Differential Stimulation of Cholesterol and Unsaturated Fatty Acid Biosynthesis in Cells Expressing Individual Nuclear Sterol Regulatory Element-binding Proteins*

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Three sterol regulatory element-binding proteins (SREBP-1a, -1c, and -2) stimulate transcription of genes involved in synthesis and receptor-mediated uptake of cholesterol and fatty acids. Here, we explore the individual roles of each SREBP by preparing lines of Chinese hamster ovary (CHO) cells that express graded amounts of nuclear forms of each SREBP (designated nSREBPs) under control of a muristerone-inducible nuclear receptor system. The parental hamster cell line (M19 cells) lacks its own nSREBPs, owing to a deletion in the gene encoding the Site-2 protease, which releases nSREBPs from cell membranes. By varying the concentration of muristerone, we obtained graded expression of individual nSREBPs in the range that restored lipid synthesis to near physiologic levels. The results show that nSREBP-2 produces a higher ratio of synthesis of cholesterol over fatty acids than does nSREBP-1a. This is due in part to a selective ability of low levels of nSREBP-2, but not nSREBP-1a, to activate the promoter for squalene synthase. nSREBP-1a and -2 both activate transcription of the genes encoding stearyl-CoA desaturase-1 and -2, thereby markedly enhancing the production of monounsaturated fatty acids. nSREBP-1c was inactive in stimulating any transcription at the concentrations achieved in these studies. The current data support the emerging view that the nSREBPs act in complementary ways to modulate the lipid composition of cell membranes.

Cholesterol and fatty acids, the building blocks of cell membranes, are synthesized by regulated pathways in animal cells. Both pathways are influenced by a single family of transcription factors designated sterol regulatory element binding proteins (SREBPs), whose concerted actions optimize the lipid content of membranes (reviewed in Brown and Goldstein (1)). Appropriate to their role in modulating membrane composition, the SREBPs are bound intrinsically to cell membranes.

* This work was supported in part by National Institutes of Health Research Grant HL20948 and by the Perot Family Foundation. The abbreviations used are: CHO, Chinese hamster ovary; HMG, 3-hydroxy-3-methylglutaryl; LDL, low density lipoprotein; PCR, polymerase chain reaction; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein; nSREBP, nuclear form of SREBP; N-BP cells, transfected CHO cell lines that express the individual nuclear forms of SREBP-1a, -1c, or -2; SCD, stearyl-CoA desaturase; EcR, ecdysone receptor; RXR, retinoid-X receptor; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; CMV, cytomegalovirus; PMCA, 3-pyrenemethyl-23,24-dinor-5-cholen-22-oate-3β-y1.

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They are synthesized as long precursors of ~1150 amino acids with three domains. The NH2-terminal domain of ~480 amino acids is a classic basic helix-loop-helix-leucine zipper transcription factor. This is followed by a membrane attachment domain of ~80 amino acids and a COOH-terminal domain of ~590 amino acids that performs a regulatory function. The SREBPs are attached to membranes of the nuclear envelope and endoplasmic reticulum in a hairpin fashion with their NH2-terminal and COOH-terminal domains projecting into the cytosol and the membrane attachment domain projecting into the lumen.

In order to reach the nucleus, the NH2-terminal domains of the SREBPs must be released from the membranes by proteolysis. This is accomplished by sequential cleavage at two peptide bonds, designated Site-1 and Site-2 (1). The proteolytic release of the SREBP NH2-terminal domains is subject to negative feedback regulation by cholesterol (3). When the cholesterol content of the cells declines, the Site-1 protease becomes activated, thereby initiating proteolytic release. When cholesterol builds up in the endoplasmic reticulum, the activity of the Site-1 protease is reduced, and the SREBPs remain bound to the membranes (1, 4).

Three SREBPs are known to exist in animal cells. Two of these, designated SREBP-1a and -1c, are synthesized from a single gene through the use of alternate promoters and first exons (5–7). These proteins differ in the length of an acidic sequence at the extreme NH2 terminus that activates transcription. SREBP-1a has a long acidic sequence of 42 amino acids, 12 of which are negatively charged. In SREBP-1c this sequence is reduced to 24 amino acids, of which 6 are acidic (1). SREBP-1c was cloned independently in Dallas (5) and Boston (8), where it was designated as ADD1. SREBP-2 is synthesized from a different gene (9, 10). Overall, it is 47% identical to SREBP-1a, and it also has a long transcription activating domain. The SREBPs can function as homodimers (9), although heterodimerization has not been excluded.

Tissue culture cells and animal organs generally produce all three SREBPs, but they do so in differing ratios. In most tissue culture cells, the SREBP-1a transcript far exceeds that for SREBP-1c (7). However, in most organs of adult animals, including liver and adipose tissue, the SREBP-1c transcript predominates (7). The SREBP-2 transcript is present in all cells at levels that are approximately equal to the total of SREBP-1a and -1c (1).

The reason for the existence of three closely related SREBPs and the reason for their differing ratios in different cell types remain obscure. As far as is currently known, all of the SREBPs activate transcription of the same family of target genes. The identified targets can be divided into two pathways, genes relevant to cholesterol metabolism and those relevant to fatty acid metabolism (1).

In the cholesterol biosynthetic pathway, the SREBPs directly...
activate transcription of the genes encoding 3-hydroxy-3-methylglutaryl (HMG) coenzyme A synthase, HMG-CoA reductase, farnesyl diphosphate synthase, and squalene synthase (1, 5, 9, 11, 12). In the fatty acid and triglyceride biosynthetic pathways, the direct targets of SREBPs include acetyl-CoA carboxylase, fatty acid synthase, and glycerol-3-phosphate acyltransferase (13–17). The SREBPs also directly activate transcription of the gene encoding the low density lipoprotein (LDL) receptor, which provides cholesterol and fatty acids from external sources (5, 9).

Various cells and tissues have been forced to overproduce nuclear forms of SREBPs as a result of transfection or transgenic expression. Under these conditions many other genes are activated, but it is not known whether this activation is direct or indirect, i.e., as a result of activating other genes. This class includes the genes that encode the isoform of squalene synthase (SCD), the enzyme that converts saturated to monounsaturated fatty acids.

### TABLE I

| cDNA probe | Plasmid | Restriction enzyme | Sizea | Reference |
|------------|---------|--------------------|-------|-----------|
| SREBP-1    | pRC-CMV-SREBP-1 | NolI/SalI | 3.8   | 49        |
| SREBP-2    | pRC-CMV-SREBP-2 | NolI/SalI | 5.0   | 37        |
| HMG CoA reductase | pRED-227 | BamHI | 4.5   | 50        |

a kb, kilobase pair.

### TABLE II

| cDNA probe | Primer pair | Primer sequences | Size of PCR productb | Reference |
|------------|-------------|------------------|----------------------|-----------|
| HMG-CoA synthase | 5’ | 5’-GGACACAGAGACAACTCATCGCAAG-G3’ | 634 | 51 |
| Farnesyl diphosphate synthase | 3’ | 5’-CATCCCGAACGCGTCGGGCAAG-G3’ | 690 | 52 |
| Squalene synthase | 5’ | 5’-GTGGTATGGCAGAGGAG-A3’ | 550 | 53 |
| Lanoster synthase | 5’ | 5’-AGAGATAGCGAGAAGGCGC-G3’ | 540 | 54 |
| Lanoster 14-alpha demethylase | 5’ | 5’-AGAGCTGAGTTGCTCATGCTGAG-G3’ | 662 | 55 |
| LDL receptor | 5’ | 5’-CAGAGGACCATCTGCCAGGAG-G3’ | 1600 | 56b |
| Fatty acid synthase | 5’ | 5’-GGTTTGGCCTGGAACTGGCCCGG-G3’ | 1286 | 19,57 |
| Stearoyl-CoA desaturase-1 | 5’ | 5’-ATGCCGCGCCCATAGCTCCAAG-G3’ | 425 | 19,58 |
| Caveolin | 5’ | 5’-CGCTCAGTATAAGGATATCTCGGGAGG-G3’ | 373 | 59,60 |

b bp, base pair.

### EXPERIMENTAL PROCEDURES

**Materials**—Standard molecular biology techniques were used (25). DNA sequencing was performed with the dideoxy chain termination method on an Applied Biosystems model 373A DNA sequencer. Fetal calf lipoprotein-deficient serum (d = 1.215 g/ml) was prepared by ultracentrifugation (26). Human LDL (d = 1.019–1.063 g/ml) was radiolabeled with sodium 125I by the iodine monochloride method (26). We obtained 125I-labeled sheep anti-mouse immunoglobulin, Rapid-hyb buffer, Hybond N+, Hybond C extra membranes, [3H]dCTP (800 Ci/mmol), and Redivue [α-32P]dCTP (3000 Ci/mmol) from Amersham Pharmacia Biotech; anti-FLAG M2 monoclonal antibody from Eastman Kodak Co.; [1,2-14C]acetic acid, sodium salt (110 mCi/mmol) from American Radiolabeled Chemicals, Inc.; [1-14C]stearic acid (55 mCi/mmol) from NEN Life Science Products; [3H] and [14C]-labeled fatty acids for use as standards from NEN Life Science Products and American Radiolabeled Chemicals, Inc.; muristerone A, Zeocin, pCDNA3 vector, pCR-Blunt vector, and the edcysone-inducible expression kit containing plasmid.
Fig. 1. Schematic representation of inducible system for graded expression of nSREBPs. This system is based on the ecdysone system described by No et al. (24) and modified by Invitrogen. The ecdysone receptor (VgEcR) and its partner RXR are both encoded by the plasmid pVgXR. The two proteins heterodimerize and transactivate E/GRE-containing promoters in the presence of muristerone A. An E/GRE is placed upstream of a minimal promoter that can drive expression of the nSREBP-1a, -1c, or -2. These nSREBPs are epitope-tagged with the FLAG epitope and are encoded by plasmids pIND-BP-FLAG. The induced nSREBP-1a, -1c, or -2 in turn drives expression of endogenous target genes whose promoters contain SREs.

Table III

### Sequences of PCR primers used for generating hamster cRNA probes

| cRNA probe       | Primer pair | Primer sequences                                   | Restriction enzyme | References |
|------------------|-------------|---------------------------------------------------|--------------------|------------|
| HMG-CoA reductase | 5′-TTAAGCTTCTGGCATGGGCTTAGAGGACCTAGGAGCC-3′ | SmaI               | 50, 61          |
| Squalene synthase | 5′-TTGAATTCCTGACTTCGACACCCATTACCAGGTCA-3′ | EcoRI              | 53               |
| Stearoyl-CoA desaturase-1 | 5′-TTGAATTCCTGACTTCGACACCCATTACCAGGTCA-3′ | SmaI               | 50, 61          |
| Stearoyl-CoA desaturase-2 | 5′-TTGAATTCCTGACTTCGACACCCATTACCAGGTCA-3′ | SmaI               | 62               |
| β-Actin          | 5′-TTGGAATTCTCGACCCATTACCAGGTCA-3′          | KpnI               | 63, 64          |

**Table III**

| cDNA probe       | Primer pair | Primer sequences                                   | Restriction enzyme | References |
|------------------|-------------|---------------------------------------------------|--------------------|------------|
| HMG-CoA reductase | 5′-TTAAGCTTCTGGCATGGGCTTAGAGGACCTAGGAGCC-3′ | SmaI               | 50, 61          |
| Squalene synthase | 5′-TTGAATTCCTGACTTCGACACCCATTACCAGGTCA-3′ | EcoRI              | 53               |
| Stearoyl-CoA desaturase-1 | 5′-TTGAATTCCTGACTTCGACACCCATTACCAGGTCA-3′ | SmaI               | 50, 61          |
| Stearoyl-CoA desaturase-2 | 5′-TTGAATTCCTGACTTCGACACCCATTACCAGGTCA-3′ | SmaI               | 62               |
| β-Actin          | 5′-TTGGAATTCTCGACCCATTACCAGGTCA-3′          | KpnI               | 63, 64          |
Differential Expression of Nuclear SREBP-1α, -1c, and -2

FIG. 2. Amounts of nuclear SREBP proteins in N-BP cell lines after induction with varying concentrations of muristerone A. (A) N-BP cell lines M19, 5, and 10 were used to prepare a nuclear extract, and the other two groups were used for assays of 14C-lipid synthesis and LDL receptor activity (Figs. 3 and 5, respectively). Aliquots of the nuclear extract protein (25 μg/lane) were subjected to SDS-PAGE and immunoblot analysis with anti-FLAG M2 antibody followed by incubation with 125I-labeled sheep anti-mouse immunoglobulin as described under "Experimental Procedures." The filter was exposed to BioMax MS-1 film (Sigma) at room temperature for 4 days. The same filter was also exposed to a Fuji PhosphorImager, and the amount of radioactivity in the nSREBs was quantified using a Bio-Imaging analyzer with BAS 100 MacBAS software (Fuji Medical Systems) (see Figs. 3–5).

once with phosphate-buffered saline and then harvested in buffer containing 50 mM Hepes-KOH at pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, 1 mM diithiothreitol, 1 mM sodium EDTA, and 1 mM sodium EGTA supplemented with a mixture of protease inhibitors (O). The cell suspension was passed through a 22-gauge needle 15 times and centrifuged at 1000 × g at 4 °C for 5 min. Each pellet from two pooled 60-mm dishes or one 100-mm dish was resuspended in 0.12–0.15 ml of buffer containing 50 mM Hepes-KOH at pH 7.5, 0.42 mM NaCl, 2.5% (v/v) glycerol, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM EGTA, 1 mM diithiothreitol, and the protease inhibitor mixture, followed by rotation at 4 °C for 30 min and centrifugation at 105,000 × g in a Beckman TLS 100.2 rotor for 30 min at 4 °C. The resulting supernatant was used as the nuclear extract. Protein concentration was measured with a BCA kit.

Samples of the nuclear extract fractions were mixed with 4× SDS loading buffer (29), subjected to SDS-PAGE on 8% gels, and transferred to a Hybond-C membrane. The membrane was blocked by incubation in phosphate-buffered saline containing 5% (v/v) Tween 20, 5% (v/v) heat-inactivated newborn calf serum, and 0.05% (v/v) nonfat dry milk, and 5% (v/v) heat-inactivated newborn calf serum. Each membrane was then incubated with 5 μg/ml anti-FLAG M2 antibody for 2 h at room temperature. After washing five times with phosphate-buffered saline and 0.05% Tween 20, the membranes were incubated for 1 h at room temperature with 125I-labeled sheep anti-mouse immunoglobulin (3 × 10⁶ cpm/ml). The blots were washed at room temperature (four washes; 5 min/wash) with phosphate-buffered saline, 0.05% Tween 20. They were then exposed to film as indicated in the figure legends.

Assays of Lipid Synthesis and LDL Receptor Activity—Intact monolayers were incubated with 0.5 mM sodium [14C]acetate (20–29 dpm/pmol) or 0.1 mM sodium [14C]stearate-albumin (6 dpm/pmol) as described in the figure legends. Incorporation into lipids was determined as described previously (30) except that each sample received, before saponification, −10⁶ dpm [1H]oleic acid as a recovery standard for calculating fatty acid synthesis. The data are expressed as nanomoles of [14C]acetate incorporated into cholesterol or fatty acids per mg of cell protein per h. Receptor-mediated degradation of 125I-LDL by intact cells was measured as described previously (31). Degradation activity represents the high affinity receptor-dependent rate of proteolysis and is expressed as micrograms of 125I-labeled acid-soluble (non-iodide) material released into the culture medium per mg of total cell protein per 5 h. The protein content of cells was measured by the method of Lowry et al. (32).

HPLC Analysis of 14C-Labeled Individual Fatty Acids—Cell monolayers were incubated with sodium [14C]acetate or sodium [14C]stearate-albumin as described above. Prior to saponification, a recovery standard consisting of −10⁶ cpm [14C]linoleic acid was added to each sample. After extraction with petroleum ether, the fatty acid fraction was dried under nitrogen gas, dissolved in 0.1 ml of methanol containing 0.1% (v/v) acetic acid, and analyzed by reverse-phase HPLC using a Waters computer-controlled apparatus with a Nova-Pak C₁₈ reverse-phase column (3.9 × 150 mm) coupled to a Radiometer Flo-I radioactive flow detector. Samples (80 μl representing 0.3 mg of protein from 0.8 dish of cells) were injected automatically by a WISP injector, and the 14C-labeled fatty acids were resolved by isocratic chromatography with a mixture of 84.9% methanol, 15% water, and 0.1% acetic acid at a flow rate of 1 ml/min. Each species of fatty acid was identified by comparison of its retention time with that of a known standard. Recoveries of total fatty acids in different experiments ranged from 30 to 47%, as judged by the recovery of the added [14C]linoleic acid.

cDNA Probes—cDNA probes for Chinese hamster SREBP-1, SREBP-2, and HMG-CoA reductase were prepared using the plasmids and restriction enzymes listed in Table I. cDNA probes for nine other Chinese hamster mRNAs were prepared by PCR as follows. First-strand cDNA was prepared from CHO-K1 total RNA using a first-strand cDNA synthesis kit primed with random hexadeoxynucleotides. The cDNA was used as template in PCR reactions with the primer pairs listed in Table II. The resulting PCR products were subcloned into either pCR-Blunt (LDL receptor probe) or pN0TA/TT (all other probes). The fragments were released by EcoRI (LDL receptor, 1-kilobase pair fragment), XhoI (lanoster demethylase and lanoster synthase), or BamHI (all other probes) and purified on agarose gels. All cDNA probes were radiolabeled with α-[32P]dCTP using Prime-IT II random primer labeling kit (all other probes).

Blot Hybridization of mRNA—Total RNA was prepared from monolayers of stably transfected cell lines using the RNeasy Midi kit. For Northern gel analysis, total RNA was mixed with RNA sample loading buffer (containing 50 μg/ml ethidium bromide) (Sigma), denatured with formaldehyde and formamide, subjected to electrophoresis in a 1% agarose formaldehyde gel, and transferred to Hybond N+ membranes.
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Visualization of the membranes under UV light showed equivalent transfer of 25 and 18 S RNA in all samples. The membranes were hybridized with the indicated 32P-labeled probes (2 × 10⁶ cpm/ml) for 2 h at 65 °C using Rapid-hyb buffer, washed twice with either 0.1% (w/v) SDS/0.2× SSC at 65 °C for 20 min (HMG-CoA reductase) or 0.1% SDS/0.1× SSC at 65 °C for 30 min (all other mRNAs); and exposed at −80 °C to Reflection NEF 495 film (NEN Life Science Products) with intensifying screens for the indicated time. The amount of radioactivity was quantified by exposure of the filter to a BAS1000 Fuji PhosphorImager.

FIG. 4. Ratio of cholesterol/fatty acids synthesized in N-BP cell lines in relation to the amount of muristerone A-induced expression of nSREBP-1a, -1c, or -2. Values from Fig. 3 were used to calculate the ratio of cholesterol/fatty acid synthesis. The × on the vertical axis denotes the value obtained in CHO-K1 cells studied in the same experiment.

FIG. 5. LDL receptor activity in N-BP cell lines in relation to the amount of muristerone A-induced expression of nSREBP-1a, -1c, or -2. N-BP cells (Fig. 2) were switched on day 2 to Dulbecco’s modified Eagle’s medium (without glutamine) containing the indicated amount of muristerone A, 2 mg/ml bovine serum albumin, and 10 µg/ml 2-131I-LDL (135 cpm/mg of protein) in the absence or presence of 500 µg/ml unlabeled LDL. After incubation for 5 h at 37 °C, the amount of high affinity degradation of 2-131I-LDL was measured as described previously (26). The results are plotted against the amount of expressed nSREBP protein (Fig. 2). Each value represents the average of triplicate incubations.

allows production of precise amounts of single nSREBPs in the M19 cells, which lack their own SREBPs as a result of the absence of the SREBP-2 promoter. Fig. 2 shows an experiment in which we added varying concentrations of muristerone to the culture medium of the stably transfected cells and measured the amounts of each nSREBP by SDS-PAGE and immunoblotting with the anti-FLAG antibody. By adjusting the concentration of muristerone, we obtained comparable levels of expression of each of the three SREBPs. For an unknown reason, transcription of the SREBP-1a gene was more sensitive than that of the other two genes, and hence in future experiments we used lower concentrations of muristerone when inducing this gene. In each experiment we harvested dishes of cells and measured the amounts of nSREBPs by immunoblotting and PhosphorImager quantification. The results are plotted as a function of the amount of muristerone expressed.
fold higher than the effective level of nSREBP-2 and -1a.

We calculated the ratio of the synthesis of $^{14}$C-cholesterol/$^{14}$C-fatty acids at varying levels of the three nSREBPs, and the results are plotted in Fig. 4. In the absence of nSREBP, this ratio was extremely low, reflecting the selective loss of cholesterol synthesis in the M19 cells. nSREBP-2 restored this ratio to the same level that was observed in wild-type CHO cells (indicated by the $^3$ on the vertical axis in Fig. 4). Once this threshold was reached, the ratio remained constant even at higher levels of nSREBP-2, reflecting parallel increases in the synthesis of cholesterol and fatty acids. In contrast, nSREBP-1a produced a ratio that remained below the value in wild-type CHO-7 cells, even at high expression levels. nSREBP-1c did not affect the cholesterol/fatty acid ratio, consistent with its failure to alter the synthesis of either cholesterol or fatty acids in these cells.

In the absence of nSREBP, the M19 cells failed to take up and degrade $^{125}$I-LDL with high affinity, indicating a deficiency of LDL receptors (Fig. 5). nSREBP-2 and nSREBP-1a were equally effective in increasing the rate of $^{125}$I-LDL degradation.

Again, nSREBP-1c had no effect.

To measure the effects of the nSREBPs on the level of mRNA transcripts, we performed a series of Northern blots on RNA preparations from N-BP cell lines that were incubated with varying amounts of muristerone (Fig. 6B). In the same experiment we measured the levels of nSREBP proteins in nuclear extracts by immunoblotting (Fig. 6A). All of the blots were quantified on a PhosphorImager, and the mRNA levels were plotted as a function of the amount of expressed nSREBP (Fig. 7). nSREBP-1a and nSREBP-2 were equipotent in raising the mRNAs for HMG-CoA synthase, farnesyl diphosphate synthase, lanosterol synthase, lanosterol 14α-demethylase, and the LDL receptor. nSREBP-1a was somewhat less active than was nSREBP-2 in raising the mRNA for HMG-CoA reductase, and it was much less active with regard to squalene synthase. nSREBP-1a and nSREBP-2 were equipotent in stimulating transcription of the gene for fatty acid synthase. nSREBP-1a appeared somewhat more effective than nSREBP-2 in raising the total mRNA for SCD, but the probe failed to distinguish between SCD-1 and SCD-2 on the Northern blots.
In contrast to the above 3–20-fold stimulations in mRNA expression, the increase in mRNA levels for several genes was either negligible (caveolin and SREBP-1) or less than 2-fold (SREBP-2) (Figs. 6 and 7).

The difference between nSREBP-1a and -2 with respect to squalene synthase was striking. The Northern blotting experiment was repeated three times on different days using different cell extracts and different clones of cells expressing nSREBP-1a. In each case nSREBP-1a was less than 0.1 as effective as nSREBP-2 in raising the level of squalene synthase mRNA. To quantify these data more precisely, we established an RNase protection assay for squalene synthase, and we used HMG-CoA reductase as a control (Fig. 8). The results confirmed the failure of nSREBP-1a to enhance the squalene synthase mRNA.

To distinguish between the two isozymes of SCD, we employed an RNase protection assay using probes that were specific either for SCD-1 or SCD-2 (Figs. 6, A and B). In the absence of muristerone, the amount of mRNA encoding SCD-2 was much less than that encoding SCD-1 in all three N-BP cell lines. Both nSREBP-1a and -2 increased the levels of SCD-1 and SCD-2 mRNA. The relative effect on SCD-2 was greater, so that at high levels of nSREBP expression the absolute amount of SCD-2 mRNA was similar to that for SCD-1.

The increase in the SCD mRNAs would be expected to increase the production of unsaturated fatty acids. To test this hypothesis, we induced the expression of nSREBP-1a with muristerone, and we then incubated the cells with [14C]acetate (Table IV and Fig. 10). After 3 h we saponified the cell lipids, extracted the fatty acids, separated them by HPLC, and detected the products with a continuous radioactivity monitor.

For quantitative purposes, we added an internal standard of [14C]linoleic acid (18:2), which is not produced in hamster cells. When the N-BP-1a cells were incubated in the absence of muristerone, they produced only saturated fatty acids (mostly 16:0 and a small amount of 14:0) (Fig. 10A). After muristerone induction, the synthesis of the saturated fatty acids increased (18:0 as well as 16:0 and 14:0) (Fig. 10B). Even more dramatically, we observed the appearance of relatively large amounts of monounsaturated fatty acids (18:1 and 16:1) that were not detected in the untreated cells.

The quantitative results of the experiment of Fig. 10 are shown in Table IV (Experiment A). For this purpose, the peak area for each of the major fatty acids was measured and expressed as a percent of the total. The [14C]-fatty acids in the M19 cells consisted almost entirely of palmitate (16:0), and there was no effect of muristerone. In the N-BP-1a and N-BP-2 cells, the addition of muristerone produced a dramatic shift toward the monounsaturated varieties. Muristerone had no effect on fatty acid synthesis in the N-BP-1c cells.
If SREBPs affect the synthesis of unsaturated acids, one would predict that this synthesis should be regulated in wild-type CHO-K1 cells when nuclear SREBPs are induced by sterol deprivation or repressed by sterol addition. Indeed, as shown in Experiment B of Table IV, the addition of sterols reduced the percentage of palmitoleate (16:1) and oleate (18:1) in CHO-K1 cells. This experiment was performed on two other occasions with similar results.

To demonstrate the effect of nSREBP-1a on fatty acid desaturation directly, we incubated N-BP-1a cells with [14C]stearate (18:0) for varying times in the absence and presence of muristerone. The cells were harvested, and the relative amounts of [14C]stearate and [14C]oleate (18:1) were measured by HPLC (Fig. 11). In the absence of muristerone, the N-BP-1a cells converted relatively little of the [14C]stearate to oleate (12% at 5 h). This conversion was elevated by 3.6-fold in the presence of muristerone (43% at 5 h). This experiment was performed on one other occasion with similar results.

**DISCUSSION**

In the current studies we used an inducible promoter system to produce graded expression of the nSREBPs in permanently transfected M19 cells, which cannot produce their own nSREBPs, owing to an absence of the Site-2 protease (22). Each of the three nSREBPs was tagged with two tandem copies of the FLAG epitope, thereby allowing the levels of protein to be compared directly by immunoblotting with a common antibody. We attempted to keep the level of expression within the physiologic range as reflected by restoration of lipid synthesis to rates that were similar to those in wild-type cells that were induced by sterol deprivation. The results indicate that nSREBP-1a and nSREBP-2 can both stimulate transcription of genes involved in cholesterol and fatty acid biosynthesis, but they do so with different relative efficiencies. In contrast, nSREBP-1c had little, if any, stimulatory activity in these hamster cells at concentrations that were equivalent to those of nSREBP-1a and -2.

The differences between nSREBP-1a and -2 were apparent in the overall ratio of synthesis of 14C-fatty acids and 14C-cholesterol from [14C]acetate (Figs. 3 and 4). Although both transcription factors stimulated both pathways, nSREBP-2 produced a higher ratio of 14C-cholesterol to 14C-fatty acids. One reason for this difference became apparent when we measured the effects of the two nSREBPs on the levels of individual mRNAs (Figs. 6 and 7). nSREBP-1a and 2 both induced the mRNAs encoding fatty acid synthase and early enzymes in the cholesterol biosynthetic pathway, including HMG-CoA synthase, HMG-CoA reductase, and farnesyl diphosphate synthase. They also had similar inducing effects on two of the late enzymes in the cholesterol biosynthetic pathway, i.e. lanosterol synthase and lanosterol 14α-demethylase (CYP51). However, nSREBP-2 was much more potent than nSREBP-1a in inducing transcription of the key branch point enzyme squalene synthase. These findings suggest that cells may produce relatively high levels of SREBP-1a when they have a demand for nonsterol products derived from farnesyl pyrophosphate, such as prenylated proteins or dolichol (33). Indeed, SREBP-1a is expressed at relatively high levels in rapidly growing tissue culture cells which have a relatively large demand for these nonsterol products (7).

Differential effects of nSREBP-1a and nSREBP-2 on the squalene synthase promoter have also been observed by Guan et al. (12, 34), who expressed high levels of the nuclear forms of these transcription factors by transient transfection under control of the strong CMV promoter. At these high levels of expression, both nSREBPs activated a reporter gene driven by the squalene synthase promoter, but they did so by interacting at different sites. nSREBP-2 bound to more sites than did
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TABLE IV
Incorporation of [14C]acetate into fatty acids in M19, N-BP, and CHO-K1 cells

| Expt. | Cell line | Addition A to medium | 14C-Fatty acid distribution |
|-------|-----------|----------------------|-----------------------------|
|       |           |                      | Saturated                   | Unsaturated                |
|       |           |                      | 14:0 | 16:0 | 18:0 | 16:1 | 18:1 | % of total |
| A*    | M19       | None                 | 0.0  | 98.8 | 1.2  | 0.0  | 0.0  |
|       |           | Muristerone A        | 0.1  | 99.5 | 0.4  | 0.0  | 0.0  |
|       | N-BP-1a   | None                 | 1.2  | 98.8 | 0.0  | 0.0  | 0.0  |
|       |           | Muristerone A        | 16.2 | 58.7 | 3.2  | 1.4  | 10.6 |
|       | N-BP-1c   | None                 | 0.0  | 100.0| 0.0  | 0.0  | 0.0  |
|       |           | Muristerone A        | 0.0  | 100.0| 0.0  | 0.0  | 0.0  |
|       | N-BP-2    | None                 | 8.4  | 52.5 | 7.7  | 2.7  | 28.7 |
|       |           | Muristerone A        | 2.5  | 44.1 | 14.0 | 7.1  | 32.4 |
| B**   | CHO-K1    | None                 | 2.9  | 52.2 | 23.4 | 4.3  | 17.2 |

* M19 and N-BP cells were set up as described in Fig. 2. On day 1, the medium was switched as described in Fig. 2, and the monolayers were incubated in the absence or presence of 10 μM muristerone A except for the NB-1a cells, which were incubated with 0.8 μM muristerone A (see Fig. 9A). After incubation for 24 h at 37 °C, the cells were pulse-labeled for 3 h with 0.5 mM sodium [14C]acetate (29 dpm/pmol) and subjected to fatty acid distribution analysis as described under “Experimental Procedures.”

** CHO-K1 cells were set up as described in Fig. 2. On day 1, the monolayers were switched to medium B supplemented with 5% fetal calf lipoprotein-deficient serum in the absence or presence of 10 μg/ml cholesterol plus 1 μg/ml 25-hydroxycholesterol. After incubation for 18 h at 37 °C, the cells were pulse-labeled for 5.5 h with 0.5 mM sodium [14C]acetate (29 dpm/pmol) and processed as described in experiment A. Each value is the average of duplicate incubations.

The finding that SREBP-2 favors cholesterol synthesis is consistent with several previous observations from this laboratory. In one set of experiments, livers of hamsters (35, 36) and mice2 were deprived of cholesterol by the feeding of a bile acid binding resin and an HMG-CoA reductase inhibitor. The amount of nSREBP-2 increased, but the amount of nSREBP-1 (now known to be nSREBP-1c) decreased. Similarly, when cultured CHO cells were mutagenized and selected for resistance to killing by 25-hydroxycholesterol, which kills cells by reducing the activity of the Site-1 protease, several of the mutant cell lines survived by producing truncated forms of SREBP-2 (37, 38). None produced truncated SREBP-1. Similar results have recently been obtained by Rosenfeld and Osborne (39) in experiments in which CHO cells were individually transfected with cDNAs encoding the nuclear forms of SREBP-1a, -1c, and -2 and were then subjected to selection with 25-hydroxycholesterol. Only those cells transfected with nSREBP-2 survived the selection.

The relative inactivity of nSREBP-1c in the current studies is profound. As discussed above, nSREBP-1c does have the capacity to enhance transcription when it is expressed at high levels in liver (19) and in a variety of cultured cells (7, 19, 40). In view of its short acidic transcriptional activation domain, nSREBP-1c may have to interact with another transcription factor in order to enhance transcription. Such a factor may be relatively tissue-specific, allowing nSREBP-1c to function in selected tissues under selected developmental or metabolic conditions. The other factor might be another basic helix-loop-helix-leucine zipper protein that heterodimerizes with nSREBP-1c (and presumably nSREBP-1a). Alternatively, it might be another factor that interacts with nSREBP-1c in a different fashion. Indeed, the actions of all nSREBPs are known to be dependent on interaction with other transcription factors such as Sp1 in the case of the LDL receptor promoter (41) and NF-Y in the case of the farnesyl diphosphate synthase (42) and HMG-CoA synthase (43) promoters. In addition, nSREBP-1c might have a modulating role by forming heterodimers with nSREBP-1a and -2. The current studies were specifically designed to avoid such heterodimerization. In the future it will be necessary to study the actions of each of these nSREBPs under conditions where heterodimerization with other nSREBPs is possible.

A striking finding in the current studies was the failure of the M19 cells to synthesize unsaturated fatty acids and the restoration of this synthesis when either nSREBP-1a or nSREBP-2 was expressed (Fig. 10 and Table IV). This restoration is attributable to the 3.5-fold induction of the mRNAs for SCD-1 and the even larger 13-fold induction of SCD-2. The

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2 H. Shimano, J. Horton, J. L. Goldstein, and M. S. Brown, unpublished observations.
cells. Cholesterol and unsaturated fatty acids can modulate functional properties of cell membranes, particularly those carried out by lipid microdomains such as caveolae (47, 48). The SREBP's, therefore, appear to be central organizers of the composition and function of the membranes of animal cells.

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Fig. 11. Increased conversion of [14C]stearic acid to [14C]oleic acid in muristerone A-treated cells expressing nSREBP-1a. N-BP-1a cells were set up for experiments as described in Fig. 2. On day 1, the medium was switched as described in Fig. 2, and the monolayers were incubated in the absence or presence of 0.8 μM muristerone A (see Fig. 9A). After incubation for 20 h at 37°C, the cells were pulse-labeled for the indicated time with 0.1 mM sodium [14C]stearate-albumin (6.3 dpm/μmol). The addition of [14C]stearate was made in a staggered fashion so that all the cells were harvested at the same time. Samples were subjected to fatty acid distribution analysis as described under “Experimental Procedures.” Each value is the average of duplicate incubations, which showed an overall mean variation of 6.4% (range, 1–17%).
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