Original Paper

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Promoter analysis of the DHCR24 (3β-hydroxysterol Δ^{24}-reductase) gene: characterization of SREBP (sterol-regulatory-element-binding protein)-mediated activation

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Synopsis

DHCR24 (3β-hydroxysterol Δ^{24}-reductase) catalyses the reduction of the C-24 double bond of sterol intermediates during cholesterol biosynthesis. DHCR24 has also been involved in cell growth, senescence and cellular response to oncogenic and oxidative stress. Despite its important roles, little is known about the transcriptional mechanisms controlling DHCR24 gene expression. We analysed the proximal promoter region and the cholesterol-mediated regulation of DHCR24. A putative SRE (sterol-regulatory element) at $-98/-90$ bp of the transcription start site was identified. Other putative regulatory elements commonly found in SREBP (SRE-binding protein)-targeted genes were also identified. Sterol responsiveness was analysed by luciferase reporter assays of approximately 1 kb 5′-flanking region of the human DHCR24 gene in HepG2 and SK-N-MC cells. EMSAs (electrophoretic mobility-shift assays) and ChIP (chromatin immunoprecipitation) assays demonstrated cholesterol-dependent recruitment and binding of SREBPs to the putative SRE. Given the presence of several CACCC-boxes in the DHCR24 proximal promoter, we assessed the role of KLF5 (Krüppel-like factor 5) in androgen-regulated DHCR24 expression. DHT (dihydrotestosterone) increased DHCR24 expression synergistically with lovastatin. However, DHT was unable to activate the DHCR24 proximal promoter, whereas KLF5 did, indicating that this mechanism is not involved in the androgen-induced stimulation of DHCR24 expression. The results of the present study allow the elucidation of the mechanism of regulation of the DHCR24 gene by cholesterol availability and identification of other putative cis-acting elements which may be relevant for the regulation of DHCR24 expression.

Key words: androgen, cholesterol, 3β-hydroxysterol Δ^{24}-reductase (DHCR24), Krüppel-like factor 5 (KLF5), promoter analysis, sterol-regulatory-element-binding protein (SREBP)

INTRODUCTION

Cholesterol biosynthesis is tightly regulated. The rates of cholesterol biosynthesis vary over a great range as a func-tion of cholesterol availability and, actually, it has long been known that this pathway is subjected to feedback inhibition by its end product, cholesterol [1]. The key mechanism for this feedback regulation takes place at the transcriptional level and it is controlled by the SREBP

[SRE (sterol regulatory element)-binding protein] family of transcription factors. SREBPs are intrinsic proteins of the endoplasmic reticulum membrane that require a proteolytic processing, which takes place in the Golgi complex, to release the N-terminal region from the membrane so that it can enter the nucleus and activate the transcription of target genes. The processing of SREBPs is controlled by cholesterol availability: it is stimulated upon cellular cholesterol depletion and is inhibited when cellular cholesterol rises. In the nucleus, SREBPs bind to specific non-palindromic nucleotide sequences known as SREs located in the promoters of target genes, thereby activating their transcription. SREBPs also have the ability to transactivate genes that contain an E-box palindromic motif in their promoter [2].

There are three SREBP isoforms in mammals designated SREBP-1a, SREBP-1c and SREBP-2 [2], of which the two former are derived from a single gene. SREBP-2 preferentially increases transcription of genes for cholesterol biosynthesis and uptake, SREBP-1c principally activates genes for fatty acid biosynthesis, and SREBP-1a has a less restricted role since it activates genes for both cholesterol and fatty acid biosynthesis [3]. Maximal stimulation of transcription by SREBPs requires binding of one or more accessory transcription factors to the promoter in the vicinity of the SRE. These factors include members of the Sp1 (specificity protein 1), NF-Y/CBF (nuclear factor-Y/CCAAT-binding factor) and CREB (cAMP-response-element-binding protein) families. On the other hand, the expression of SREBP targets can be suppressed by the transcription factor YY1 (Yin and Yang 1) [4].

DHCR24 encodes the microsomal enzyme DHCR24, which catalyses the C-24 reduction of sterol intermediates containing a 24,25-double bond, a reaction that may occur at each of the 19 steps of cholesterol biosynthesis from lanosterol [5]. The deficiency of this enzyme causes desmosterolosis, an autosomal recessive disease characterized by elevated levels of desmosterol, the immediate Δ^{24}-unsaturated precursor of cholesterol, in plasma and tissues, developmental malformations and neuropsychological alterations [6]. Interestingly, in addition to its enzymatic activity in cholesterol biosynthesis, other roles have been assigned to this protein. Thus, DHCR24 is down-regulated in brain regions affected by AD (Alzheimer's disease), which led to the identification of this gene as seladin-1 (selective AD indicator-1), and confers resistance against β-amyloid and oxidative stress-induced apoptosis [7]. Moreover, it has been reported that DHCR24 plays an important role in the cellular response to oncogenic and oxidative stress and is a key regulator of Ras-induced senescence [8,9]. Consistently, DHCR24 mRNA expression has been shown to be altered in different tumours [10–15]. Moreover, DHCR24 is an important mediator of lipid raft formation [16] and plays a significant role in HCV (hepatitis C virus) replication [17]. Previously, it has been reported that the anti-inflammatory effects of reconstituted HDL (high-density lipoprotein) [18] and ApoA-I (apolipoprotein A-I) [19] are partly mediated by an up-regulation of DHCR24.

It is reasonable to assume that, among the different roles assigned to DHCR24, its enzymatic function in cholesterol biosynthesis is pre-eminent. Nevertheless, the knowledge about the regulation of DHCR24 by sterols is scarce. Feeding rats a diet containing the cholesterol-lowering drugs cholestyramine and lovastatin enhanced the liver Δ^{24}-reductase activity [5]. We have described that Δ^{22}-unsaturated phytosterols are effective competitive inhibitors of DHCR24 activity [20], and Zerenturk et al. [21] have recently reported that certain side-chain oxysterols reduce DHCR24 activity without altering the enzyme's protein levels. Regarding gene expression, some evidence suggests that SREBPs control the transcription of DHCR24. Horton et al. [22] reported that SREBP-2 transgenic mice have increased liver expression of DHCR24. Moreover, Reed et al. [23], using a genome-wide promoter occupancy approach, found that SREBP-1 binds to the promoter of DHCR24 in HepG2 (human hepatocellular carcinoma) cells. Indeed, a very recent study by Zerenturk et al. [23a] has identified two functional SREs and two NF-Y sites in the promoter of DHCR24.

Previous studies have described different regulatory elements in the DHCR24 gene. The distal promoter region of DHCR24 contains putative ER (oestrogen receptor) [24] and AR (androgen receptor)-binding sites [14]. Recently, a DR4 motif has been identified in the DHCR24 distal promoter as a binding site for the CAR/RXR (constitutive androstane receptor/retinoid X receptor) and PXR (pregnane X receptor)/RXR heterodimers [25]. Additionally, an LXR (liver X receptor)-binding site has been found in the second intron [26]. The localization and a first analysis of the proximal promoter have not been reported until recently, and showed that it is a single CpG-rich promoter that can be regulated by DNA methylation [27].

In the present study, we performed a detailed analysis of the proximal promoter of DHCR24 that allowed the identification of a functional SRE as well as putative *cis*-acting elements that may modulate the SREBP-mediated regulation.

EXPERIMENTAL

Materials

HepG2 (human hepatocellular carcinoma, A.T.C.C. CRL-11997), SK-N-MC (human neuroblastoma, A.T.C.C. HTB 10) and LNCaP (human prostate adenocarcinoma, A.T.C.C. CRL-1740) cells were obtained from the A.T.C.C. FBS (fetal bovine serum), antibiotics and L-glutamine were purchased from Gibco BRL. DMEM (Dulbecco's modified Eagle's medium) was purchased from PAA Laboratories GmbH. CS-FBS (charcoal-stripped FBS) was purchased from HyClone-Thermo Scientific. LPDS (lipoprotein-deficient serum) was obtained by ultracentrifugation of FBS at a density of 1.21 kg/l. Lovastatin was from Merck, Sharp & Dohme and DHT (dihydrotestosterone) was from Sigma. LDLs (low-density lipoproteins) were isolated from humans by sequential ultracentrifugation [28].

Cell culture

HepG2 and SK-N-MC cells were maintained in DMEM supplemented with 10% (v/v) FBS, 0.1 mM non-essential

amino acid solution, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 units/ml streptomycin and 10 μg/ml gentamicin at 37 °C in a humidified atmosphere containing 5 % (v/v) CO_2. LNCaP cells were maintained in RPMI 1640 medium containing 10 % FBS, 100 units/ml penicillin, 100 units/ml streptomycin and 10 μg/ml gentamicin at 37 °C in a humidified atmosphere containing 5 % CO_2. For experiments, HepG2 and SK-N-MC cells were incubated with medium containing 10 % (v/v) LPDS. In experiments assessing the effects of DHT, 1 day prior to the experiments the serum-containing medium was replaced by CS-FBS.

Determination of the 5′ ends of the human DHCR24 mRNA

Rapid amplification of the 5′ cDNA ends [5′ RACE (rapid amplification of cDNA ends)] was performed using the 5′ RACE System from Invitrogen V2.0 according to the manufacturer's recommendations. First-strand cDNA synthesis was performed using 5 μg of total RNA from HepG2 cells and 200 units of SuperScript II reverse transcriptase; the DHCR24 gene-specific primer used was 5′-AAGGAGCAGGGTAGCAAGAC-3′. After RNA degradation and TdT (terminal deoxynucleotidyl transferase) tailing of the first-strand cDNA, a PCR was performed using the Abridged Anchor Primer supplied by the manufacturer and the DHCR24 GSP2 5′-CGAGAGCGGCAGGAGGAAG-3′. The PCR product was analysed by agarose gel electrophoresis and a second PCR was performed using the AUAP (Abridged Universal Amplification Primer) supplied by the manufacturer and the DHCR24 GSP3 5′-AGGCACACGAACACCCAGCG-3′. The PCR product was analysed by agarose gel electrophoresis, cloned into the pCR 2.1-TOPO vector (Invitrogen), transformed into *Escherichia coli* XL1-Blue cells and sequenced.

Cloning of the 5′ upstream region of the human DHCR24 gene and reporter plasmids

The promoter region of the human DHCR24 gene was generated by PCR amplification using HepG2 cell genomic DNA as a template and Platinum® Pfx DNA Polymerase (Invitrogen). The primers used were 5′-ATCTCGAGGGCAGAGATGAATGGAGAGG-3′ for sense, and 5′-ATAAGCTTCAGTGACAGGAGGCGCGAAC-3′ for antisense. To facilitate subsequent cloning of the PCR-derived fragments, XhoI and HindIII restriction sites were added respectively, to the 5′ end of these primers. An initial denaturation at 94 °C for 2 min was followed by 35 cycles of denaturation (94 °C, 15 s), annealing (60 °C, 30 s) and extension (68 °C, 90 s), and a final extension of 68 °C for 10 min was applied. The amplified fragment was separated on an agarose gel, recovered using the QIAquick Gel Extraction kit (Quiagen), XhoI- and HindIII-digested and cloned into the pBlueScript KS (−) vector. The fragment containing the region between − 1012 and + 6 nucleotides of the human DHCR24 gene was subcloned into the XhoI and HindIII sites of the pGL3-basic vector (Promega), sequence-verified and named pH DHCR24. Unidirectional serial

deletion of the pH DHCR24 construct were generated using the Exo III-S1 nuclease system (Fermentas) using KpnI, which was used to generate the 3′-overhang resistant to Exo III, and XhoI digestion. After treatment with Exo III (500 units) containing 75 mM NaCl, 2 μl samples were removed at 1 min intervals up to 25 min and put into 7.5 μl of S1 nuclease mix to remove the resulting single-stranded DNA overhangs. Fragment length was analysed by agarose gel electrophoresis and the appropriate fragments were recircularized using Fast-Link DNA Ligase (Epicentre Biotechnologies) and sequenced. The fragments generated were: − 643/ + 6, − 520/ + 6, − 348/ + 6, − 258/ + 6, − 198/ + 6, − 178/ + 6, − 166/ + 6, − 149/ + 6 and − 90/ + 6 pH DHCR24.

The site-directed mutagenesis construct mut SRE was produced by PCR with the following primers: Mut SRE KpnI sense 5′-GGTCGCCGCCCGGGTACCGGCCGGCCGAACCTCG-3′, Mut SRE KpnI antisense 5′-CGAGGTTCGGCCGGCCGGTAC-CCGGGCGGCGACC-3′, and the pGL3-basic vector primers RV3 5′-CTAGCAAAATAGGCTGTCCC-3′ and GL2 5′-CTTT-ATGTTTTTGGCGTCTTCCA-3′. The core SRE sequence TC-GGCCCAC (− 98 to − 90) of the pH DHCR24 was replaced by the sequence <u>C</u>CGGCC<u>GG</u>C, which generates a new KpnI restriction site. The sequence of the plasmid resulting from the above mutation was confirmed by KpnI digestion and DNA sequencing.

Transient transfection and reporter gene assay

The plasmids for transfection were prepared using the PureYield™ Plasmid Midiprep system (Promega). A luciferase assay was performed using a Dual-Glo Luciferase assay system (Promega) with pSG5-*Renilla* as an internal control for normalization of transfection efficiency.

For cholesterol-dependent transcriptional activation assays of the promoter constructs, 4×10^6 HepG2 and SK-N-MC cells were resuspended in 400 μl of OPTi-Mem and co-transfected with 10 μg of the luciferase reporter gene constructs and 0.1 μg of the pSG5-*Renilla* by electroporation. Cells were electroporated in 4-mm cuvettes at 200 V for 70 ms for HepG2 cells and 140 V for 70 ms for SK-N-MC cells, using a square waveform generator (ECM 830 Electro Square Porator; BTX). The electroporated cells were then diluted in DMEM with 10 % FBS and without antibiotics and transferred into 12-well plates. At 24 h after transfection the medium was replaced by DMEM with 10 % LPDS, with antibiotics and containing 10 μM lovastatin dissolved in DMSO (final concentration 0.044 %), 30 μg of cholesterol/ml of LDL or placebo. After 24 h of treatment, cells were harvested by scraping the plates in 50 μl of 1×passive lysis buffer (Promega), and 10 μl of the cell lysate was used for performing the dual-luciferase assay using a Sirius dual-injector luminometer (Berthold). Promoter activity was calculated as firefly/*Renilla* luciferase activity ratios after subtracting the background (non-transfected cells).

For androgen-dependent transcriptional activation assays, LNCaP cells were cultured at a density of 0.3×10^6 cells/ml in RPMI 1640 medium with 10 % FBS and without

antibiotics in 12-well plates. After 18 h, cells were transfected with 800 ng of the luciferase reporter gene constructs and 8 ng of pSG5-*Renilla* using Lipofectamine™ 2000 (Invitrogen). After 6 h of incubation, the cell medium was changed for RPMI 1640 medium containing 5% CS-FBS. At 24 h after transfection the medium was replaced by RPMI 1640 medium with 5% CS-FBS containing 10 μM lovastatin, the indicated concentrations of DHT dissolved in DMSO (final concentration 0.044%) or placebo. After 24 h of treatment, plates were scraped and cells were harvested in 50 μl of 1× passive lysis buffer (Promega), and 10 μl of the cell lysate was used for performing the dual-luciferase/*Renilla* assay.

The eukaryotic expression plasmids encoding full-length KLF5 (Krüppel-like factor 5) expression (hKLF5–pSG5) plasmid was kindly provided by Dr Min-Young Lee from Yonsei University (Seoul, South Korea) and expression plasmids encoding the 68-kDa transcriptionally active fragment of SREBP-1a (aminio acids 1–490) into pcDNA3.1 FLAG vector was a gift from Dr Yajaira Suárez from the New York University Medical Center (New York, U.S.A.). LNCaP cells were cultured at a density of 0.3×10^6 cells/ml in RPMI 1640 medium containing 10% FBS and without antibiotics in 12-well plates. After 18 h, cells were transfected with 800 ng of the expression plasmids using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's instructions. After 6 h of incubation, the cell medium was changed for RPMI 1640 medium containing 10% FBS and antibiotics and samples were collected after 24 h.

Fluorescence EMSA (electrophoretic mobility-shift assay)

HPLC-purified oligonucleotides were annealed with the specific complementary strand representing the putative SRE in the human DHCR24 promoter or the SRE in the human LDLR (LDL receptor) promoter as a control. The oligonucleotides used were: DHCR24 sense 5′-[Cy5]GCCCGGGTCTCGGCCCACCGAACCTCG-3′, DHCR24 antisense 5′-[Cy5]CGAGGTTCGGTGGGCCGAG-ACCCGGGC-3′, LDLR sense 5′-[Cy5]GATCAAAATCAC-CCCACTGCAAACT-3′, LDLR antisense 5′-[Cy5]AGTTTG-CAGTGGGGTGATTTTGATC-3′. The TNT-coupled reticulocyte lysate system (Promega) was used to prepare the *in vitro*-translated active fragment of SREBP-1a (SREBP-1a-pcDNA3.1 FLAG). The reaction mixture (20 μl) for the EMSA contained 2 μl of 10× binding buffer [500 mM KCl, 10 mM DTT (dithiothreitol) and 100 mM Tris/HCl, pH 7.5], 10% (v/v) glycerol, 0.05% Nonidet P40, 0.125% Triton X-100, 1 mM DTT, 0.5 mM MgCl$_2$, 0.2 μg of poly(dI-dC)·(dI-dC), the indicated amounts of *in vitro*-translated SREBP-1a and 75 μM of fluorescence double-stranded DNA oligonucleotides. The reaction was carried out at 25°C for 20 min. In competition assays, the indicated amounts of unlabelled double-stranded DNA oligonucleotides were added 5 min before the addition of the fluorescence probe. The anti-SREBP-1 antibody (H-160, Santa Cruz Biotechnology) was preincubated for 60 min at 4°C before adding the labelled probes. As a negative control, a reticulocyte lys-

ate without SREBP-1a-pcDNA3.1 FLAG was used. The DNA–protein complexes were resolved by electrophoresis using 2.4% non-denaturing PAGE. Subsequently, the gel was scanned and resolved wet, using a Typhoon 9400 scanner (GE Healthcare).

ChIP (chromatin immunoprecipitation) assay

ChIP assays were performed according to a procedure described previously [29] with minor modifications and using the ChampionChIP One-Day Kit (SABiosciences). Briefly, 20×10^6 HepG2 cells were treated with 10 μM lovastatin or 30 μg/ml LDL-cholesterol for 24 h and then cross-linked with 1% (v/v) formaldehyde in DMEM for 10 min at 37°C. Cross-linking was stopped by the addition of glycine to a final concentration of 125 mM. After 5 min, the cells were washed twice with PBS and harvested with PBS containing a protease inhibitor cocktail. The cells were lysed and sonicated on ice with a Branson Digital Sonifier at 10% amplitude for six 10-s pulses to shear the chromatin to an average fragment length of approximately 500 bp and then microcentrifuged. The sonicated chromatin was precleared with Protein A beads/60 μg of salmon sperm DNA (Sigma). After centrifugation, supernatants were incubated in a rotor with 4 μg of anti-SREBP-2 antibody (ab30682, Abcam) or normal-rabbit IgG (SABiosciences) at 4°C overnight. A 10 μl aliquot of each precleared chromatin was used to measure total input chromatin (input DNA fraction).

The immunoprecipitated DNA–protein complexes were washed four times and treated with Proteinase K for 30 min at 45°C. The immunoprecipitated and input DNA fractions were purified and used as a template for PCR. The primers used for analysis of the human DHCR24 proximal promoter containing the SRE were 5′-GGGGAGAAAAGGGTGGAG-3′ for sense and 5′-GTTCGCGCCTCCTGTC-3′ for antisense. The primers for human LDLR-containing SRE, used as a positive control, were 5′-GTGGGAATCAGAGCTTCACG-3′ for sense and 5′-GACCTGCTGTGTCCTAGCTG-3′ for antisense. Real-time PCR analyses were performed using the SYBR Green I Master kit and LightCycler 480 II (Roche Applied Science). The melting curves were evaluated and the PCR products were separated on a 2% (w/v) agarose gel and stained with ethidium bromide to confirm the presence of a single product. The relative occupancy of the immunoprecipitated factor at a locus was estimated using the following equation: $2^{(C_t\,\text{specific}\,-\,C_t\,\text{input specific})}/2^{(C_t\,\text{IgG}\,-\,C_t\,\text{input IgG})}$, where C_t (threshold cycle) specific and C_t IgG are the mean C_t of the PCR done in triplicate with the DNA sample from the specific immunoprecipitation (anti-SREBP-1 antibody) or mock immunoprecipitacion (normal-rabbit IgG) respectively, and C_t input specific and C_t input IgG are the mean C_t of the PCR done in triplicate with the DNA samples from the corresponding input DNA fractions.

Real-time RT–PCR (reverse transcription–PCR) analysis

Total RNA was extracted with TriReagent (Sigma) according to the manufacturer's recommendation and 500 ng was used to
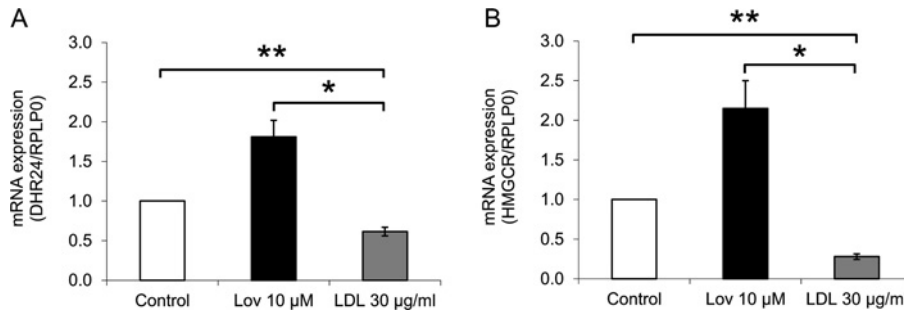
Figure 1   Regulation of HMGCR and DHCR24 mRNA expression by cholesterol availability
HepG2 cells were incubated with lovastatin (Lov, 10 μM) or LDL (30 μg of cholesterol/ml) in a lipoprotein deficient medium. Samples were harvested after 24 h and the expression of DHCR24 (**A**) and HMGCR (**B**) was measured by real-time RT–PCR using the relative quantification method by comparison with the expression of the housekeeping gene RPLP0 [30]. The results are expressed as the relative amount of mRNA compared with the level under control conditions. Results are the means$\pm$S.E.M. for three independent experiments. *$P < 0.05$ and **$P < 0.01$ by the paired Student's t test.

generate cDNA by reverse transcription using a Prime Script RT reagent kit (Takara Bio Inc.). RT–PCR amplification was performed using the SYBR Green I Master kit and LightCycler 480 II (Roche Applied Science). The initial denaturation step was at 95°C for 5 min, followed by 45 cycles of amplification at 95°C for 10 s, 60°C for 15 s and 72°C for 15 s. The melting curves were evaluated and the PCR products were separated on a 2% agarose gel and stained with ethidium bromide to confirm the presence of a single product. The efficiency of the reaction was evaluated by amplifying serial dilutions of cDNA (1:10, 1:100, 1:1000 and 1:10000 dilution). We ensured that the relationship between the C_t value and the log(RNA) was linear ($-3.6 <$ slope < 3.2). All analyses were performed in triplicate, and the relative amounts of target genes were normalized against the expression of the housekeeping gene RPLP0 (encoding ribosomal protein large P0) according to the ΔC_t method [30]. The primers used in the RT–PCR were: DHCR24 sense 5′-GCCGCTCTCGCTTATCTTCG-3′, DHCR24 antisense 5′-GTCTTGCTACCCTGCTCCTT-3′, HMGCR [HMG (3-hydroxy-3-methylglutaryl)-CoA reductase] sense 5′-GGACCCCTTTGCTTAGATGAAA-3′, HMGCR antisense 5′-CCACCAAGACCTATTGCTCTG-3′, RPLP0 sense 5′-CC-TCATATCCGGGGGAATGTG-3, and RPLP0 antisense 5′-GC-AGCAGCTGGCACCTTATTG-3.

Computational analysis
DHCR24 promoter analysis of potential transcription-factor-binding sites was performed with the 'Transcription Element Search System' from the University of Pennsylvania (TESS, http://www.cbil.upenn.edu/tess) [31].

Statistical analyses
Statistical comparisons were performed by paired Student's t test or two-way ANOVA using SigmaStat, version 2.3 (Jandel Corporation). Statistical significance was set at $P < 0.05$.
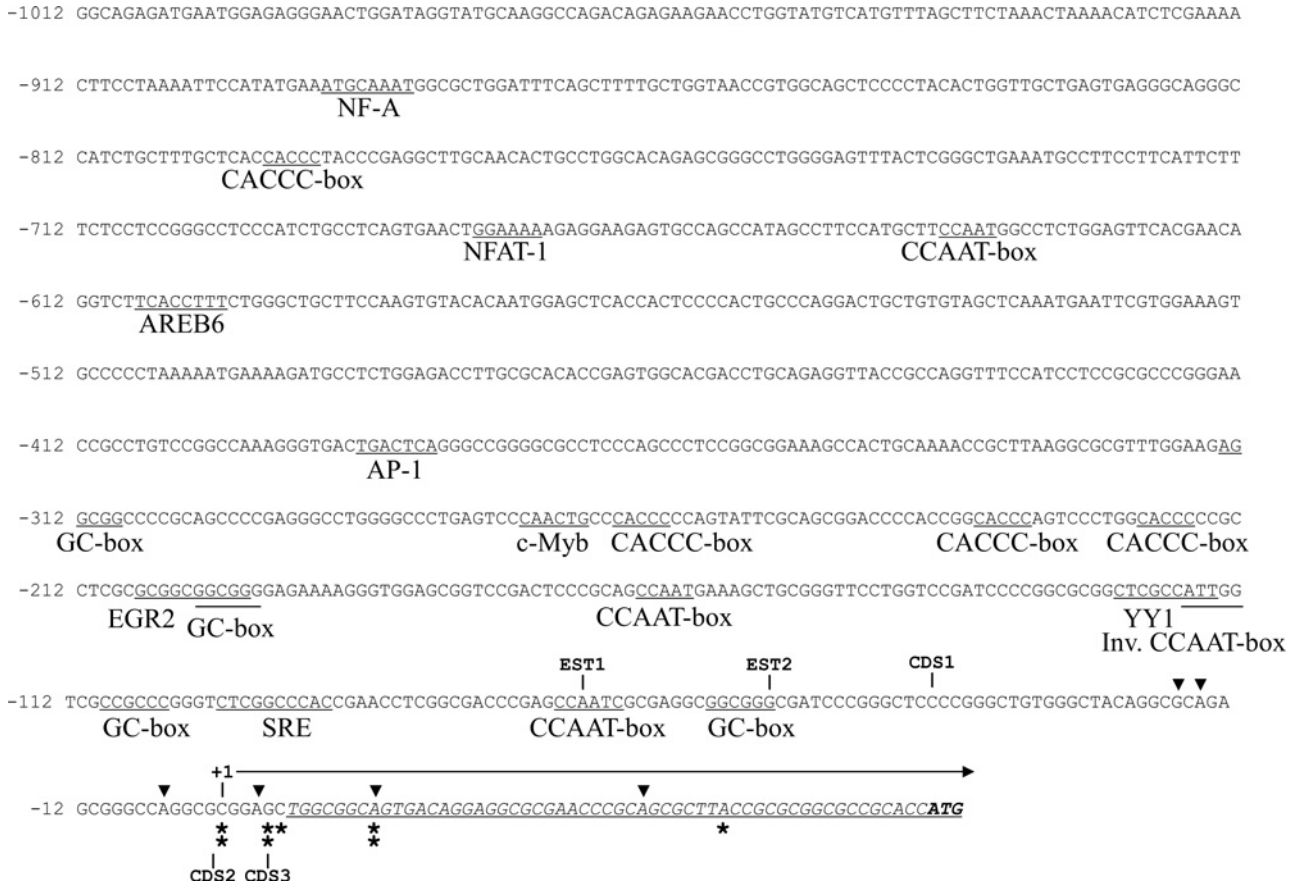
RESULTS

DHCR24 gene expression regulation by cholesterol availability
To determine whether the human DHCR24 gene is regulated by cholesterol availability, RNA was isolated from HepG2 cells incubated in the absence or presence of LDL, as a source of cholesterol, or lovastatin, an inhibitor of HMGCR, the rate-limiting enzyme in the cholesterol biosynthesis pathway. RT–PCR analysis showed that DHCR24 mRNA levels increased 1.8 times in cells incubated in the presence of lovastatin and decreased 1.6 times in the presence of LDL as compared with the control (Figure 1A). Similar results were obtained for HMGCR, a well-characterized cholesterol-regulated gene (Figure 1B). These results suggest that, similarly to HMGCR, the transcription of DHCR24 is activated by cholesterol depletion through a SREBP-dependent mechanism.

Structure of the proximal DHCR24 promoter
We located the DHCR24 promoter in HepG2 cells by 5′ RACE PCR. Sequencing of nine RACE PCR clones revealed five potential TSSs (transcription start sites) upstream (C^{-60}, G^{-56}, C^{-55}, A^{-47} and A^{-18}) (Figure 2) and one downstream (T^{+38}, not shown) of the ATG codon. The C^{-60} TSS was chosen as the $+1$ position for numbering the human DHCR24 promoter. Analysis of full-length cDNA entries in the GenBank® database and the Database of Transcriptional Start Sites (DBTSS; http://dbtss.hgc.jp/) revealed multiple other proximal start sites in the same region (Figure 2). There was no apparent TATA box, which is consistent with the presence of multiple TSSs, as occurs in TATA-less genes. Interestingly, there was a consensus CCAAT-box 65 bp upstream of the TSS as located by RACE PCR (Figure 2). Such a sequence is responsible for positive transcriptional control of many genes transcribed by RNA polymerase II and is often localized in this region. The proximal promoter region (-50 bp to -300 bp) has a G+C content of 75.64% (Supplementary Figure S1

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-1012  GGCAGAGATGAATGGAGAGGGAACTGGATAGGTATGCAAGGCCAGACAGAGAAGAACCTGGTATGTCATGTTTAGCTTCTAAACTAAAACATCTCGAAAA

 -912  CTTCCTAAAATTCCATATGAAATGCAAATGGCGCTGGATTTCAGCTTTTGCTGGTAACCGTGGCAGCTCCCCTACACTGGTTGCTGAGTGAGGGCAGGGC
                           NF-A

 -812  CATCTGCTTTGCTCACCACCCTACCCGAGGCTTGCAACACTGCCTGGCACAGAGCGGGCCTGGGGAGTTTACTCGGGCTGAAATGCCTTCCTTCATTCTT
                      CACCC-box

 -712  TCTCCTCCGGGCCTCCCATCTGCCTCAGTGAACTGGAAAAGAGGAAGAGTGCCAGCCATAGCCTTCCATGCTTCCAATGGCCTCTGGAGTTCACGAACA
                              NFAT-1                                           CCAAT-box

 -612  GGTCTTCACCTTTCTGGGCTGCTTCCAAGTGTACACAATGGAGCTCACCACTCCCCACTGCCCAGGACTGCTGTGTAGCTCAAATGAATTCGTGGAAAGT
             AREB6

 -512  GCCCCCTAAAAATGAAAAGATGCCTCTGGAGACCTTGCGCACACCGAGTGGCACGACCTGCAGAGGTTACCGCCAGGTTTCCATCCTCCGCGCCCGGGAA

 -412  CCGCCTGTCCGGCCAAAGGGTGACTGACTCAGGGCCGGGGCGCCTCCCAGCCCTCCGGCGGAAAGCCACTGCAAAACCGCTTAAGGCGCGTTTGGAAGAG
                           AP-1

 -312  GCGGCCCCGCAGCCCCGAGGGCCTGGGGCCCTGAGTCCCAACTGCCCACCCCCAGTATTCGCAGCGGACCCCACCGGCACCCAGTCCCTGGCACCCCCGC
        GC-box                              c-Myb  CACCC-box                CACCC-box   CACCC-box

 -212  CTCGCGCGGCGGCGGGGGAGAAAAGGGTGGAGCGGTCCGACTCCCGCAGCCAATGAAAGCTGCGGGTTCCTGGTCCGATCCCCGGCGCGGCTCGCCATTGG
        EGR2 GC-box                                      CCAAT-box                              YY1
                                                                                            Inv. CCAAT-box

                                    EST1              EST2          CDS1
                                     |                 |             |
 -112  TCGCCGCCCGGGTCTCGGCCCACCGAACCTCGGCGACCCGAGCCAATCGCGAGGCGGCGGGCGATCCCGGGCTCCCCGGGCTGTGGGCTACAGGCGCAGA
        GC-box       SRE                           CCAAT-box   GC-box                                    ▼ ▼

            +1
             |
  -12  GCGGGCCAGGCGCGGAGCTGGCGGCAGTGACAGGAGGCGCGAACCCGCAGCGCTTACCGCGCGGCGCCGCACCATG
           ▼    |  ▼        ▼                                   ▼
                *  **        *                                  *
                *  *         *
                |  |         |
               CDS2 CDS3
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Figure 2   DNA sequence of the 5′-flanking region of the human DHCR24 gene

The sequence shown contains 1012 nucleotides 5′ to the TSS of the longest mRNA transcript found by RACE PCR (C+1) plus 60 nucleotides 5′ to the ATG initiation codon. The potential TSS found by RACE PCR upstream of the ATG initiation codon are marked by asterisks (*); ▼ represents the major TSS found in the DBTSS. EST1 corresponds to dbEST Id:25435736 and EST2 corresponds to dbEST Id: 12525335. CDS1 corresponds to GenBank® accession number AF261758, CDS2 corresponds to GenBank® accession number BC011669, and CDS3 corresponds to GenBank® accession number AB073391. Putative transcription-factor-binding sites are underlined and identified below the sequence.

at http:www.bioscirep.org/bsr/033/bsr033e006add.htm). Therefore, DHCR24 gene transcription appears to be driven by a TATA-less GC-rich promoter.

Approximately 1 kb of the 5′-flanking region containing the promoter of DHCR24 was analysed for potential transcription-factor-binding sites using the TESS software. This analysis revealed the presence of several binding sites, but did not show any SRE (Figure 2). The transcription-factor-binding sites predicted with high score were: four GC-boxes ($-57/-52$, $-109/-104$, $-202/-197$ and $-314/-309$), three direct CCAAT-boxes ($-70/-65$, $-166/-160$ and $-638/-634$), one inverted CCAAT-box ($-118/-114$), four CACCC-boxes ($-221/-217$, $-235/-231$, $-266/-262$ and $-797/-792$) and one binding site for the following transcription factors: YY1 ($-123/-115$), EGR2 (early growth-response 2) ($-207/-198$), c-Myb ($-274/-269$), AP-1 (activator protein 1) ($-388/-382$), AREB6 ($-607/-600$), NFAT-1 (nuclear factor of activated T-cells 1) ($-678/-673$) and NF-A ($-891/-884$).

Despite the fact that TESS did not predict any SRE, visual inspection of the DHCR24 promoter sequence reveals a putative SRE at $-98/-90$ (Figure 2). Flanking this element TESS predicted two CCAAT-boxes and two GC-boxes, which are potential binding sites for NF-Y and Sp1 respectively, and the YY1 binding site overlapping the inverted CCAAT-box (Figure 2), all of which are commonly found in SRE-responsive genes. This region is highly conserved between human and mouse, with a 90% of sequence identity between both species (Supplementary Figure S1).

DHCR24 promoter activity assay

To identify the transcriptional regulatory regions in the human DHCR24 promoter, including the region involved in the response to cholesterol availability, a luciferase reporter driven by the DHCR24 promoter was cloned and serial deletion constructs of the 5′-flanking region of the promoter were prepared (Figure 3). We analysed their responsiveness to cholesterol addition, in the form of LDL, and cholesterol depletion by lovastatin in HepG2 and SK-N-MC cells.
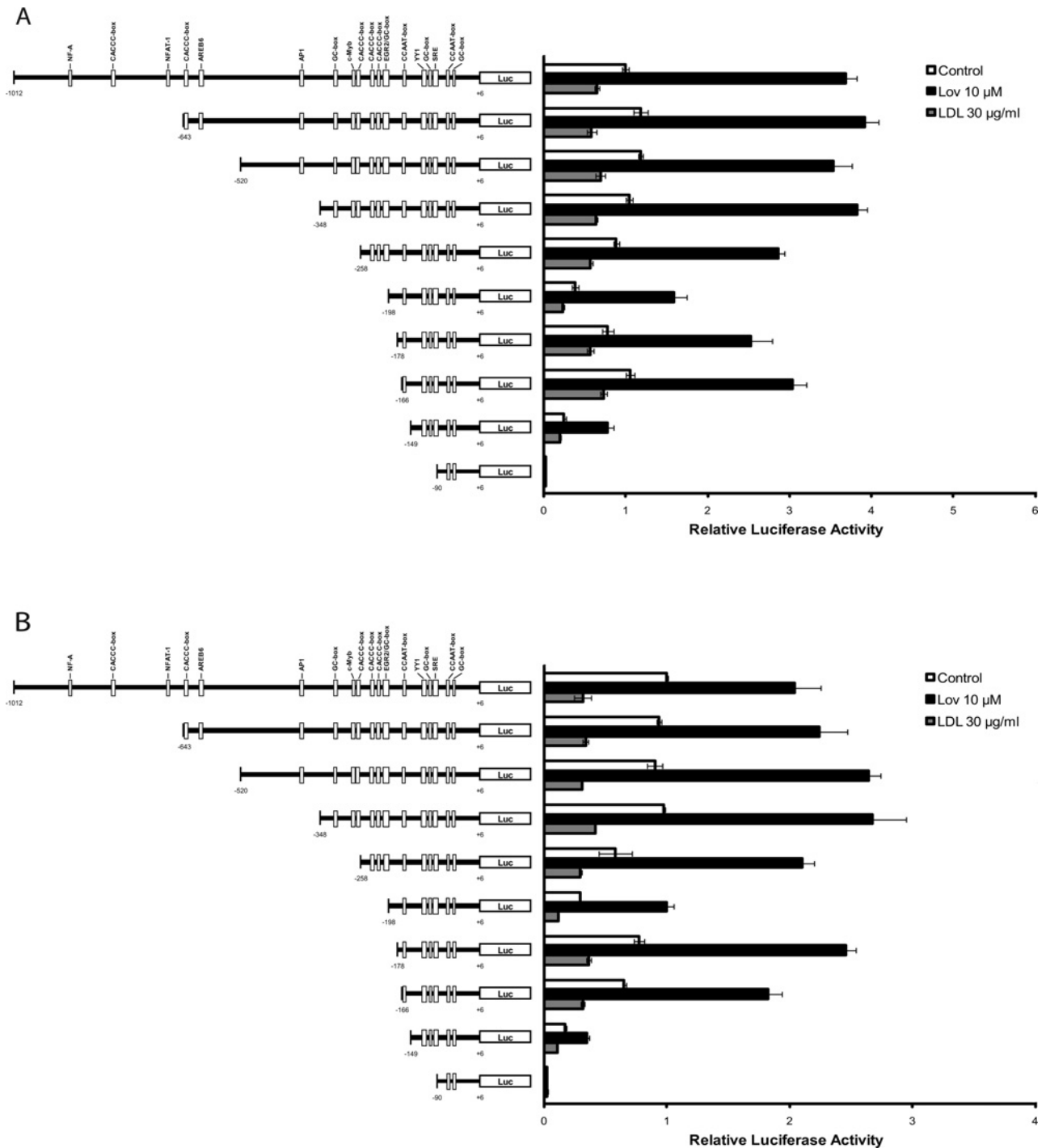
Figure 3   Effect of cholesterol availability on the transcriptional activity of the human DHCR24 proximal promoter
A fragment of 1018 bp of the 5′ flanking region containing the promoter of DHCR24 was cloned into the pGL3-basic vector as described in the Experimental section. Unidirectional serial deletions of the pH DHCR24 construct were generated and various reporter gene plasmids were transfected into HepG2 (**A**) or SK-N-MC (**B**) cells. Plasmid constructs containing different sequential portions of the DHCR24 promoter are schematized on the left and their corresponding luciferase activities are shown in the histograms on the right. The transcription factor-binding sites are identified. Cholesterol availability was modified by the incubation of cells with lovastatin (Lov, 10 μM) or LDL (30 μg of cholesterol/ml) for 24 h. Transcriptional activation was measured by the dual-luciferase reporter assay. Bars indicate the relative firefly luciferase activity normalized to *Renilla* luciferase activity used as the internal control of transfection efficiency. Results are the means$\pm$S.E.M. for three independent experiments.

Human DHCR24 promoter $-1012/+6$ reporter activity in HepG2 cells increased 3.7 times upon cholesterol depletion and decreased 1.5 times upon LDL addition (Figure 3A). Deletion of regions spanning nucleotides -1012 to -348 caused no significant change in either basal promoter activity or the response to lovastatin or LDL. The next two deletions (constructs $-258/+6$ and $-198/+6$) exhibited a progressive decrease in the promoter basal activity, but conserved the cholesterol-mediated regulation. This suggests the loss of some positive *cis*-acting sequences in the region between positions -348 and -198, which are important for basal transcription. Three CACCC-boxes, two GC-boxes, one c-Myb site and one EGR2 site were predicted in this region. Constructs $-178/+6$ and $-166/+6$ exhibited a slight increase in basal activity, suggesting the presence of a *cis*-acting element that could act as a silencing element. Deletion of the nucleotides between positions -166 and -149 caused a 4.3-fold reduction in the promoter basal activity, with no alteration of the ability to respond to cholesterol availability. This sharp reduction in basal DHCR24 promoter activity could be due to the loss of a CCAAT-box in this region. The last deletion (up to -90) completely abrogated both the basal and the cholesterol-regulated activity of DHCR24. Similar results were obtained in SK-N-MC cells (Figure 3B).

These results suggest that the elements between -166 bp and -90 bp are crucial for basal activity of the DHCR24 promoter and, notably, for the transcriptional regulation by cholesterol. In this region the presence of one YY1 ($-123/-115$), one inverted CCAAT-box ($-118/-114$), one GC-box ($-109/-104$) and one SRE ($-98/-90$) *cis*-acting elements was predicted.

Mutational analysis of the DHCR24 SRE

To further characterize the function of the putative SRE in the DHCR24 promoter, the *cis*-acting sequence TCGGCCCAC of the SRE ($-98/-90$) of the luciferase reporter DHCR24 promoter was mutated to CCGGCCGGC (Figure 4A). HepG2 cells were transiently transfected with the wild-type promoter–reporter construct (pH DHCR24), the mutant promoter–reporter construct (mut SRE) or the empty vector (pGL3) and the response to the addition of LDL or lovastatin was analysed. As shown in Figure 4(B), mutation of the SRE decreased the basal activity of the DHCR24 promoter and abrogated the cholesterol-mediated regulation. These results indicate that the SRE identified in the present study is responsible for the cholesterol-mediated response of the DHCR24 promoter and that it also plays an essential role in the promoter basal activity.

Interaction of SREBP with the putative SRE in the DHCR24 promoter

Next, we performed an EMSA to determine whether the putative SRE present in the DHCR24 promoter has the ability to bind mature SREBP. As a control we used a probe containing the SRE present in the LDLR promoter. As shown in Figure 5, mature SREBP-1a formed a complex with a 27-mer double-stranded DNA probe containing the putative SRE of the DHCR24 promoter (-107 bp to -81 bp). SREBP-1a bound
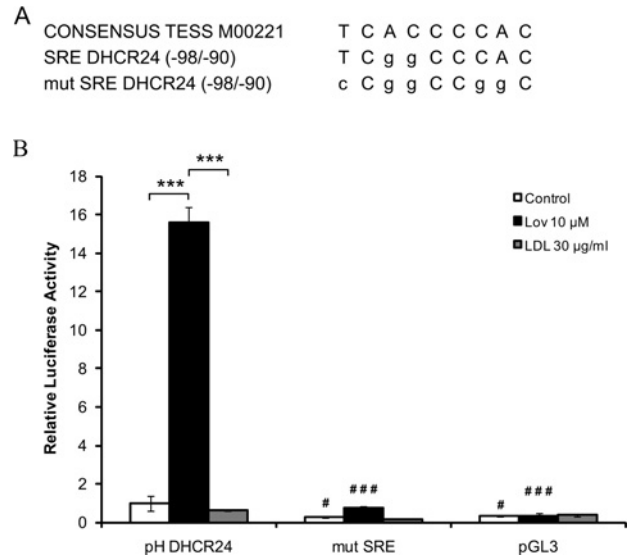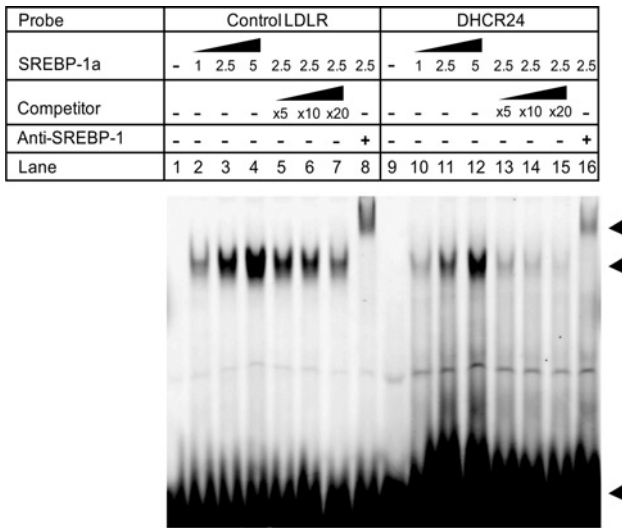
Figure 4 Mutational analysis of the putative SRE in the DHCR24 promoter

(**A**) Sequence of the consensus SRE, SRE in the DHCR24 promoter and in the mutated DHCR24 promoter. (**B**) Transcriptional activity assay of the wild-type and mutated SRE in the DHCR24 promoter. The wild-type or the SRE-mutated vectors were transfected into HepG2 cells. Cholesterol availability was modified by the incubation of cells with lovastatin (Lov, 10 μM) or LDL (30 μg of cholesterol/ml) for 24 h, and transcriptional activation was measured by the dual-luciferase reporter assay. Bars indicate the relative firefly luciferase activity normalized to *Renilla* luciferase activity used as an internal control of transfection efficiency. Results are the means$\pm$S.E.M. for three independent experiments. ***$P < 0.001$ against different treatment within the same plasmid; #$P < 0.05$ and ###$P < 0.001$ against pH DHCR24 within the same treatment by two-way ANOVA.

in a dose-dependent manner to the DHCR24 probe (Figure 5, lanes 10–12) and the complexes formed were approximately the same size as those formed with the LDLR probe (Figure 5, lanes 2–4). Unlabelled DHCR24 probe effectively competed in a dose-dependent manner with the labelled DHCR24 probe (Figure 5, lanes 13–15), as also observed in competition studies between the unlabelled and labelled LDLR probe (Figure 5, lanes 5–7). The addition of an anti-SREBP-1 antibody resulted in a supershifted band with both the LDLR and the DHCR24 probe, consistent with the binding of SREBP to these probes (Figure 5, lanes 8 and 16). Together, these findings indicate that SREBP-1a is able to bind to the putative SRE in the DHCR24 promoter.

Finally, to verify the functionality of the SRE *in vivo* and to determine whether the binding of SREBP-2 to the DHCR24 promoter could be modified by cholesterol availability we performed a ChIP assay. As shown in Figure 6, SREBP-2 effectively bound to the DHCR24 promoter in cells treated with lovastatin and the binding decreased in the presence of LDL. Similar results were obtained when the SREBP binding to the LDLR promoter was analysed. These results indicate that the putative SRE present in the DHCR24 promoter is a functional SREBP-binding site and that cholesterol regulates DHCR24 expression through this family of transcription factors *in vivo*.

Probe	Control LDLR								DHCR24							
SREBP-1a	–	1	2.5	5	2.5	2.5	2.5	2.5	–	1	2.5	5	2.5	2.5	2.5	2.5
Competitor	–	–	–	–	x5	x10	x20	–	–	–	–	–	x5	x10	x20	–
Anti-SREBP-1	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+
Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

Figure 5 SREBP binding to the SRE of the DHCR24 promoter
A double-stranded DNA probe corresponding to the wild-type SRE in the DHCR24 (right) and in the LDLR (left) promoter were end-labelled with Cy5 and used in a mobility-shift experiment as described in the Experimental section. An increasing amount (1, 2.5 or 5 μg) of purified FLAG-tagged SREBP-1a was incubated with the labelled SRE probes (lanes 2–4 and 10–12 for the LDLR and DHCR24 probes, respectively). The unlabelled SRE probes for both LDLR and DHCR24 were used as competitors and were added at 5-, 10- and 20-fold molar excess of the corresponding labelled probe (lanes 5–7 and 13–15, respectively). A reticulocyte lysate without SREBP-1a-pcDNA3.1 FLAG was used as a negative control (lanes 1 and 9). The specificity of the SREBP–SRE binding was assessed by the incubation with an anti-SREBP antibody (lanes 8 and 16 for the LDLR and DHCR24 probes, respectively). The top arrowhead indicates the super-shifted band corresponding to the anti-SREBP antibody–SREBP-1a–SRE probe complex. The middle arrow indicates the SREBP1-a–SRE complex and the bottom arrowhead indicates the free probes.

Androgen regulation of DHCR24 transcription and co-operation between SREBP and KLF5

It has been previously shown that testosterone regulates DHCR24 gene expression, probably through an ARE (androgen response element) present in the distal promoter region [14]. Also, it has been reported that androgens regulate the expression of genes involved in cholesterol and fatty acid biosynthesis through a co-ordinated indirect mechanism that involves the transcription factors SREBP and KLF5 [32–34]. Given that the DHCR24 proximal promoter contained multiple CACCC-boxes (Figure 2), which can bind KLF5 [35], we next investigated the contribution of such elements to the androgen-mediated regulation of DHCR24 expression.

First, to confirm that androgens regulate DHCR24 expression, we treated LNCaP cells, an androgen-sensitive human prostate adenocarcinoma cell line, with different concentrations of DHT and analysed DHCR24 expression by real-time PCR. As shown in Figure 7(A), DHT produced a dose-dependent increase in DHCR24 mRNA levels. Interestingly, when 1 nM DHT was combined with 10 μM lovastatin a synergistic effect on DHCR24 mRNA levels was observed (figure 7A). To ascertain whether the SRE present in the DHCR24 promoter was involved in the regulation by androgens, we performed transient transfection as-
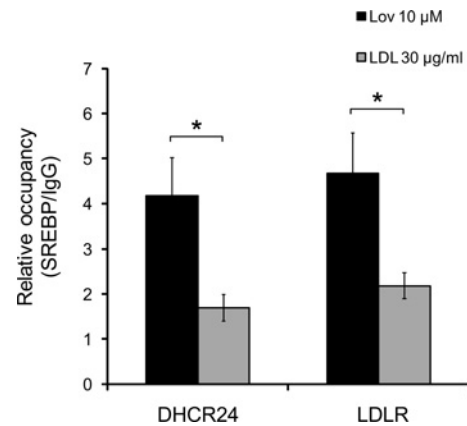
Figure 6 SREBP binding to the SRE in the DHCR24 promoter and response to cholesterol availability
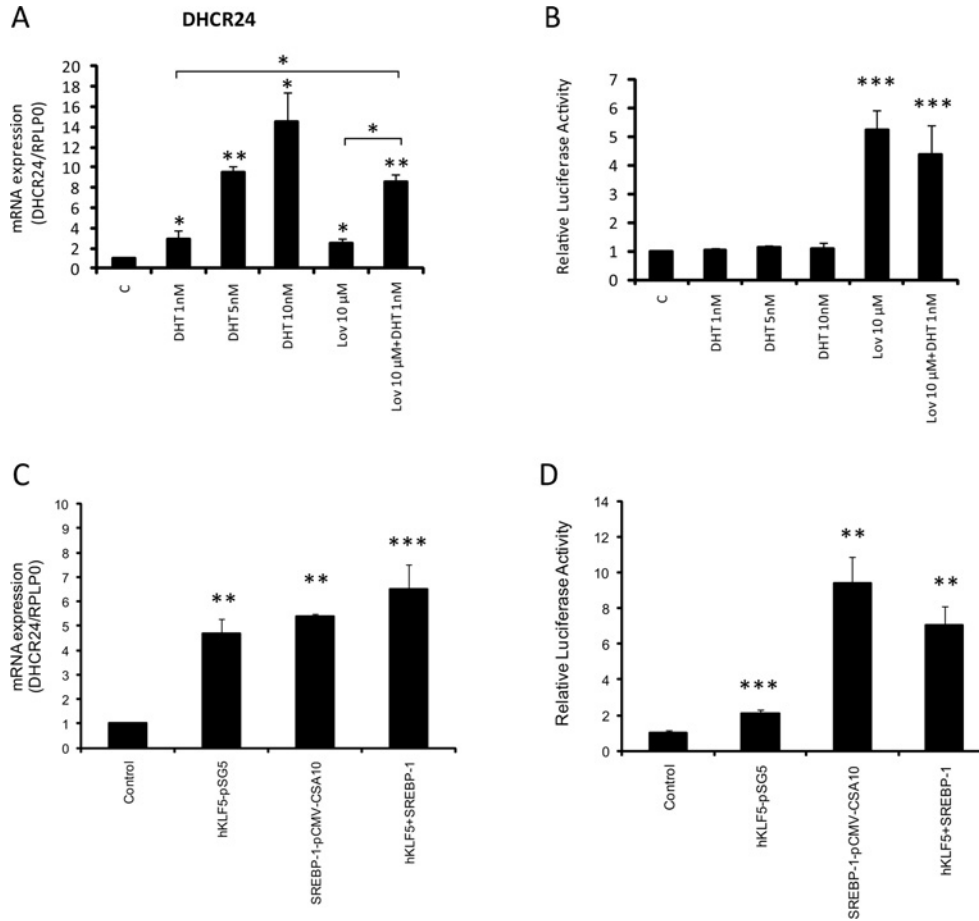HepG2 cells incubated with lovastatin (Lov, 10 μM) or LDL (30 μg of cholesterol/ml) were subjected to ChIP using an antibody against SREBP-2 or IgG as a negative control. Co-precipitated DNA was amplified by PCR using primers flanking the SRE-binding site in the DHCR24 promoter as described in the Experimental section. The specific amplification of the immunoprecipitated SRE of the LDLR promoter was used as a positive control. Results are the means±S.E.M. for four independent experiments. *$P < 0.05$ against the control condition by the paired Student's t test.

says using the reporter construct of the DHCR24 promoter. As shown in Figure 7(B), none of the DHT doses used had an effect on DHCR24 promoter activity, nor did 1 nM DHT alter the stimulatory effect of lovastatin. This indicates that the element mediating the regulation of DHCR24 expression by androgens is not located in the proximal promoter region of this gene.

Finally, we questioned whether KLF5 is able to regulate DHCR24 promoter activity. To investigate this, we overexpressed KLF5 alone or in combination with SREBP-1a in LNCaP cells and measured the DHCR24 mRNA levels and promoter activity. When KLF5 and SREBP-1a were overexpressed separately there was a similar increase in DHCR24 mRNA levels (Figure 7C). However, the simultaneous overexpression of these transcription factors did not significantly increase DHCR24 mRNA levels above those observed overexpressing KLF5 and SREBP-1a separately (Figure 7C). On the other hand, the overexpression of any one of these transcription factors stimulated DHCR24 promoter activity, although SREBP-1a overexpression had a much greater effect than that of KLF5. The overexpression of both transcription factors together did not increase promoter activity above the levels reached by overexpressing SREBP-1a alone (Figure 7D). These results suggest that the DHCR24 promoter responds to KLF5, but there is not a synergistic effect between this factor and SREBP-1a.

DISCUSSION

Cholesterol biosynthesis is a highly regulated process, the main regulatory mechanism involving the SREBP family of

Figure 7   Effect of DHT and KLF5 on DHCR24 expression
LNCaP cells were incubated with 1, 5 or 10 nM DHT in the presence or the absence of lovastatin (Lov, 10 μM) for 24 h and, subsequently, the mRNA level (**A**) and the promoter activity (**B**) of DHCR24 were measured by RT–PCR and the dual-luciferase reporter assay, respectively. LNCaP cells were transfected with hKLF5–pSG5, SREBP1–pCDNA3.1 FLAG or both and, subsequently, the mRNA level (**C**) and the promoter activity (**D**) of DHCR24 were measured by RT–PCR and the dual-luciferase reporter assay, respectively. Results are the means$\pm$S.E.M. of three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ against the control condition by the paired Student's t test.

transcription factors, whose activity is modulated by cholesterol availability. DHCR24 catalyses the reduction of the C-24 double bond of sterol intermediates in cholesterol biosynthesis. This enzyme generated great interest owing to the down-regulation of DHCR24 in regions of the brain affected by AD [7]. DHCR24 also plays an important role in cellular responses to oncogenic and oxidative stress and is a key regulator of Ras-induced senescence [8,9]. On the other hand, DHCR24 is involved in lipid raft formation [16] and HCV replication [17]. In addition to some studies indicating that DHCR24 is under the transcriptional control of SREBP [22,23], a recent study [23a] has confirmed that SREBP-2 regulates DHCR24, and has identified two functional SREs and two NF-Y sites in the DHCR24 proximal promoter. The present study investigated in detail the regulation of the DHCR24 proximal promoter by cholesterol availability through SREBPs.

Our analysis revealed the presence of an SRE motif in the DHCR24 promoter located at position $-98/-90$ and which is similar to the SRE consensus sequence in the LDLR pro-

moter (TCACCCCAC, TRANSFAC Matrix Record M00221) and differs from this in two nucleotides (TCGGCCCAC). Mutational analysis revealed the importance of this SRE in both the sterol-regulated and the basal activity of the DHCR24 promoter. Moreover, we demonstrated that this site mediates the binding of SREBP in response to cholesterol availability. Reed et al. [23], studying the genome-wide promoter occupancy of SREBP-1 in HepG2 cells, found DHCR24 among the 1141 potential SREBP-1-targeted genes. They also identified an E-box (CAN-NTG, TRANSFAC Matrix Record M00220) as the most common SREBP-1 DNA-binding motif in SREBP-1 target promoters. In the present study, we identified a functional SRE motif in the DHCR24 promoter.

We also addressed the potential TSS in the DHCR24 promoter in HepG2 cells by RACE PCR, and found multiple TSSs in the region between 18 and 128 bp upstream from the ATG codon, which is in agreement with the previously published bioinformatics analysis of TSSs reported in the DBTSS [27]. This is a

common feature in TATA-less promoters, which exhibit closely arranged multiple TSSs distributed along a broad region that can extend 100 bp or more [36]. Also frequent in TATA-less promoters is that they are associated with CpG islands, which contain multiple binding sites for the transcription factor Sp1. It has been suggested that Sp1 sites direct the basal transcriptional machinery and are often located 40–80 bp upstream from the TSS [36]. In agreement with previous work [24,27], we described the presence of four GC-boxes in the proximal region of the DHCR24 promoter, two of them located at the appropriate distance of the TSS (− 57/ − 52 and − 109/ − 104) and are conserved in human and mouse (Figure 1 and Supplementary Figure S1).

The DHCR24 promoter has several other features in common with the promoters of other SREBP target genes. SREBPs are known to be weak transcriptional activators and require the presence of additional transcription factors like Sp1 and NF-Y to elicit maximal activation [2,37,38]. Consistently with this, aside from the two Sp1-binding GC-boxes mentioned above, we found two NF-Y-binding CCAAT-boxes in the vicinity of the SREBP-binding site of the DHCR24 core promoter.

The promoter of several SREBP target genes also contains a YY1-binding site, which is either overlapping or adjacent to binding sites for NF-Y, Sp1 or SREBP [4]. YY1 is a multi-functional transcription factor that may operate as both a positive or a negative regulator of gene expression as well as an initiator-binding protein, interacting directly with both TFIIB (transcription factor IIB) and RNA polymerase II in the absence of the TBP (TATA-box-binding protein) [39]. It has been proposed that YY1 is a negative regulator of transcription of at least three SREBP-responding genes: LDLR, farnesyl diphosphate synthetase and HMGCS (HMG-CoA synthetase) [4]. The presence in the HMGCS promoter of one YY1 element overlapping an inverted CCAAT-box (ATTGG) may explain the negative effect of YY1 by competing with NF-Y for the same binding site [4]. In the proximal promoter region of DHCR24 we found one YY1 element overlapping an inverted CCAAT-box similarly to what was found in the HMGCS promoter. Thus, YY1 may have a similar negative effect on DHCR24 promoter activity, an issue that needs verification.

Previous reports have demonstrated that several enzymes involved in cholesterol and fatty acid biosynthesis are up-regulated in prostate cancer cells by androgens [40]. It has been proposed that androgens regulate the expression of many of these genes through an indirect mechanism that involves an increase of SREBP expression and the level of the nuclear active form of this transcription factor [32,33]. In contrast with other enzymes involved in lipid metabolism, DHCR24 seems to be a direct target of the androgen receptor through an ARE found in the distal promoter region [14]. In the present study, we found that the proximal promoter of DHCR24 was unable to respond to DHT and, consequently, the ARE contained in the distal region appears to be essential for the androgen response of this gene. Interestingly, we found a synergistic effect between DHT and the cholesterol-synthesis inhibitor lovastatin on DHCR24 expression, which suggests co-operation between DHT and SREBP in the regulation of DHCR24.

It has been described that KLF5 physically interacts with SREBP to mediate the androgen-dependent activation of FASN (fatty acid synthase) in LNCaP prostate cancer cells [34]. KLF5 exerts this effect through its binding to multiple CACCC-boxes present in the FASN gene, but which are absent in other lipogenic and cholesterogenic genes that are unresponsive to KLF5 [34]. In the present study we described four CACCC-boxes in the proximal promoter of DHCR24 and the ability of KLF5 to stimulate mRNA expression and promoter activity of DHCR24. However, in contrast with what occurs with FASN expression, KLF5 appears not to be involved in the androgen-induced stimulation of DHCR24 expression. Consequently, we hypothesize that the synergism between androgens and SREBP is due to an interaction between the ARE previously described in the distal region of the promoter [14] and the SRE currently identified in the proximal promoter of DHCR24.

DHCR24 expression has been reported to be altered in different tumours, such as adrenal gland, ovary, testis and prostate tumours [10–15,41]. The increase in DHCR24 expression by KLF5 may have a role in these alterations, since this transcription factor regulates proliferation, cell cycle, survival, migration, angiogenesis, apoptosis, differentiation and stemness [42].

Another important region for DHCR24 promoter activity spans nucleotides − 348 to − 198, since its deletion markedly reduced the basal transcriptional activity, without affecting the cholesterol-mediated regulation. In this region, three CACCC-boxes, two GC-boxes and one c-Myb and one EGR2 response element were predicted. Among these sites, the EGR2 response element may be particularly relevant in the control of DHCR24 expression in the nervous system. EGR2 plays a critical role in Schwann cell development and myelination [43]. During myelination Schwann cells require a large amount of lipids and, thus, the expression of many genes involved in lipid biosynthesis is up-regulated, including the majority of the enzymes involved in cholesterol biosynthesis [44]. Interestingly, EGR2-null mice exhibit an important down-regulation of cholesterol biosynthetic genes, which is consistent with a direct regulation of this pathway by EGR2 [44]. Moreover, it has been suggested that EGR2 co-operates with SREBP and synergistically activates the promoter of several SREBP target genes such as HMGCR, CYP51 and SCD2 [45]. EGR2 has also been involved in tumour suppression, macrophage differentiation and bone formation [46–48]. Although the DHCR24 regulation by EGR2 needs to be verified, the presence of an EGR2-binding site in the DHCR24 promoter is consistent with the important role of DHCR24 and desmosterol in brain development and the severe malformations produced by DHCR24 deficiency [6,49].

Finally, in agreement with previous results published by Drzewinska et al. [27], we found a negative element in the DHCR24 promoter. In the present study, we localized more precisely this element between nucleotides − 198 and − 178, but we did not find any putative transcription-factor-binding site in this region that can explain the increment of the promoter activity when these 20 nucleotides were deleted.

In conclusion, in the present study we have identified a functional SRE in the core promoter region of the DHCR24 gene,

which mediates the regulation of DHCR24 expression in response to cholesterol availability. The characterization of the proximal promoter region of this gene revealed the presence of other putative binding sites that may be relevant for the regulation of DHCR24 by different factors. Among these, KLF5 is able to increase DHCR24 transcription through the activation of the proximal promoter region, a mechanism that is not involved in the androgen-induced up-regulation of DHCR24 expression.

AUTHOR CONTRIBUTION

Lidia Daimiel performed the experiments, analysed the data and wrote the paper. María Fernández-Suárez, Sara Rodríguez-Acebes and Lorena Crespo performed the experiments. Miguel Lasunción wrote the paper. Diego Gómez-Coronado conceived and designed the experiments and wrote the paper. Javier Martínez-Botas conceived and designed the experiments, analysed the data and wrote the paper.

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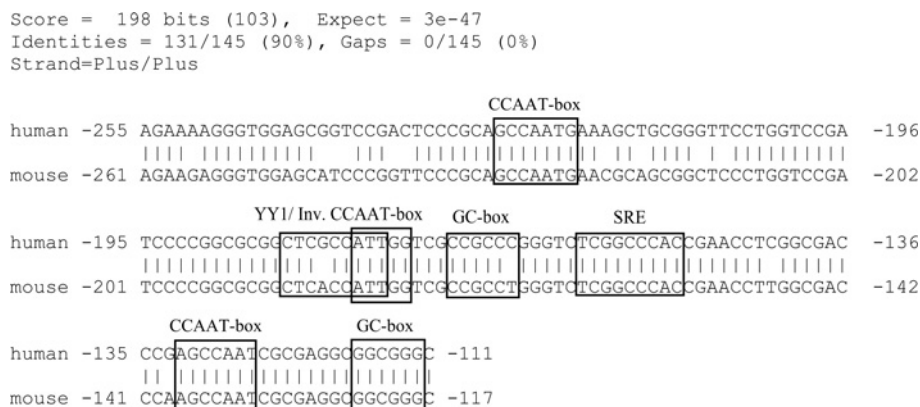
SUPPLEMENTARY DATA

Promoter analysis of the DHCR24 (3β-hydroxysterol Δ^{24}-reductase) gene: characterization of SREBP (sterol-regulatory-element-binding protein)-mediated activation

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Figure S1   Alignment of the conserved 5′-flanking regions of human and mouse DHCR24 gene
Human and mouse DHCR24 promoter sequences were obtained from GenBank®. Nucleotides are numbered from the TSS. The conserved transcription-factor-binding sites, including the SRE, are highlighted.

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