Isolation and characterization of the human stearoyl-CoA desaturase gene promoter: requirement of a conserved CCAAT *cis*-element

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Stearoyl-CoA desaturase is the rate-limiting enzyme in the production of mono-unsaturated fatty acids. We have recently cloned and characterized the human *Scd* cDNA and *SCD* (the stearoyl-CoA desaturase structural gene) on chromosome 10, as well as the non-transcribed pseudogene on chromosome 17. In order to further define *SCD* regulation and function, we have isolated and characterized the promoter of the structural gene. Screening of chromosome-10-specific libraries resulted in the isolation of 4.1 kb of *SCD* sequence upstream of the translation start site. Binding sites for transcription factors critical for mouse *Scd1* and *Scd2* promoter activity, such as sterol-regulated-element-binding protein and nuclear factor Y, were present in the human *SCD* promoter (*Scd* is the mouse stearoyl-CoA desaturase gene). Deletion analysis in HaCaT keratinocytes identified a critical region for promoter activity between nts 496–609 upstream of the translation start site. Site-directed mutagenesis of binding sites in this region identified the CCAAT

box as the critical *cis*-element for *SCD* promoter activity. An electrophoretic mobility-shift assay confirmed that this element binds nuclear proteins from HaCaT keratinocytes. The polyunsaturated-fatty-acid (PUFA) response element, previously identified in the promoters of mouse *Scd1* and *Scd2*, was found to be conserved in the human *SCD* promoter, and contained the critical CCAAT *cis*-element. A minimal promoter construct including this region was responsive to fatty acids, with oleate and linoleate decreasing transcription and stearate increasing it. These studies indicate that CCAAT-box-binding proteins activate *SCD* transcription in cultured keratinocytes and that fatty acids modulate transcription, most likely through the conserved PUFA response element.

Key words: lipid metabolism, monosaturated fatty acids, palmitoleate, transcription.

INTRODUCTION

Stearoyl-CoA desaturase is a lipid-metabolic enzyme that catalyses the insertion of a double bond between carbon atoms 9 and 10 in the saturated fatty acids (SFAs) palmitoyl-CoA and stearoyl-CoA to generate the mono-unsaturated fatty acids (MUFAs) palmitoleoyl-CoA and oleoyl-CoA respectively. MUFAs play important roles in many processes, including energy metabolism (triacylglycerol storage in adipose tissue) [1], membrane fluidity [2], anti-oxidation [3], signal transduction [4,5], apoptosis [6] and senescence [7]. Moreover, a critical role in differentiation is evidenced by the lack of development of the sebaceous and meibomian glands in the *asebia* mutant mouse, due to loss of *Scd1* function [8]. Altered stearoyl-CoA desaturase gene expression is associated with various diseases such as cancer [9], obesity [10], and aging [11]. The pleiotropic nature of MUFAs requires complex regulation of stearoyl-CoA desaturase genes that includes diverse signals such as insulin [12], carbohydrate [13,14], fatty acids [15] and temperature [16]. Furthermore, tight regulation of the rat *Scd* protein is indicated by a 3–4 h half-life, due to the action of an integral membrane-bound protease in the endoplasmic reticulum [17].

In order to gain an insight into the complex regulation of *SCD* genes, nucleotide sequences have been isolated from many organisms, including plants, bacteria, yeasts, fish, cows, rats, mice [18], fruitflies (*Drosophila melanogaster*) [19], nematode worms (*Caenorhabditis elegans*) [20], hamsters [21], sheep [22] and humans [23]. At the levels of nucleotide and amino acid sequences, the stearoyl-CoA desaturase gene family exhibits both similarities and differences across the phylogenetic Kingdoms [24]. The similarities are (1) the high levels of sequence identity of the open reading frames (ORF) among the orthologous genes, (2) the presence of conserved amino acids (and their spacing) involved in catalysis of the Δ^9 desaturation reaction, and (3) the presence of a long 3'-untranslated region (3'-UTR) in the vertebrate genes. The major difference is the variable number of stearoyl-CoA desaturase genes between different species. Those organisms with a single structural gene include cyanobacteria [25], the yeast *Saccharomyces cerevisiae* [26], sheep [22] and human [23], whereas those with more than one structural gene include the yeast *Mortierella alpina* [27], *D. melanogaster* [19], *C. elegans* [20], mouse [28,29] and rat [30]. The reason for the multiple stearoyl-CoA desaturase genes is unclear, although there is evidence to suggest that stearoyl-CoA desaturase proteins

Abbreviations used: AP-2, activator protein-2; RFX-1, X-box-binding regulatory factor-1; FES2, fat-specific element-2; *SCD*, stearoyl-CoA desaturase; *Scd*, human stearoyl-CoA desaturase gene; *Scd*, mouse stearoyl-CoA desaturase gene ('stearoyl-CoA desaturase' indicates non-human and non-murine genes, as well as cDNA, mRNA and protein from any species); EMSA, electrophoretic-mobility-shift assay; UFA, unsaturated fatty acid; MUFA, mono-unsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; ORF, open reading frame; UTR, untranslated region; SREBP, sterol-regulated-element-binding protein; NF, nuclear factor; CDS, coding sequence; Sp1, stimulatory protein 1, all sequences in the present paper are numbered relative to the translation start site, designated +1.

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The nucleotide sequence reported in this paper has been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AF320307.

in *C. elegans* [20] and mouse [31] have different substrate specificities. This hypothesis is further supported by the finding of tissue-specific expression of the rodent stearoyl-CoA desaturase homologues [28–30,32], whereas stearoyl-CoA desaturase in human [23] and sheep [22] is ubiquitously expressed. Further species-specific regulatory mechanisms are indicated by the use of tandem polyadenylation signals by human *SCD* to generate two alternate mRNA transcripts [23], each encoding the identical enzyme, whereas the rodent *SCD* genes each generate a single mRNA transcript [28–30].

Owing to the diverse nature of the processes in which stearoyl-CoA desaturase functions, the regulation of stearoyl-CoA desaturase gene expression involves both transcriptional and post-transcriptional mechanisms [15,33]. Studies of transcriptional regulation have focused on functional analysis of promoters, these having been isolated from yeast *OLE1* [18], mouse *Scd1* [28], mouse *Scd2* [29], rat stearoyl-CoA desaturase 1 [30] and sheep stearoyl-CoA desaturase [22]. The mouse *Scd1* and *Scd2* promoters have been extensively analysed and *cis*-elements identified that regulate transcription [15,34], particularly through the transcription factors sterol-regulated-element-binding protein (SREBP) and nuclear factor (NF) Y [33].

In order to understand the role of *SCD* in human health and disease, we recently isolated the full-length human stearoyl-CoA desaturase cDNA and *SCD* structural gene, demonstrating considerable homology with the mouse *Scd* genes, particularly with respect to the ORF, intron–exon junctions and the long 3'-UTR [23]. Unlike the mouse and the rat, which have at least three stearoyl-CoA desaturase loci, humans have only two loci, one being the structural gene and the other a fully processed, transcriptionally silent pseudogene [23]. In the present study we characterized the human *SCD* promoter from the structural gene on chromosome 10. The human *SCD* promoter is similar to the mouse *Scd* promoters in that the polyunsaturated-fatty-acid (PUFA) response region is conserved and contains within it the critical SREBP and NF-Y binding sites required for mouse *Scd1* and *Scd2* transcription. Further, we demonstrate that the CCAAT box (putative NF-Y binding site) binds nuclear proteins and is required for transcriptional activation in cultured human keratinocytes. A minimal promoter, bearing this region, is responsive to unsaturated fatty acid (UFA)-mediated transcriptional repression, as well as transcriptional activation by SFA. Finally, many putative transcription-factor binding sites are identified that may play a role in regulating the diverse functions of *SCD*.

EXPERIMENTAL

Genomic fragment isolation

Isolation of the λ -phage encompassing the 5' end of the *SCD* gene (4 kb of promoter, exon 1, intron 1, exon 2 and part of intron 2) from a chromosome-10-specific genomic library was previously obtained using standard techniques [23]. The 5.6 kb insert was cloned as an *EcoRI* fragment into pBlueScript (Stratagene, La Jolla, CA, U.S.A.) (clone 19) and subsequently used to generate both nuclease-protection-assay probes and luciferase-reporter-assay constructs as described below.

Luciferase reporter construction

To generate p(–3348/–150*)*SCD*-luc (c1), clone 19 was digested with *PstI* and *SacII* and blunted with T4 polymerase. The 3.2 kb fragment containing only the 5' end of the gene was

cloned into a blunted *HindIII* site of the luciferase vector pGL3-basic (Promega, Madison, WI, U.S.A.). p(–2328/–150)*SCD*-luc (c2) and p(–1295/–150)*SCD*-luc (c4) were generated by digesting the clone 19 with *TaqI/SacII* and *KpnI/SacII* respectively, and the resulting fragments were subcloned into a blunted *HindIII* site of pGL3-basic. p(–1537/–150)*SCD*-luc (c3) and p(–882/–150)*SCD*-luc (c5) were subsequently generated by digesting c1 with *SacI* and *SmaI* respectively. The resulting fragments were re-ligated using T4 ligase. p(–461/–150)*SCD*-luc (c6) was generated by digesting c4 with *EaeI/NcoI* and the resulting fragment was ligated to blunted *NcoI/HindIII* sites of pGL3-basic. p(–270/–150)*SCD*-luc (c7) was created by digesting c4 with *HincII* and *NcoI*. The resulting fragment was subcloned into *NcoI/HindIII* digested blunted pGL3-basic. p(–753/–150)*SCD*-luc (c8), p(–609/–150)*SCD*-luc (c9) and p(–496/–150)*SCD*-luc (c10) constructs were assembled by PCR using one of the three specific sense oligonucleotides (bp –753 to –732, c8), 5'-GGTTCACCACTGTTTCCTGAGA-3'; (–609 to –688, c9) 5'-GATGCCGGGCAGAGGCCAGCG-3'; (–496 to –474, c10), 5'-GGCAA-CGGCAGGACGAGGTGGCA-3'; and a common antisense oligonucleotide (–166 to –145) 5'-CCGCGGTGCGTGGAGGTCCCCG-3'. All PCR reactions were conducted with proof-reading Turbo *PFU* DNA polymerase (Stratagene), and the original c5 construct was used as template. Amplification products were phosphorylated with T4 kinase and subcloned into the *HindIII*-blunted site of pGL3-basic. The sequence of the PCR products were examined for accuracy using the dideoxy-nucleotide-chain-termination method.

RNAse protection analysis

Total RNA was extracted, using RNeasy (Tel-Test Inc., Friendswood, TX, U.S.A.), from human hair-follicle biopsies and the human keratinocyte cell line, HaCaT [35]. Total RNA from human brain and mRNA from human liver were purchased from Clontech, Palo Alto, CA, U.S.A. A *SCD* fragment was amplified with PCR, using clone 19 as a template with the following primers: sense (–275 to –256), 5'-GCCAGTCAACTCCTCGCACT-3'; antisense (+7 to +27), 5'-ATCGTCTGCAGCAAGTGGGC-3'. This resulted in a 302-bp fragment (nucleotides –275 to +27) which was subcloned into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA, U.S.A.). The plasmid (NPA1-pCR2.1) was further digested with *XbaI* and *EcoRV* to release unnecessary vector sequence between the insert and the T7 promoter and re-ligated with T4 ligase. The final construct (NPA2-pCR2.1) was linearized by *HindIII* and a labelled antisense RNA (400 bp) was synthesized from the T7 promoter of the plasmid using T7 RNA polymerase (Ambion, Austin, TX, U.S.A.). Following synthesis, the full-length probe was gel-purified. Total RNA from human hair follicles (15 μ g), human brain (10 μ g), and HaCaT keratinocytes (2, 5 and 10 μ g) and mRNA from human liver (5 μ g) was hybridized to the labelled transcript for 16 h at 42 °C. The RNA samples were digested with an RNase mixture (Ambion), and the sizes of the protected RNA were determined by electrophoresis on a denaturing 5%-(w/v)-polyacrylamide gel, using a DNA sequencing ladder generated from NPA2-pCR2.1.

Nuclear extracts and electrophoretic-mobility-shift assays (EMSA)

Nuclear extracts of HaCaT keratinocytes were prepared using standard procedures [36]. Protein concentrations of the extract

were determined with BCA assay (Pierce, Rockford, IL, USA). Electrophoretic mobility shift assays were performed with a Gel Shift assay system according to the manufacturer's instructions (Promega, Madison, WI, U.S.A.). Briefly, hybridization was performed at room temperature for 20 min in a volume of 20 μ l consisting of 0.1 pmol of double-stranded CCAAT oligonucleotide probe (70000 c.p.m.) and 5 μ g of nuclear extract. The gel shift mixture was incubated for 20 min at room temperature, and samples were immediately electrophoresed at 150 V for 3 h on a non-denaturing 5%-polyacrylamide gel using 0.5 $\times$ TBE running buffer (34 mM Tris/borate/0.75 mM EDTA) pre-run at 100 V for 100 min. The gel was then dried for autoradiography. For competition studies, radioinert DNA competitor was added as a 100-fold molar excess and preincubated with the nuclear extract at room temperature for 5–10 min. The 32 P-labelled DNA was then added to the mixture and incubated at room temperature. The competitor oligonucleotides used for EMSAs were as follows: activator protein-2 (AP-2) (sense, –595 to –575), 5'-GCCAGCGCGGGTGAAGAG-3'; AP-2 (antisense), 5'-CTCTTCCACCCGCCGCTGGGC-3'; stimulatory protein 1 (Sp1) (sense, –559 to –539), 5'-AACAGAGGGGAGGGGGAGCGA-3'; Sp1(antisense), 5'-TCGCTCCCCCTCCCCTCTGTT3'; CCAAT (sense, –509 to –489), 5'-GCGCCGAGCCAATGGCAACGG-3'; CAAT (antisense), 5'-CCGTTGCCATTGGCTCGGCGC-3'.

Transfection and dual luciferase assay

HaCaT keratinocytes were used as the recipient cells for transient transfection assays. On the day prior to transfection, cells were seeded into six-well plates at 400000 cells/well. Transient transfections were performed with 2 μ g of test plasmids using SuperFect Reagent (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's protocol. As a control for transfection efficiency and cell density, HaCaT cells were co-transfected with a 50 ng of *Renilla* luciferase control reporter vector driven by the thymidine kinase promoter (pRL-TK). At 48 h post-transfection, adherent cells were washed once with 3 ml of PBS, dissolved in 500 μ l of cell culture lysis reagent (Promega, Madison, WI, USA), and harvested after 30 min at room temperature. Luciferase assays were performed immediately using a Dual-Luciferase Reporter Assay System kit (Promega, Madison, WI, USA), and measurements were taken using a TD-20/20 luminometer (Turner Designs, Inc., Sunnyvale, CA, U.S.A.). The promoter activity was reflected by the ratio of firefly/*Renilla* luciferase for each construct. Duplicate or triplicate readings were taken for each assay, and all assays were replicated a minimum of three times over a minimum of two independent experiments. Results of statistical tests are indicated in the Table and Figure legends.

Mutagenesis

Mutation in the binding sites for transcription factors Sp1 and CCAAT were introduced into the plasmid *c9*, containing the 0.5 kb *SCD* promoter subcloned into the blunted *Hind*III site of pGL-3 basic, using the Quick Change Site-Directed Mutagenesis kit (Stratagene). Two double-stranded oligonucleotide primers containing the desired mutations are as follows (underlined base-pairs denote mutant substitutions): Sp1 (–566 to –532), 5'-AAGGAGAAACAGAGGAAAGGGGGAGCGAGGAGCTG-3', and CCAAT (–517 to –483), 5'-AGCAGATTGCCCGGAGAAAATGGCAACGGCAGGAC-3'.

Cell culture

All reagents were from Gibco BRL (Rockville, MD, U.S.A.) unless otherwise stated. HaCaT keratinocytes were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal-calf serum (Sigma, St Louis, MO, U.S.A.), 2 mM L-glutamine and 50 units/ml penicillin + streptomycin. The cells were incubated in humidified incubators at 37 °C and 5% CO₂. For fatty acid treatments, HaCaT keratinocytes were exposed to fatty acid-BSA conjugate (100 μ M) for 24 h prior to harvest. Stock solutions (2.75 mM) of fatty acid-BSA solutions were prepared as follows: 8.5 mg of stearate (Sigma) was dissolved in 1 ml of 0.1 M NaOH, then heated to 90 °C for dissolution. This solution was added dropwise to 6 ml of 10% fatty-acid-free BSA (Sigma) with stirring at 37 °C. This solution was adjusted to 10 ml with distilled water, filtered through a 0.22 μ m-pore-size filter, and stored at 4 °C. The same procedure was followed for oleate (Sigma); however, heating was kept to a minimum. The BSA solution used for controls was prepared following above procedure, but without added fatty acids. Linoleate-BSA conjugate was purchased from Sigma and reconstituted to 3 mM with distilled water. All stock solutions were diluted to 100 μ M in cell-culture media for application to cells.

DNA sequencing

The DNA sequence in both directions was determined by Howard Hughes Medical Institute Biopolymer/Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT, U.S.A.) using PerkinElmer Biosystems (Foster City, CA, U.S.A.) model 337 gel DNA sequencer and model 3700 capillary DNA sequencer.

Sequence comparison of the 5' ends of the human and mouse *SCD* genes

The mouse *Scd1* sequence used for alignment was obtained from GenBank® and used as follows: the promoter, 5'-UTR and proximal coding sequence (CDS) (1–885; M21280) had a G inserted at nt 698 in order to conform to the published sequence [28,37] to generate a 886-nt sequence. Mouse *Scd2* sequence used for alignment was obtained from GenBank® and used as follows: the promoter (nts 1–587; M26269) was joined to the 5'-UTR/proximal CDS of the mRNA (nts 1–324; M26270) to generate a 911-nt sequence. A 910-nt section of human *SCD* sequence was used for alignment, consisting of 887 nts 5' to the ATG start site, plus 24 nts of ORF (nts 3127–4126; AF320307). The three sequences were aligned using the ClustalW algorithm from the Vector NTI sequence analysis software. In the alignment window, various subregions (as described in the Results section) were highlighted and percentage identity calculated for the three-way and pairwise comparisons. Pairwise comparisons were made by removing one of the three sequences from the three-way alignment, then recalculating the percentage identity between the remaining pair, a procedure that preserves the original alignment.

RESULTS

Characterization of the 5'-region of the *SCD* gene

Previously we have shown that there are two *SCD* loci in human genome, with one being the functional gene present on chromosome 10, and the other a pseudogene located on chromosome 17 [23]. To obtain the upstream sequence of the functional *SCD*

Table 1 Putative transcription-factor binding sites*

Further abbreviations: CREB, cAMP-response-element-binding protein; Oct-6, octamer-binding protein-6; TATA-BP, TATA box-binding protein; HNF-3 β , hepatocyte nuclear factor 3 β ; Whn, winged-helix nude; C/EBP, CAAT-enhancer-binding protein; PPAR, peroxisome-proliferator-activated receptor; ER, (o)estrogen receptor; RAR- α 1, retinoic acid receptor α 1; T₃R, tri-iodothyronine receptor; VDR, vitamin D receptor; IRF-1, interferon regulatory factor 1; STAT, signal transduction and activators of transcription; AP-1, activator protein 1.

Transcription factor	Binding site	Position(s)
Ubiquitous		
AP-2	CCCS†	−165‡
CREB	TGAC	−1377, −1145
NF-1	TGGC	−1872, −1744, −1651, −1241, −827, −502, −459, −244, −194
NF-Y	CCAA	−3981, −3898, −501, −458
Oct-6	AATT	−177
RFX-1	GYAA	−522, −495, −196
SP-1	GGCG	−1038, −930, −551, −314, −304
TATA-BP	TAAA	−234, −179
Tissue — specific		
HNF-3 β	TRTT	−2730, −2424, −1958, −217
Whn	ACGC	−2290, −942, −858, −811, −615, −291, −155
Lipid metabolism		
C/EBP	GMAA	−2913, −1946, −1124, −440, −420, −219
PPAR	AAAG	−1186, −257
SREBP	TCAC	−4027, −3692, −3484, −3612, −2236, −2010, −517§
Steroid-hormone nuclear receptors		
ER	GTCA	−1738, −1359
RAR- α 1	GGTC	−3080, −2228, −1904, −1762
T ₃ R	GGTC	−2228
VDR	GAGG	−324, −222
Immune system		
IRF-1	GAAA	−3612, −2521
NF- κ B	GGGA	−2566, −2525, −1047, −1041, −868, −862, −727, −281
c-Rel	TTCC	−1689, −220
STAT	GGAA	−1138
Proliferation/differentiation		
AP-1	TGAC	−3116, −1678, −454, −271, −204
c-Myc	CACG	−941, −291
N-Myc	CGTG	−292

* Matinspector Professional output from Transfac database v4.1 with core similarity at ≥ 0.75 and matrix similarity at ≥ 0.85 .

† Core binding site, 5' to 3' direction (IUPAC nucleotide code).

‡ Numbering is relative to translation initiation site (+1).

§ Novel SRE indentified in the promoters of *Scd1* and *Scd2*.

gene, chromosome-10-specific libraries were screened with a 1.9 kb fragment (from clone 34) containing the 5' end of the *Scd* cDNA [23]. One of the genomic clones obtained from this screen had a 5.6 kb insert [23]. Analysis of the insert indicated that there was approx. 4.1 kb upstream of the ATG translation start site.

Putative transcription-factor binding sites were identified using the Matinspector Professional algorithm with the Transfac database v4.1 [38] using core similarity setting of 0.75 and matrix similarity setting of 0.85. Many classes of transcription-factor binding sites were identified, consistent with the pleiotropic function of *SCD* genes (Table 1, Figure 1).

Identification of the transcription initiation site

Screening for *Scd* in a cDNA library from cultured human foreskin keratinocytes resulted in two clones which extend 5' from the ATG translation initiation site, these being clone 34 to −235 and clone 45 to −229 [23] (arrowheads, Figure 1). Further definition of the transcriptional start site was carried out for cultured human keratinocytes, human hair-follicle keratinocytes (the major human skin cell type to express *Scd* mRNA *in vivo*; results not shown), human brain, and human liver. Primer extension and RNase protection were carried out with total RNA isolated from HaCaT keratinocytes, hair-follicle biopsies,

brain, and mRNA from liver. Owing to the high GC content in this region, primer extension did not yield consistent results. For the RNase protection assays, a cRNA probe, consisting of 302 nts of *SCD* (excluding vector sequence) from −275 to +27 was used in the protection assay. A major band of 172 nts was detected in RNA isolated from liver and hair follicles, whereas a major band of 300 nts was seen in brain and HaCaT keratinocyte RNA (Figure 2). No bands of the size of the full-length probe (400 nts, including vector sequence) were detected, indicating lack of contaminating sense DNA template and *in vitro*-transcribed sense RNA. Liver showed the same protected fragments as hair follicles, whereas brain had a similar protection pattern to that of HaCaT cells. This indicates that, for liver and hair follicles, the major site of transcription initiation is at −145 (single-lined box in Figure 1) from the translation start site. This position is 35 nts downstream of the proximal TATA box at −179, similar to mouse *Scd1* in 3T3-L1 cells [28]. In brain and cultured keratinocytes, the transcription start site is 5' to that of hair follicles, as indicated by the 300-nt protected fragment in brain and HaCaT cell RNA and the termination of cDNA clones 34 and 45 in the region of the distal TATA box located at −234 (Figure 1). There are minor protected bands in brain, liver, and HaCaT cells, with a common band of 220 nts (Figure 2). This indicates the presence of a minor transcription start site at −193,

Figure 1 Characterization of the 5' end of the human *SCD* gene

Sequence of the 5' end of the human *SCD* gene. Numbering is relative to the translation initiation codon (+1). Nts -4152 to +27 are present in the GenBank® Nucleotide Sequence Database under accession number AF320307; however, only residues -902 to +27 are shown. Amino acids are indicated using the single-letter code. Intron 1 is indicated in lower case. The start site of transcription in liver and hair-follicle tissue is boxed. A minor transcription site common to liver, brain and HaCaT keratinocytes is shown as a box bounded by a broken line. The FSE2 element is shown as a box bounded by a double line. Termination of *Scd* cDNA clones 34 (filled arrowhead) and 45 (open arrowhead) are indicated. The downward-pointing arrow (↓) indicates the 5'-most extent of RNase-protected fragment in brain and HaCaT keratinocytes. The core sequence of putative transcription-factor binding sites are single-underlined and notated above the sequence with rightward (>) or leftward (<) arrowheads indicating 5' to 3' direction of the element. Overlapping core sequences are double-underlined. SREBP binding site at -517 is a novel SRE identified in mouse *Scd1* and *Scd2* promoters [33]. See Table 1 for core sequence of putative binding sites within the FSE2 element. Restriction-enzyme sites are indicated by tildes above the sequence. **Bold** letters and numbers indicate the 5' extent of the clones used for reporter assays.

a position 42 nts downstream of the distal TATA box ('broken-line' box in Figure 1). Overall, these results suggest that the *SCD* gene has different major transcription start sites that depend on tissue-specific factors.

Functional analysis of the *SCD* promoter

To determine which promoter sequences are sufficient to direct transcription, a 3.2 kb *PstI*-*SacII* fragment of the 5' flanking region was fused in both orientations to a promoterless luciferase reporter gene and examined for the ability to mediate basal transcription. In addition, progressive 3' deletions in the putative promoter region were also examined for their effect on reporter activity. The resulting constructs were transiently transfected into HaCaT keratinocytes, and cell extracts were assayed for

Figure 2 Determination of transcription initiation site

Total RNA from human hair follicles (15 µg, lane 1), mRNA from human liver (5 µg, lane 2), total RNA from human brain (10 µg, lane 3) and total RNA from HaCaT keratinocytes (10 µg, lane 4; 5 µg, lane 5; 2.5 µg, lane 6) was hybridized with a 400-nt *Scd* riboprobe (302 nts are *SCD* sequence) at 42 °C. After digestion of single-stranded RNA with RNase A/T1, the protected fragments were resolved along with a sequencing ladder of the probe-containing vector NPA2-pCR2.1. Filled arrowheads indicate major protected fragments. Arrowhead at 220 nt indicates minor protected fragment common to liver, brain, and HaCaT keratinocytes. Sizes (nts) of the protected fragments are indicated on right.

luciferase activity at 48 h post-transfection. A total of seven different constructs were initially generated using convenient restriction-enzyme sites in the 3.2 kb flanking region (Figure 3). Significant transcriptional activity was contained with 5 constructs covering the region between -3348 to -882. Further deletion to -461 resulted in complete loss of activity. *SCD* sequences fused in opposite transcriptional orientation did not induce any significant level of reporter activity. The highest promoter activity was obtained with construct c5, which corresponded to bp -882 to -150. These results demonstrated that, of the constructs tested, c5 contained the minimal required nucleotide sequence information for *SCD* transcription, and that essential *cis*-elements lay between -882 and -461.

To further delineate *cis*-elements responsible for human *SCD* gene expression, the c5 construct was deleted further to create three more constructs: c8, c9 and c10 (Figure 4; see also Figure 1). These constructs were transiently transfected into HaCaT

Figure 3 Functional analysis of human *SCD* promoter

Left panel: a panel of deletion constructs. Restriction sites are indicated. Sequence numbering is relative to translational start site (+1). 'c1-' is the reverse orientation of c1. Right panel: promoter activity of constructs transfected into HaCaT keratinocytes. Constructs were transfected in duplicate, cell lysates harvested 48 h post-transfection, and readings taken in triplicate. Values are plotted as the means $\pm$ S.E.M. for four samples from two independent experiments, and represent the ratio of the relative light units (RLU) of firefly luciferase to *Renilla* luciferase. $P < 0.0001$, one-way ANOVA; $P < 0.0001$, Tukey-Kramer HSD test for all comparisons of c1, c2, c3, c4, c5 versus c6, c7, c1-, pGL3 + pRL, pGL3, none. PGL3, firefly luciferase vector; pRL, *Renilla* luciferase vector; none, no DNA.

keratinocytes, in addition to the parental construct c5. Deletion of 129 nts from the 5'-end of the c5 construct (to generate c8) did not have any major effect on the promoter activity. Similarly, deletion of a further 144 nts, to generate c9, did not affect *SCD* promoter activity. However, deletion of a further 113 nts, to generate c10, completely eliminated all promoter activity as indicated by the reduced luciferase activity to background level (Figure 4). Taken together, these results narrowed the region that contains essential *cis*-elements required for *SCD* transcription in HaCaT keratinocytes to -609 to -496.

Examination of the sequences of this region revealed several potential transcription-factor binding sites (Figure 4, lower panel); AP-2 (-594), interferon-stimulated response element (-561), Sp1 (-551), X-box-binding regulatory factor-1 (RFX-1; -522, -495), SREBP (-517), NF-1 (-502) and NF-Y (-501). Considering the co-operative role of Sp1 and NF-Y in the regulation of the fatty acid synthase promoter [39], and NF-Y [33] in the regulation of mouse *Scd* genes, the effect of mutations in the Sp1 binding site and CCAAT box on *SCD* promoter activity was determined using site-directed mutagenesis. Using c9, the core binding site for Sp1 at -551, was changed from GGAG to AAAG (boxed in Figure 4) and the CCAAT box binding site for NF-Y at -501 was changed from CCAA to AAAA (boxed in Figure 4). The mutation in the CCAAT box also changes the core binding site for NF-1 (-502 on the non-coding strand) from TGGC to TAAC. It is unlikely that this NF-1 site is functional, as it contains the two most detrimental deviations (substitution at position 3 in the 5'-half-site of the palindrome and a shortened spacer of four nucleotides between half-sites), as indicated by a recent, more reliable prediction method for functional NF-1 sites [40]. Relative to the

promoter activity of c9, mutation of the Sp1 site has no effect (Table 2). However, mutation of the CCAAT box completely removes *SCD* promoter activity (Table 2). These results, taken together with the requirement of the NF-Y-binding CCAAT element for mouse *Scd1* transcription, strongly suggest that a critical *cis*-element for *SCD* promoter activity is the CCAAT box located at -501.

EMSA for CCAAT-box-binding proteins

To determine whether the CCAAT box critical for *SCD* promoter activity was actually occupied by DNA-binding proteins present in nuclei of HaCaT keratinocytes, we prepared nuclear extracts and performed EMSAs (Figure 5). A specific gel shift was obtained when nuclear extract from HaCaT keratinocytes was assayed with radiolabelled oligonucleotide corresponding to the CCAAT-box-binding site at -501. The gel shifts were competed with non-radiolabelled oligonucleotide containing the CCAAT element (see Figure 4), but not with the oligonucleotides containing either AP-2 or Sp1 binding sites. There are a few lower bands that were partially competed by CCAAT oligonucleotide, but not by the AP-2 and Sp1 oligonucleotides. Since NF-Y is the major protein that recognizes the CCAAT box [41] and binds as a trimer, it is possible that the lower, partially competed, bands represent partially assembled NF-Y complexes.

Regulation of the *SCD* promoter by fatty acids

Previous work identified a region of the mouse *Scd1* and *Scd2* promoter that was responsible for down-regulation of promoter

Figure 4 Fine deletion analysis of the human *SCD* promoter

Upper left: panel of deletion constructs. Restriction sites and 5' ends of primer sites are indicated. Upper right: promoter activity of constructs transfected into HaCaT keratinocytes. Constructs were transfected in duplicate, cell lysates harvested 48 h post-transfection, and readings performed in duplicate. Values are plotted as the means $\pm$ S.E.M. for four samples from two independent experiments, and represent the ratio of the relative light units (RLU) of firefly luciferase to *Renilla* luciferase. $P < 0.0001$, one-way ANOVA; $P < 0.0001$, Tukey–Kramer HSD test for all comparisons of c5, c8, c9 versus c10, c6. Lower panel: promoter region showing location of c9, c10, c6 (**bold**), location of transcription-factor binding sites (as annotated in Figure 1), nucleotides (boxed) subjected to site-directed mutagenesis in Table 2, and competitor oligonucleotides (lower case) used for EMSA in Figure 5.

Table 2 Site-directed mutagenesis of the SP-1 site and CCAAT box

c9-derived constructs containing mutations for SP-1 (–551) and CCAAT (–501) were generated, transfected into HaCaT keratinocytes, and analysed as described in the Experimental section. Values are expressed as the means of the relative light units (RLU) of firefly luciferase to *Renilla* luciferase from samples (n) from three independent experiments. $P < 0.0001$, one-way ANOVA; $P < 0.0001$, Tukey–Kramer HSD test for all comparisons of c9 (CCAAT), pGL3 + pRL versus c9, c9 (SP-1).

Construct	RLU (mean $\pm$ S. E.M) (n)
pGL3 + pRL	0.05 $\pm$ 0.01 (3)
c9	31.46 $\pm$ 0.34 (4)
c9 (SP1)	28.18 $\pm$ 1.62 (5)
c9 (CCAAT)	0.47 $\pm$ 0.03 (5)

activity by UFAs [42]. To examine whether the minimal *SCD* promoter could respond to UFA, c9 was transfected into HaCaT

keratinocytes, and the cells subsequently exposed to various fatty acids conjugated to BSA (Table 3). BSA alone had no effect on *SCD* promoter activity. In contrast, stearate up-regulated promoter activity, whereas oleate (a MUFA) and linoleate (a PUFA) down-regulated promoter activity to the same extent. It is notable that stearate and oleate are the substrate and product, respectively, of the *Scd* enzyme.

Comparison of the promoters of human *SCD*, mouse *Scd1* and mouse *Scd2*

In order to compare possible functional similarities and differences in *SCD* gene regulation between human and mouse, a multiple sequence alignment of the 5' ends of human *SCD*, mouse *Scd1*, and mouse *Scd2* genes was performed using sequences approx. 900 nts upstream of the ATG translation start

Figure 5 EMSA of CCAAT-box-binding proteins

Nuclear proteins from HaCaT keratinocytes were mixed with 32 P-labelled CCAAT oligonucleotide, with and without competitor oligonucleotide, and analysed using non-denaturing gel electrophoresis as described in the Experimental section. The sense strands of the double-stranded oligonucleotides used for EMSA are indicated in lower case in Figure 4; AP-2, -595 to -575; Sp1, -559 to -539; CCAAT, -509 to -489. Arrowhead indicates the band demonstrating specific CCAAT-box-binding proteins. The bar indicates partially competed CCAAT box-binding proteins.

Table 3 *SCD* promoter activity in response to fatty acid treatment

BSA or BSA-conjugated fatty acids were added to c9-transfected HaCaT keratinocytes and analysed as described in the Experimental section. Values are expressed as the means of the relative light units (RLU) of firefly luciferase to *Renilla* luciferase from samples (*n*) from three independent experiments. $P < 0.0001$, one-way ANOVA; $P < 0.05$ for all treatments versus BSA, Tukey–Kramer HSD test and Dunnett's test.

Treatment	RLU (mean $\pm$ S. E.M) (<i>n</i>)
No DNA	0.02 $\pm$ 0.00 (3)
pGL3 + pRL	0.05 $\pm$ 0.01 (3)
BSA	35.07 $\pm$ 3.72 (4)
Stearate	51.79 $\pm$ 3.06 (4)
Oleate	21.24 $\pm$ 3.11 (3)
Linoleate	21.42 $\pm$ 1.47 (3)

sites. Sub-regions of the aligned sequences were selected for further analysis, the largest of which is shown in Figure 6. This region (*Scd1*, -626 to +1; *Scd2*, -500 to +1; *SCD*, -565 to +1) has 41.8% identity, indicating a homologous relationship between the three *SCD* genes (Figure 7). When pairwise comparisons are made over the same region, *SCD* is more similar to *Scd1* (59.7% identity) as compared with *Scd2* (55.9% identity) (Figure 7). A comparison of the proximal regions of *SCD* (-297

to +1), *Scd1* (-301 to +1), and *Scd2* (-235 to +1) genes demonstrate 36.4% identity, indicating an increase in overall variability between the three sequences in this region (Figure 7). However, pairwise comparison of this proximal region indicates the striking similarity of *SCD* to *Scd1* (62.6% identity) as compared with *Scd2* (44.5% identity) (Figure 7). This similarity between *SCD* and *Scd1* is due to the presence (and positioning) of the following putative *cis*-elements: two TATA boxes, an fat-specific element-2 (FSE2) element, and binding sites for NF-1, AP-2, Myc, winged-helix nude ('Whn'), and AP-1 (Figure 6). The notable differences between the *SCD* and *Scd1* promoters in this region are the presence in the human of c-Rel, nuclear factor κ B, neuroblastoma-derived Myc and RFX-1 binding sites (Figure 1), and of the extended UTR in *Scd2* (Figure 6). Comparison of a distal region of the promoters of *SCD* (-565 to -451), *Scd1* (-626 to -511) and *Scd2* (-500 to -385) demonstrate 78.4% identity, indicative of a highly conserved regulatory region (Figure 7). Pairwise comparison of this region indicates that *SCD* is equally similar to both *Scd1* (87.9% identity) and *Scd2* (86.2% identity) (Figure 7). This high degree of similarity between all three genes is partly due to the presence and spacing of two CCAAT box elements and a novel SRE (Figure 6). In addition, a conserved RFX-1 site is present in all three promoters. This distal region of the promoters contain the PUFA-response element (Figure 6).

DISCUSSION

A sequence comparison of the 5' end of human and mouse *SCD* genes indicates two regions of high homology, these being (1) the 5'-UTR and basal/proximal promoter (-297 to +1 in human) and (2) a region containing the PUFA-response element (-565 to -451 in human). Pairwise comparisons of these regions, particularly the proximal portion, indicate that human *SCD* is the homologue of mouse *Scd1*. The similarities include the 5' UTR, the major transcription start site in human skin and liver relative to the proximal TATA box, and the presence and spacing of several putative transcription-factor binding sites. The NF-1 [43] and AP-2 [37] elements are required for *Scd1* transcription during adipogenesis in 3T3-L1 cells, whereas the FSE2 [binds CCAAT-enhancer-binding protein ('C/EBP')] [44] and AP-1 [45] elements are implicated in transcription of several lipogenic genes, including *Scd1* [46]. Deletion of the human *SCD* promoter, which leaves these elements intact but removes the upstream CCAAT box (-501), causes the loss of all transcriptional activity, indicating dispensability of the proximal promoter for basal transcription in HaCaT keratinocytes. This is consistent with a major transcription start site in HaCaT keratinocytes 5' to these elements. However, these elements may have a more central role in human hair-follicle keratinocytes and liver, since the major transcription start site in these tissues is 3' to these elements. Notably, *Scd1* (expressed in liver) requires some of these sites for transcription, and the FSE2 element contains a conserved putative hepatocyte nuclear factor-3 β binding site. *Scd2* (expressed in brain) does not contain these proximal elements and initiates transcription from an upstream site, similar to transcription initiation of human *SCD* in brain. Thus the single *SCD* gene in human uses multiple transcription initiation sites that are tissue-specific and that reflect the transcription initiation patterns of the individual tissue-specific mouse *Scd* homologues.

The second region of high homology between human *SCD* and mouse *Scd1/Scd2* is the region from -565 and -461 (in human) which contains the PUFA-response region, an element previously shown to mediate the down-regulation of mouse *Scd*

Figure 6 Sequence alignment of the regulatory regions of human and mouse *SCD* genes

Multiple sequence alignment of 5' UTR and promoter regions of human *SCD*, mouse *Scd1*, and mouse *Scd2* as described in the Experimental section. Numbering is relative to translational start site (+1). Grey boxes indicate sequence identity. Single-lined boxes with overlying filled arrowheads indicate start site of transcription for *Scd1* [28], *Scd2* [29], and in liver and hair follicle tissue for *SCD*. The double-lined box indicates the FSE2 element. **Bold** letters indicate the PUFA-response element [42]. Circled numbers indicate sub-regions used for comparisons in Figure 7. The core sequence of putative transcription-factor binding sites are annotated as in Figure 1. See Table 1 for core sequence of putative binding sites within the FSE2 element. The SREBP-binding site is the novel SRE element from mouse *Scd1* and *Scd2* [33]. The AP-2 binding site in mouse *Scd1* is defined in [37].

mRNA transcription in response to PUFAs [42]. The very high sequence identity is partly due to the presence and spacing of two CCAAT boxes and a novel binding site for SREBP. This SREBP-binding site represents a functional, non-canonical SRE first identified in the promoters of mouse *Scd1* and *Scd2* [33], and is located within the *cis*-acting PUFA-response region. The finding that SREBP activates *Scd* genes [33,47] and that SREBP mRNA [48] and protein activation [49] are negatively regulated by PUFAs suggest that PUFAs down-regulate *Scd* transcription by interfering with SREBP function [33]. That the human *SCD* promoter activity is decreased by linoleate is consistent with this mechanism. The PUFA-response region also contains a CCAAT box, located 5 nts downstream of the SRE element. This CCAAT element is required for full activation of the mouse *Scd1* and *Scd2* promoters, and was shown to bind the NF-Y transcription factor [33]. The human *SCD* promoter is also dependent on this CCAAT site. Although the HaCaT cell proteins that bind to this CCAAT element were not characterized, based on the similarity to the mouse *Scd* promoters and the well-established SREBP–NF-Y interaction in various lipogenic genes [41], it is likely that NF-Y is involved in transcriptional activation of human *SCD*.

The MUFA oleate decreased human *SCD* transcription in HaCaT keratinocytes and decreased transcription of both *Scd1* and *Scd2* in CHO-K1 cells [50]. Oleate has been found to prevent the proteolytic activation of SREBP [51,52], and thereby decrease transcription from SRE-dependent promoters. In contrast with its effect in CHO-K1 cells, oleate does not down-regulate *Scd1* in liver or adipose tissues [13,15], suggesting a cell-type- and/or tissue-specific effect of MUFA suppression for *Scd1* [50]. The extent of these effects on MUFA regulation of human *SCD* remain to be tested. Stearate, a saturated-fatty-acid substrate of *Scd*, was found to increase transcription of the human *SCD* promoter, via the same minimal construct that is down-regulated by MUFA and PUFA. Conflicting data exist for the effect of stearate on *SCD*: no effect on *Scd1* mRNA in mouse liver [13] or 3T3-L1 adipocytes [53], and increased *Scd* mRNA in pig adipose tissue [54] and enzyme activity in mouse liver [55]. It is possible that differential regulation of *SCD* transcription in response to stearate is dependent on the type of cell and/or tissue, as may be the case for oleate-mediated transcriptional suppression. In summary, we have isolated and characterized the human *SCD* promoter, and have demonstrated similarities as well as differences in sequence and activity as compared with mouse *Scd*

	③	②	①	
<i>SCD</i>	-565	-451	-235	+1
<i>Scd1</i>	-626	-511	-301	+1
<i>Scd2</i>	-500	-385	-297	+1
<i>SCD-Scd1-Scd2</i>	78.4	41.8	36.4	
<i>SCD-Scd1</i>	87.9	59.7	62.6	
<i>SCD-Scd2</i>	86.2	55.9	44.5	
<i>Scd1-Scd2</i>	81.9	52.8	49.3	

Figure 7 Similarity comparisons of the regulatory regions of human and mouse SCD genes

Nucleotide sequence comparison of the promoters and 5'-UTR regions of human *SCD*, mouse *Scd1* and mouse *Scd2*. All comparisons are based on the sequence alignment from Figure 6. Boxes indicate regulatory regions of the genes to the left, and numbering is relative to the translational start site (+1). Circled numbers correspond to those on the multiple sequence alignment of Figure 6, and serve to highlight regions used for comparisons. Braces indicate regions used for comparisons. Values under the braces indicate percentage identity for the various comparisons that are indicated to the left.

promoters. The similarities most likely reflect the common function of SCD in regulating endogenous MUFA production, whereas the differences may be related to the different requirements for gene regulation in species with different numbers of functional SCD genes.

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Received 28 February 2001/2 April 2001; accepted 10 May 2001