ABSTRACT

The sodium/iodide symporter (NIS), which is essential for iodide concentration in the thyroid, is reported to be transcriptionally regulated by sterol regulatory element-binding proteins (SREBP) in rat FRTL-5 thyrocytes. The SREBP are strongly activated after parturition and throughout lactation in the mammary gland of cattle and are important for mammary epithelial cell synthesis of milk lipids. In this study, we tested the hypothesis that the NIS gene is regulated also by SREBP in mammary epithelial cells, in which NIS is functionally expressed during lactation. Regulation of NIS expression and iodide uptake was investigated by means of inhibition, silencing, and overexpression of SREBP and by reporter gene and DNA-binding assays. As a mammary epithelial cell model, the human MCF-7 cell line, a breast adenocarcinoma cell line, which shows inducible expression of NIS by all-trans retinoic acid (ATRA), and unlike bovine mammary epithelial cells, is widely used to investigate the regulation of mammary gland NIS and NIS-specific iodide uptake, was used. Inhibition of SREBP maturation by treatment with 25-hydroxycholesterol (5 μM) for 48 h reduced ATRA (1 μM)-induced mRNA concentration of NIS and iodide uptake in MCF-7 cells by approximately 20%. Knockdown of SREBP-1c and SREBP-2 by RNA interference decreased the mRNA and protein concentration of NIS by 30 to 50% 48 h after initiating knockdown, whereas overexpression of nuclear SREBP (nSREBP)-1c and nSREBP-2 increased the expression of NIS in MCF-7 cells by 45 to 60%, respectively, 48 h after initiating overexpression. Reporter gene experiments with varying length of NIS promoter reporter constructs revealed that the NIS 5′-flanking region is activated by nSREBP-1c and nSREBP-2 approximately 1.5- and 4.5-fold, respectively, and activation involves a SREBP-binding motif (SRE) at −38 relative to the transcription start site of the NIS gene. Gel shift assays using oligonucleotides spanning either the wild-type or the mutated SRE at −38 of the NIS 5′-flanking region showed that in vitro-translated nSREBP-1c and nSREBP-2 bind only the wild-type but not the mutated SRE at −38 of NIS. Collectively, the present results from cell culture experiments with human mammary epithelial MCF-7 cells and from genetic studies show for the first time that the NIS gene and iodide uptake are regulated by SREBP in cultured human mammary epithelial cells. Future studies are necessary to clarify if the regulation of NIS expression and iodide uptake by SREBP also applies to the lactating bovine mammary epithelium.

Key words: sodium/iodide symporter, sterol regulatory element-binding proteins, mammary gland, iodide transport

INTRODUCTION

The sodium/iodide symporter [Na⁺/I⁻ symporter (NIS)] is an integral glycoprotein in the basolateral membrane mediating the efficient uptake of iodide from the bloodstream into cells. The NIS is best known for its essential role in the concentration of iodide in the thyroid, an important prerequisite for the synthesis of iodine-containing thyroid hormones in the thyroid (Carrasco, 1993). The NIS gene expression in the thyroid is principally regulated by thyroid-stimulating hormone (TSH), the main hormonal regulator of the thyroid (Vassart and Dumont, 1992), via the TSH receptor/cAMP pathway (Laglia et al., 1996; Endo et al., 1997).

Recent evidence indicates that the thyroid NIS gene is also subject to regulation by non-TSH signaling pathways (Nicola et al., 2010; Ringseis et al., 2013). In this regard, it has been recently reported that the NIS gene is regulated by 2 transcription factors, sterol regulatory element-binding protein (SREBP)-1c and SREBP-2 (Hua et al., 1993; Yokoyama et al., 1993), in rat thyrocytes (Ringseis et al., 2013). The SREBP are highly conserved through evolution from fungi to mammals (Osborne and Espenshade, 2009) and play a key role in the regulation of lipid homeostasis. In all mammals, the SREBP-1c isoform preferentially activates genes involved in fatty acid and triacylglycerol synthe-
sis, whereas SREBP-2 mainly activates genes involved in cholesterol metabolism (Osborne and Espenshade, 2009). Both, SREBP-1c and SREBP-2 are synthesized as inactive precursors (pSREBP) with a molecular weight of about 125 kDa and are located as complexes with other proteins in the endoplasmic reticulum membrane. Upon a decrease of cellular cholesterol content, pSREBP are escorted to the Golgi apparatus, where they are proteolytically processed leading to the release of their active N-terminal domain, called nuclear SREBP (nSREBP), with a molecular weight of about 70 kDa (Espenshade et al., 2002; Yang et al., 2002). The nSREBP bind to sterol regulatory elements (SRE) in the regulatory region of target genes and, thereby, initiate gene transcription (Horton et al., 2002).

Using molecular biological techniques a functional SRE could be identified in the NIS 5′-flanking region indicating that the rat NIS gene is a SREBP target gene (Ringseis et al., 2013). Interestingly, transcriptional activation of the NIS 5′-flanking region by nSREBP occurred independent of the presence of nucleotide sequences essential for thyroid-specific NIS gene regulation like the NIS upstream enhancer region and the proximal promoter (Ringseis et al., 2013), indicating that SREBP-dependent regulation of the NIS gene may be of greater importance in extra-thyroidal tissues, in which NIS is also functional but TSH is not the primary regulator. One of these tissues is the mammary gland.

In contrast to the constitutive expression of NIS in the thyroid, investigations in rats and mice showed that mammary gland NIS is functionally expressed in the mammary epithelium from the end of pregnancy and throughout lactation until weaning (Tazebay et al., 2000). In the lactating mammary gland, NIS is responsible for the secretion of iodide into the milk and thus essential for providing iodide for thyroid hormone biosynthesis in the neonate in general and in the newborn calf during the Colostral phase in specific. According to studies in laboratory rodents, the inducible expression of NIS in mammary epithelial cells during lactation involves the cooperative action of the lactogenic hormones oxytocin, prolactin, and estrogens (Tazebay et al., 2000), yet the mechanism of NIS gene regulation by these hormones is not completely clear. Studies about NIS gene regulation and iodide uptake in bovine mammary epithelial cells or bovine mammary gland are completely lacking. However, it has long been known that feedstuffs containing glucosinolates, like coproducts from oil production (rapeseed meal, rapeseed press cake) and feed from other cruciferous plants (kale), lowers iodine excretion via the milk of cows (Piironen and Virtanen, 1963). Earlier studies with dairy cows showed that feeding a ration with rapeseed products from old conventional rapeseed varieties with glucosinolate concentrations of 50 to 100 mmol/kg reduced iodine content of cow milk by 50 to 75% (Iwarsson, 1973; Papas et al., 1979) compared with a ration containing no rapeseed but having the same iodine concentration. This effect is mediated by the degradation products of glucosinolates in the animal’s body like thiocyanates and isothiocyanates, which are known to competitively inhibit iodide uptake by NIS in the thyroid and the mammary gland (Yoshida et al., 1998; Rillema et al., 2000). Thus, these findings indicate that mammary gland NIS of cattle plays also a role for the provision of iodide to the calf, in modern dairy production at least during the colostral phase. Due to the unique function of mammary gland NIS for iodide transfer into the milk, it is very likely that NIS in the mammary gland of cattle is regulated at least similarly as in other mammals.

Noteworthy, SREBP are strongly induced, activated, or both after parturition and throughout lactation in the mammary gland as shown in cattle (Bionaz and Loor, 2008), sheep (Barber et al., 2003), and mice (Rudolph et al., 2010). The physiological significance of SREBP activation in the lactating mammary gland is to stimulate the synthesis of fatty acids, triacylglycerols, phospholipids, and cholesterol (Rudolph et al., 2007; Rudolph et al., 2010; Mani et al., 2010) and thus to maintain lipid content in the milk and to provide lipophilic nutrients to the sucking neonate. In line with the key function of SREBP-1c for fatty acid and triacylglycerol synthesis, CLA isomers, which cause a reduction of milk fat content in dairy cows (Loor and Herbein, 1998; Perfield et al., 2007) and many other species, such as rats (Ringseis et al., 2004), sheep (Lock et al., 2006), and goats (Lock et al., 2008), were found to inhibit SREBP-1c maturation (Peterson et al., 2004) and decrease expression of SREBP-1c (Harvatine and Bauman, 2006) in mammary epithelial cells. Whether SREBP may also play a role in the secretion of nonlipid nutrients like iodide through regulation of mammary gland NIS and thus are generally important for maintaining the nutrient content of the milk is currently unknown. In view of recent evidence that the NIS gene is transcriptionally regulated by SREBP in the thyroid, the present study aimed to test the hypothesis that SREBP are regulators of the NIS gene and iodide uptake in mammary epithelial cells. As a mammary epithelial cell model the human breast adenocarcinoma cell line MCF-7 was used because it is a well-established mammary epithelial cell model to investigate the regulation of mammary gland NIS and NIS-specific iodide uptake (Kogai et al., 2000). In contrast, bovine mammary epithelial cell lines, such as BMEG + HM (Schmid et al., 1983), HH2a (Huynh and Pollak, 1995), ET-C (Zavizion et al., 1996), and Mac-T (Huynh et al., 1991), have not been characterized yet for iodide uptake.
or NIS expression. In addition, in spite of their bovine
origin these cell lines are not necessarily better mod-
els of the bovine mammary gland because they either
do not express lactation-specific proteins (BMEC+H,
HH2A, ET-C) or in the case of Mac-T cells show a poor
lactation phenotype compared with bovine mammary
tissue (Hosseini et al. 2013).

MATERIALS AND METHODS

Cell Culture

Cells of MCF-7, a breast adenocarcinoma cell line
(Soule et al., 1973) and with inducible expression of
NIS by all-trans retinoic acid (ATRA; Kogai et al.,
2000), were obtained from Cell Lines Service (Eppel-
heim, Germany) and grown at 37°C in a humidified
atmosphere composed of 95% air and 5% CO2 in Dul-
becco’s modified Eagle medium (DMEM; Gibco/Life
Technologies, Darmstadt, Germany) and 10% fetal bo-
vine serum (Gibco/Life Technologies). Growth medium
was changed every 2 d. After reaching a confluence of
80%, the cells were either sub-cultivated or used for
experiments. Experimental conditions [concentrations
of chemicals, such as ATRA and 25-hydroxycholesterol
(25-HC), and duration of experiment] are indicated in
the Results section and figure legends.

Cell Viability Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-
zolium bromide (MTT) assay was used to assess cell
viability in response to ATRA, 25-HC, small interfering
RNA (siRNA) molecules, and expression plasmids.
The MCF-7 cells were seeded in 96-well and 24-well cul-
ture plates at a density of 1 × 10^4 and 5 × 10^4 cells per
well, respectively, and after reaching 80% confluence
incubated with different concentrations of ATRA (0,
0.25, 0.5, 1, 2, 5, and 10 μM) or 25-HC (0, 2.5, 5, 10, 15,
25, and 50 μM) or transfected with siRNA molecules
as described in the RNA interference section or with
reporter gene plasmids, SREBP expression plasmids or
empty plasmid and selected luciferase reporter vectors
as described in the transient transfection section. All
cells were treated with the same vehicle concentration
[1% vol/vol, ethanol, or dimethyl sulfoxide (DMSO)].
The MTT assay was performed as described recently
(Gessner et al., 2012), with the exception that MCF-7
cells were incubated with the MTT solution for 4 h.
Cell viabilities of treated cells are presented relative to
that of control cells, which was set to 100%.

RNA Isolation and Quantitative Real-Time PCR

For quantitative real-time PCR (qPCR) analysis,
MCF-7 cells were seeded in 24-well plates and incu-
bated as indicated. Total RNA extraction, cDNA syn-
thesis, and qPCR were performed as described recently
for FRTL-5 cells (Ringseis et al., 2013). Gene-specific
primer pairs and their features are listed in Table 1.

Western Blotting

For Western blotting of precursor and nuclear SREBP-1 and SREBP-2 and NIS, MCF-7 cells were
seeded in 6-well plates and incubated as indicated. To
prevent degradation of SREBP, MCF-7 cells were treat-
ed with 25 μg/mL of the calpain inhibitor N-acetyl-Leu-
Leu-Norleucinal 3 h before cell harvesting according to
Hua et al. (1996). Cytosolic and nuclear fractions from

Table 1. Characteristics of gene-specific primers used for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Reference gene</th>
<th>Primer sequence (forward, reverse; 5’ to 3’)</th>
<th>Product size (bp)</th>
<th>Gene accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target gene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FASN</td>
<td>GCCGCTGAGTCTCTGAGAGATG, TATTTGCTCTGTCGCCGATTG</td>
<td>189</td>
<td>NM_004104</td>
</tr>
<tr>
<td>GPAM</td>
<td>AATGTGCCAATCGTGGCAAC, ATCCACTTCTGACAAACATAG</td>
<td>105</td>
<td>NM_00124949</td>
</tr>
<tr>
<td>HMGCR</td>
<td>GACAGGATGCAACGACAAAGT, TTGAACATCTGAGCTCGCACAAC</td>
<td>170</td>
<td>NM_000859</td>
</tr>
<tr>
<td>LDLR</td>
<td>GTGATCGGCAACGCGGTAAG, CCCAGGCTTGGTGAGAGCATTG</td>
<td>128</td>
<td>NM_000527</td>
</tr>
<tr>
<td>NIS</td>
<td>TGCGGACTTTTGCGACTTACTT, TGCAGATCATCTGCTTGCAAC</td>
<td>133</td>
<td>NM_000453</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>CGGACGCTATGGAATTGCACACTTTC, GATGCTAGTGGACATGACTCTTCC</td>
<td>328</td>
<td>NM_001005291</td>
</tr>
<tr>
<td>SREBP-2</td>
<td>TAGGAGTCGGGGAGAATG, GTCTGGGTCAAGTCCAGTTTGGAAGCT</td>
<td>179</td>
<td>NM_004599</td>
</tr>
</tbody>
</table>

cells were prepared using the Nuclear Extract Kit from Active Motif (La Hulpe, Belgium). Protein concentrations of cell fractions were determined by the bicinchoninic acid protein assay kit (Interchim, Montluçon, France) with BSA as the standard. From the fractions, 10 μg (from the cytosolic fraction for NIS) and 20 μg of protein (from both the cytosolic and nuclear fraction for precursor and nuclear SREBP-1 and SREBP-2) were separated by SDSPAGE (7.5–12.5%) and electrotransferred onto nitrocellulose membranes (Pall Corporation, Pensacola, FL). Loading of equal amounts of protein in each lane was verified by Ponceau S (Carl Roth, Karlsruhe, Germany) staining. After incubating the membranes overnight at 4°C in blocking solution, membranes were incubated with primary antibodies anti-rabbit SREBP-1 and anti-rabbit SREBP-2 (both 1:2,000; St. Cruz, Heidelberg, Germany), anti-rabbit NIS [1:2,000; kindly provided by Nancy Carrasco, Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT (Levy et al., 1997)] and anti-mouse β-actin (1:10,000; Abcam, Cambridge, UK) as a reference protein to control for adequate normalization at room temperature (RT) for 2 h. The membranes were washed and then incubated with horseradish peroxidase-conjugated secondary antibodies anti-rabbit IgG (1:10,000; Sigma-Aldrich, Taufkirchen, Germany) for SREBP-1, SREBP-2 and NIS, and anti-mouse IgG (1:10,000; Abcam) for β-actin at RT for 2 h. Afterward, blots were developed using enhanced chemiluminescence (ECL) Plus (GE Healthcare, München, Germany). The signal intensities of specific bands were detected with a Bio-Imaging system (Syngene, Cambridge, UK) and quantified using Syngene GeneTools software (nonlinear dynamics; Syngene).

Iodide Uptake

For iodide uptake, MCF-7 cells were seeded in 24-well plates and incubated as indicated. Iodide uptake was carried out as described recently (Ringseis et al., 2013) with minor modifications. In brief, the medium was removed and cells were washed with warm (37°C) Hanks’ buffered salt solution (HBSS). Subsequently, 0.5 mL of HBSS containing Na125I (0.3 μCi/mL) was immediately added to each well and cells incubated at 37°C for 1 h. To distinguish between total iodide uptake and NIS-specific uptake, the incubation was performed both in the absence (total iodide uptake) and presence (NIS-specific uptake) of 1 mM KClO4, a specific inhibitor of iodide uptake via NIS. The incubation was terminated by aspiration of the medium. After rinsing 2 times with ice-cold HBSS, cells were lysed with 0.5 mL of 0.5 M NaOH with shaking at RT for 30 min. The cell lysate from each well was transferred in a counter vial for scintillation counting (Perkin Elmer Liquid Scintillation Analyzer Tri-Carb 2900TR, Rodgau, Germany).

RNA interference

Knockdown variants of MCF-7 cells expressing low levels of SREBP-1c and SREBP-2, respectively, were produced by siRNA-mediated gene knockdown. For this purpose, MCF-7 cells were seeded in 24-well and 6-well plates for qPCR and Western blotting, respectively, and after reaching a confluence of 70 to 80%, cells were transfected using Lipofectamine 2000 Reagent (Invitrogen) and gene-specific Stealth RNAi molecules (SREBP-1c/-2 siRNA; Invitrogen) targeting human SREBP-1c and SREBP-2, respectively, at a final concentration of 50 nM for 24 h according to the manufacturer’s protocol. To control for unspecific knockdown effects, control cells were transfected with a control RNAi molecule (control siRNA) that was not homologous to any human gene sequence. After 24 h, transfection medium was aspirated and cells were incubated with growth medium for 24 h. The knockdown effect was assessed by qPCR and Western blotting. The siRNA sequences were as follows (sense, antisense): SREBP-1c siRNA (5’-UCAGAUAACACCACCCGUCAAAU-3’, 5’-UAUGGUAGACCGUCUGGU-GUAUCUGA-3’), SREBP-2 siRNA (5’-GAGGCAG-GCUUUGAAGACGAAGCUA-3’, 5’-UAGCUCUGC-GCUUCAAGCCCUCCUC-3’), and control siRNA (5’-UCUCGGAGCUUGUCACGdTdT-3’, 5’-ACGUGAGACGUUCGGAGAAdTdT-3’).

Bioinformatics

For the identification of putative SREBP-binding sites, in silico analysis was performed using TFBIND software developed for searching transcription factor binding sites including TATA boxes, GC boxes, CCAAT boxes, and transcription start sites (Tsunoda and Takagi, 1999). The TFBIND tool uses weight matrix of transcription factor database TRANSFAC (Wingender et al., 2001).

Plasmid Construction and Site-Directed Mutagenesis

Using cDNA and genomic DNA sequences from National Center for Biotechnology Information GenBank (accession numbers NM_000453 and NC005796), a 630-bp hNIS promoter fragment (from −280 to +350 relative to transcription start site) was PCR amplified from a genomic DNA resource (BAC RP11–343E23;
Table 2. Sequences of 5′-primers used for PCR amplification of human sodium/iodide symporter (hNIS) reporter constructs

<table>
<thead>
<tr>
<th>hNIS construct</th>
<th>Oligonucleotide sequence (5′ to 3′)</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−244/+350</td>
<td>AAACCTGAGGATCAGCTTGGTGGGATG</td>
<td>594</td>
</tr>
<tr>
<td>−160/+350</td>
<td>AAACCTGAGGATGAGCGCTTGGTGGGATG</td>
<td>510</td>
</tr>
<tr>
<td>+164/+350</td>
<td>AAACCTGAGGATGAGCGCTTGGTGGGATG</td>
<td>186</td>
</tr>
<tr>
<td>+263/+350</td>
<td>AAACCTGAGGATGAGCGCTTGGTGGGATG</td>
<td>87</td>
</tr>
</tbody>
</table>

The sequence of the common 3′-primer is presented in the corresponding methods description in Plasmid Construction and Site-Directed Mutagenesis.

REGULATION OF THE SODIUM/IODIDE SYMPORTER

BACPAC Resources, Oakland, CA) using the following primer pair: 5′-AGGAAGCCCTCATGAGGCGGCGGT-GCGGAG-3′ (forward) and 5′-AAACTCGAGGCTGCTCCCTCCCTCCTGCGGAG-3′ (reverse). The generated PCR fragment with XhoI and HindIII restriction sites introduced at the 5′ and 3′ ends was subcloned into the XhoI and HindIII digested pGL4.10 [luc2] vector (Promega, Mannheim, Germany) upstream of the luciferase reporter gene. The hNIS promoter truncation constructs (−244/+350, −160/+350 and +164/+350 and +263/+350) were PCR amplified from the parental pGL4.10-hNIS-280/+350 promoter construct using the abovementioned 3′-primer and different 5′-primers (Table 2). All generated PCR fragments were subcloned into XhoI and HindIII sites of pGL4.10 [luc2] vector (Promega) upstream of the luciferase reporter gene. Mutated hNIS reporter gene constructs were generated by selectively introducing a mutation in the putative SRE using the QuickChange XL Site-Directed Mutagenesis Kit from Stratagene Europe (Amsterdam, the Netherlands) according to the manufacturer’s protocol using the following oligonucleotides: SRE-179mut (forward: 5′-GGCCCTCCCCGATACCgttCCCTGCTCCC-3′, reverse: 5′-CTTACGGGAGCAGCTTacaATTGTGGGTTGCAG-3′), E-box-like SRE-38mut (forward: 5′-CTGGAGAGTGGTGCGTGCAGGGaacGGTATCGGGGAGGGC-3′, reverse: 5′-CTTACGGGAGCAGCTTacaATTGTGGGTTGCAG-3′). The artificial reporter gene constructs containing 2 copies of either wild-type or mutated E-box-like SRE-38 were prepared by annealing the oligonucleotides (wild-type E-box-like SRE-38: XhoI-TCGAAGCCAGACAACTACAGGCTGGTGCTCCCTCCCCACCAGAATCAAGGAGTCGCTCCCTGTAAG, HindIII-AGCTCTCTACCGGGGGACAGCTTGCTCCCTCCCTCCCTCCTGCAG; mutated E-box-like SRE-38: XhoI-TCGAAGCCAGACAACTACAGGAGTCGCTCCCTCCCTCCCTCCTGCAG) and subcloning into XhoI and HindIII sites of pGL4.23 [luc2/minP] vector (Promega), which contains the minimal promoter minP followed by the luciferase reporter gene luc2. To confirm the integrity of all plasmids and to control for the intended mutations and the absence of any unexpected mutations, the cloned DNA fragments were sequenced. The generation of the positive control reporter gene construct containing 2 copies of the human LDLR (hLDLR)-SRE and expression plasmids for nSREBP-1c (AA 1–448) and nSREBP-2 (AA 1–460) has been described recently (Ringseis et al., 2013).

Transient Transfection and Dual Luciferase Assay

For transient transfection, MCF-7 cells were seeded in 96-well plates and transiently transfected with 50 ng of the generated reporter gene constructs and co-transfected with 50 ng of either nSREBP-1c or nSREBP-2 expression plasmids or 50 ng of the empty expression plasmid (pcDNA3.1) using FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s protocol. For normalization of transfection efficiency, cells were co-transfected with 5 ng of pGL4.74 [luc2] vector (Promega), which encodes for the Renilla luciferase, as an internal control. In addition, cells were transfected with 50 ng of either pGL4.10 [luc2] vector or pGL4.23 [luc2/minP] vector (both from Promega) or the 2× hLDLR-SRE luciferase reporter vector, containing 2 copies of the SRE-1 from hLDLR, as negative and positive controls. After 12 h, transfection medium was aspirated and cells were incubated for 12 h with growth medium. Subsequently, medium was aspirated from the cell layer and cells were washed with 1× PBS and lysed with passive lysis buffer (Promega). Luciferase activities of cell lysates were measured using Beetle-Juice and Renilla-Juice Kits from PJK (Kleinblittersdorf, Germany). Normalized luciferase activities were calculated by dividing the luciferase activity of each construct by that of the corresponding empty vectors, pGL4.10 or pGL4.23 (Inoue et al., 2011). Results are shown relative to cells transfected with the empty expression plasmid pcDNA3.1, which were set to 1.
Electrophoretic mobility shift assays were performed as described recently in detail (Wen et al., 2010). In brief, recombinant rat nSREBP-1c and nSREBP-2 were in vitro translated from the corresponding expression plasmids using the TNT T7 Quick Coupled Transcription/Translation System (Promega) according to the manufacturer’s protocol. Annealed oligonucleotides spanning either the wild-type or the mutated hNIS E-box-like SRE-38 of hNIS 5′-flanking region were end-labeled with digoxigenin (DIG) using the DIG Gel Shift Kit, 2nd Generation (Roche). In addition, annealed and DIG-labeled oligonucleotides spanning either the wild-type or the mutated hLDLR-SRE were used as specific and nonspecific control. Sequences of oligonucleotides were as follows: wild-type hNIS E-box-like SRE-38 (forward: TCGATGCAACCCACAAT- CACGAGCTGCTCCCGTAA, reverse: AGTCT- TACGGGAGCAQCTGATTGTGGGTGCA), mutated hNIS E-box-like SRE-38 (forward: TCGATG- CAACCCACAATgtaAGCTGCTCCCGTAA, reverse: AGTCTTACGGGACCACTacaATTTGTTGGT- GCA), wild-type hLDLR-SRE (forward: GTAG- ATTTTTGAAAATCAACCCACGTGAAAATCC, reverse: GGGAGGAGTtTGACATGGGT- GATTTCACAAAAAT), mutated hLDLR-SRE (forward: GTAGATTTTTGAAAAGTCAaatGCGTGTA- CAAA, reverse: GGGAGGAGTtTGCAacG- GttTGACATTTCAAAAAT). For competition experiments, recombinant proteins were incubated with DIG-labeled hNIS E-box-like SRE-38 probe and increasing excess of unlabeled specific probe (hLDLR-SRE).

Statistical Analysis

All data represent means and SD. In all figures showing numerical data, with the exception of data from 125I-iodide uptake, the means and SD were calculated from the means for the same treatments of 3 independent experiments. The means and SD for 125I-iodide uptake were calculated from 3 replicates within one independent experiment, but the data from the one experiment shown are representative for the 2 other independent experiments. Statistical analysis was performed using the Minitab statistical software (Rel. 13.0, State College, PA). Data (residuals) of each independent experiment were analyzed for normality of distribution (Anderson-Darling test). Because all data showed normal distribution, one-way ANOVA was applied to evaluate the effect of treatment. Effects were considered significant if P < 0.05. If one-way ANOVA revealed significant effects, a post-hoc comparison (Fisher’s multiple comparison) was applied. 

RESULTS AND DISCUSSION

Sterol-Mediated Inhibition of SREBP Maturation

To test the hypothesis that the NIS gene is regulated by SREBP in MCF-7 cells, we first studied the effect of reducing the cellular levels of transcriptionally active nSREBP by the oxysterol 25-HC. Oxysterols such as 25-HC serve as biologically active molecules for regulation of lipid metabolism in all animal cells (Gill et al., 2008), and are formed endogenously from intracellular cholesterol by mitochondrial sterol 27-hydroxylase following delivery of cholesterol into the mitochondria (Li et al., 2006, 2007). Thus, when intracellular cholesterol levels are increased, cholesterol is transported into the mitochondria and 25-HC is synthesized. However, 25-HC does not accumulate in the cell because 25-HC is subsequently sulfated by sulfotransferase 2B1b to form 25-HC 3-sulfate (Ren et al., 2006), which inhibits proteolytic processing of SREBP-1 and SREBP-2 and thereby inhibits lipid biosynthesis (Ma et al., 2008). Thus, 25-HC is a useful approach to study the effect of decreased cellular levels of nSREBP-1 and nSREBP-2 (Nohturfft et al., 2000; DeBose-Boyd et al., 2001; Sato, 2010). Incubation experiments with 25-HC in MCF-7 cells were performed at a concentration of 5 μM, which is within the concentration range shown to be successful in reducing levels of nSREBP-1 and nSREBP-2 in other cell types, such as hepatocytes (DeBose-Boyd et al., 2001) and thyrocytes (Ringseis et al., 2013). The MTT assay revealed that this concentration of 25-HC did not impair the viability of MCF-7 cells (data not shown).

Incubation experiments with 25-HC in MCF-7 cells were performed in the presence of ATRA (1 μM), which is a known inducer of NIS gene transcription and iodide uptake in this cell line (Kogai et al., 2000; Alotaibi et al., 2010). This was necessary because NIS mRNA is only barely detectable and iodide uptake is very low in the absence of ATRA (Kogai et al., 2000). The ATRA was also found not to be toxic for MCF-7 cells up to a concentration of 10 μM as shown by an MTT assay (data not shown). As illustrated in Figures 1A and 1B, treatment of MCF-7 cells with ATRA alone had no effect on the mRNA concentrations of SREBP-1c and SREBP-2 and the protein concentrations of nSREBP-1 and nSREBP-2 compared with treatment with DMSO (control), indicating that ATRA does not interfere with SREBP maturation. Combined treatment of MCF-7 cells with ATRA and 25-HC strongly reduced the mRNA concentrations of SREBP-1c and SREBP-2 and the protein concentrations of nSREBP-1 and nSREBP-2 compared with treatment with either ATRA or DMSO (Figures 1A and 1B).
In addition, the 25-HC-induced reduction of mRNA concentrations of SREBP and protein concentrations of nSREBP was accompanied by a decrease of the mRNA concentrations of the known SREBP-2 target genes 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) and LDLR and of the SREBP-1c target gene fatty acid synthase (FASN) compared with treatment with either ATRA or DMSO (Figure 1C). These findings indicated that 25-HC inhibits SREBP maturation in MCF-7 cells as in hepatocytes (DeBose-Boyd et al., 2001) and thyrocytes (Ringseis et al., 2013).

Regarding NIS expression, Figure 1D demonstrates that ATRA alone strongly increased NIS mRNA concentration and combined treatment with ATRA and 25-HC resulted in a further increase in NIS mRNA concentration compared with either treatment alone (Figure 1D). These findings indicated that NIS expression is regulated by SREBP-1c and SREBP-2 in MCF-7 cells.

Figure 1. Sterol-mediated inhibition of sterol regulatory element-binding proteins (SREBP) maturation reduces expression of sodium/iodide symporter (NIS) and NIS-specific iodide uptake in MCF-7 cells. The MCF-7 cells were grown in Dulbecco’s modified Eagle medium with 10% fetal calf serum until 70% confluent, and subsequently treated with either dimethyl sulfoxide (DMSO; 1% vol/vol), all-trans-retinoic acid (ATRA; 1 μM), or ATRA (1 μM) and 25-hydroxycholesterol (25-HC; 5 μM) for 48 h, and analyzed for relative mRNA concentrations of SREBP-1c and SREBP-2 (A), relative protein concentrations of nuclear SREBP (nSREBP)-1 and nSREBP-2 (B), relative mRNA concentrations of target genes of SREBP-1c (FASN, GPAM) and SREBP-2 (HMGCR, LDLR) (C), and NIS (D) and iodide uptake (E). (A, C, D) Bars represent relative mRNA concentrations expressed as fold of DMSO and are means ± SD from 3 independent experiments. (B) For Western blot analysis of nSREBP-1 and nSREBP-2, MCF-7 cells were treated with the calpain inhibitor N-acetyl-Leu-Leu-Norleucinal (ALLN; 25 mg/mL) 3 h before cell lysis to prevent degradation of SREBP. Relative protein levels of nSREBP-1 and nSREBP-2 were determined in nuclear extracts. Bars represent relative protein concentrations expressed as fold of DMSO and are means ± SD from 3 independent experiments. One representative immunoblot for nSREBP-1, nSREBP-2, and β-actin is shown. (E) For iodide uptake measurement, MCF-7 cells were incubated with Na125I (0.15 μCi/well) for 60 min in the presence and absence of the NIS-specific inhibitor KClO4 (1 mM). Bars represent counts per minute per well and are means ± SD from 3 replicates from 1 of 3 independent experiments. *Different from DMSO (P < 0.05), #Different from ATRA (P < 0.05).
25-HC reduced the ATRA-induced increase of NIS mRNA concentration by approximately 20% in MCF-7 cells. To confirm this finding on the functional level we also studied the effect of ATRA alone and of ATRA and 25-HC on Na\(^{125}\)Iodide uptake into MCF-7 cells. Uptake experiments were performed in the presence and absence of KClO\(_4\) (1 mM), a specific inhibitor of iodide uptake via NIS. As shown in Figure 1E, total iodide uptake (absence of KClO\(_4\)) into MCF-7 cells was dramatically increased by treatment with ATRA alone. Treatment of MCF-7 cells with 25-HC attenuated the ATRA-induced increase of total iodide uptake by approximately 20%. In the presence of KClO\(_4\) in the culture medium, neither ATRA alone nor ATRA and 25-HC stimulated iodide uptake, indicating that ATRA stimulates and 25-HC inhibits NIS-specific iodide uptake. Collectively, the results from this experimental approach provided first evidence that NIS expression and iodide uptake are regulated by SREBP in a human mammary epithelial cell line.

**RNA Interference-Mediated Knockdown of SREBP-1c and SREBP-2**

In a second approach of investigating the regulation of NIS expression by SREBP in MCF-7 cells, we studied the expression of NIS in MCF-7 cells with a targeted knockdown of either SREBP-1c or SREBP-2. Knockdown was induced by transfection of cells with siRNA molecules targeting either SREBP-1c or SREBP-2. Preliminary MTT assays revealed that siRNA molecules specific for SREBP-1c and SREBP-2 reduced the viability of MCF-7 cells only slightly by approximately 10% compared with cells treated with a nonspecific control siRNA for 24 h (data not shown). Transfection of MCF-7 cells with siRNA targeting SREBP-1c caused a reduction in the mRNA level of SREBP-1c by about 40% (Figure 2A) and in the protein levels of pSREBP-1 and nSREBP-1 by about 25 to 40% (Figure 2B) after 48 h compared with transfection of cells with control siRNA. Likewise, transfection of MCF-7 cells with siRNA targeting SREBP-2 resulted in a decrease in the mRNA level of SREBP-2 by about 60% (Figure 2A) and in the protein levels of pSREBP-2 and nSREBP-2 by about 80 and 40%, respectively (Figure 2B). The mRNA levels of known SREBP-1c target genes \([FASN, \text{glycerolphosphate-acyltransferase (GPAM)}]\) and SREBP-2 target genes \([HMGCR, LDLR]\) were reduced by about 30 to 40% in MCF-7 cells transfected with siRNA targeting SREBP-1c and SREBP-2, respectively (Figure 2C). Similar as observed in response to 25-HC-mediated inhibition of SREBP maturation, siRNA-mediated knockdown of SREBP-1c and SREBP-2 reduced the mRNA level of NIS by about 45 and 25%, respectively (Figure 2D). The protein level of NIS in MCF-7 cells was reduced by 25 and 40% in response to siRNA targeting SREBP-1c and SREBP-2, respectively (Figure 2E). In summary, these results provided further evidence to substantiate the hypothesis that SREBP are regulators of the NIS gene in human mammary epithelial cells.

**Overexpression of nSREBP-1c and nSREBP-2 Increases Expression of NIS in MCF-7 Cells**

To provide further evidence for the regulation of NIS expression by SREBP in MCF-7 cells, we explored the effect of overexpression of either nSREBP-1c or nSREBP-2 in MCF-7 cells. Successful overexpression of nSREBP-1c and nSREBP-2 was evident from markedly elevated protein concentrations of nSREBP-1c and nSREBP-2 following transient transfection of MCF-7 cells with expression plasmids for either nSREBP-1c or nSREBP-2 compared with empty plasmid (pcDNA3.1; Figure 3A). In line with this, MCF-7 cells overexpressing nSREBP-1c had approximately 40 to 60% increased mRNA concentrations of the SREBP-1c target genes FASN and GPAM compared with control cells transfected with empty plasmid (Figure 3B). In addition, the mRNA concentrations of the SREBP-2 target genes HMGCR and LDLR were slightly increased by about 30% in MCF-7 cells overexpressing nSREBP-1c (Figure 3B). The MCF-7 cells overexpressing nSREBP-2 had approximately 80% elevated mRNA concentrations of the SREBP-2 target genes HMGCR and LDLR. The mRNA concentrations of SREBP-1c target genes did not differ between MCF-7 cells overexpressing nSREBP-2 and control cells. Finally, we studied the effect of nSREBP overexpression on NIS expression in MCF-7 cells. As shown in Figure 3C, the mRNA concentration of NIS was 50 to 60% higher in MCF-7 cells overexpressing either nSREBP-1c or nSREBP-2 than in control cells, indicating again that NIS expression is regulated by both nSREBP-1c and nSREBP-2. In connection with the results obtained from the first 2 experimental approaches, these results suggested that mammary gland NIS is a target gene of SREBP-1c and SREBP-2, at least in the human MCF-7 cell line. Because SREBP have been initially identified as master regulators of lipid synthesis and uptake and due to this play an important role in the lactating mammary gland, our finding that SREBP regulate also NIS gene expression and iodide uptake in mammary epithelial cells may indicate that SREBP influence additional physiological functions of the mammary gland beyond lipid biosynthesis.
To investigate whether the hNIS gene is transcriptionally regulated by SREBP via specific SREBP binding sites, a 630-bp sequence upstream of the translation start site (5’-flanking region) of hNIS was screened for the existence of putative SREBP responsive sequences using TFBIND software. This search revealed 5 putative SREBP responsive regions at positions −250, −179, −38, +207, and +242 relative to the transcription start site. Three of them were more or less homolog to the classic SRE from hLDLR called SRE-1 (consensus sequence: 5’-ATCACCCCAC-3’; Briggs et al., 1993), whereas 2 of them showed characteristics of both a SRE and an E-box. Such elements are called E-box-like SRE or SRE-2 (consensus sequence: 5’-ATCACGTGA-3’) and frequently found in the promoter region of lipogenic genes (Amemiya-Kudo et al., 2002; Nara et al., 2002). The sequence, position, and degree of homology

Figure 2. RNA interference-mediated knockdown of sterol regulatory element-binding proteins (SREBP)-1c and SREBP-2 reduces expression of sodium/iodide symporter (NIS) in MCF-7 cells. The MCF-7 cells were grown in Dulbecco’s modified Eagle medium with 10% fetal calf serum until 70% confluent, and subsequently transfected with siRNA targeting SREBP-1c or SREBP-2 or control siRNA for 24 h. Afterward, MCF-7 cells were incubated with growth medium for an additional 24 h and then analyzed for relative mRNA concentrations of SREBP-1c and SREBP-2 (A), relative protein concentrations of nuclear SREBP (nSREBP)-1 and precursor SREBP (pSREBP) (B), relative mRNA concentrations of target genes of SREBP-1c (FASN, GPAM) and SREBP-2 (HMGCR, LDLR; C), NIS mRNA concentration (D), and NIS protein concentration (E). (A, C, D) Bars represent relative mRNA concentrations expressed as fold of control siRNA and are means ± SD from 3 independent experiments. (B, E) Bars represent relative protein concentrations expressed as fold of control siRNA and are means ± SD from 3 independent experiments. One representative immunoblot for pSREBP-1, nSREBP-1, pSREBP-2, nSREBP-2, NIS, and β-actin is shown. *Different from control siRNA (P < 0.05).
Figure 3. Overexpression of nuclear sterol regulatory element-binding protein (nSREBP)-1c and nSREBP-2 increases expression of sodium/iodide symporter (NIS) in MCF-7 cells. The MCF-7 cells were transiently transfected with expression plasmids for either nSREBP-1c or nSREBP-2 or empty plasmid (control) for 24 h and analyzed following a 24-h incubation period for relative protein concentrations of nSREBP-1 and nSREBP-2 (A), and relative mRNA concentrations of target genes of SREBP-1c (FASN, GPAM), SREBP-2 (HMGCR, LDLR; B), and NIS (C). (A) Relative protein levels of nSREBP-1 and nSREBP-2 were determined in nuclear extracts. Bars represent relative protein concentrations expressed as fold of control and are means ± SD from 3 independent experiments. One representative immunoblot for nSREBP-1, nSREBP-2, and β-actin is shown. (B, C) Bars represent relative mRNA concentrations expressed as fold of control and are means ± SD from 3 independent experiments. *Different from control ($P < 0.05$).
Figure 4. The E-box-like sterol regulatory element (SRE)-38 is critical for activation of the 5'-flanking region of human sodium/iodide symporter (hNIS) by nuclear sterol regulatory element-binding protein (nSREBP)-1c and nSREBP-2. (A) Sequence, position, and degree of homology relative to the consensus SRE of putative SRE as indicated by MathInspector software (Genomatix, Munich, Germany) in a 630-bp sequence of the hNIS 5'-flanking region (from −280 to +350). (B, C) MCF-7 cells were transiently transfected with either hNIS 5'-flanking region luciferase reporter gene constructs containing different regions between −280, −244, −160, +164, and +263, respectively, and +350 (ATG translation start site; B) or a positive control vector containing 2 copies of the hLDLR-SRE (C). (B, C) MCF-7 cells were co-transfected with a renilla luciferase plasmid for normalization and either expression plasmids for nSREBP-1c or nSREBP-2 or empty plasmid (control) for 12 h. Following a 12-h incubation period with growth medium, cells were lysed and luciferase activities were measured. Bars represent relative luciferase activities expressed as fold of control and are means ± SD from 3 independent experiments each performed in triplicate. *Different from control (P < 0.05). LUC = luciferase; minP = minimal promoter.

To next examine whether this 630-bp sequence of the hNIS 5'-flanking region is responsive to nSREBP-1c and nSREBP-2, we cloned this fragment (from −280 to +350) in front of a firefly luciferase reporter gene, transfected this construct together with expression vectors for either nSREBP-1c or nSREBP-2 or empty vector (pcDNA3.1) into MCF-7 cells, and measured the luciferase reporter activity following a 12-h incubation period with growth medium. Initial MTT experiments showed that 12 h transfection of cells with either expression vectors or empty vector together with selected luciferase reporter constructs (pGL4.10 and pGL4.10-hNIS-280/+350) reduced viability of MCF-7 cells by no more than 9% compared with cells treated with vehicle only for 12 h (data not shown), indicating that effects from reporter gene assays are not strongly biased by the
transfection procedure. As shown in Figure 4B, a 1.5- and 4.5-fold increase occurred in the luciferase activity in response to nSREBP-1c and nSREBP-2, respectively, relative to empty vector in MCF-7 cells transfected with the hNIS construct containing the region from -280 to +350. This observation indicated that the hNIS 5′-flanking region is responsive to nSREBP, even though the response of the luciferase reporter of hNIS construct to nSREBP-1c and nSREBP-2 was relatively small when compared with the positive control vector 2× hLDLR-SRE (Figure 4C).

To further clarify the role of the putative SRE for regulation of the hNIS 5′-flanking region, we generated 4 hNIS 5′-end deletion reporter gene constructs, in which the SRE of the hNIS 5′-flanking region were serially deleted from the 5′ end. Figure 4B shows that the response of the luciferase reporter of the deletion constructs containing the region -244 to +350 and -160 to +350 to nSREBP-1c and nSREBP-2 was similar to that containing the region -280 to +350, indicating that the E-box-like SRE-250 and the SRE-179 are not decisive for the responsiveness of the hNIS 5′-flanking region to nSREBP. In contrast, the deletion construct containing the region +164 to +350, which lacked the E-box-like SRE-38 and contained only 2 putative SRE in close proximity to the translation start site at positions +207 and +242, showed no response at all to nSREBP-1c and nSREBP-2, indicating that the E-box-like SRE-38 might be a functional SRE. Also, the deletion construct containing only the region +263 to +350, which contains no putative SRE, exhibited almost no response to nSREBP-1c and nSREBP-2 (Figure 4B).

**E-Box-Like SRE-38 Is Critical for Activation of the 5′-Flanking Region of hNIS**

To clarify whether the E-box-like SRE-38 is of importance for SREBP-dependent regulation of the hNIS 5′-flanking region, we compared the luciferase reporter response of different hNIS deletion reporter constructs containing the region from -244 to +350, in which either SRE-179 or E-box-like SRE-38 or both of them were mutated by site-directed mutagenesis, with that of the nonmutated hNIS deletion construct. As shown in Figure 5A, the reporter response of each of the hNIS deletion constructs containing a mutated E-box-like SRE-38 exhibited a markedly lower response to nSREBP-1c and nSREBP-2 than those with a nonmutated E-box-like SRE-38. Similarly, the shorter hNIS deletion construct (region from -160 to +350) containing the mutated E-box-like SRE-38 was clearly less responsive to nSREBP-1c and nSREBP-2 than the corresponding nonmutated deletion construct. Figure 5A also demonstrates that mutation of the SRE-179 did not influence the reporter response of the hNIS deletion construct to nSREBP-1c and nSREBP-2, thus confirming the observations from the abovementioned deletion experiments (Figure 4C). To further strengthen our findings of a critical role of E-box-like SRE-38 for regulation of hNIS 5′-flanking region, we designed 2 reporter gene constructs containing 2 copies of either nonmutated or mutated E-box-like SRE-38 of hNIS in front of the luciferase reporter. As shown in Figure 5B, the reporter response of the construct containing 2 copies of the mutated E-box-like SRE-38 exhibited only a weak response to nSREBP-1c and nSREBP-2 compared with the construct with the nonmutated E-box-like SRE-38. Collectively, these findings strengthened the assumption that the E-box-like SRE-38 is functional.

**nSREBP-1 and nSREBP-2 Bind to the E-Box-Like SRE-38 of hNIS 5′-Flanking Region**

Because functional SRE are able to bind nSREBP, we next investigated whether nSREBP-1c and nSREBP-2 bind specifically to the E-box-like SRE-38 in the hNIS 5′-flanking region. For this end, electrophoretic mobility shift assays using a DIG-labeled double-stranded 34-bp oligonucleotide containing the E-box-like SRE-38 of hNIS 5′-flanking region (hNIS E-box-like SRE-38) and in vitro translated nSREBP-1c or nSREBP-2 were performed. Reliability of electrophoretic mobility shift assays was tested using DIG-labeled oligonucleotides containing either the wild-type (positive control) or the mutated hLDLR-SRE (negative control). Figure 6 demonstrates that a shifted protein-DNA complex was formed between the wild-type hLDLR-SRE and either nSREBP-1c (lane 2, Figure 6A) or nSREBP-2 (lane 2, Figure 6B) being indicative of binding of nSREBP to hLDLR-SRE, but not between the mutated hLDLR-SRE and either nSREBP-1c (lane 3, Figure 6A) or nSREBP-2 (lane 3, Figure 6B). As illustrated in lane 4, a shifted protein-DNA complex was also formed between nSREBP-1c (Figure 6A) and nSREBP-2 (Figure 6B) and the wild-type hNIS E-box-like SRE-38, but not with the mutated hNIS E-box-like SRE-38 (lane 5 in Figure 6A and 6B), in which 4 nucleotides of the putative SRE were mutated. Competition experiments revealed that complex formation between nSREBP-1c or nSREBP-2 and hNIS E-box-like SRE-38 was successively reduced with increasing molar excess of unlabeled specific probe (wild-type hLDLR-SRE; lanes 6–8 in Figure 6A and 6B), indicating that nSREBP-1c and nSREBP-2 bind specifically to the E-box-like SRE-38 of hNIS 5′-flanking region. Collectively, these results from genetic and molecular biological studies demonstrated clearly that the mammary gland NIS gene is a direct target gene of SREBP-1c and SREBP-2, at
least in a human mammary epithelial cell line. Thus, it may be postulated that SREBP are more than just lipid regulators in mammary epithelial cells and even regulate the uptake of iodide into mammary epithelial cells. Provided that NIS is also regulated by SREBP in the mammary gland epithelium of cattle, the iodide content of the cow’s colostrum and mature milk may be influenced by the activity of mammary gland SREBP. Thus, it appears possible that feeding CLA, which inhibit the maturation (Peterson et al., 2004) or expression (or both) of SREBP-1c (Harvatine and Bauman, 2006) in mammary epithelial cells, to cows not only reduces milk fat content but also iodide content. Albeit speculative, this may reduce the iodide supply to the newborn calf during the colostral phase and the iodide intake from milk by the consumer.

CONCLUSIONS

The present study shows for the first time that the NIS gene and iodide uptake is regulated by SREBP in the human mammary epithelial cell line MCF-7. Tran-
Figure 6. Nuclear sterol regulatory element-binding protein (nSREBP)-1c and nSREBP-2 bind to the E-box-like sterol regulatory element (SRE)-38 of human sodium/iodide symporter (hNIS) 5'-flanking region. Electrophoretic mobility shift assay was performed using in vitro translated nSREBP-1c (A) and nSREBP-2 (B), respectively, and digoxigenin (DIG)-labeled oligonucleotide corresponding to either wild-type (wt) or mutated (mut) E-box-like SRE-38 of hNIS (hNIS E-box-like SRE-38). For competition, 10-, 20-, and 50-fold molar excess of unlabeled specific probe [human low-density lipoprotein receptor (LDLR)-SRE] was used. The DIG-labeled specific probe (hLDLR-SRE) and nonspecific probe (mutated hLDLR-SRE) are also indicated.
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