required because SREBP is a very weak transcriptional activator by itself and it functions effectively with Sp1 in the LDL receptor promoter (18). This synthetic construct was activated to a higher level than the wild type reductase promoter (5) (Fig. 3A). To evaluate the specificity of this activation we prepared a double point mutant derivative of this synthetic plasmid with mutations at −156 and −155 in all three copies of the reductase DNA element. The wild type bases at these sites are important for both sterol regulation (15) and recognition by SREBP-1 (Fig. 1). This mutant construct was not activated in the transient assay by SREBP-1 (Fig. 3A). Thus, SREBP binds to non-canonical sites in the reductase promoter and the important sterol regulatory footprint 2 also is a target for SREBP-1 activation in a transient DNA transfection assay.

SREBP proteins are members of the bHLHZip family of transcription factors that play a critical role in regulating gene expression in response to changes in the sterol concentration.
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transcription factors. These proteins typically recognize the "E-box" element which is the palindromic sequence 5'-CANNTG-3' (22). Individual members display unique preferences for both the internal and flanking bases and the SREBP-1 "E-box" element which is the palindromic sequence 5'-CACGTG-3' (16). In addition, the SREBP-1 protein derivatives used in these studies. The SREBP-1 coding sequence from amino acid 1 to 490 is shown at the top of the figure. This region contains all of the information required to activate transcription. There is an acidic activation domain at the extreme amino terminus, a proline-rich domain, and the bHLHZip domain as indicated. The extended basic domain protruding in the amino-terminal direction from the classic bHLH domain is also indicated. All of the amino-terminal deletion derivatives were made by polymerase chain reaction and expressed as 6X His fusion proteins in pRSET (Invitrogen) and purified by metal chelation chromatography.

to study the interaction of SREBP-1 with its different sites in more detail we have analyzed binding of the wild type and mutant forms to the different cis-acting sites. In the above studies, the protein fragment used contained amino acids 1-490 of the human SREBP-1 protein. This corresponds to all of the information required for DNA binding and transcriptional activation. In Fig. 4 we analyzed binding of SREBP-1 derivatives that retain different extents of the amino-terminal amino acids that flank the bHLHZip motif (Fig. 4B) to five different SREBP recognition sites. The full-length protein (amino acids 1-490) recognized all five different cis-acting recognition sites similarly (Fig. 4A, lanes 1-5). However, different results were obtained when we performed DNA binding studies with a protein fragment that lacked all of the amino acids upstream of the bHLHZip motif (Fig. 4A, lanes 6-10). This truncated version recognized the high affinity E-box site and the LDL receptor SRE-1 similar to the full-length protein. In contrast, this shorter protein recognized the 3'-SRE-1 site of the HMG-CoA synthase promoter weakly and failed to appreciably recognize the companion SRE-1 element of the synthase or the SREBP-1 site shown in Fig. 1 of the reductase promoter (compare lanes 3-5 with 8-10).

These results were more carefully documented by performing protein titration assays (Fig. 5) and the data were used to calculate the apparent dissociation constant (Kd) for SREBP-1 binding at each site (Table I). In these studies we also compared the binding of other SREBP-1 truncation mutants that retain intermediate amounts of the protein upstream of the bHLHZip region as indicated in Fig. 4B.

Table I shows the sequence of the five different sites used and the Kd values obtained for several derivatives containing the indicated portion of the coding sequence of SREBP-1. All of the DNA sites were recognized similarly by protein derivatives containing amino acids 299 to 320 in addition to the bHLH
were used to calculate the Kd values displayed in Table I.  

**Table I**  
Amino acids 299–320 increase DNA binding site repertoire for SREBP-1

| Binding site               | Sequence                          | SREBP-1 1–490 | SREBP-1 234–490 | SREBP-1 299–490 | SREBP-1 321–490 |
|---------------------------|-----------------------------------|---------------|----------------|----------------|----------------|
| LDL receptor SRE-1        | 5’-ATCACCCCAC-3'                  | 6.3           | 6.6            | 4.0            | 2.1            |
| HMG-CoA reductase         | 5’-CCGCCCCAT-3’                   | 6.6           | 11.6           | 6.7            | >500           |
| HMG-CoA synthase SRE-1    | 5’-CTACCCCCAC-3’                  | 6.0           | 15.0           | ND*            | >500           |
| HMG-CoA synthase SRE-1    | 5’-GCCACCTAC-3’                   | 11.0          | 13.0           | ND             | >500           |
| Consensus E-Box           | 5’-ATCACGTGAT-3’                   | 2.5           | ND             | 3.1            | 4.6            |

*ND indicates these values were not determined.*

DISCUSSION

Both SREBP-1 and -2 were identified as proteins that recognized the LDL receptor SRE-1 element and the recombinant proteins bound to the same DNA sites and activated the same target genes (6–9, 12). In addition, they were both regulated by sterol dependent proteolysis in cultured cells (9). Oxysterol-resistant mutant cells line arise from DNA rearrangements at the SREBP-2 locus that result in production of a truncated SREBP-2 protein lacking the carboxyl-terminal domain that anchors the precursor to the membrane of the endoplasmic reticulum (11, 13). This prevents the normal sequestration of the protein from the nucleus in sterol replete cells and leads to continuous accumulation of nuclear SREBP-2 independent of the level of regulatory oxysterols in the culture medium. Thus, cholesterol regulated genes including the LDL receptor, HMG-CoA synthase, and reductase, are expressed at a high level that is refractory to the sterol regulation. However, the SRE-1 homology originally proposed for the HMG-CoA reductase promoter (5) does not correspond to the sterol regulatory site that was identified by an exhaustive single-nucleotide substitution analysis (15).

The fact that reductase was activated in the oxysterol-resistant cells prompted us to re-investigate the possibility that it was activated directly by SREBP. In the experiments presented here we show that SREBP-1 binds to the reductase promoter and mutations that abolish sterol regulation also abolish SREBP-1 binding. In addition, we show that transient expression of the mature-soluble form of SREBP-1 activates the reductase promoter through the identified sterol regulatory SREBP binding sites. We have also demonstrated that transient expression of the mature-soluble form of SREBP-2 activates the reductase promoter in a similar manner.

In earlier reports the reductase promoter was not significantly activated by transiently expressed SREBPs in the human 293 cell line (8, 11). The difference with our results is likely because we used a different cell line (HepG2) and we used a higher level of plasmid DNA expressing the mature form of SREBP-1. When analyzed in parallel, the LDL receptor promoter was activated to a much greater absolute level even in our assays (Fig. 3). Since the magnitude of activation for the reductase promoter by SREBP is lower, it is likely that another factor is required in addition to SREBP for high level activation. This co-regulator may be the Red 25 protein we previously identified which also binds to the sterol regulatory-SREBP site between –195 and –150 of the reductase promoter (17).

There are three regions of SREBP binding in the reductase promoter defined by DNase I protection studies: footprints 1–3 in Figs. 1 and 2. The previously identified sterol regulatory region corresponds to footprint 2 (15). This interval is divided into two mutation sensitive regions that each constitute the core recognition site for SREBP (Fig. 2B). Footprints 1 and 3

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1. H. S. Sanchez, S. Millinder Vallett, and T. F. Osborne, unpublished observations.

2. H. S. Sanchez, S. Millinder Vallett, and T. F. Osborne, unpublished observations.
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share the core element of 5'-PyCAppY-3'. The footprint 1 region has not previously been tested by mutagenesis, however, it is unlikely to be critical for sterol regulation since synthetic constructs that contain the footprint 2 region fused to the herpes TK promoter (and lack the footprint 1 region) are regulated efficiently by sterols (5). Also, the present studies have been performed with the hamster reductase promoter and the footprint 1 region exhibits extensive sequence divergence when compared to the corresponding region of the human promoter (23). In fact, the SREBP half-site of footprint 1 is not conserved. The footprint 3 region is conserved with the human promoter and a mutation of this region in the hamster resulted in a severely crippled promoter that was still regulated by sterols (5). Thus, the footprint 2 region was still able to support transcriptional activation upon sterol deprivation.

An auxiliary role for SREBP binding at footprints 1 and 3 is suggested by the phenotype of one class of sterol regulatory mutants in the footprint 2 region of the reductase promoter that resulted in high constitutive promoter activity independent of the sterol level of the culture medium. These mutants were also expressed at a high level when transfected into the hamster resistant SRD-2 cells that overexpress nuclear SREBP-2 (24). In contrast, all of the sterol regulatory mutations of the LDL receptor and HMG-CoA synthase promoters resulted in low constitutive promoter activity even in the SRD-2 cells (3, 4, 6, 24–26). The low unregulated expression is consistent with the inactivation of a simple site for a transcriptional activator such as SREBP. The high constitutive expression of the unusual reductase mutants is likely due to the fact that footprint 2 mutations retain the additional SREBP binding sites of footprints 1 and 3 which could support activation by the high level of nuclear SREBP in the SRD-2 cells. The high activity of the reductase promoter mutants in normal cells cultured in the presence of sterols can only be simply explained by the action of an inhibitory protein that binds to the footprint 2 region and suppresses promoter activity.

Taken together, all of the available data indicate the SREBP footprint 2 region is the key sterol regulatory site for HMG-CoA reductase. This element contains two separate SREBP recognition half-sites (Figs. 1 and 2) and both are required for normal sterol regulation (15). This region is also directly responsible for activation by SREBP-1 in transient DNA transfection studies (Fig. 3).

The sites that are recognized by SREBP in the reductase promoter do not exhibit a high degree of sequence similarity with the known SREBP recognition sites of the LDL receptor or the high affinity E-box recognition site (Fig. 2). In fact, the original SRE-1 homology region predicted previously by sequence inspection of the reductase promoter (4) does not correspond to the element that is responsible for sterol regulation (15) or SREBP binding (Figs. 1 and 2). Interestingly, using techniques similar to those described here we have also demonstrated that the PI1 promoter for acetyl-CoA carboxylase, the rate controlling enzyme of fatty acid biosynthesis, is regulated by sterols and SREBP through non-canonical SREBP recognition sites (27). In fact, one of the acetyl-CoA carboxylase half-sites is 5'-ACCTA-3' which is identical to one of the critical reductase half-sites in footprint 2.

In a recent report Ericsson et al. (28) have shown that SREBP-1 binds to a non-canonical SREBP recognition site that is responsible for sterol regulation of the promoter for farnesyl diphosphate synthase, another sterol regulated gene of cholesterol biosynthesis. These studies along with our current observations suggest that the DNA binding specificity for SREBP needs to be investigated in more detail. Indeed, the Myc protein, another member of the bHLH family which binds to classic E-box sequences, also recognizes several non-canonical variant sites (29).

In the current study we also demonstrate that a small domain of 22 amino acids from SREBP-1 is required for recognition of the reductase "non-canonical" recognition site. This domain is also required for efficient recognition of the two synthase elements (Fig. 4) and for recognition of non-canonical sites in the acetyl-CoA carboxylase promoter. The potential significance of this observation is that different forms of SREBP-1 may be expressed with the capacity to recognize only a subset of recognition elements. The amino acids within this region extend the basic domain (predicted pI = 8.7) and there is a predicted a-helical stretch as well. These features could provide additional protein-DNA contacts that are required for recognition of the variant DNA sites by SREBP-1.

Acknowledgments—We thank Mary Bennett, Edith Sanchez, and John Han for help with gel mobility shift assays and protein purification and we thank Marian Waterman for comments on the manuscript.

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