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Multiple DNA Elements for Sterol Regulatory Element-Binding Protein and NF-Y Are Responsible for Sterol-Regulated Transcription of the Genes for Human 3-Hydroxy-3-Methylglutaryl Coenzyme A Synthase and Squalene Synthase[1]

Jun Inoue, Ryuichiro Sato,[2] and Masatomo Maeda

Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871

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The expression of the human SREBP-2 gene is transcriptionally regulated in a cooperative manner by sterol regulatory element-binding proteins (SREBPs) and the general transcription factor NF-Y [Sato, R., Inoue, J., Kawabe, Y., Kodama, T., Takano, T., and Maeda, M. (1996) *J. Biol. Chem.* 271, 26461-26464]. To understand the sterol-dependent transcriptional regulation by these factors in detail, we have examined the regulation of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase and squalene synthase genes, whose promoters have multiple potential sterol regulatory elements (SRE, SREBP binding site) and NF-Y binding sites. The promoter of the human HMG CoA synthase gene was cloned, sequenced, and functionally characterized by means of reporter gene assays. The results indicate that an inverted CCAAT box, two SRE motifs and two Sp1 sites localized in a 90-bp region coordinately regulate the transcription. In the case of the human squalene synthase promoter, two SRE motifs and an inverted CCAAT box between the motifs localized in a 51-bp region are responsible for the sterol-regulated transcription of the gene. Gel mobility shift assay reveals that these two inverted CCAAT boxes are recognized by NF-Y. The involvement of multiple responsive elements in the transcription of HMG CoA synthase and squalene synthase seems to induce a higher level of sterol-dependent regulation (3.5 to 5.8-fold) compared with that of the SREBP-2 promoter, which contains a single pair of SRE motif and CCAAT box (1.8 to 2.6-fold). Reporter gene assays using constructs containing various nucleotide spacing lengths between the SRE motif and the CCAAT box demonstrate that the 16 to 20-bp spacing range is required for maximal transcriptional regulation. These results agree with the findings that the distances between the two motifs in the known sterol responsive elements in several genes, including the human HMG CoA synthase and squalene synthase genes, are in this range.

Key words: cholesterol, HMG CoA synthase, NF-Y, squalene synthase, SREBP.

The intracellular cholesterol level is well regulated by a feedback control mechanism which governs the transcription of genes for enzymes of cholesterol synthesis, as well as the low density lipoprotein (LDL) receptor. Increases in intracellular cholesterol content elicit the suppression of transcription of the genes for HMG CoA synthase, HMG CoA reductase, farnesyl diphosphate (FPP) synthase, squalene synthase, and the LDL receptor (*1-5*). The 5′-flanking regions of these genes contain either a 10-bp stretch of nucleotides (5′-ATCACCCCAC-3′), designated the sterol regulatory element-1 (SRE-1), or related motifs. Two structurally related transcription factors, designated sterol regulatory element binding protein (SREBP)-1 and -2, bind to the element and activate transcription of these genes (*6, 7*). SREBPs are synthesized as 125-kDa membrane-bound precursors which are localized on the nuclear envelope and the endoplasmic reticulum (*8, 9*). In sterol-depleted cells the precursors are proteolytically cleaved to generate soluble NH_2-terminal fragments containing an acidic transactivation domain and a basic-helix-loop-helix-leucine zipper (bHLH-Zip) region that mediates protein dimerization and DNA binding. The NH_2-terminal fragments translocate to the nucleus and activate transcription. When sterols accumulate within cells, the precursors are no longer proteolyzed but rather remain bound to the membranes, resulting in a decline in transcription of sterol-regulated genes.

The binding of SREBPs to the SRE motif is not in itself

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[2] To whom correspondence should be addressed. Tel: +81-6-879-8187, Fax: +81-6-879-8189, E-mail: rsato@phs.osaka-u.ac.jp

Abbreviations: bHLH-Zip, basic-helix-loop-helix-leucine zipper; CB, CCAAT box; FCS, fetal calf serum; FPP, farnesyl diphosphate; HEK, human embryonic kidney; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; PCR, polymerase chain reaction; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein.

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sufficient for the sterol-dependent transcriptional regulation of these genes. The promoter of the LDL receptor gene has two binding sites for the universal transcription factor Spl, and an SRE-1 site between them. Mutations which abolish Spl binding to either of the two sites significantly reduce transcription of the gene (*10*). Gel mobility shift analyses have revealed that SREBP binding to SRE-1 stimulates Spl binding (*11*). Recent studies have further demonstrated that the transcription of the genes for fatty acid synthase and acetyl CoA carboxylase are also regulated by SREBPs and Spl (*12, 13, 15*).

Another general transcription factor NF-Y (also named CBF, CCAAT-binding factor), instead of Spl, is required for the sterol-dependent transcriptional regulation of certain genes. We previously found that the gene for SREBP-2 is regulated by sterol levels in cultured cells and that the promoter has an SRE-1 site and a CCAAT box that is recognized by NF-Y (*14*). Both sites are required for efficient sterol regulation of transcription. An example of coordinate transcriptional regulation by SREBPs and NF-Y is FPP synthase (*3*). The binding of SREBP-1 to an SRE-like site within the FPP synthase promoter is enhanced by the binding of NF-Y to the CCAAT box (*16*). It has also been demonstrated that overexpression of a dominant negative form of NF-Y suppresses transcription of HMG CoA synthase (*3*). NF-Y forms a heterotrimeric molecule composed of three different subunits, NF-YA, NF-YB, and NF-YC, all of which are needed for DNA binding. These subunits show a high degree of sequence identity with *Saccharomyces cerevisiae* HAP3, HAP2 and HAP4, respectively (*17*).

The promoters of the SREBP-2 and FPP synthase genes contain only a single pair of SREBP and NF-Y binding sites. The promoter of human squalene synthase, in contrast, has an approximately 90-bp nucleotide-stretch that contains two CCAAT boxes and three SRE-like motifs (*4*). The promoter of hamster HMG CoA synthase also has three SRE-like motifs and a CCAAT box localized between the first and second SRE-like motifs (*1*). Although it seems likely that both the SREBPs and NF-Y control the transcription of these genes, little is known about which motifs are required for this regulation. The present studies were designed to address the roles of the multiple motifs, especially SRE and the CCAAT box, involved in the sterol regulation of these genes. We have cloned the promoter of human HMG CoA synthase and we show here that two of the three SRE-like motifs in the hamster gene are conserved in human and that NF-Y is essential for efficient transcriptional regulation. We have also characterized the human squalene synthase promoter and defined the region responsible for sterol-dependent regulation. Furthermore, we have shown, by means of luciferase assays, that the specific nucleotide spacing between the SREBP and NF-Y binding sites is critical for maximal transcriptional activation.

MATERIALS AND METHODS

Cloning of the 5′-Flanking Region of the Human HMG CoA Synthase Gene—A 5′ primer (named S1) was designed to hybridize to a 17-base region beginning at nucleotide number −511 (Fig. 1) in the 5′-flanking region of the hamster HMG CoA synthase gene (the first letter of the ATG initiation codon is base number +1). Two 3′ primers (named S2 and S3) were designed to hybridize to 17 bases of the opposite strand beginning at nucleotides number −70 and −87, respectively (*18*). The first PCR was performed with the S1 and S2 primers using human genomic DNA (*14*). The second PCR was performed with the S1 and S3 primers. The 430-bp PCR product was cloned into the PCR II vector (Invitrogen). The resulting clone, designated pHMG S-TA, was sequenced by the dideoxy chain termination method (*19*) using a Silver Sequence™ DNA sequencing system (Promega).

Cloning of the 5′-Flanking Region of the Human Squalene Synthase Gene—A 5′ primer (named Primer A) was designed to hybridize to a 16-base region beginning at nucleotide number −200 of the 5′-flanking region of human squalene synthase gene (the cytidine at the transcription start site is base number +1) (*4*). A 3′ primer (named Primer B) was designed to hybridize to 20 bases of the opposite strand beginning at nucleotide number +67. The resulting 270-bp PCR product was cloned directly into the PCR II vector. The clone, designated pSS200-TA, was sequenced.

Tissue Cultures and Cell Transfection—Human embryonic kidney (HEK) 293 cells were cultured as described previously (*14*) with a minor modification. To diminish the detachment of the cells during transfection, the culture dishes were pre-coated with type I collagen derived from porcine skin (Cellmatrix) according to the manual provided by the Nitta Gelatin (Osaka). Cell transfection and luciferase assay were performed as described previously (*14*).

Construction of Reporter Genes Containing the Human HMG CoA Synthase Promoter for Luciferase Assay—An expression plasmid for an active form of SREBP-2, pSREBP2(1-481), was described previously (*14*). To generate pHMG S-WT, the 430-bp *Bgl*II-*Hin*dIII fragments were ligated to the same restriction sites in the reporter basic vector (PicaGene™ Basic Vector 2, Toyo, Tokyo, identical to pGL3 basic vector by Promega). To disrupt CB1, ATTGGC was replaced by the *Eco*RI recognition sequence. The antisense primer S4 (5′-CCAGTTGAATTC-GACGGAGCTGCG-3′, nucleotides −436 to −416 in Fig. 1) containing five point mutations (underlined) was coupled with S1 to produce a 100-bp fragment (named Megaprimer1). To generate pHMG S-CB1KO, the second PCR was performed with S3 and Megaprimer1 using pHMG S-WT as a template to produce a 430-bp fragment containing the CB1 mutation. The antisense primers S5 (5′-GAGAGACT-GACCTCGAGATCAACGAGAGCCCAG-3′, nucleotides −416 to −384) for pHMG S-SRE1KO (GCCACCTCAC→TGATCTCGAG, mutated nucleotides underlined) and S6 (5′-CAGGGCCGAGGAAACTAGTATCAAGACTGACGT-GA-3′, nucleotides −398 to −364) for pHMG S-SRE2KO were designed to generate pHMG S-SRE1KO and pHMG S-SRE2KO, respectively. To disrupt the two Spl binding sites, 5′-Spl was replaced by the *Xba*I site and 3′-Spl was replaced by the *Dra*I site. The antisense primer S7 (5′-AT-ATCTAGAGACAAAGTCTCAGGGC-3′, nucleotides −369 to −354 in Fig. 1) was coupled with S1 to produce a 170-bp fragment. The sense primer S8 (5′-TATTCTAGA-TCTTCTCTTTAAACTTCC-3′, nucleotides −347 to −330) was coupled with S3 to produce a 200-bp fragment. These two fragments were combined using the *Xba*I site and inserted into the basic vector (named pHMG S-Sp1KO).

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Construction of Reporter Genes Containing the Human Squalene Synthase Promoter for Luciferase Assay—pSS200 was constructed by PCR using Primer A with a *Xba*I site at the 5′ end and Primer B with a *Hin*dIII site at the 3′ end. The PCR fragments using pSS200-TA as a template were digested with *Xba*I and *Hin*dIII, and re-cloned into the *Nhe*I-*Hin*dIII site of a luciferase reporter basic vector. To generate pSS90, the PCR fragments were digested with *Nhe*I and *Hin*dIII, and re-cloned into the *Nhe*I-*Hin*dIII site of a luciferase reporter basic vector. To disrupt SRE1, Primer C with 4 mutations (ATCA→TGAT) starting from nucleotide −200 and Primer B were used to generate pSS200-SRE1KO. To disrupt CB1, Primer D with 5 mutations (ATTGG→GAATT) starting from nucleotide −200 and Primer B were used to generate pSS200-CB1KO. To disrupt SRE2, GTGTGA was replaced by the *Sal*I recognition sequence (GTGA→CGAC). The antisense primer (Primer E) with GTCGAC starting from nucleotide −143 was coupled with Primer A to produce a 70-bp fragment. The sense primer (Primer F) with GTCGAC starting from nucleotide −138 was coupled with Primer B to produce a 210-bp fragment. These two fragments were combined using the *Sal*I site and inserted into the reporter vector (named pSS200-SRE2KO). To generate pSS200-SRE1•2KO, PCR was performed with Primers C and B using pSS200-SRE2KO as a template to produce a 270-bp fragment containing the SRE1 and SRE2 mutations. To generate pSS200-CB1•SRE2KO, PCR was performed with Primers D and B using pSS200-SRE2KO as a template to produce a 270-bp fragment containing the CB1 and SRE2 mutations.

Gel Mobility Shift Assay—A double-stranded DNA fragment of human SREBP-2 gene corresponding to nucleotides −140 to −57 (*14*) was 3′ end-labeled with a Digoxigenin-11-ddUTP using Dig gel shift kit (Boehringer Mannheim). The nuclear extract from HEK293 cells was prepared as described (*14*). Anti-NF-YB polyclonal antibodies recognizing the B subunit of NF-Y were generously provided by Dr. Roberto Mantovani (*20*). The reaction conditions were described previously (*14*). In competition assays, an excess amount of an unlabeled 24-bp fragment containing CB1 in the human squalene synthase promoter (SS-WT, nucleotides −172 to −149 in Fig. 3), the mutant inverted CCAAT box (SS-Mut, ATTGG→GAATT), CB1 in the human HMG CoA synthase promoter (HMG S-WT, nucleotides −433 to −410 in Fig. 1) or the mutant inverted CCAAT box (HMG S-Mut, ATTGG→GAATT) were added prior to addition of the labeled probe.

Construction of Reporter Genes with Various Lengths of Spacing between the SRE Motif and the CCAAT Box—To generate the reporter gene constructs containing various lengths of nucleotide spacing, p140-Luc (*14*) was manipulated. A restriction site for *Eco*RV was introduced between SRE-1 and an inverted CCAAT box (nucleotides −118 to −113). The first PCR was performed with the sense primer with five mutations (5′-C***ATCACCCCA-C***GATATCCTTCGCTCGC-3′, the SRE-1 sequence written in bold italic letters) starting from nucleotide −129 and the antisense primer from nucleotide +172 (S9) using p140-Luc as a template to produce Megaprimer 2. The sense primer from nucleotide −140 (S10) was coupled with Megaprimer 2 to produce a 300-bp fragment containing an *Eco*RV recognition sequence. The resulting construct was designated pLuc*Eco*RV. Subsequently, three primers S11: (5′-ATATAGTACTTTTAAAGATATCGTTAACCTCC***ATT-GG***CTGAGATGAGCC-3′; inverted CCAAT box in bold italic letters); S12:(5′-ATATAGTACTTTTAAAGATAT-CGTTAACGCCTCC***ATTGG***CTGAGATGAGCC-3′); S13:

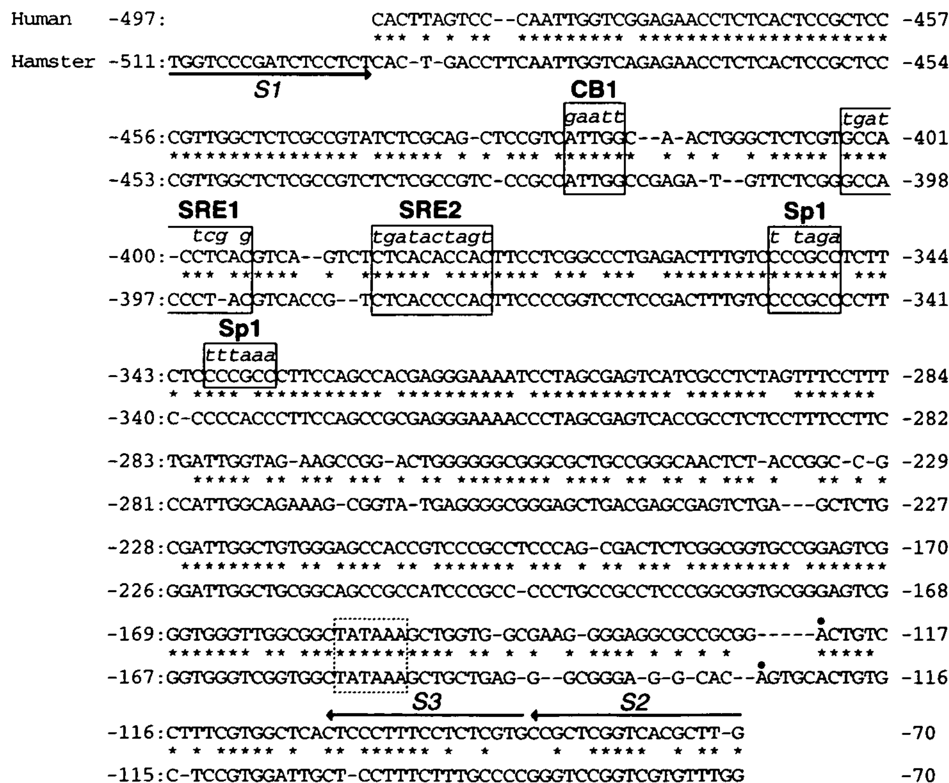

Fig. 1. **Conservation of DNA sequences of the human and hamster HMG CoA synthase promoter region.** The promoter region sequences for the human and hamster HMG CoA synthase (*28*) were aligned for determination of homology. Asterisks denote identical nucleotides. Position +1 is assigned to the A of the ATG codon specifying the initiator methionine; negative numbers refer to the 5′-untranslated sequence. The sites of transcription initiation (*1, 18*) are indicated by dots (●). The dashed box denotes a possible TATA box. The potential regulatory elements are boxed. The arrows indicate the primers used for the cloning. The SRE-2 found in the hamster sequence is underlined. The mutant sequences for reporter gene constructs used in the following experiments are shown by italic letters above the individual original sequence.

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(5′-ATATAGTACTTTTAAAGATATCGTTAACAGGCC-TCC*ATTGG*CTGAGATGAGCC-3′) containing *Sca*I, *Dra*I, *Eco*RV, *Hinc*II, and *Stu*I recognition sites (underlined) were coupled with S9 (containing the *Hin*dIII recognition site at the 5′ end) to produce 300-bp fragments. To generate pLuc derivatives containing 8-32 nucleotide spacings, these 300-bp fragments were digested with *Sca*I-*Hin*dIII, *Dra*I-*Hin*dIII, *Eco*RV-*Hin*dIII, *Hinc*II-*Hin*dIII, or *Stu*I-*Hin*dIII and subcloned into the *Eco*RV-*Hin*dIII sites of pLuc*Eco*RV.

RESULTS

Cloning of the 5′-Flanking Region of the Human HMG CoA Synthase Gene—To analyze the transcriptional regulation of the human HMG CoA synthase gene, we first cloned its 5′-flanking region by PCR (*14*). The nucleotide sequence is compared with that of hamster sequence (Fig. 1). Pairwise comparison reveals that 82% of the nucleotides are identical and two of the three SRE-like motifs in the hamster gene are conserved in humans. The human SRE motifs (SRE1 and SRE2) fit with a consensus sequence (5′-PyCAPyNPyCAPy-3′, Py=pyrimidine) present in the known sterol responsive regions of several genes (*21*), suggesting that SREBPs are likely to recognize these motifs. Two adjacent Spl sites exist between the SRE motifs and a potential TATA box in the human gene, whereas only the upstream Spl site is conserved in the hamster promoter. Two CCAAT box sequences (ATTGG around −485-bp and CB1) are found upstream of the SRE motifs in both species.

Identification of the Elements Responsible for Sterol-Regulated Transcription of Human HMG CoA Synthase—We next studied the promoter function of the cloned 5′-flanking region. HEK 293 cells were transfected with one of the reporter gene constructs and cultured with 5% lipoprotein-deficient serum (LPDS) in the presence of either cholesterol plus 25-hydroxycholesterol (suppressing conditions) or a HMG CoA reductase inhibitor, pravastatin, plus mevalonate (inducing conditions) for 2 days. The amount of induction of luciferase activity by cholesterol depletion was calculated (fold regulation by sterols in Fig. 2A). The wild-type construct (pHMG S-WT) yielded a 3.5-fold regulation by sterols. Mutation of CB1 (pHMG S-CB1KO) or SRE1 (pHMG S-SRE1KO) abolished sterol responsiveness, suggesting that these motifs are essential for sterol-mediated regulation. These results thus indicate that neither the inverted CCAAT box around −485-bp, the SRE2 site, nor the Spl site is able to compensate for the roles played by CB1 and SRE1. Disruption of SRE2 (pHMG

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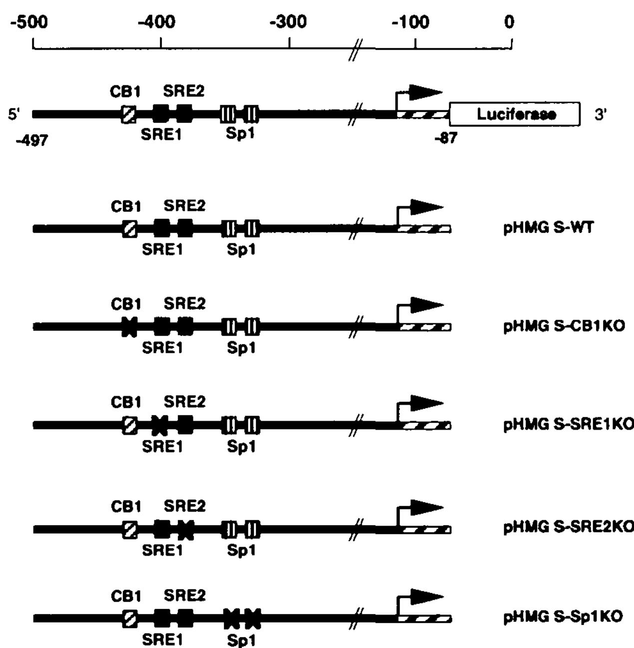


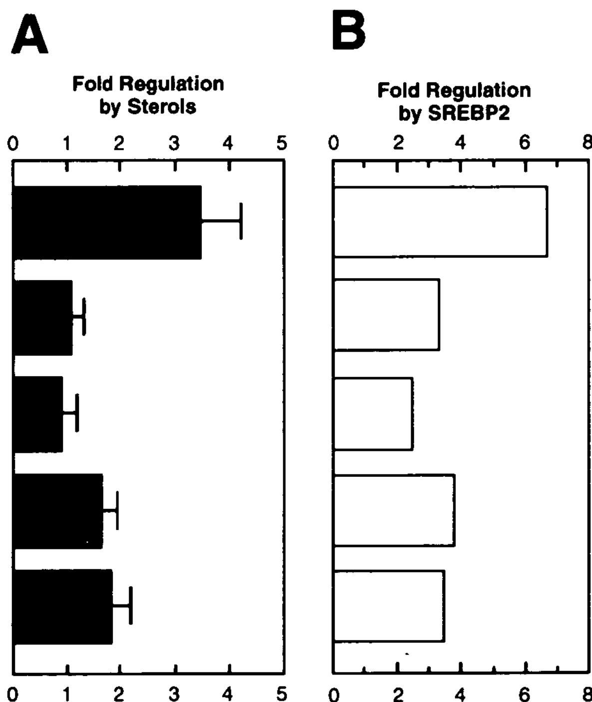


Fig. 2. **Sterol-mediated regulation of reporter genes containing the HMG CoA synthase promoter in transfected HEK293 cells.** Reporter gene constructs are shown together with the scale on the top. Potential regulatory elements are indicated in marked boxes. The site of transcription initiation is indicated by an arrow. (A) HEK 293 cells were transfected with one of the reporter constructs (200 ng) and cultured with 5% LPDS in the presence of either 10 μg/ml of cholesterol plus 1 μg/ml of 25-hydroxycholesterol (S, suppressing conditions) or 50 μM pravastatin plus 50 μM mevalonate (I, inducing conditions) for 2 days. The fold activation (luciferase activity under inducing *versus* suppressing conditions) is shown. The luciferase activity was normalized with respect to the protein content of cell extracts. Data are the mean±SD of 9 independent transfection experiments. The increase in fold regulation by pHMG S-WT, -SRE2KO, or -SP1KO is statistically significant. (B) HEK 293 cells were transfected with the indicated reporter constructs and either an expression plasmid for the active form of SREBP-2 or the vector without the insert. The cells were incubated in the medium containing 7% FCS. The fold activation (luciferase activity with the active SREBP-2 *versus* without it) is shown. Data are the mean of 3 independent transfection experiments. The luciferase activities obtained by the reporter genes used in (A) under suppressing conditions and (B) without the active SREBP-2 were in the range of about 2,000 to 4,000 relative light units/μg protein.

S-SRE2KO) reduced sterol responsiveness but still retained a 1.7-fold induction by sterol depletion. Furthermore, double mutation of the Sp1 sites (pHMG S-Sp1KO) resulted in a similar reduction of the increased induction, suggesting that the SRE2 and the two Sp1 sites participate in the upregulation of sterol responsiveness only when both CB1 and SRE1 are intact. To confirm the involvement of SREBP in this regulation, HEK 293 cells were cotransfected with one of the above reporter genes and an expression plasmid encoding the active form of SREBP-2 (amino acids 1-481). Similar results were obtained by co-expression of the active SREBP-2 (Fig. 2B). Although more than 2-fold induction of luciferase activity was observed in all the reporter gene constructs due to overexpression of the active SREBP, mutation of any one of the elements resulted in a reduction of the increased induction. Thus, CB1 and SRE1 are the sites mainly responsible for sterol-dependent transcriptional regulation of human HMG CoA synthase and the SRE2 and the two Sp1 sites may assist in the regulation.

Characterization of the Human Squalene Synthase Promoter—We cloned the human squalene synthase promoter, which contains several CCAAT boxes and SRE motifs, so as to characterize which motifs are functional in

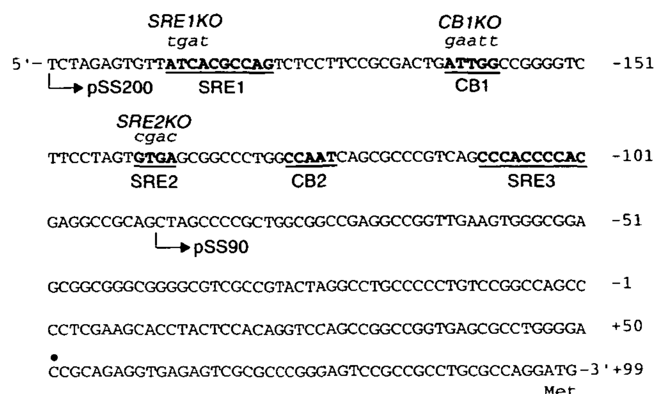
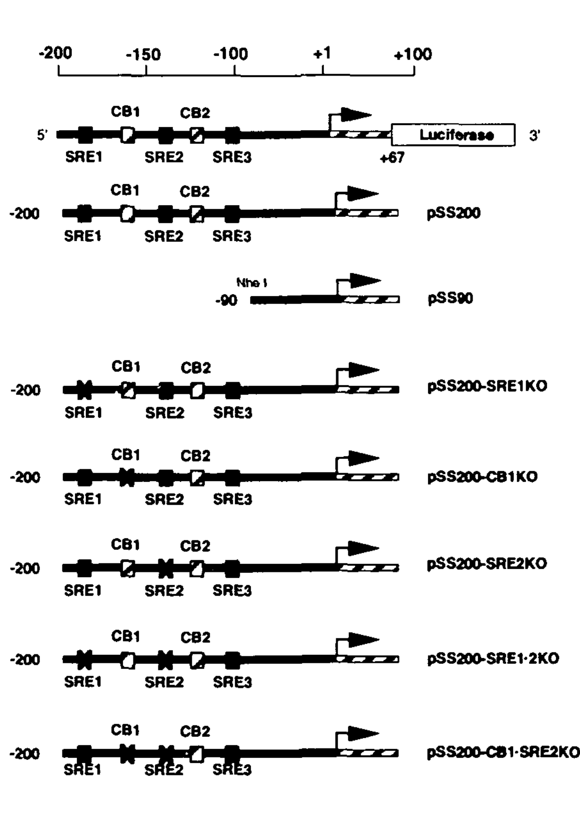


Fig. 3. **Sequences of the human squalene synthase promoter and its mutant derivatives.** The nucleotide sequence from −200 to +99 (the third letter of the initiation codon) is shown. The site of transcription initiation (●) is indicated. Potential regulatory elements are underlined. The sites used for preparation of truncated reporter gene constructs are indicated by the arrows. Compared with the previously published sequence (*4*), a mismatch nucleotide in SRE3 (5′-cCCACCCCAC-3′, a thymidine reported substituted for the underlined cytidine) was found. The mutant sequences for reporter gene constructs are shown by italic letters above the individual original sequence.



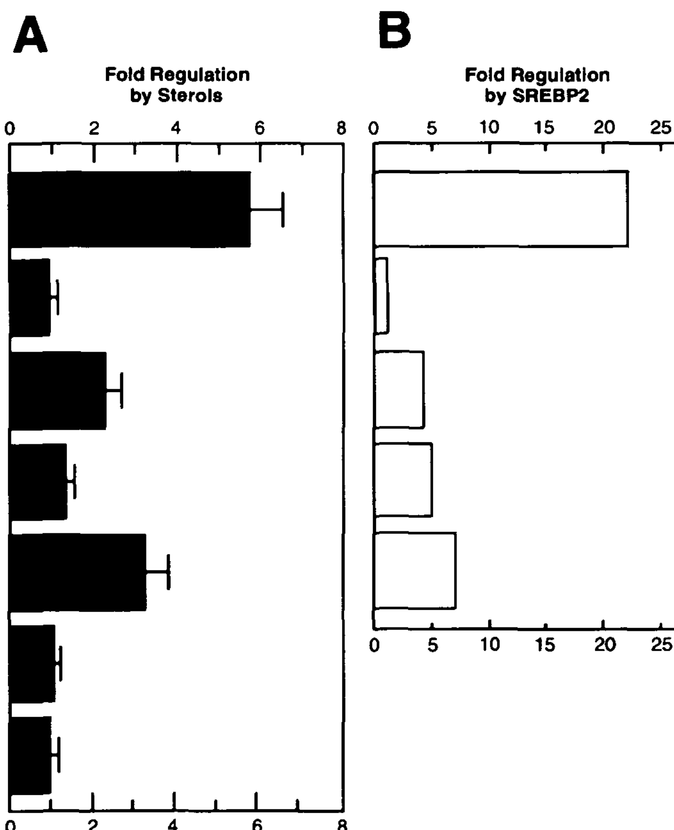


Fig. 4. **Sterol-mediated regulation of reporter genes containing the squalene synthase promoter in transfected HEK293 cells.** Reporter gene constructs are shown together with the scale on the top. Potential regulatory elements are indicated in marked boxes. The site of transcription initiation is indicated by an arrow. (A) HEK293 cells were transfected and cultured as described in the legend to Fig. 2. Data are the mean±SD of 8-12 independent transfection experiments. The increase in fold regulation by pSS200, pSS200-SRE1KO, or -SRE-2KO is statistically significant. (B) Cells were cotransfected with the indicated reporter gene constructs and either an expression plasmid for the active form of SREBP-2 or the vector without the insert, and cultured as described in the legend to Fig. 2. Data are the mean of 3 independent transfection experiments. The luciferase activities obtained with the reporter genes used in (A) under suppressing conditions and (B) without the active SREBP-2 were in the range of about 7,000 to 24,000 relative light units/μg protein.

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the sterol-regulated transcription of this gene. It has been demonstrated that an approximately 200-bp segment of this gene is important for sterol-responsiveness (*4*). The cloned 5′-flanking region is shown in Fig. 3 and the putative SRE motifs and CCAAT boxes are underlined. Previous analyses (*4, 22*) revealed that recombinant SREBP-1a or -2 bound to a sequence containing SRE1 (5′-ATCACGCCAG-3′), SRE2 (5′-GTAG-3′), or SRE3 (5′-TCCACCCCAC-3′). We also confirmed, by gel mobility shift analysis, that a double-stranded 24-bp probe containing SRE2, but not the 4-bp mutant SRE2 (5′-CGAC-3′), was recognized by recombinant SREBP-2 protein (data not shown).

The luciferase assays demonstrated that the highest induction of activity was observed after transfection with pSS200 (Fig. 4A). Deletion of all the elements (pSS90) abolished cholesterol-dependent transcriptional regulation, suggesting that the 110-bp region is essential for regulation. To characterize further the role of each element, various reporter genes with one or two mutated motifs introduced into them were constructed. Mutation of SRE1 (pSS200-SRE1KO) or SRE2 (pSS200-SRE2KO) similarly maintained a 2- to 3-fold induction. Disruption of both SRE1 and 2 (pSS200-SRE1·2KO), however, abolished sterol responsiveness, suggesting that these two SRE motifs are essential for regulation. Furthermore, mutation of CB1 (pSS200-CB1KO) also abolished sterol responsiveness. A combination of SRE2, CB2, and SRE3 (pSS200-CB1KO) did not stimulate transcription. Similar results were obtained by co-expression of the active SREBP-2 (Fig. 4B). Mutation of any one of SRE1, 2, and CB1 resulted in a significant reduction of the increased induction. These results indicate that SRE1, 2, and CB1 are essential for sterol-dependent transcriptional regulation of the human squalene synthase gene and that multiple bindings of SREBPs to SRE motifs close to a CCAAT box induce an additively higher level of regulation.

NF-Y Binds to the CCAAT Box—In a previous paper we showed that the promoter of the human SREBP-2 gene contains an inverted CCAAT box that binds to NF-Y. To determine whether NF-Y binds to the inverted CCAAT box in the HMG CoA synthase or squalene synthase promoter, we performed competition assays using gel mobility shift analysis. When digoxigenin-labeled probes encoding the CCAAT box in the promoter region of the human SREBP-2 gene were incubated with nuclear extracts of HEK 293 cells, a shifted band due to DNA-NF-Y complex formation was detected (Fig. 5, lane 1). This band was supershifted by antibodies against the B subunit of NF-Y (lane 2) and disappeared upon the addition of an excess of unlabeled probe with the first CCAAT box in the squalene synthase promoter or the CCAAT box in the HMG CoA synthase promoter (lanes 3 and 5), but not upon addition of mutant versions of the probes (lanes 4 and 6). These results indicate that a general transcription factor, NF-Y, is probably involved in sterol-regulated transcription of these two genes, carrying out this activity by binding to the CCAAT boxes present in their promoter regions.

Effect of the Specific Spacing between the SRE Motif and the CCAAT Box on Promoter Activity—Although a direct interaction of SREBPs with NF-Y in solution has not yet been shown, it is likely that SREBPs interact with NF-Y on DNA for coordinate regulation of transcription (*16*). Such interaction might require a specific spacing between the SRE motif and the CCAAT box. In order to study the effect of the length of spacing on transcriptional activation, we

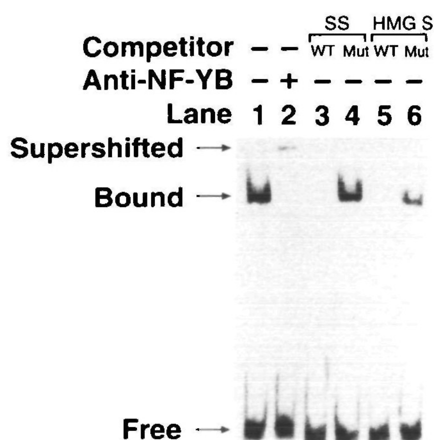

Fig. 5. **NF-Y binds to the ATTGG sequence in the squalene synthase (SS) and HMG CoA synthase (HMG S) promoter.** Double-stranded DNA corresponding to nucleotide −140 to −57 of the SREBP-2 promoter was 3′ end-labeled with digoxigenin 11-ddUTP and used in the presence of 6 μg of HEK293 nuclear extract (lane 1). Following the addition of 0.5 μg of antibody to NF-YB, the reaction mixture was placed on ice for 30 min (lane 2). In lanes 3-6, a 1,000-fold excess amount of unlabeled 24-bp fragment was added to the reaction (WT, a wild-type fragment; Mut, a mutant fragment with GAATT instead of ATTGG). DNA-protein complexes transferred to nitrocellulose membrane were detected with anti-digoxigenin antibodies (*14*).

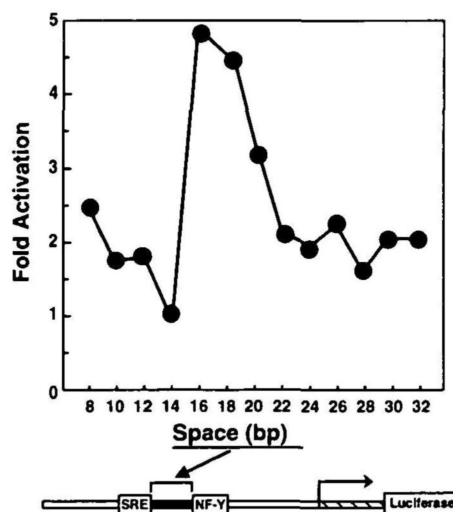

Fig. 6. **Effect of length of the spacing between the SRE motif and the inverted CCAAT box on activation of the SREBP-2 promoter by SREBP-2.** The diagram at the bottom depicts the relevant features of the plasmids used in the study. The horizontal axis shows the length of the spacing between the SRE motif (SRE) and the inverted CCAAT box (NF-Y). HEK293 cells were cotransfected with the indicated plasmid (200 ng) and either pSREBP2(1-481) or the vector without the insert (300 ng), and cultured with a medium containing 7% fetal calf serum for 2 days. The fold activation (luciferase activity in the presence of the active form of SREBP-2 *versus* that in the absence) is shown. The luciferase activities obtained by the reporter plasmids in the absence of the active form of SREBP-2 were in the range of about 1,400 to 3,600 relative light units/μg protein.

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chose the promoter of the human SREBP2 gene, which has a single pair of sites, the SRE-1 motif and the inverted CCAAT box, with an 18-bp spacing. We constructed 13 versions of reporter genes with 8-32 nucleotide spacings between the SRE motif and the inverted CCAAT box. HEK 293 cells were transfected with one of the reporter gene constructs together with an expression plasmid as an active form of SREBP-2 in order to produce higher induction, since the original reporter gene containing the SREBP-2 promoter produced only a 2-fold induction in response to cholesterol depletion. One big peak of fold-increase induction (3.2 to 4.8-fold) was observed in reporter genes with a 16-20 nucleotide spacing (Fig. 6). These results are in good accord with the facts that the distances between the two motifs in the known sterol responsive elements found in the human SREBP-2, HMG CoA synthase, squalene synthase, and FPP synthase genes are all in this range.

DISCUSSION

We have shown that both a general transcription factor NF-Y and SREBPs are involved in sterol-dependent transcriptional regulation of human HMG CoA synthase and squalene synthase, and we have identified the DNA elements responsible for this regulation. In the promoter of the human HMG CoA synthase gene, hamster SRE-2 (5′-CGTCCCGC-3′, underlined in Fig. 1), which had previously been thought to be the putative sterol regulatory element (*1*), is not conserved. The remaining two SRE motifs conserved in human HMG CoA synthase (SRE1 and 2) work together with an adjacent CCAAT box as critical *cis*-elements (Fig. 2). Interestingly, two Sp1 sites localized 23-bp downstream from the second SRE motif are partly involved in sterol-dependent transcriptional regulation (Fig. 2A). In an earlier study, in contrast, mutation of the Sp1 site in the hamster gene, which has only a single Sp1 site corresponding to the first site in the human promoter, did not affect sterol responsiveness (*1*). In this study, when cells were transfected with a reporter gene together with an expression plasmid to give an active form of SREBP-2, mutation of the two Sp1 sites (pHMG S-Sp1KO) attenuated the increase in the luciferase activity produced by the wild-type reporter gene (pHMG S-WT), indicating that these Sp1 sites assist in SREBP-dependent regulation (Fig. 2B).

The current results show that the first and second SRE motifs (SRE1 and 2) and the first inverted CCAAT box (CB1) participate in the sterol-dependent transcriptional regulation of human squalene synthase (Fig. 4). A similar combination of SRE2, 3, and CB2 did not respond to cholesterol depletion (pSS200-CB1KO in Fig. 4). This might be due to the shorter spacing between CB2 and SRE2 or SRE3 (*i.e.*, less than 13 nucleotides). Guan *et al.* recently demonstrated that an inverted Y-box sequence containing CB2 together with SRE1 and 2 can participate in sterol-dependent transcriptional regulation of the human squalene synthase gene (*22*). The results reported here clearly show that CB1 rather than CB2 is critical for such regulation. This discrepancy might be due to differences in the host cells (Hep G2 *versus* HEK 293) used for the luciferase assays, since they are derived from different organs. Based on the results of current investigation into the requisite spacing between the CCAAT box and the SRE motif (Fig. 6), the distance from CB2 to SRE1 (51 bp) might be rather far apart for these sites to communicate with each other.

It has been reported that expression of endogenous mRNA for HMG CoA synthase or squalene synthase is greatly increased when cultured cells are grown under inducing conditions (cholesterol starvation), as compared with expression of the LDL receptor or SREBP-2 (*23, 24, 14*). The data presented here clearly show that multiple SRE motifs and the CCAAT box localized in the promoter of HMG CoA synthase or squalene synthase are responsible for this phenomenon. Two SRE motifs, a CCAAT box and two Sp1 sites coordinately upregulated the transcription of the HMG CoA synthase gene by 3.5-fold (Fig. 2A). In the case of the squalene synthase promoter, two SRE motifs and a CCAAT box regulated transcription sterol-dependently, resulting in a 5.8-fold increase of induction (Fig. 4A). The promoter of the human SREBP-2 gene, in contrast, has a single paired SRE motif and CCAAT box, thereby generating only an 1.8- to 2.6-fold upregulation by sterols (*14*). Briggs *et al.* have also demonstrated this using chloramphenicol acetyltransferase assays in which a reporter gene containing four copies of the SRE motifs plus a single copy of the Sp1 site yields a 14-fold greater sterol-dependent transcriptional activation, whereas only a 2.5-fold increase in induction is observed for a reporter gene containing a single pair of the SRE motif and the Sp1 site (*25*). These results indicate that multiple SRE motifs close to a CCAAT box induce a higher level of sterol-dependent transcriptional regulation of the genes for human HMG CoA synthase and squalene synthase.

It is clear from the current results that the combination of SREBPs and NF-Y is critical for sterol-dependent transcriptional regulation of these two genes. Our next task was to identify how many nucleotides between the CCAAT box and the SRE motif are needed for sterol-dependent transcriptional regulation. To answer this question, we constructed a series of reporter genes with various nucleotide spacing lengths from 8 to 32 nucleotides, because the distances between the two motifs in the known sterol responsive elements are in this range. We found that a 16 to 20-bp spacing is the range which gives the highest induction of the luciferase activity. Because a 14-bp spacing did not give significant induction (Fig. 6), we prepared another reporter gene construct with a 15-bp spacing, as found in the human HMG CoA synthase promoter. This construct indeed induced luciferase activity to the same level as the construct with a 20-bp spacing (data not shown). These results are in good accordance with the findings that the distances between the two motifs in the known sterol responsive elements [*i.e.*, human SREBP-2 (Ref. *14*), 18 bp; human HMG CoA synthase, 15 bp; human squalene synthase, 16 and 16 bp; human FPP synthase (Ref. *26*), 21 bp] are all in this range, except for a singular 9-nucleotide spacing observed in the promoter of the glycerol-3-phosphate acetyltransferase gene (*27*). Furthermore, the current results clearly support the previous findings that a 4-bp insert between two motifs in the FPP synthase promoter, generating a 25 nucleotide spacing, abolished sterol-regulated transcription (*16*). When cells were co-transfected with an expression plasmid encoding an active form of SREBP-1a instead of SREBP-2, similar results were obtained (data not shown), suggesting that either isoform might require a 16 to 20 nucleotide spacing

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range for interaction with NF-Y. Further experiments are required to answer the question as to whether less than 8 or more than 32 nucleotide spacing permits the interaction.

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REFERENCES

1. Smith, J.R., Osborne, T.F., Brown, M.S., Goldstein, J.L., and Gil, G. (1988) Multiple sterol regulatory elements is promoter for hamster 3-hydroxy-3-methylglutaryl-coenzyme A synthase. *J. Biol. Chem.* **263**, 18480-18487
2. Osborne, T.F., Gil, G., Goldstein, J.L., and Brown, M.S. (1988) Operator constitutive mutation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase promoter abolishes protein binding to sterol regulatory element. *J. Biol. Chem.* **263**, 3380-3387
3. Jackson, S.M., Ericsson, J., Osborne, T.F., and Edwards, P.A. (1995) NF-Y has a novel role in sterol dependent transcription of two cholesterogenic genes. *J. Biol. Chem.* **270**, 21445-21448
4. Guan, G., Jiang, G., Koch, R.L., and Shechter, I. (1995) Molecular cloning and functional analysis of the promoter of the human squalene synthase gene. *J. Biol. Chem.* **270**, 21958-21965
5. Smith, J.R., Osborne, T.F., Goldstein, J.L., and Brown, M.S. (1990) Identification of nucleotides responsible for enhancer activity of sterol regulatory element in low density lipoprotein receptor gene. *J. Biol. Chem.* **265**, 2036-2310
6. Yokoyama, C., Wang, X., Briggs, M.R., Admon, A., Wu, J., Hua, X., Goldstein, J.L., and Brown, M.S. (1993) SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. *Cell* **75**, 187-197
7. Hua, X., Yokoyama, C., Wu, J., Briggs, M.R., Brown, M.S., and Goldstein, J.L. (1993) SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element. *Proc. Natl. Acad. Sci. USA* **90**, 11603-11607
8. Sato, R., Yang, J., Wang, X., Evans, M.J., Ho, Y.K., Goldstein, J.L., and Brown, M.S. (1994) Assignment of the membrane attachment, DNA binding, and transcriptional activation domains of sterol regulatory element-binding protein-1 (SREBP-1). *J. Biol. Chem.* **269**, 17267-17273
9. Wang, X., Sato, R., Brown, M.S., Hua, X., and Goldstein, J.L. (1994) SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. *Cell* **77**, 53-62
10. Sudhof, T.C., van der Westhuyzen, D.R., Goldstein, J.L., Brown, M.S., and Russell, D.W. (1987) Three direct repeats and a TATA-like sequence are required for regulated expression of the human low density lipoprotein receptor gene. *J. Biol. Chem.* **262**, 10773-10779
11. Sanchez, H.B., Yieh, Lynn., and Osborne, T.F. (1995) Cooperation by sterol regulatory element-binding protein and Sp1 in sterol regulation of low density lipoprotein receptor gene. *J. Biol. Chem.* **270**, 1161-1169
12. Bennett, M.K., Lopez, J.M., Sanchez, H.B., and Osborne, T.F. (1995) Sterol regulation of fatty acid synthase promoter. Coordinate feedback regulation of two major lipid pathways. *J. Biol. Chem.* **270**, 25578-25583
13. Lopez, J.M., Bennett, M.K., Sanchez, H.B., Rosenfeld, J.M., and Osborne, T.F. (1996) Sterol regulation of acetyl coenzyme A carboxylase: a mechanism for coordinate control of cellular lipid. *Proc. Natl. Acad. Sci. USA* **93**, 1049-1053
14. Sato, R., Inoue, J., Kawabe, Y., Kodama, T., Takano, T., and Maeda, M. (1996) Sterol-dependent transcriptional regulation of sterol regulatory element-binding protein-2. *J. Biol. Chem.* **271**, 26461-26464
15. Kawabe, Y., Sato, R., Matsumoto, A., Honda, M., Wada, Y., Yazaki, Y., Endo, A., Takano, T., Itakura, H., and Kodama, T. (1996) Regulation of fatty acid synthase expression by cholesterol in human cultured cells. *Biochem. Biophys. Res. Commun.* **219**, 515-520
16. Ericsson, J., Jackson, S.M., and Edwards, P.A. (1996) 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. *J. Biol. Chem.* **271**, 24359-24364
17. Sinha, S., Maity, S.N., Lu, J., and de Crombrugghe, B. (1995) Recombinant rat CBF-C, the third subunit of CBF/NFY, allows formation of a protein-DNA complex with CBF-A and CBF-B and with yeast HAP2 and HAP3. *Proc. Natl. Acad. Sci. USA* **92**, 1624-1628
18. Gil, G., Smith, J.R., Goldstein, J.L., and Brown, M.S. (1987) Optional exon in the 5′-untranslated region of 3-hydroxy-3-methylglutaryl coenzyme A synthase gene: Conserved sequence and splicing pattern in humans and hamsters. *Proc. Natl. Acad. Sci. USA* **84**, 1863-1866
19. Sanger, F. (1981) Determination of nucleotide sequences in DNA. *Science* **214**, 1205-1210
20. Mantovani, R., Pessara, U., Tronche, F., Li, X.-Y., Knapp, A.M., Pasquali, J.L., Benoist, C., and Mathis, D. (1992) Monoclonal antibodies to NF-Y define its function in MHC class II and albumin gene transcription. *EMBO J.* **11**, 3315-3322
21. Magana, M.M. and Osborne, T.F. (1996) Two tandem binding sites for sterol regulatory element binding proteins are required for sterol regulation of fatty-acid synthase promoter. *J. Biol. Chem.* **271**, 32689-32694
22. Guan, G., Dai, Pei-Hua., Osborne, T.F., Kim, J.B., and Shechter, I. (1997) Multiple sequence elements are involved in the transcriptional regulation of the human squalene synthase gene. *J. Biol. Chem.* **272**, 10295-10302
23. Kawabe, Y., Shimokawa, T., Matsumoto, A., Honda, M., Wada, Y., Yazaki, Y., Endo, A., Itakura, H., and Kodama, T. (1995) The molecular mechanism of the induction of the low density lipoprotein receptor by chenodeoxycholic acid in cultured human cells. *Biochem. Biophys. Res. Commun.* **208**, 405-411
24. Sheng, Z., Otani, H., Brown, M.S., and Goldstein, J.L. (1995) Independent regulation of sterol regulatory element-binding proteins 1 and 2 in hamster liver. *Proc. Natl. Acad. Sci. USA* **92**, 935-938
25. Briggs, M.R., Yokoyama, C., Wang, X., Brown, M.S., and Goldstein, J.L. (1993) Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. I. Identification of the protein and delineation of its target nucleotide sequence. *J. Biol. Chem.* **268**, 14490-14496
26. Ericsson, J., Jackson, S.M., Lee, B.C., and Edwards, P.A. (1996) Sterol regulatory element binding protein binds to a cis element in the promoter of the farnesyl diphosphate synthase gene. *Proc. Natl. Acad. Sci. USA* **93**, 945-950
27. Ericsson, J., Jackson, S.M., Kim, J.B., Spiegelman, B.M., and Edwards, P.A. (1997) Identification of glycerol-3-phosphate acyltransferase as an adipocyte determination and differentiation factor 1- and sterol regulatory element-binding protein-responsive gene. *J. Biol. Chem.* **272**, 7298-7305
28. Gil, G., Brown, M.S., and Goldstein, J.L. (1986) Cytoplasmic 3-hydroxy-3-methylglutaryl coenzyme A synthase from the hamster. *J. Biol. Chem.* **261**, 3717-3724

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