Sterol-dependent Transcriptional Regulation of Sterol Regulatory Element-binding Protein-2*

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We show in this manuscript that expression of the mRNA for sterol regulatory element-binding protein-2 (SREBP-2) is regulated by the cellular sterol level in cultured HeLa cells. We have cloned the 5′-flanking region of the gene encoding human SREBP-2. Characterization of this region shows the minimum 50-base pair segment, which contains a 10-base pair sterol regulatory element-1 (SRE-1) identical to the one in the human LDL receptor promoter, confers sterol responsiveness when fused to the luciferase reporter gene. Enforced expression of the truncated SREBP-2 protein (amino acid residues 1–481) also shows that this upstream segment contains the information required for transcriptional activation. The luciferase assays using mutant versions of the reporter genes reveal that the sterol-dependent transcriptional regulation is mediated by two nearby motifs, the SRE-1 and the NF-Y binding site (the inverted CCAAT box, ATTTGCC); the latter is reported to play a critical role in sterol-dependent regulation of 3-hydroxy-3-methylglutaryl-coenzyme A synthase and farnesyl diphosphate synthase genes (Jackson, S. M., Ericsson, J., Osborne, T. F., and Edwards, P. A. (1995) J. Biol. Chem. 270, 21445-21448). Gel mobility shift assays demonstrate that the transcription factor NF-Y truly binds to the ATTTGCC sequence. These findings suggest that the activity of SREBP-2 is controlled not only post-translationally but proteolytically of the precursor protein but also transcriptionally by itself together with NF-Y.

Two structurally related sterol regulatory element-binding proteins, designated SREBP-1 and SREBP-2,1 are implicated to be central regulators in cholesterol and fatty acid metabolisms; the binding of SREBPs to a 10-bp sterol regulatory element (SRE-1) elicits the transcriptional activation of downstream genes such as HMG CoA synthase and the LDL receptor (1, 2). Recent studies have also demonstrated that SREBPs are involved in transcriptional control of the genes for farnesyl diphosphate synthase (3), squalene synthase (4), fatty acid synthase, and acetyl CoA carboxylase (5–7).

SREBPs are synthesized as 125-kDa membrane-bound precursors that are localized on the nuclear envelope and the endoplasmic reticulum (8, 9). In sterol-depleted cells the precursors are proteolytically cleaved to generate soluble NH2-terminal fragments (designated as the mature form with ~480 amino acids) containing an acidic transactivation domain and a basic helix-loop-helix-leucine zipper region that mediates protein dimerization and DNA binding. The mature form translocates to the nucleus and activates transcription. When sterols accumulate within cells, the precursors are no longer proteolysed but remain on the membranes, resulting in the decline in transcription of sterol-regulated genes.

In addition to the proteolytic activation of precursor proteins, here we report that mRNA expression for SREBP-2 is regulated by the cellular cholesterol level. The 5′-flanking region of the human SREBP-2 gene has been found to contain the SRE-1 and the NF-Y binding site. We further demonstrate that both sites are necessary for sterol-mediated regulation of the SREBP-2 gene transcription.

EXPERIMENTAL PROCEDURES

RNase Protection Assay—Monolayers of human HeLa cells were set up on day 0 (1.5 × 106 cells/100-mm dish) in medium A (Dulbecco’s modified Eagle’s medium, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml of fungizone) supplemented with 7% (v/v) fetal calf serum (FCS). On day 1, the cells were refed with medium A containing 5% lipoprotein-deficient serum (LPDS, from Sigma) supplemented with either 1 μg/ml of 25-hydroxycholesterol (Sigma) plus 10 μg/ml of cholesterol (Sigma) or 50 μM of a HMG CoA reductase inhibitor, pravastatin (Sankyo CO. Ltd., Japan) plus 50 μM of sodium mevalonate (Sigma).

On day 3, the cells were harvested, and total RNA was prepared by the method of Chomczynski and Sacchi (10). Riboprobes for glyceraldehyde-3-phosphate dehydrogenase, the LDL receptor, and SREBP-2 were prepared and subjected to RNase protection assays as described previously (11, 12).

Cloning of the 5′-Flanking Region of the SREBP-2 Gene—Human genomic DNA (13) was digested with PvuI and ligated to the PvuI cassette according to the manual provided by Takara Biomedicals (Kyoto, Japan). The first PCR was performed with the C1 primer corresponding to the 5′ half of the cassette and an antisense primer (S1) beginning at the 11th codon of the human SREBP-2 cDNA. The second PCR was performed with the C2 primer corresponding to the 3′ half of the cassette and an antisense primer (S2) beginning at the 5th codon of the cDNA. PCR products were subcloned into a TA vector (Invitrogen), and their sequences were determined by the dideoxy chain termination method (14) using either a Silver Sequence method (Promega) or an Applied Biosystems model 373A DNA sequencer.

5′ RACE Analysis—The 5′ RACE System was purchased from Life Technologies, Inc. First strand cDNA was produced using the total RNA of human hepatoma Hep G2 cells with an antisense primer correspond-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) D86746.

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1 The abbreviations used are: SREBP, sterol regulatory element-binding protein; RACE, rapid amplification of cDNA ends; bp, base pair; PCR, polymerase chain reaction; SRE, sterol regulatory element; LDL, low density lipoprotein; FCS, fetal calf serum; LPDS, lipoprotein-deficient serum; HMG, 3-hydroxy-3-methylglutaryl; kb, kilobase pair.
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Fig. 1. SREBP-2 mRNA expression is regulated by cellular sterol levels. Total RNA was prepared from HeLa cells cultured in the presence of other sterols (S, suppressing conditions) or a HMG CoA reductase inhibitor, pravastatin (I, inducing conditions) as described under “Experimental Procedures.” A, 5-μg aliquots of RNA were analyzed for the LDL receptor (LDLR) and SREBP-2 mRNA, and 2-μg aliquots of RNA were analyzed for glyceraldehyde-3-phosphate dehydrogenase mRNA by ribonuclease protection assay. B, the intensity of the bands was measured with a BAS 2000 (Fuji Film). The relative mRNA levels were normalized to 100 for each mRNA under suppressing conditions. Data are the mean ± S.D. of three samples. In three separate experiments the same relative mRNA levels were obtained.

Fig. 2. Nucleotide sequence of the 5′-flanking region of the human SREBP-2 gene. A, the 5′-flanking region was cloned, and its nucleotide sequence was determined. The nucleotide sequence from the initiation site was deposited in GenBank (accession number AF035992). B, nucleotide sequence of the 3′-untranslated region of SREBP-2 containing amino acids 1–481, replaced the same restriction sites of a pGL2 vector (Promega Corp.). To generate truncated SREBP-2 containing amino acids 1–481, replaced the same fragments coding the 5′-untranslated region of SREBP-2 gene into the reporter plasmids were constructed by cloning the NotI fragment obtained by reverse transcription-PCR using total RNA from HeLa cells and oligonucleotides containing the above restriction sites are not determined exactly in the three sites, G/C, G/T, and C/T, respectively. The bands was measured with a BAS 2000 (Fuji Film). The relative mRNA levels were normalized to 100 for each mRNA under suppressing conditions.

RESULTS AND DISCUSSION

HeLa cells were cultured with LPDS in the presence of either cholesterol plus 25-hydroxycholesterol or with pravastatin plus mevalonate and their total RNA was prepared. Ribonuclease protection assay demonstrated that the amounts of mRNA for the LDL receptor and SREBP-2 were significantly augmented under inducing conditions (Fig. 1, A and B); sterol depletions elicited 6- and 2-fold increases, respectively. These results in—

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In order to identify the sequence motifs in the 5′-flanking region of the gene and search for the potential sequence motifs responsible for the activation, a 1.6-kb PsI restriction fragment of genomic DNA was amplified by PCR. The nucleotide sequence of the 0.84-kb 3′ end of the clone is shown in Fig. 2A. The nucleotide start site of the longest clone analyzed by 5′ RACE is designated +1 (Fig. 2, A and B). Interestingly, a SRE-1 identical to that in the human LDL receptor promoter (16) is found around −120 nucleotides from the transcription initiation site.

In order to identify the sequence motifs in the 5′-flanking region responsible for sterol-mediated transcriptional regulation of SREBP-2 gene, we carried out luciferase assay using various deletion constructs of reporter genes (Fig. 3A). HEK 293 cells were transfected with these reporter genes and cultured under the suppressing or inducing conditions. A 2–3-fold statistically significant increase in luciferase activity was observed under the inducing conditions with p1400-Luc, p624-Luc, or p140-Luc, whereas there was no increase with p91-Luc. This magnitude of sterol regulation was similar to that observed for the endogenous SREBP-2 mRNA (Fig. 1). These results strongly suggest that the 50-bp segment between −140 and −91 contains the cis-acting element(s) necessary for the sterol-mediated transcriptional regulation.

Because the SRE-1 locates in the 50-bp segment, we examined whether SREBP-2 itself activates the transcription of the gene. HEK 293 cells were cotransfected with one of the above reporter genes and an expression plasmid encoding the active form of SREBP-2. Coexpression of the active SREBP-2 with p624-Luc or p140-Luc resulted in 10-fold elevation of luciferase activity but with p91-Luc did not result in stimulation (Fig. 3B). Furthermore, the LDL receptor promoter reporter carrying the SRE-1 also exhibited similar stimulation of the activity. These results suggest that the 50-bp segment in the 5′-flanking region contains the information required for the transcriptional activation of SREBP-2 gene and that the SRE-1 may have a critical role in regulation.

It has been demonstrated that the sterol-dependent activation of the LDL receptor, fatty acid synthase, and acetyl CoA carboxylase genes requires SREBPs and the ubiquitous transcription factor Sp1 (5, 7, 18). In the case of the HMG CoA synthase and farnesyl diphosphate synthase genes, both SREBPs and NF-Y are necessary (3, 19). These observations suggest that the synergistic interaction of the ubiquitous transcription factor and SREBPs is important for transcriptional regulation. Because the 50-bp segment of the 5′-flanking region of the SREBP-2 gene contains the SRE-1 and the NF-Y binding site, we hypothesized that this gene may also be regulated by a functional interaction of SREBP-2 with NF-Y (or related factors). To test this hypothesis, we constructed two versions of mutant reporter genes. The pSREKO-Luc contains the 140-bp 5′-flanking sequence with ATCA → TGAT mutations at the SRE-1 (ATCACCCAC). It is demonstrated that each point mutation among the four nucleotides (underlined) abolishes sterol-mediated regulation of LDL receptor transcription (20). In the pNF-YKO-Luc, the ATTTGC sequence is replaced by the EcoRI sequence. When HEK 293 cells transfected with p140-Luc were cultured under the inducing conditions, the luciferase activity was significantly elevated (Fig. 4, left). The deletion of one of the two motifs reduced the response to the cellular sterol level. Cotransfection of the plasmid encoding the active form of SREBP-2 together with p140-Luc resulted in a 15-fold increase in luciferase activity, whereas no or only slight (2-fold) induction was observed in the cells cotransfected with the pSREKO-Luc or the pNF-YKO-Luc (Fig. 4, right). These results indicate that the two binding sites are required for the sterol-mediated transcriptional regulation of the SREBP-2 gene.

To confirm the binding of NF-Y to the ATTTGC sequence, gel mobility shift assay was performed. Incubation of the 85-bp probe with Hep G2 nuclear extract produced a band (Fig. 5, lane 1). This band almost completely disappeared in the presence of an excess of unlabeled probe (lane 2) and was supershifted by antibodies (Anti-NF-YB) recognizing the B subunit of NF-Y, which is composed of three subunits, but not by antibodies to the LDL receptor (lanes 3 and 4). The same results were obtained using HEK 293 nuclear extract (data not shown). These results suggest that the general transcription factor NF-Y binds to the ATTTGC sequence in the promoter of the SREBP-2 gene and are consistent with the findings that NF-Y plays an important role for sterol-dependent regulation of the farnesyl diphosphate synthase and HMG-CoA synthase genes (3, 19). The SRE-1 in the SREBP-2 promoter is recog-
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**FIG. 4.** Effect of the mutation in the SRE-1 or the NF-Y binding site on the expression of reporter gene. Left, HEK 293 cells were transfected with the indicated reporter plasmid. In pSREKO-Luc, the SRE-1 of p140-Luc is replaced. In pNF-YKO-Luc, the NF-Y binding site of p140-Luc is replaced. The cells were cultured under either suppressing or inducing conditions as described in the legend to Fig. 3. The fold activation (luciferase activity under inducing versus suppressing conditions) is shown. Right: HEK 293 cells were co-transfected with the indicated plasmid and either pSREBP2(1–481) or the vector without the insert. The fold activation (luciferase activity in the presence of the active form of SREBP-2 versus in the absence) is shown. Data are the mean ± S.D. of three dishes.

The sterol-dependent transcriptional induction of the SREBP-2 gene was smaller than that of the LDL receptor gene in both the in vivo studies using HeLa cells (Fig. 1) and the in vitro studies using the reporter genes (Fig. 3A). Because SREBP-2 is further activated by proteolytic processing to generate the mature form, which translocates to the nucleus and activates transcription, in sterol-depleted cells, modest transcriptional induction would probably be expected, and the 5'-flanking region of the SREBP-2 gene indeed appears to be designed to activate its expression moderately. By comparing the promoter sequences of genes regulated by sterols, it will be possible to clarify the mechanism by which the magnitude of the transcriptional induction by SREBP's is determined.

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**FIG. 5.** NF-Y binds to the ATTTGCC sequence in the SREBP-2 promoter. Double-stranded DNA corresponding to nucleotides −140 to −57 of the SREBP-2 promoter was 3' end-labeled with Digoxigenin 11-ddUTP and used in gel shift studies in the presence of 6 μg of Hep G2 nuclear extract. In lane 2, a 1,000-fold molar excess of competitor (an unlabeled probe) was added to the reaction. Following the addition of 0.5 μg of antibodies to NF-YB and the LDL receptor, the reaction mixture was placed on ice for 30 min (lanes 3 and 4). The bound (B) and supershifted (SS) probes are indicated. The free (F) probe runs at the bottom of the gel. DNA-protein complexes transferred to nitrocellulose membrane were detected with anti-Digoxigenin antibodies (Boehringer Mannheim).
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