Low-temperature effect on the sterol-dependent processing of SREBPs and transcription of related genes in HepG2 cells

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Abstract **Lowering the growth temperature of HepG2 cells from 37°C to 20°C results in a 73% reduction in human squalene synthase (HSS) protein, a 76% reduction in HSS mRNA, and a 96% reduction in promoter activity of a secreted alkaline phosphatase-HSS reporter gene. A similar decrease in either mRNA or protein levels is observed for 3-hydroxy-3-methylglutaryl CoA reductase, farnesyl diphosphate synthase, the LDL receptor, and fatty acid synthase. All these proteins and mRNAs show either a decrease or a complete loss of sterol-dependent regulation in cells grown at 20°C. In contrast, sterol regulatory element binding proteins (SREBPs)-1 and -2 exhibit a 2- to 3-fold increase in mRNA levels at 20°C. The membrane-bound form of the SREBPs is dramatically increased, but the proteolytic processing to the nuclear (N-SREBP) form is inhibited under these conditions. Overexpression of the N-SREBP or SREBP cleavage-activating protein (SCAP), but not site-1 or site-2 proteases, restores the activation of the HSS promoter at 20°C, most likely by liberating the SCAP-SREBP complex so that it can move to the Golgi for processing. These results indicate that the cholesterol synthesizing machinery is down-regulated at low temperatures, and points to the transport of the SCAP-SREBP complex to the Golgi as the specific down-regulated step.**—Shechter, I., P. Dai, M. A. Roseman, S. D. Gupta, B. B. Boyer, and G. Guan. **Low-temperature effect on the sterol-dependent processing of SREBPs and transcription of related genes in HepG2 cells. *J. Lipid Res.* 2003.** 44: **1581–1590.**

Supplementary key words sterol regulatory element binding protein • transcriptional regulation • cholesterol metabolism

Cholesterol is an essential component of eukaryotic cellular membranes and is a precursor in the synthesis of steroid hormones and bile acids (1–3). Cholesterol is regarded as a modulator of membrane fluidity. The effect of cholesterol on membrane fluidity and phase behavior was recognized and studied in detail in the early seventies (4). Perhaps its most important physico-chemical effect with respect to hypothermic adaptation is to broaden or even abolish the endothermic gel-liquid crystalline phase transition of glycero- and sphingolipids. In this way, cholesterol helps to maintain the physiologically essential liquid crystalline phase over a wide range of temperatures (5). There is also strong evidence that eukaryotic plasma membranes contain lipid domains (rafts) that are rich in cholesterol and lipids with relatively saturated acyl chains (i.e., sphingolipids), and coexist with domains rich in phospholipids attached to unsaturated acyl chains (6–8).

Cholesterol synthesis is regulated mainly by coordinated transcription involving sterol regulatory elements (SREs), which are the target of three bHLHLZ transcription factors called SRE binding proteins (SREBPs) (9). These proteins are synthesized as endoplasmic reticulum (ER) membrane-bound precursors (M-SREBPs) with two membrane-spanning domains (10). After synthesis, the SREBPs are bound to an SREBP cleavage-activating protein (SCAP). The NH2-terminal region of SCAP contains eight membrane-spanning helices that serve as the sterol-sensing domain; the COOH-terminal region contains several copies of a WD40 sequence that binds to the SREBPs (10–14). At low membrane cholesterol levels, SCAP escorts the SREBPs to the Golgi compartment (15) where the SREBPs are processed by site-1 and site-2 proteases (S1P and S2P, respectively). Proteolytic cleavage releases the C-terminal (C-SREBPs) and the soluble, transcription-

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Abbreviations: CMV, cytomegalovirus; FAS, fatty acid synthase; FPPS, farnesyl diphosphate synthase; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HSS, human squalene synthase; LDLR, low density lipoprotein receptor; pCMV-βGAL, pCMV-β-galactosidase; S1P, site-1 protease; S2P, site-2 protease; SCAP, SREBP cleavage-activating protein; SQS, squalene synthase; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein.

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ally active N-terminal (N-SREBPs) into the cytosol. The latter can then enter the nucleus and activate sterol synthesis. The sterol-regulated movement of the SCAP-SREBPs complex from the ER to the Golgi is a pivotal event that controls cholesterol homeostasis in eukaryotic cells (10, 16–18). Several studies suggest that sterols regulate the cleavage of SREBPs by modulating the ability of SCAP to transport SREBPs to a post-ER compartment that houses active S1P (19, 20). The transport of ER proteins to the Golgi occurs at a wide range of temperatures. For instance, it was demonstrated that 20°C incubation of cells does not block ER-to-Golgi transport of marker proteins such as vesicular stomatitis virus G protein, but this temperature prevents the movement from the *trans* Golgi to the plasma membrane (21–23). Recently, two additional ER-bound proteins, INSIG-1 (24) and INSIG-2 (25), were shown to be involved in the regulated transport of the SCAP-SREBP complex to the Golgi. These two proteins have 59% identity with five putative *trans* membrane domains. In the presence of high membrane cholesterol content, the INSIG proteins bind to the sterol-sensing domain of SCAP, causing its retention in the ER and preventing SREBPs-SCAP complex transport to the Golgi. At low cholesterol levels, the INSIGs dissociate from the SCAP, allowing the migration of the SREBPs-SCAP complex to the ER budding region for transport to the Golgi (24–27).

The N-SREBPs display differential activation of gene transcription. It was first reported that various *cis* elements in the human hepatic squalene synthase (HSS) promoter differentially bind N-SREBP-1a and N-SREBP-2. Based on this, distinct functional specificity for the two transcription factors was predicted (28, 29). Subsequent studies by Pai et al. established the differential stimulation of cholesterol and unsaturated fatty acid biosynthesis in chinese hamster ovary (CHO) cells expressing the individual SREBPs. There again, an increase in the mRNA for squalene synthase (SQS) was affected by SREBP-2 and not by the two isoforms of SREBP-1 (30). These observations, together with studies in genetically manipulated mice (31), indicate that SREBP-1 may be selectively involved in the activation of genes associated with fatty acid metabolism, while SREBP-2 is more specific in cholesterol homeostasis, primarily because of its selective activation of the SQS gene. The number of genes found to be activated by the SREBPs is constantly increasing, and includes enzymes involved in cellular cholesterol homeostasis and fatty acid synthesis, as well as SCD-1 and -2 and caveolin-1 (32–34).

In this study, we show the down-regulation of the transcription of genes responsible for cellular cholesterol homeostasis at low temperatures. We also identify the regulatory step most affected by the low temperature and discuss possible mechanisms in the regulation of cholesterol production under hypothermic conditions.

EXPERIMENTAL PROCEDURES

Plasmids and DNA probes

pHSS1kb-Luc is a luciferase reporter driven by a fully functional HSS promoter, as described previously (35). pHSS1kb-secreted alkaline phosphatase (SEAP) contains the same HSS promoter that in this construct controls the expression of SEAP as a reporting system. pHSS1kb-SEAP was constructed by inserting HSS promoter into the *Hind*III and *Kpn*I sites of the vector pTAL-SEAP (Clontech). p-Cytomegalovirus (pCMV)-SCAP, pCMV-Myc-S1P, and pCMV-HSV-S2P are constructs that express functional SCAP, S1P, and S2P, respectively, and were obtained from American Type Culture Collection (12–14, 36). pCMV-CSA10 and pCMV-CS2 are expression vectors encoding the truncated nuclear forms of human SREBP-1a and SREBP-2, respectively. These constructs were provided by Timothy Osborne and expressed as described previously (37). DNA probes used in Northern blot analysis were obtained as follows: 1.4 kb probes for human SREBP-1a and SREBP-2 were released by restriction digestion of plasmids pCMV-CSA10 and pCMV-CS2 with *Bam*HI and *Eco*RI; the probe for HSS was a cDNA fragment (38); the probe for 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) was a 1.2 kb *Hind*III fragment from the plasmid pHRed102 (39); the probe for LDL receptor (LDLR) was a 0.9kb *Xho*I-*Bgl*II fragment obtained from a plasmid containing the full-length cDNA of human LDLR (pLDLR3, ATCC #57004); farnesyl diphosphate synthase (FPPS) probe was a 1.1 kb PCR product from rat FPPS cDNA; and the 1.2 kb probe for GAPDH was obtained by *Bam*HI-*Sal*I digestion of the human GAPDH cDNA.

Cell culture and transfection

Human hepatoma HepG2 cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1 mM glutamine, 1 mM pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO_2. HepG2 HSS-SEAPs are permanently transfected cells with the pHSS1kb-SEAP reporter construct. Transient transfections of cells were conducted in 6-well plates. For each transfection, 0.5 μg of pHSS1kb-Luc reporter plasmid and 0.1 μg of pCMV-β-galactosidase (pCMV-βGAL) as a control for transfection efficiency, plus 0.5 μg of one of the following plasmids were transfected into HepG2 cells using Fugene6 Reagent (Roche): pCDNA3.1 (as pCMV vector control), pCMV-CSA10, pCMV-CS2, pCMV-SCAP, pCMV-Myc-S1P, or pCMV-HSV-S2P. Twenty-four hours after the transfections, the cells were treated with sterol(+) or sterol(−) medium as defined below. Then, one set of plates was maintained at the original culture condition while the second set of plates was transferred into a CO_2 incubator set at 20°C. After a 24 h incubation period, the cell extracts were assayed for luciferase activity. Transfections into HepG2 HSS-SEAP cells and subsequent treatments were the same except that at the end of the treatment time, the culture medium was collected and assayed for SEAP activity using the Great EscAPe SEAP Reporter System kit (Clontech). Luciferase and β-GAL assays were performed as described previously (35). SEAP and luciferase activities are expressed as the ratio of the SEAP or luciferase activity to β-GAL activity. Sterol(+) and sterol(−) conditions were achieved by supplementation of 1 μg/ml 25-OH cholesterol + 10 μg/ml cholesterol or 5 μg/ml lovastatin, respectively, in MEM with 10% lipid-depleted serum.

Western blot analysis

HepG2 cells (3×10^5) were plated into 100 mm plates and grown for 2 days at 37°C. Two sets of the plates were then treated with sterol(+) or sterol(−) medium as mentioned above. After the treatment, one set of plates was maintained at 37°C and the other set was transferred to 20°C. After 24 h incubation, the cells were lysed in 50 mM Tris-HCl (pH 7.9), 1 mM EDTA, 10 mM DTT, and Protease Inhibitor Cocktail Tablets

(Roche). Following sonication, whole-cell lysates were collected and centrifuged (5×10^4 *g* for 5 min). For Western blots, 40–70 μg of each protein sample was run on NuPAGE Tris-Acetate Gel and transferred onto a nitrocellulose membrane. The membrane was then probed with different primary antibodies, and the specific proteins were detected by horseradish peroxidase-conjugated secondary antibodies using the Electrochemiluminescence (ECL) Western Blotting Analysis System (Amersham Biosciences). Quantitation of the signals was obtained by densitometry and ImageQuant software (Molecular Dynamics). Anti-human HMGR antibody is a purified monoclonal antibody obtained from the hybridoma cell line A9. Anti-HSS antibody is a polyclonal antisera against rat SQS (40). Anti-human fatty acid synthase (FAS) antibody is a monoclonal antibody from Immuno-Biological Laboratories Co., LTD. Anti-human LDL receptor antibody (IgG-4A4), anti-N-terminal of human SREBP-1a antibody (IgG2A4), anti-N-terminal of human SREBP-2 antibody (IgG-1D2), and anti-C-terminal of human SREBP-2 antibody (IgG-1C6) were obtained from corresponding hybridoma lines purchased from ATCC. Anti-GAPDH antibody is a monoclonal antibody purchased from Advanced ImmunoChemical, Inc.

Northern blot analysis

HepG2 cells (3×10^5) were plated into 100 mm plates and grown for 2 days at 37°C. The cells were then treated as described above in "Western blot analysis." At the end of the treatment period, the cells were harvested and the total RNA was prepared using Tryzol Reagent (Invitrogen Corporation). For Northern blotting, 20 μg total RNA from each sample was separated on 1.2% agarose gel, transferred onto a Nylon membrane, and probed with different probes as described above in "Plasmids and DNA probes." Quantitation of the signals was also obtained by densitometry and ImageQuant software.

RESULTS

Temperature-dependent transcriptional regulation of HSS promoter-reporter gene

In general, the differential effects of low temperature on enzyme expression and gene transcription shown below were proportional to the decrease in temperature. However, in the following studies we chose to demonstrate the differences between 37°C and 20°C as a representative change in the sterol-dependent regulation of cholesterol metabolism in the cold.

The effects of low temperature were first examined on the promoter activity of HSS using the permanently transfected HepG2 HSS-SEAP cell line. As shown in **Fig. 1**, this 1 kb HSS promoter activity is highly responsive to sterol regulation at 37°C and, similar to the effect of sterols on the HSS promoter-reporter pHSS1kb-Luc (35), shows a 5.9-fold increase in the response to cholesterol deprivation. However, promoter activity, as represented by the SEAP activity, is minimized at 20°C and represents about 23% of that observed at 37°C in the presence of sterols. In addition, sterol regulation at 20°C is no longer observed, and promoter activity is not enhanced by depletion of sterols from the cells. As the temperature shifts from 20°C to 37°C, an increase in the regulatory response to sterols is observed (data not shown).

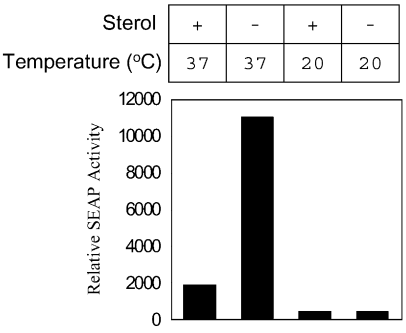

Fig. 1. Squalene synthase (SQS) promoter activity at 37°C and 20°C. Permanently transfected HepG2 human squalene synthase (HSS)-secreted alkaline phosphatase (SEAP) cell lines were incubated in 6-well plates in normal MEM for 48 h before treatment. The plates were then divided into two sets that were treated with either sterol(+) or sterol(−) medium as described in Experimental Procedures. Half of the plates from each set were maintained at 37°C, whereas the second half were transferred to a CO_2 incubator set at 20°C. After 24 h incubation at the indicated temperature and media conditions, the culture media were collected from each well and assayed for SEAP activity. Protein concentrations were determined in total cell lysates of each of the four culture groups. The relative SEAP activity is expressed as the ratio of SEAP activity to protein concentration for each sample.

Temperature-dependent sterol regulation of the LDL receptor and lipid-synthesizing enzyme protein levels

The relative amounts of the LDLR, HMGR, HSS, and FAS proteins in HepG2 cells grown at 37°C and 20°C in the presence and absence of sterols were determined by Western blots (**Fig. 2**). As anticipated, at 37°C the amounts of all of these cellular proteins are increased by sterol deprivation. Similar to the observed regulation of the HSS promoter (Fig. 1), the HSS protein level is low at 20°C and does not display sterol regulation. Similar low-temperature effect is observed for the LDLR and, most pronouncedly, for HMGR. Here again, protein expression of these genes at 20°C is fully suppressed and is similar to, or lower than, the suppressed expression of these proteins in the presence of sterols at 37°C. Unlike the sterol-synthesizing enzymes, FAS is less responsive to sterol or temperature regulation. Although there is a decrease in the level of FAS at 20°C, it is relatively low (20% in the presence of sterols and 32% in their absence), and sterol regulation is still apparent.

Temperature- and sterol-dependent regulation of the mRNA levels of the LDL receptor and cholesterol biosynthesis-related enzymes

Sterol regulation of the relative amounts of the mRNAs of HMGR, HSS, FPPS, and the LDLR from cells grown at 37°C and 20°C was analyzed by Northern blots (**Fig. 3**). At the low temperature, regulation of all these mRNA levels by sterols is greatly diminished. However, unlike in the proteins (see Fig. 2), a low level of sterol regulation can still be observed. At 20°C, there is a 1.2- to 2.1-fold increase in mRNA levels in cholesterol-deprived cells as compared with cells grown in sterol-supplemented medium. In contrast, a 14.6- to 55.3-fold increase is observed for cells grown at 37°C. Interestingly, the basal, sterol-suppressed levels of the various mRNAs is higher in cells grown at 20°C.

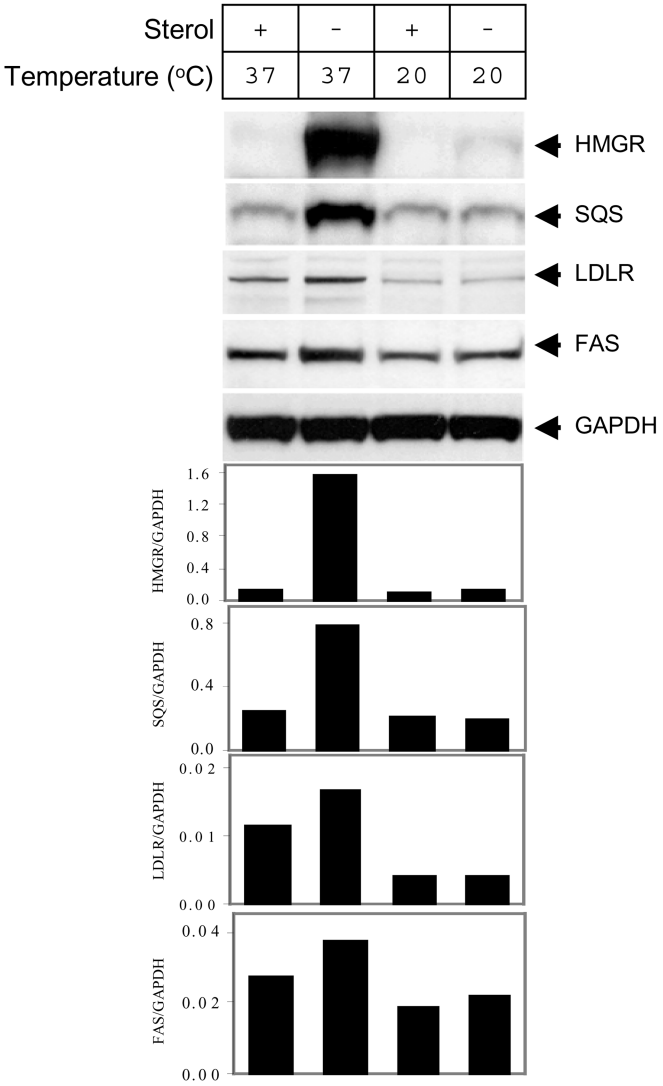

Fig. 2. Western blot analysis of lipid-regulated enzymes in HepG2 cells grown at 37°C and 20°C. HepG2 cells (3×10^5) were plated into 100 mm plates and grown for 2 days at 37°C. Two sets of the plates were then treated with either sterol(+) or sterol(−) medium as described in Experimental Procedures. Half of the plates from each set were maintained at 37°C, whereas the second half were transferred to a CO_2 incubator set at 20°C. After 24 h of incubation, the cells were harvested and whole-cell lysates were prepared. For Western blot analyses, 40–70 μg of protein from each sample was separated on NuPAGE Tris-Acetate Gel and transferred onto nitrocellulose membranes. The target proteins were detected by various specific primary antibodies as indicated, followed by horseradish peroxidase conjugated secondary antibodies using the ECL Western Blotting Analysis System. Quantitation of the signals was obtained by densitometry and ImageQuant software. The bar graph indicates the relative intensity of each band after normalization to the GAPDH signal. Similar results were obtained in three separate experiments.

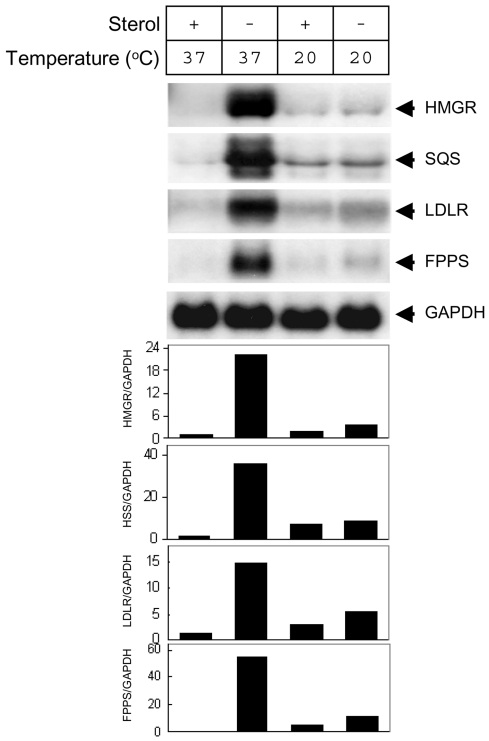

Fig. 3. Northern blot analysis of mRNA Level for lipid-regulated genes in HepG2 cells grown at 37°C and 20°C. HepG2 cells (3×10^5) were plated into 100 mm plates and grown for 2 days at 37°C. Then two sets of the plates were treated with either sterol(+) or sterol(−) medium as described in Experimental Procedures. Half of the plates from each set were maintained at 37°C, whereas the second half were transferred to a CO_2 incubator set at 20°C. After 24 h incubation, the cells were harvested and total RNA was prepared from each experimental group. For Northern blot analysis, 20 μg of total RNA from each sample was separated on 1.2% agarose gel and transferred onto Nylon membranes. The membranes were hybridized with different DNA probes as labeled. All probes were prepared by restriction enzyme digestion of plasmids containing respective cDNAs. Quantitation of the signals was obtained by densitometry and ImageQuant software. Radiolabel intensities for each of the probes were normalized to the GAPDH signal and represented as fold increases to the relative intensity for sterol(+) at 37°C.

Temperature- and sterol-dependent regulation of the processing and the mRNA levels of SREBP-1a and SREBP-2

Since low temperature seems to primarily affect the mRNA levels of genes involved in cholesterol homeostasis, we next analyzed the effect of temperature on the processing of the SREBPs, the transcription factors primarily responsible for the expression of these genes. **Figure 4A** demonstrates the effect of the two different temperatures on the processing of SREBP-1. Since the available anti-SREBP-1 antibody cannot differentiate between the two isoforms of this protein (SREBP-1a and SREBP-1c), it is at present not possible to determine which of them is shown and, therefore, is generally labeled as SREBP-1. Significant processing of the M-SREBP-1 to the N-SREBP-1 form is observed in cholesterol-deprived cells at 37°C, as was reported for other mammalian cells (18). Under these conditions, M-SREBP-1 is the predominant form in the presence of sterols, and N-SREBP-1 predominates in cholesterol-deprived cells. A 5.8-fold increase in the amount of N-SREBP-1 is observed in cholesterol-deprived cells at this temperature. However, at 20°C, M-SREBP-1 is the pre-

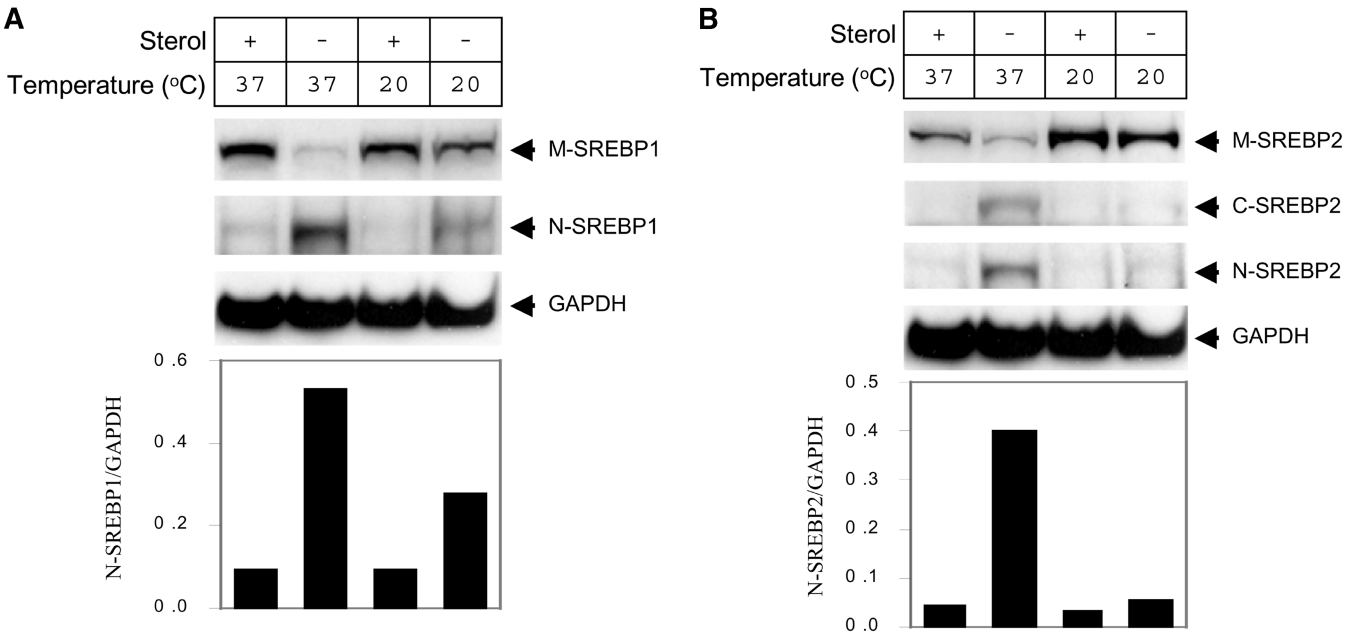

Fig. 4. Western blot analysis of sterol regulatory element binding protein (SREBP)-1 and SREBP-2 in HepG2 cells grown at 37°C and 20°C. HepG2 cells (3×10^5) were plated into 100 mm plates and grown for 2 days at 37°C. Then two sets of the plates were treated with either sterol(+) or sterol(−) medium as described in Experimental Procedures. Half of the plates from each set were maintained at 37°C, whereas the second half were transferred to a CO_2 incubator set at 20°C. After 24 h incubation, the cells were harvested and whole-cell lysates were prepared. For Western blot analyses, 40–70 μg of protein from each sample was separated on NuPAGE Tris-Acetate Gel and transferred onto nitrocellulose membranes. The target proteins were detected by various specific primary antibodies as indicated followed by horseradish peroxidase-conjugated secondary antibodies using the ECL Western Blotting Analysis System. Quantitation of the signals was obtained by densitometry and ImageQuant software. The bar graph indicates the relative intensity of each band after normalization to the GAPDH signal. Similar results were obtained in three separate experiments. The blots depict the membrane (M-SREBP-1) and the nuclear (N-SREBP-1) forms of SREBP-1 (A), and the membrane (M-SREBP-2), C-terminal protein (C-SREBP-2), and nuclear (N-SREBP-2) forms of SREBP-2 (B).

dominant form of the protein in either sterol-supplemented or -deprived cells, indicating a decrease in the proteolytic maturation of M-SREBP-1. When the cellular level of the nuclear form of SREBP-1 is compared under all these conditions, it is apparent that, although there is a 40% decrease in the amount of N-SREBP-1 in the absence of sterols, there is still a significant (3-fold) sterol-dependent regulation at the low temperature.

The effect of temperature on the maturation of SREBP-2 is more dramatic (Fig. 4B). The availability of two different antibodies directed to either the C-terminal or the N-terminal domains of SREBP-2 enables a more precise analysis of this regulation. Similar to the sterol-dependent regulation of the processing of M-SREBP-1 (Fig. 4A), the predominant form at the high temperature and in the presence of sterols is M-SREBP-2. Proteolytic processing is observed upon sterol deprivation at 37°C, as indicated by the decrease of this protein and concurrent increase in the cellular levels of C- and N-SREBP-2 forms. A 9.14-fold increase in the cellular level of N-SREBP-2 is observed in cholesterol-deprived cells as compared with sterol-fed cells under these conditions. However, at 20°C, the processing of M-SREBP-2 is almost completely inhibited regardless of the availability of sterols to the cells. At this temperature, the amount of N-SREBP-2 in cholesterol-deprived cells is only about 14% of that observed under the same growth conditions at 37°C. Similar ratios were calculated for the C-SREBP-2. Interestingly, it appears that the amount of the M-SREBP-2 form is higher in cells grown at the low temperature even in the presence of sterols.

The effect of the growth temperature on the mRNA levels of the SREBPs is shown in **Fig. 5**. It is evident that there is a general increase in the mRNA levels for both SREBP-1 and SREBP-2 in the cells grown at 20°C as compared with those in cells grown at 37°C. Thus, the temperature effect on the mRNA levels appears to be inversely related to the proteolytic activation of the SREBPs. In addition, it appears that, unlike the loss of regulation of mRNA levels for the genes associated with cholesterol synthesis at the low temperature, transcriptional regulation by sterols is hardly affected, and at both temperatures, an increase in the mRNAs for the SREBPs is observed in cholesterol-deprived cells.

Temperature-independent activation of HSS transcription by SREBPs

Although it appears from the increase in SREBP mRNA (Fig. 5) that transcription of the SREBP genes is not suppressed at the low temperature, it was still necessary to eliminate the possibility that the observed suppression of the HSS promoter activity (Fig. 1) and the mRNA and protein levels for the genes in the cholesterol biosynthetic pathway (Figs. 2, 3) is not merely a thermodynamic effect of the low temperature on the kinetics of transcrip-

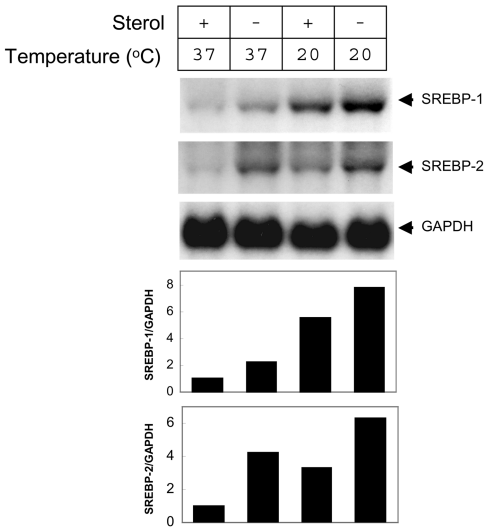

Fig. 5. Northern blot analysis for SREBP1 and SREBP2 expression at 37°C and 20°C under sterol(+) and sterol(−) conditions. Preparation of RNA and Northern blot analysis procedures were done as described in the legend for Fig. 4, except that the radiolabeled probes used were cDNAs for SREBP-1 and SREBP-2. Radiolabel intensities for each of the probes were normalized to the GAPDH signal and represented as fold increase to the relative intensity for sterol(+) at 37°C.

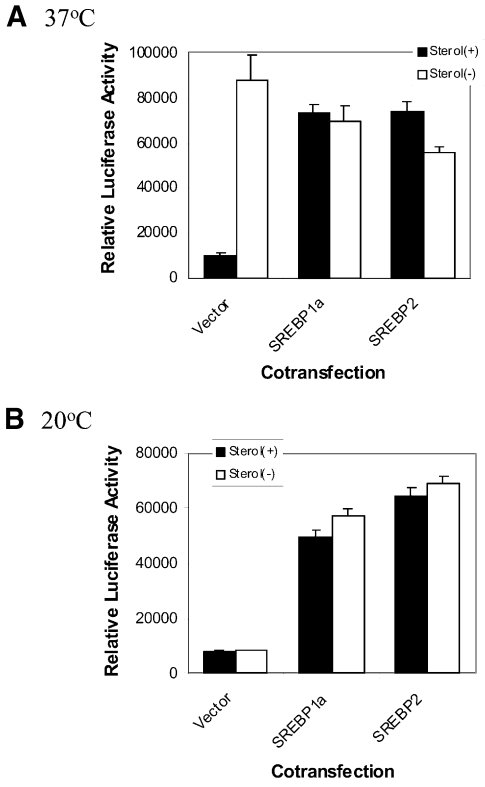

Fig. 6. Induction of SQS promoter activity by overexpression of SREBP-la and SREBP-2 at 37°C and 20°C. On Day 1, HepG2 cells were plated into 6-well plates in normal MEM medium at 1×10^5 cells/well and were grown at 37°C. On Day 2, the cells were transfected with 0.5 μg pHSS-1kb-Luc (HSS promoter-luciferase reporter), 0.1 μg p-cytomegalovirus (pCMV)-β-galactosidase (βGAL), and 0.5 μg of one of the following plasmids: pCMV vector, pCMV-CSA10, or pCMV-CS2. On Day 3, the media was changed for each of the transfected groups to sterol(+) and sterol(−). Half of the cultures from each experimental group were then transferred to a 20°C incubator. On Day 4, the cells were lysed and the total cell extracts were assayed for luciferase and βGAL activity. Relative luciferase activity is expressed as the ratio of luciferase activity to βGAL activity. The result represents the mean of three separate experiments. A: Relative luciferase activity in cell extracts cultured at 37°C. B: Relative luciferase activity in cell extracts cultured at 20°C. Solid bars and open bars represent activities in cells grown in sterol(+) and sterol(−) media, respectively.

tion. For this, we have tested the temperature effect on the activation of the HSS promoter in HepG2 cells cotransfected with the HSS luciferase promoter reporter gene pHSS1kb-Luc and either one of the constructs expressing the nuclear form of the SREBP-1a or SREBP-2 (pCMV-CSA10 or pCMV-CS2, respectively). As reported earlier (28), expression of either of these SREBPs can overcome the suppression of the promoter activity by sterols (**Fig. 6A**). Under these conditions, sterol suppression is almost completely reversed by the SREBPs. Similar to the HSS-SEAP reporter gene (Fig. 1), the activation of the HSS promoter is fully suppressed at 20°C regardless of the presence or absence of sterols in the growth media (Fig. 6B). However, this suppression of promoter activity at the low temperature can be fully reversed by the expression of the SREBPs at 20°C. This result indicates that the suppression of HSS (and most likely all the other sterol related genes) expression is not merely a thermodynamic reduction in the rate of transcription. It also strongly indicates that the low-temperature suppression of these genes' transcription is most likely the result of a deficiency in the cellular N-SREBPs, as observed above (Fig. 4). Since there are no decreases in levels of the mRNAs of the SREBPs or the membrane M-SREBPs forms, it is concluded that suppression of the protein expression and the mRNAs levels (Fig. 2, 3) must be the consequence of an inhibition of the proteolytic processing of the M-SREBPs to the nuclear-soluble forms at 20°C. Having evaluated that, we have tested the potential of the three proteins known to be involved in the transport and proteolytic activation of the SREBPs SCAP, S1P, and S2P (10, 16–18) in reversing the cold effects on the HSS promoter activity. At 37°C, over-expression of the two proteases did not result in the activation of the HSS promoter. In fact, inhibition of HSS promoter activity by S1P and S2P is observed in cholesterol-deprived cells (50% in S1P-expressing cells and 70% in S2P-expressing cells). However, overexpression of SCAP can overcome this sterol suppression, and an increased HSS promoter activity is observed (**Fig. 7A**), most likely due to the reported noninhibitable SREBP processing under these conditions (14). At 20°C, HSS promoter activity is suppressed and is no longer regulated by lipids (Fig. 7B), as also shown in Fig. 1. Similar to the effect at high temperatures, S1P and S2P failed to restore promoter activity. However, under these conditions, expression of SCAP fully restores promoter activity to the level observed in cells grown at 37°C.

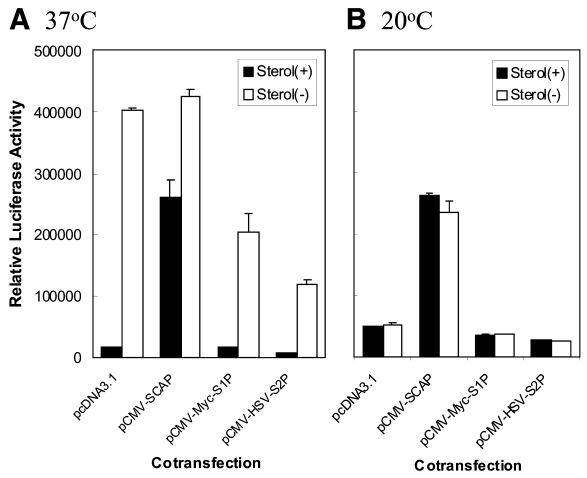

Fig. 7. Activation of SQS promoter at 20°C by overexpression of SREBP cleavage-activating protein (SCAP). On Day 1, HepG2 cells were plated into 6-well plates in normal MEM medium at 1×10^5 cells/well and were grown at 37°C. On Day 2, the cells were transfected with 0.5 μg pHSS-1kb-Luc (HSS promoter-luciferase reporter), 0.1 μg pCMV-βGAL, and 0.5 μg of one of the following plasmids: pCMV vector, pCMV-SCAP, pCMV-Myc-S1P, or pCMV-HSV-S2P. On Day 3, the media was changed for each of the transfected groups to sterol(+) and sterol(−). Half of the cultures from each experimental group were then transferred to a 20°C incubator. On Day 4, the cells were lysed and the total cell extracts were assayed for luciferase and βGAL activity. Relative luciferase activity is expressed as the ratio of luciferase activity to βGAL activity. The result represents the mean of three separate experiments. A: Relative luciferase activity in cell extracts cultured at 37°C. B: Relative luciferase activity in cell extracts cultured at 20°C. Solid bar and open bar represent activities in cells grown in sterol(+) and sterol(−) media, respectively.

DISCUSSION

In the current study, we have investigated the effects of temperature on cellular cholesterol synthesis and its regulation by sterols. It is well established that both prokaryotic and eukaryotic cells maintain constant membrane fluidity over a wide range of temperatures, a process known as homeoviscous adaptation, by remodeling the membrane lipids (41–49). There is also strong evidence that tolerance of animals to extreme hypothermia is accompanied by membrane remodeling (50). In a different study, we established that the activity of hepatic SQS, a key regulatory enzyme in cholesterol biosynthesis and the most specific enzyme of the biosynthetic pathway, is significantly down-regulated in hibernating Alaskan ground squirrels compared with winter-active euthermic animals (data not shown). Thus, it became interesting to investigate if a similar effect occurs in liver-derived cells from nonhibernating species. Thus, we have investigated the regulation of cholesterol synthesis during temperature adaptation of cultured HepG2 cells.

It is evident that sterol-mediated control is absent at 20°C. Thus, sterol deprivation of the cells does not result in an increase of the cellular HSS protein (Fig. 2). Similar to HSS, the levels of other known sterol-regulated proteins, HMGR and LDLR, are suppressed and are no longer subject to sterol regulation at this low temperature. Interestingly, unlike these proteins, FAS retains a rather significant level at the low temperature. Since the sterol-mediated regulation of all these genes is mainly transcriptional, the temperature effect on the mRNAs level and on a representative promoter-reporter gene was analyzed. The activity of the HSS-SEAP promoter-reporter gene in permanently transfected cells is almost completely suppressed at 20°C, and no sterol-mediated regulation is detected (Fig. 1). A similar, but not identical, profile is observed for the mRNA levels of several genes involved in cholesterol homeostasis (Fig. 3). Although substantially suppressed, residual sterol-mediated regulation of the mRNAs level is still observed at 20°C. Presently, we do not have a satisfactory explanation for this observed differences. Nevertheless, it is apparent that sterol regulation is strongly inhibited at the low temperature.

The transcriptional regulation of the various genes (or lack of it) can be explained by the degree of processing of the M-SREBPs at the different temperatures. For SREBP-1, substantial sterol-regulated processing is observed at 37°C. At 20°C, processing in sterol-depleted cells is decreased, but significant negative sterol regulation of the processing is still retained (Fig. 4A). Although the differences between the temperature-dependent regulation of SREBP-1 and SREBP-2 processing (see below) is not completely understood, the relatively limited temperature effect on the processing of SREBP-1 is essentially in agreement with the limited temperature responsiveness of the level of FAS protein, which is known to be primarily regulated by SREBP-1a (10, 31, 51–53) (Fig. 2). The temperature effect on the sterol-mediated regulation of SREBP-2 is much more pronounced than that on SREBP-1 (Fig. 4B). For SREBP-2, only residual sterol regulation is shown at 20°C. The processing of this protein is mostly suppressed at the low temperature and little, if any, of the N- and the C-SREBP-2 forms are produced.

This general response of the SREBP-2 processing to temperature and sterol regulation is strikingly similar to the response in mRNA levels of HSS and HMGR under the same conditions. It is also similar, but slightly less so, to the LDLR mRNA response (compare signal levels in Figs. 2, 4B). Therefore, it appears that the temperature effect on the sterol regulation of the maturation of SREBP-1 and SREBP-2 is not identical. Evidence for differences in the activation of these two transcription factors was presented before. Although it was demonstrated that, in cultured cells (54, 55) and in animals (56), the maturation of both SREBP-1 and SREBP-2 requires SCAP, Sheng et al. have shown that in animals, the processing of SREBP-1 and SREBP-2 is differentially regulated (57). It appears, therefore, that mechanisms other than regulation by SCAP are involved in the differential maturation of the SREBPs and are yet to be uncovered. It is also apparent that the amount of M-SREBP-2 is by far greater at 20°C than at 37°C (Fig. 4B). At the low temperature, 3- to 4-fold higher levels were observed regardless of the presence of sterols in the growth media. These elevated levels of M-SREBP-2 are most likely caused by enhanced gene tran-

scription at low temperatures, as indicated by the higher levels of the mRNAs at 20°C (Fig. 5). The results do not exclude the possibility of a suppressed activity of mRNA degradation at the low temperatures. However, unlike the strong inhibition of HSS promoter activity (Fig. 1) or sterol regulation of cholesterol related enzymes (Fig. 2), there is a significantly high level of sterol-mediated regulation of the SREBPs mRNA (Fig. 5). Both SREBP-1 and SREBP-2 are known to be self-regulated, and a classical SRE-1 element is present in the promoter of the SERBP-2 gene (58–60). The data in Fig. 5 indicate that, for the SREBPs (in particular for SREBP-2), there appears to be a strong temperature-dependent negative regulation in which a low temperature decrease in N-SREBP-2 (Fig. 4B) results in an increase in its mRNA level (Fig. 5) and the membrane form of the protein (Fig. 4B).

The decreased activation of genes involved in cholesterol synthesis can be accounted for entirely by the reduced level of the N-SREBPs at 20°C. This explanation is further supported by the observation that overexpression of the nuclear forms of the SREBPs can compensate for the low endogenous levels of the N-SREBPs and activate the HSS reporter gene at the low temperature (Fig. 6). This also points to the maturation process of this transcription factor as the temperature-regulated step.

The reduced processing activity of the SREBPs at low temperatures suggests that the transport of the M-SREBPs to the Golgi is rate limiting at 20°C. Since the overexpression of S1P and S2P proteases could not overcome the inhibitory low-temperature effect on the HSS promoter-reporter gene (Fig. 7), it is unlikely that the proteolytic release of the N-SREBPs in the Golgi is limited at this temperature. However, overexpression of SCAP, which results in a significant activation of this reporter gene in the presence of sterols at 37°C (Fig. 7A), elicits activation of the reporter at 20°C as well. The overexpression of SCAP was shown to prevent sterols from inhibiting the export of SCAP-SREBPs from the ER, presumably by competing for the INSIG proteins and saturating the ER retention mechanism (61). The activation by SCAP of the SQS promoter-reporter can overcome the presence of sterols at 20°C, indicating that the inhibited step of cholesterol biosynthesis at the low temperature is actually the transport of the SCAP-SREBP complex to the Golgi. The available evidence suggests that the low-temperature inhibition of the processing of the SREBPs is due to a specific ER-to-Golgi transport of the SCAP-SREBP complex and not to a general inhibition of the budding compartment at 20°C. It is well documented that at 20°C the ER-to-Golgi transport of marker vesicular stomatitis virus G protein is not inhibited (21–23), and further lowering of the temperature to 15°–16°C is required for the inhibition of this process, as evidenced by the accumulation of vesicular tubular transport clusters (62, 63). In addition, the activation of the SREBP processing at 20°C by overexpression of SCAP (and not of S1P or S2P) indicates a specific displacement of the SCAP-SREBP complex from the ER retention mechanism and validates the existence of an ER-to-Golgi transport in HepG2 cells at this temperature, in agreement with the above. This observation allows the formulation of an interesting model that may explain the strong anchoring of SCAP to the INSIGs at low temperatures. We have obtained indications that at low temperatures, during the remodeling of the cell's membranes, there is a dramatic decrease in the cholesterol content of the cholesterol-rich lipid rafts in the plasma membrane with no net loss of total cholesterol content (data not shown). This free cholesterol can then be transported to the ER, where the excess can be used to cement the SREBP-SCAP complex to the INSIGs (24, 25, 27). A similar effect was reported by sphingomyelinase treatment of cultured CHO cells, which prevents the nuclear entry of SREBP-2, most likely by the enrichment of the ER with free cholesterol from depleted microdomains of the plasma membrane (64). An additional explanation for the above observation is that at lower temperatures, the affinity of the SCAP-SREBP complex to the INSIGs increases either due to a change in configuration of these proteins or because of a temperature-dependent redistribution of the ER cholesterol. Further detailed study of the distribution of cholesterol in the various subcellular compartments at 20°C is necessary for the understanding of the low-temperature effect on cholesterol biosynthesis.

Whatever the explanation, we have shown here that in cultured cells, there is a dramatic shut-down of the cholesterol synthesizing machinery at low temperatures that cannot be explained by a simple thermodynamic kinetic effect. Our observations may be used in future studies in which the cellular depletion of the nuclear forms of the SREBPs is required.

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