

Transcriptional regulation of human and murine 17 β -hydroxysteroid dehydrogenase type-7 confers its participation in cholesterol biosynthesis

Thomas Ohnesorg, Brigitte Keller¹, Martin Hrabé de Angelis¹ and Jerzy Adamski¹

School of Paediatrics and Reproductive Health, Discipline of Obstetrics and Gynaecology, Adelaide University, Adelaide 5005, South Australia, Australia

¹GSF-National Research Center for Environment and Health, Institute of Experimental Genetics, Ingolstaedter Landstraße 1, 85764 Neuherberg, Germany

(Requests for offprints should be addressed to J Adamski; Email: adamski@gsf.de)

Abstract

In both humans and mice, 17 β -hydroxysteroid dehydrogenase type-7 (HSD17B7) was described as possessing dual enzymatic functionality. The enzyme was first shown to be able to convert estrone to estradiol *in vitro*. Later involvement of this enzyme in postsqualene cholesterol biosynthesis was postulated (conversion of zymosterone to zymosterol) and could be proven *in vitro*. In this work, we performed a detailed analysis of the transcriptional regulation of both the human and murine genes. Despite relatively low sequence similarity, both promoters contain similar contexts of transcription factor-binding sites. The participation of these sites in transcriptional regulation of HSD17B7 was proven by electrophoretic mobility shift assay and site-directed mutagenesis of the corresponding binding sites. We describe novel involvement of vitamin D receptor/retinoid X receptor and provide new information on the regulation of HSD17B7 expression by sterol regulatory element-binding protein and hepatocyte nuclear factor 4, the latter known from other genes of cholesterologenic enzymes. The results of our study provide unequivocal evidence for a role of HSD17B7 in cholesterol biosynthesis.

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Introduction

An important group of steroid-converting enzymes, 17 β -hydroxysteroid dehydrogenases (HSDs), are known to use a variety of substances as substrates (Peltoketo *et al.* 1999, Luu-The 2001, Mindnich *et al.* 2004). A common feature of this group is the ability to interconvert keto- and hydroxy-groups on position C17 of the steroid backbone, thereby controlling the biological action of hormones.

One of these enzymes, the 17 β -HSD type-7 (gene *HSD17B7*), was first cloned from rat as a prolactin receptor-associated protein (Duan *et al.* 1996). Besides its ability to bind the short form of the prolactin receptor, the biological function was unclear. In 1998, the murine homolog was cloned and designated as HSD17B7 (Nokelainen *et al.* 1998). The enzyme was shown to convert estrone to biologically active estradiol with the highest expression levels detected in ovaries of pregnant mice. Consequently, an important function during pregnancy was postulated. With the identification of the human homolog shortly after, *in silico*

analysis showed high levels of expression in brain and fetal liver (Krazeisen *et al.* 1999).

By phylogenetic analysis, a significant similarity with yeast 3-keto sterol reductase (ERG27) was detected (Breitling *et al.* 2001). This protein catalyzes an important step in yeast ergosterol biosynthesis (conversion of zymosterone to zymosterol), the yeast equivalent of vertebrate cholesterol biosynthesis (Gachotte *et al.* 1999). It was thereafter shown that both the human and murine HSD17B7 are able to restore growth in an ERG27-deficient yeast strain (Marijanovic *et al.* 2003). Furthermore, co-expression of HSD17b7 with Hmgcr, the rate-limiting enzyme of cholesterol biosynthesis, and other cholesterologenic enzymes in the developing mouse embryo has been shown (Laubner *et al.* 2003). Highest expression levels were found in neuronal tissues and some apoptotic regions. Recently, it could be shown that overexpression of HSD17B7 reverts cholesterol auxotrophy of NS0 cells (Seth *et al.* 2005). Therefore, several lines of evidence point to a dual functionality of HSD17B7 (both human and murine), being involved in both steroidogenesis and cholesterol biosynthesis.

In order to gain further insight into its *in vivo* functionality and due to possible differences between the human and murine enzymes, we performed a detailed analysis of the transcriptional regulation of the human and murine homologs of HSD17B7. The complex and controversial data known so far prompted us to adopt this comparative and open approach.

Transcription of genes involved in steroidogenesis is in most described cases mediated by the transcription factors (TFs) of the steroidogenic factor 1 (SF1) family (SF1 and liver receptor homolog 1, LRH-1; for review, see Val *et al.* (2003)). Numerous studies have shown that specificity protein 1 transcription factor (SP1) and cAMP-response element-binding protein (CREB) also play important roles in the regulation of steroidogenic genes (Omura & Morohashi 1995, Piao *et al.* 1997, Guo *et al.* 2003).

Genes of cholesterol biosynthesis and homeostasis are mostly regulated by sterol regulatory element-binding protein 2 (SREBP-2), in many cases also by SREBP-1a (Horton & Shimomura 1999, Horton *et al.* 2003). Due to weak DNA binding of SREBP, transcriptional regulation by SREBP requires adjacent binding sites for accessory TFs, such as SP1 (ubiquitarily GC-box TF), NF-Y (ubiquitarily CAAT-box TF), or CREB (Sanchez *et al.* 1995, Dooley *et al.* 1998, Halder *et al.* 2002, Ngo *et al.* 2002).

To date, little is known about the transcriptional regulation of both the human and murine HSD17B7 genes. The first evidence for cholesterol-dependent regulation of the murine gene was reported by Horton *et al.* (2003). The study revealed differentially expressed genes in SREBP-1a and SREBP-2 transgenic and SREBP cleavage-activating protein knockout mice by microarray analysis. The authors showed that among many other genes, HSD17B7 displayed significantly higher expression levels in the transgenic mice compared with wild-type ones. In a recent study addressing transcriptional regulation of rat HSD17b7 in luteal cell line and in dependence on luteinizing hormone (LH), two binding sites for SP1 and one for NF-Y were identified (Risk *et al.* 2005).

In our preliminary studies, we revealed the existence of several adjacent transcription start sites and the absence of a conserved TATA box for both the human and murine genes. Bioinformatic analysis of the promoter regions showed significant similarity to other genes of cholesterol biosynthesis (Ohnesorg & Adamski 2005). In the present study, we proved the involvement of bioinformatically identified TFs and examined their influence on transcriptional regulation of human and murine HSD17B7 under basal and induced conditions. We now present data which expand the knowledge on both the number and significance of other TFs and provide novel and unequivocal evidence for *in vivo* activity of HSD17B7 and its role in cholesterol biosynthesis.

Materials and methods

Bioinformatic analysis

For bioinformatic analyses, the Genomatix software package (Genomatix Software, München, Germany) was used. Identification of TF-binding sites was performed by *MatInspector* for the identification of a conserved pattern of TF-binding sites across species the FrameWorker (genomatix software) software was used (Cartharius *et al.* 2005). Both analyses were conducted with the recommended default settings at high threshold values.

Cells and cell culture

HepG2, HEK-293, HeLa, and Hepa1-6 (all DSMZ, Braunschweig, Germany) cells were grown in Dulbecco's modified Eagle's medium containing 1 mM GlutaMax (Invitrogen), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen) and, as indicated, 10% fetal calf serum (Biochrom AG, Berlin, Germany), 10% delipidized fetal or 10% delipidized/steroid-free human serum with or without cholesterol (10 μ g/ml), and 25-hydroxycholesterol (1.5 μ g/ml) or estradiol (20 nM). Forskolin was used at 20 μ M with HepG2 and Hepa1-6. Cells were cultivated at 37 °C in a humidified atmosphere containing 5% CO₂.

Cloning of the HSD17B7 promoter fragments

All HSD17B7 promoter fragments were cloned into pGL3 Basic (Promega) using HindIII and XhoI restriction sites. For restriction and ligation, NEB (Frankfurt, Germany) enzymes were used according to the manufacturer's instructions. The truncated human and murine promoter constructs were created by PCR using primers shown in Table 1. For amplification, the *Pfu Turbo* polymerase (Stratagene, Amsterdam, The Netherlands) was used. All constructs were confirmed by sequencing.

Site-directed mutagenesis

Mutant constructs of TF-binding sites were generated using the QuikChange Site-Directed Mutagenesis (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's instructions. For mutagenesis, primers shown in Table 1 and their complementary oligonucleotides were used. All mutations were confirmed by sequencing.

Dual luciferase assays and transient transfection

For analyses of transcriptional activity the Dual Luciferase Assay (Promega) was used. The cells were

Table 1 Sequences of nucleotides for amplification of promoter constructs and expression analysis

	Sequence (5'–3')
Human promoter fragments	
– 1132 Forward	GATCCTCGAGAATACAAGGGCAATCACCCAG
– 687 Forward	GATCCTCGAGAGCCCATCAAGAATTTGCCTAGGC
– 329 Forward	CAGTCTCGAGCCCACCAGGATTAAGATCAGGC
– 202 Forward	GATCCTCGAGCTATCTCACCCGACGCCGTCTC
– 99 Forward	GATCCTCGAGGTACTCTGATTGGTGACGGGTGAGG
ATG reverse	GATCAAGCTTCTTCCAAGCAGTGAAGCAAACGC
Human promoter-binding site mutagenesis	
mutHNF4	CCCCACCAGGATTA <u>ACTACA</u> AGGCGCCCGCTGAGC
mutSP1 distal	CGTCACGCAGCTC <u>AGGAC</u> CTCGGCCTATCTCAC
mutVDR	CAGCTCCGCCCTC <u>AGTG</u> TATCTCACCCGACGCC
mutSREBP	CCTCGGCCTATCTC <u>GAACC</u> AGGCCGTCTCCG
mutNF-Y distal	GGTTGGCGGACTGAG <u>AGGAG</u> AGGGATCAGCTCAGAC
mutCREBP	GGATCAGCTCAGACTCG <u>AGT</u> ACGCCAACGGGAGGC
mutSP1 proximal	CGATGACGCCAACGGGAT <u>TCGGGG</u> CGTGCCGCTACTC
mutNF-Y proximal	GCCGACTCTGATT <u>ACT</u> GACGGGTGAGGCG
Murine-promoter fragments	
– 890 Forward	ATGCCTCGAGGGATCACGGCTATCCTAGTAATTACAGC
– 497 Forward	GAATTCTCGAGGTTCCCTGCACCGAAGCCTCTCC
– 295 Forward	CTCTCTCGAGCGCTTGCTTCACTCAGAACAAAGC
– 162 Forward	GATCCTCGAGAAGATGCCGAGTCGCGATTG
– 90 Forward	GAATCTCGAGCCTTGATTGGTCGAGGTGC
ATG reverse	GATCAAGCTTGTTCTCTTAGCCTGAACCTTGATCCG
Murine promoter-binding site mutagenesis	
mutHNF4	GCTTGCTTCACTCAGAACAA <u>CTCCG</u> CCGCCGAGACTGG
mutSP1	CTTGTCACGTGGCTC <u>AGGAC</u> CTCGGCCTATCTCAC
mutVDR	GCCCCTCGGT <u>TG</u> TATCTC <u>ACAT</u> CACCCGGTCTGG
mutSREBP	CCCCTCGGCCTATCT <u>TGCCT</u> ATCCCGGTCTGGG
mutNF-Y distal	GATGCCGAGTCGCG <u>AGGAG</u> CGGGCCTAACCGC
mutCREBP	GCGCGACCCG <u>ACCAC</u> GCAGGAGAGG
mutNF-Y proximal	GGCCGAGCCTTG <u>AATG</u> ATCGAGGTGCGCG
Expression analysis of HSD17b7	
5' Primer	CCACCTGTGTTTGGCGTGATGG
3' Primer	CGCTCGCATATTTGGTCAGAGG
Expression analysis of murine β -actin	
5' Primer	GGGTCAGAAGGACTCCTATGTGGG
3' Primer	CGATCCACACAGAGTACTTGCGC
Expression analysis of HSD17B7	
5' Primer	GCTAGCAGTGGCATTGGCCTGG
3' Primer	GCATGAGCCACTGAGCCTGGC
Expression analysis of human β -actin	
5' Primer	GGATTCCTATGTGGGCGACGAGG
3' Primer	CACGGAGTACTTGCGCTCAGGAGG

Nucleotides introducing mutations are underlined.

seeded in 12-well plates and grown in medium described above. For experiments using other cell culture sera, the cells were switched after 4 h to medium containing 10% delipidized/steroid-free human serum (PAA, Coelbe, Germany) and 10% delipidized calf serum (PAN Biotech, Aidenbach, Germany) with or without 10 μ g/ml cholesterol (Sigma) and 1.5 μ g/ml 25-hydroxycholesterol (Sigma) or 20 nM estradiol (Sigma; all dissolved in dimethyl sulfoxide (DMSO)), and grown overnight in a 5% CO₂ incubator at 37 °C. The next day, the cells were transfected at 40–60% confluency with 2 μ l FuGene6 (Roche Applied Science), 0.5 μ g luciferase reporter gene constructs, and, for normalizing the transfection efficiency, 25 ng

Renilla luciferase control vector, pRL-SV40 (Promega). The cells were harvested and luciferase activity was measured in a microplate luminometer (EG&G Berthold, Bad Wildbad, Germany) 30 h after transfection. Values shown (mean \pm s.d.) are averages of at least three independent experiments each performed in triplicate. Statistical analyses were performed using Student's *t*-test and the differences were considered significant when $P < 0.05$.

Protein–DNA interactions (EMSA)

The interaction of HepG2 and Hepa 1–6 nuclear proteins (TFs) with oligonucleotides corresponding

to putative TF-binding sites was analyzed by electrophoretic mobility shift assay (EMSA). Both cell lines were grown in cell culture medium containing 10% delipidized calf serum in T-75 flasks until 100% confluency. For the preparation of nuclear extracts, the NE-PER Cytoplasmic and Nuclear Extraction Kit (Pierce, Rockford, IL, USA) was used according to the manufacturer's instructions. In order to obtain a higher concentration of nuclear proteins, only half the amount of recommended Nuclear Extraction Reagent was used. For probe labeling, the Biotin 3' End DNA Labeling Kit (Pierce, Rockford, IL, USA) was used according to the manufacturer's instructions. For analyzing DNA-protein interactions, the LightShift Chemiluminescent EMSA Kit (Pierce) was used according to the manufacturer's instructions. Briefly, nuclear cell extracts (5–10 μ g protein) or recombinant protein (250 ng) were incubated in a reaction buffer (total volume 20 μ l) of 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 2.5% glycerol, 0.05% Nonidet P-40, 5 mM MgCl₂, and 2.5 μ g BSA containing 1.5 μ g poly(dI-dC). Additional buffer components for hepatocyte nuclear factor 4 (HNF4), NF- κ B, and murine SREBP were 5% glycerol, 10 mM Hepes-KOH, and for vitamin D receptor (VDR) and human SREBP 10 mM Hepes-KOH. After 2-min incubation at room temperature, approximately 25 fmol biotin-labeled double-stranded probe containing the corresponding binding site was added. The reaction mixtures were incubated for 30 min at room temperature. For competition experiments, a 300-fold molar excess of unlabeled wild-type or mutated probe was added to the reaction mixtures before adding the labeled probe. For super-shift experiments, 2 μ g antibody (Santa Cruz Biotechnology, Heidelberg, Germany) were added to the reaction mixtures 30 min before the addition of labeled probe and incubated at 4 °C. Reaction mixtures were analyzed by native PAGE. Subsequent signal detection was carried out according to the instruction manual. Further details are given by Ebert *et al.* (2004).

RNA isolation and reverse transcriptase (RT)-PCR

For cDNA synthesis, RNA was extracted from cells using SV Total RNA Isolation Kit (Promega) and transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany) with oligo-dT18-primers. PCR-primer sequences are indicated in Table 1. Expression was tested by means of a standard PCR with Taq polymerase. Template used for PCR was applied either plain as received from first-strand cDNA synthesis or diluted five- or tenfold. Amplification was done in 30 cycles with annealing temperature of 55 °C and elongation time of 1'10".

Results

Target definition for human HSD17B7

Recently, a second form of human HSD17B7 located on chromosome 10 was described (Liu *et al.* 2005). Our sequence analysis revealed 99.2% identity within the first 500 nucleotides upstream of the translation start site in both forms (not shown). *MatInspector* analysis of this sequence provided no evidence for additional or missing putative binding sites and the existing differences affect none of the TF-binding sites as identified in our study. In this work, only the promoter of HSD17B7 residing on chromosome 1 was analyzed.

Promoter fine mapping by dual luciferase assays

Our preliminary studies showed a possibility of HSD17B7 promoter control by TFs known from genes of cholesterol biosynthesis and homeostasis, as well as genes of fatty acid metabolism (Ohnesorg & Adamski 2005, 2006).

Due to these observations and the previously described involvement of HSD17B7 in postsqualene cholesterol biosynthesis (Marijanovic *et al.* 2003), we used liver cell lines for the analysis of the mechanisms underlying the transcriptional regulation of both the human and murine HSD17B7 genes. Both human Hep-G2 and mouse Hepa 1-6 cell lines were verified for the expression of HSD17B7 by RT-PCR (Fig. 2). We used constructs containing 5'-truncated fragments of HSD17B7 promoters linked to firefly luciferase for examination of the influence of delipidized calf serum (inducing condition) on the transcriptional activity of the human and murine HSD17B7 promoter fragments compared with standard fetal calf serum containing additional cholesterol and 25-hydroxycholesterol (repressing condition). As shown in Fig. 1, the strongest induction of transcriptional activity can be seen for the fragments -329 for human and -295 for the murine promoter, the same fragments which were showing strongest induction levels under serum starvation in our previous study. The inducing effect of delipidized serum is remarkably stronger for the human promoter (human: ~fourfold; murine: ~twofold). In order to verify these results *in vivo*, we performed RT-PCR on identically grown cells. Results show upregulation of HSD17B7 mRNA under delipidized and steroid-free conditions (Fig. 2). The results are compared with the cells cultured with 10% fetal calf serum (FCS) with without the addition of cholesterol or 25-hydroxycholesterol.

Analysis of HSD17B7 promoters in different cell lines

For studies of tissue-dependent regulation of the human HSD17B7 promoter, we used HepG2 (liver),

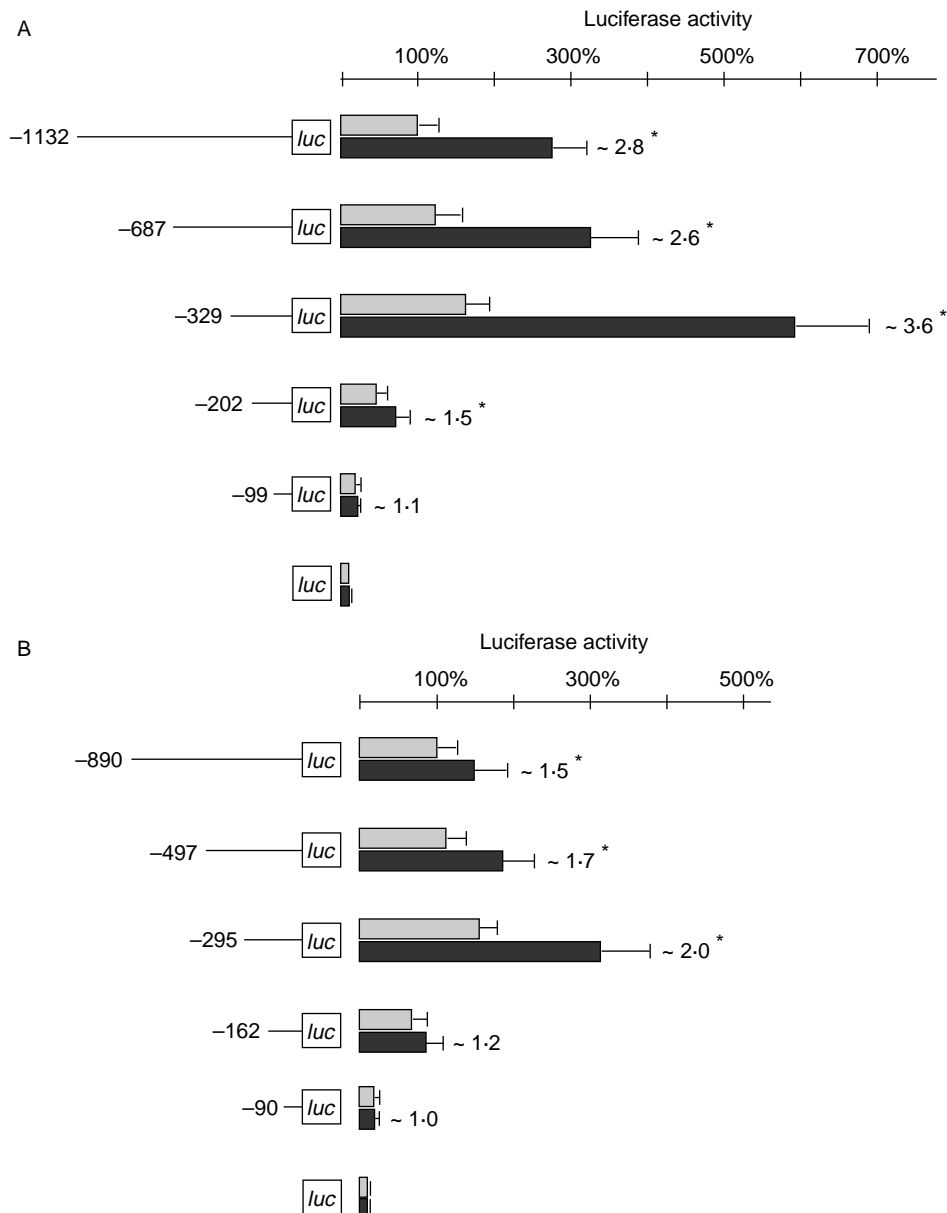


Figure 1 Results of dual luciferase assays performed with wild-type HSD17B7 promoter fragments. Constructs with promoter fragments linked to firefly luciferase were tested for transcriptional activity under basal (10% FCS + 10 μ g/ml cholesterol/1.5 μ g/ml 25-hydroxycholesterol; light gray bars) and induced (10% delipidized FCS; dark gray bars) growth conditions. Luciferase activity of constructs containing longest promoter fragments is set to 100%. Values are shown as means \pm S.D. Data were obtained from at least three independent experiments performed in triplicate; * indicate values significantly different from corresponding control in 10% FCS + cholesterol at $P < 0.05$. (A) Luciferase activity of human promoter constructs in HepG2 cells. (B) Luciferase activity of murine promoter constructs in Hepa 1-6 cells.

HEK-293 (embryonic kidney), and HeLa (cervix carcinoma) cells (Fig. 3). The construct with strongest activity (-329/-1) was transfected in these cell lines, grown under inducing or repressing conditions and luciferase activity was measured. The strongest

increase in luciferase activity by inducing growth condition can be seen in HepG2 cells (~ 3.8 -fold) compared with HEK-293 cells (~ 1.5 -fold). In HeLa cells, the effect of induction by cholesterol depletion is negligible.

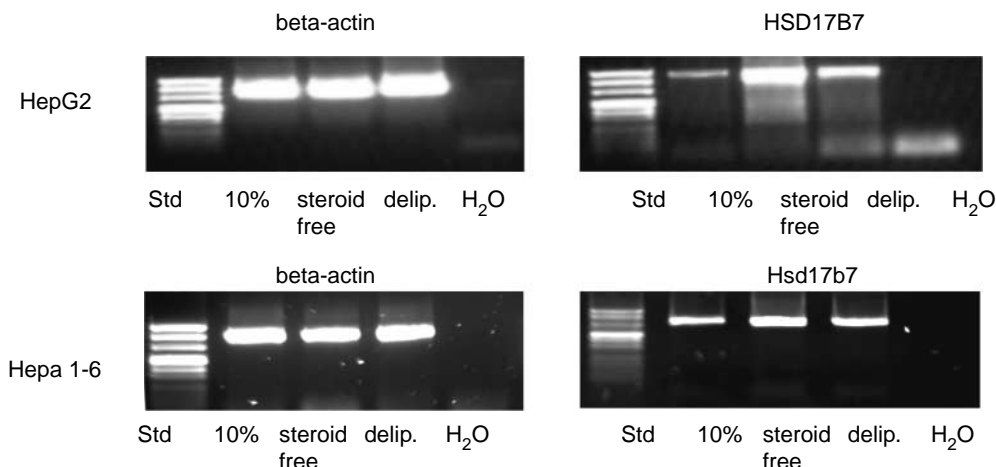


Figure 2 RT-PCR showing upregulation of HSD17B7 mRNA in both HepG2 and Hepa 1–6 cells. Cells were grown under the conditions indicated for 24 h. After harvesting, total RNA was isolated and equal amounts transcribed into cDNA. For normalization, the amplification of β -actin is shown, 10%: 10% FCS, 10 μ g/ml cholesterol/1.5 μ g/ml 25-hydroxycholesterol; steroid; free: steroid-depleted medium; delip: 10% delipidized FCS; H₂O: negative control with water as a template for the PCR. The same effects were observed with undiluted and diluted templates (five- or tenfold, not shown).

HSD17B7 promoters show significant similarities in different species

In order to identify a conserved pattern of transcriptional regulation in different species, we used the FrameWorker software. Therefore, the human and murine promoter regions showing strongest basal and induced transcriptional activity ($-329/-1$ for human promoter; $-295/-1$ for murine promoter) were analyzed. Additionally, we included the corresponding region ($-350/-1$) of the rat gene.

Despite relatively low overall sequence similarity (not shown), the promoters of all the three species examined displayed significant similarity of TF-binding motifs (Fig. 4, only human and mouse data are shown for clarity). Interestingly, the promoters of all three examined species contained a typical regulatory feature of cholesterol genes with a conserved SREBP-binding site and adjacent GC and CCAAT boxes. Additionally, we found a conserved binding site for the VDR/retinoid X receptor (RXR) heterodimer. Further common features more proximal to the start

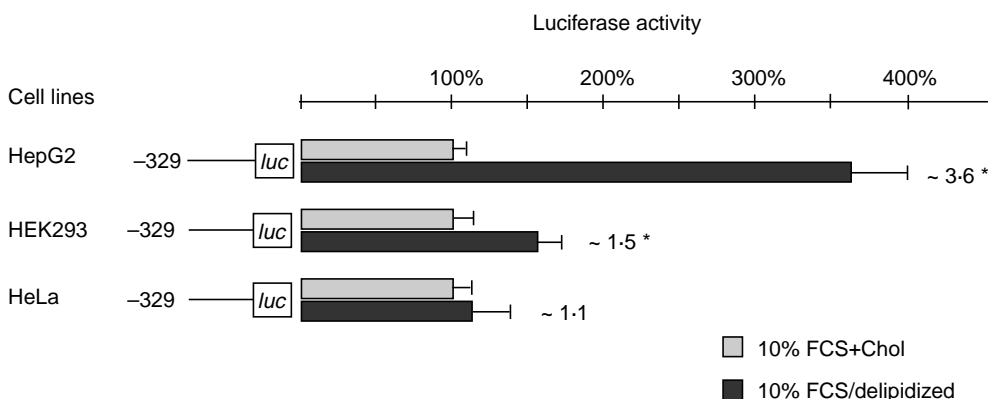


Figure 3 Activity of HSD17B7 promoter in different human cell lines. For analysis of differential expression, the construct showing strongest luciferase activity in promoter fine-mapping experiment was transfected also in HEK-293 and HeLa cells. Strongest induction of promoter activity can be seen in HepG2 cells, which is indicative for liver-specific induction by cholesterol depletion. Luciferase activity of constructs transfected in cells grown under repressing conditions is set to 100%. Values are shown as means \pm s.d. Data were obtained from at least three independent experiments performed in triplicate; * indicate values significantly different from corresponding control in 10% FCS + cholesterol at $P < 0.05$.

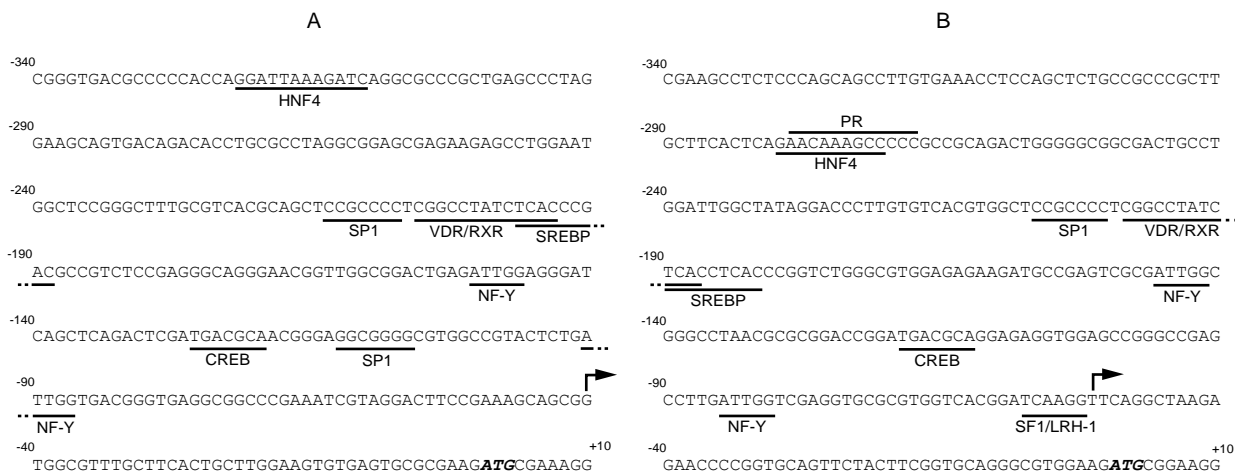


Figure 4 *MatInspector* analysis of HSD17B7 promoters. (A) Human and (B) murine HSD17B7 promoters. Putative TF-binding sites are underlined. Due to the existence of several transcription start sites, the number of bases are relative to the translation start point/start codon (boldface italic). HNF4, hepatocyte nuclear factor 4; SP1, ubiquitinary GC-box TF; VDR/RXR, vitamin D receptor/retinoid X receptor; SREBP, sterol regulatory element-binding protein; NF-Y, ubiquitinary CAAT-box TF; CREB, cAMP-response element-binding protein; PR, progesterone receptor; SF1/LRH-1, steroidogenic factor 1/liver receptor homolog 1. Arrows indicate the position of transcription start points.

codon were a binding site for the CREB and an additional CCAAT box.

Differences between murine and human promoters

In depth *MatInspector* analysis revealed more common and also distinct putative TF-binding sites. The software identified a putative HNF4-binding site for both promoters. In contrast to the murine promoter, the human contains two putative SP1-binding sites (Fig. 4A). On the other hand, *MatInspector* identified in the murine promoter, two putative binding sites for TFs known to be involved in steroidogenesis and steroid hormone action, namely SF1/LRH-1 (close to the transcription start area) and the progesterone receptor (at the site of HNF4 in minus direction) in the murine promoter (Fig. 4B). The human promoter is lacking both binding sites within 1.5 kb upstream of the start codon.

Functionality of the identified TF sites

In order to determine the biological relevance of the bioinformatically identified putative TF-binding sites, we tested the impact of mutations within these sites. For this task, we exchanged two to four bases within the TF-binding sites using oligonucleotides listed in Table 1. Luciferase activity of the constructs was again tested under inducing and repressing growth conditions as shown in Figs 5 (human promoter) and 6 (murine promoter). In most cases, the effect of mutation of each TF-binding site is at least comparable for the human and murine promoters, concerning basal or induced promoter activity. Mutation of SREBP adjacent to SP1

and NF-Y-binding sites leads to a much weaker response to the inducing condition compared with the wild-type fragment. Constructs containing double mutations of these sites show an almost complete loss of induction. On the other hand, the mutations of the proximal SP1 site in the human promoter and VDR/RXR-binding sites in both species seem to modify the basal activity. Interestingly, mutations of SREBP-binding sites also lead to a decreased basal promoter activity, whereas these constructs still exhibit remarkable increases in luciferase activity under inducing conditions. Mutations of CREB sites had no significant effect, either under basal or induced conditions.

For mutations of HNF4-binding sites, the results are species specific. While we were not able to show any effect by mutation of the corresponding binding site in the murine promoter, a weak but significant change in response to cholesterol depletion can be seen for the human fragment compared with the wild type. The factor binding to the proximal NF-Y-binding site obviously acts as a silencer, as mutation of this site leads to a strong increase in promoter activity under basal conditions.

EMSA and supershift assays

EMSA and supershift assays were carried out to define the binding of the bioinformatically identified TFs to the human and murine HSD17B7 promoters (Fig. 7). Thereby, those factors were shown to be responsible for the effects shown in mutagenesis analysis. All binding sites showing different luciferase activity due to

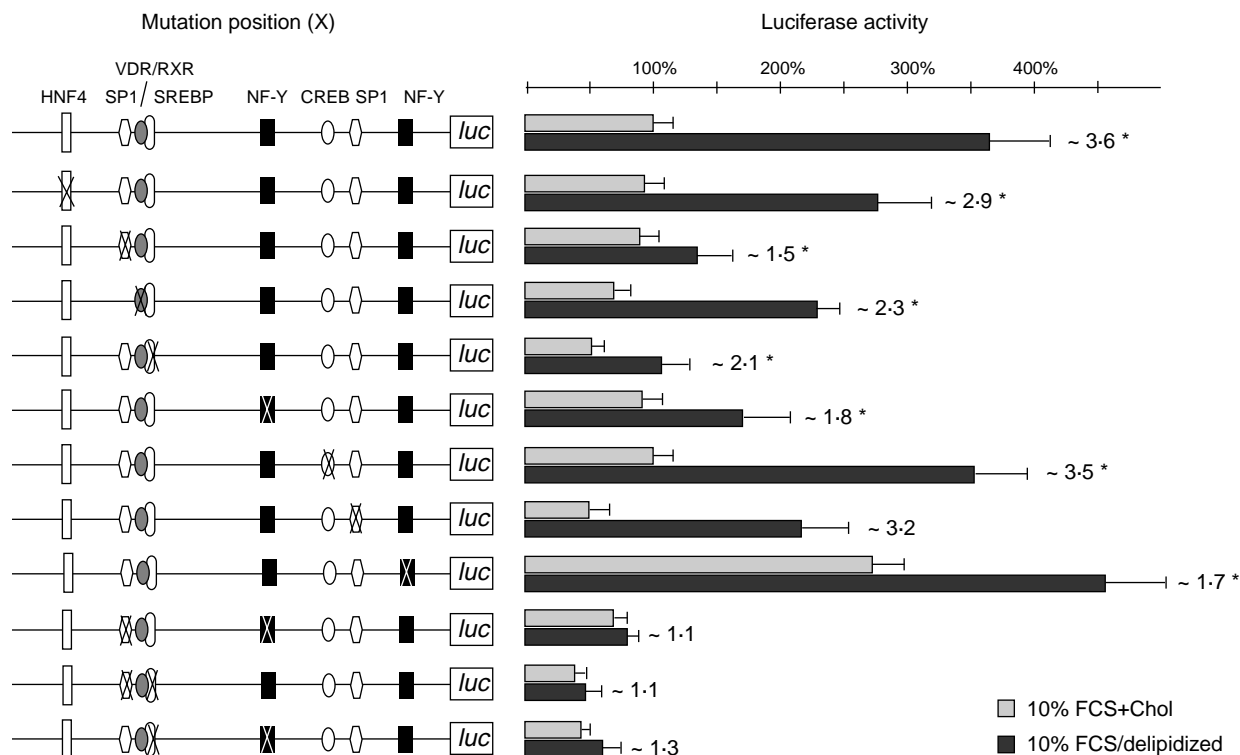


Figure 5 Effect of site-directed mutagenesis of TF-binding sites in transcriptional activity of the human HSD17B7 promoter. Constructs containing the indicated mutations were transfected in HepG2 cells and grown under basal or induced conditions respectively. Cells were lysed and luciferase activity was measured and compared with activity of the wild-type construct 30 h after transfection. Luciferase activity for the wild-type fragment under basal growth conditions is set to 100%. Values are shown as means \pm s.d. Data were obtained from at least three independent experiments performed in triplicate; * indicate values significantly different from corresponding control in 10% FCS+cholesterol at $P < 0.05$.

mutagenesis were included in these experiments. The wild-type probes used for EMSA are listed in Table 2. In order to prove the effects of mutations due to loss of TF binding, we additionally used competitors carrying the same binding site mutations as shown in Table 1.

As can be seen in Fig. 7A, both SREBP-1 and SREBP-2 are able to bind the bioinformatically predicted binding sites within the human and murine HSD17B7 promoters. The addition of specific antibodies leads to the supershifted bands in both species. Figure 7B and C shows the EMSA results for human and murine SP1-binding sites respectively. Due to the usage of recombinant SP1 protein, no supershift experiment was required. The binding of recombinant VDR (without RXR and vitamin D₃) to the predicted binding sites within human and murine promoters is shown in Fig. 7D. In contrast to the murine promoter, we could prove binding of HNF4 to the corresponding binding site in the human promoter. The addition of HNF4-specific antibody leads to a supershifted band (Fig. 7E). Finally, binding of NF-Y to the predicted distal-binding sites in both promoters is shown in Fig. 7F. In both the species, the addition of NF-Y-specific antibodies leads to a supershifted band.

Discussion

HSD17B7 participates in cholesterol biosynthesis

In our study, we identified the TFs responsible for basal- and cholesterol-dependent regulation of both the human and murine HSD17B7. Although we did not examine the corresponding promoter region of the rat experimentally, the results of the bioinformatic analysis point to a very similar regulation. Furthermore, the similarity in composition and sequence of the identified TF-binding sites within the promoters, and therefore, a high probability of similar *in vivo* regulation, leads us to the conclusion that HSD17B7 is involved in the same metabolic pathway across the examined species, namely cholesterol biosynthesis. On the other hand, our results do not disprove *in vivo* involvement of HSD17B7 in steroidogenesis. In a recent study addressing transcriptional regulation of rat HSD17B7 using a rat luteal cell line dependent on LH and forskolin, which led to a significant decrease in promoter activity, the authors were able to identify and verify two binding sites for SP1 and one for NF-Y (Risk *et al.* 2005). In contrast to our findings, a conserved

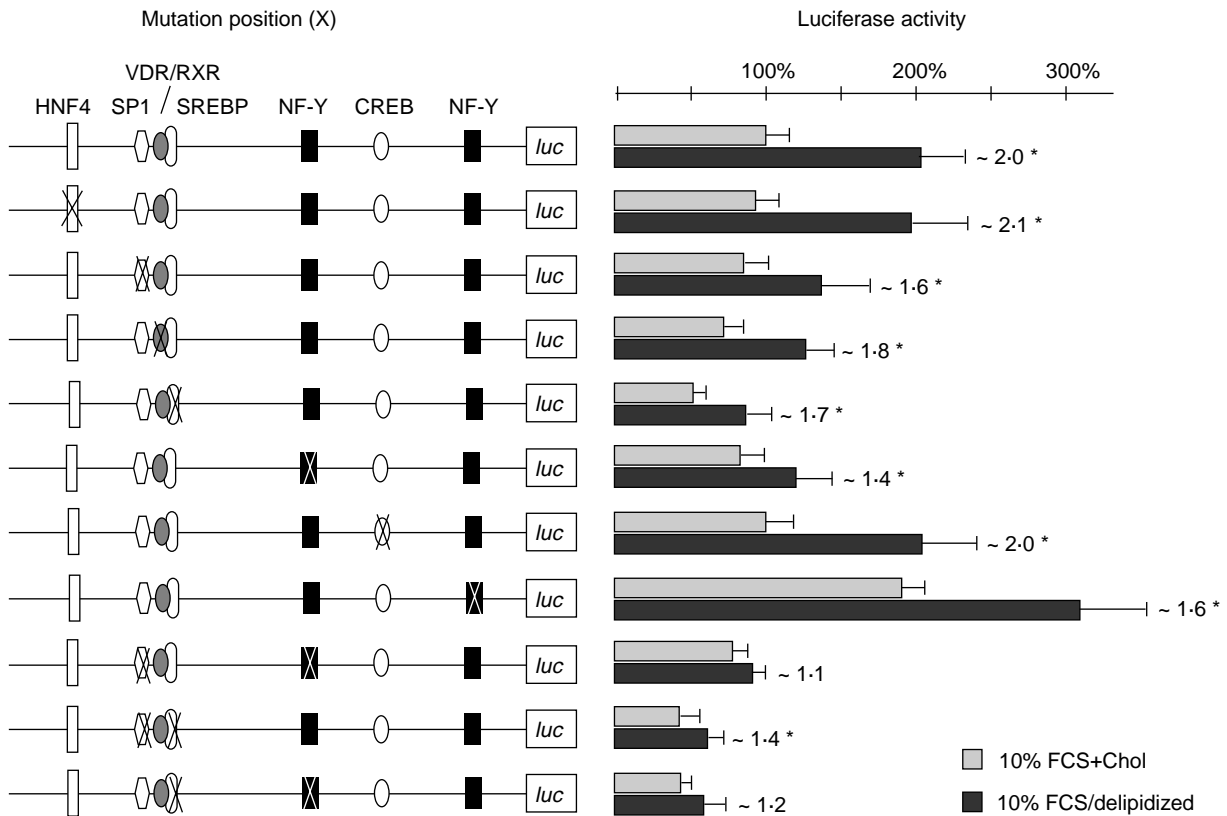


Figure 6 Effect of site-directed mutagenesis of TF-binding sites in transcriptional activity of the murine HSD17B7 promoter. Constructs containing the indicated mutations were transfected in Hepa 1–6 cells and grown under basal or induced conditions respectively. Cells were lysed and luciferase activity was measured and compared with activity of the wild-type construct 30 h after transfection. Luciferase activity for the wild-type fragment under basal growth conditions is set to 100%. Values are shown as means \pm s.d. Data were obtained from at least three independent experiments performed in triplicate; * indicate values significantly different from corresponding control in 10% FCS+cholesterol at $P < 0.05$.

CREB-binding site within the rat promoter was not detected. This might be due to the use of different algorithms for the identification of TF-binding sites. However, in our experiments, we were unable to show a significant effect mediated by this binding site, either by mutation of the binding site by or by the addition of 20 μ M forskolin (non-significant decrease only, data not shown) to the cell culture medium of liver cell lines.

Basal promoter of HSD17B7

We showed that several TFs are required for both basal and induced transcription levels. Most strikingly, HSD17B7 promoters contain a conserved and functional SREBP-binding site, accompanied by functional binding sites of typical accessory TFs of SREBP, SP1, and NF-Y. This situation is a common feature for genes involved in cholesterol biosynthesis and homeostasis, as well as in fatty acid metabolism (Kim *et al.* 2001, Schweizer *et al.* 2002). Due to their weak DNA-binding ability, TFs of the SREBP family

require adjacent binding of at least one accessory TF. All the SREBP-regulated genes so far examined contain either SP1 or NF-Y-binding sites close to the SREBP-binding site within their promoters. Although based on limited published data, genes of postsqualene cholesterol biosynthesis and fatty acid synthesis seem to be regulated by SREBPs and both SP1 and NF-Y in a cholesterol-dependent manner (Xiong *et al.* 2000, Kim *et al.* 2001, Nagai *et al.* 2002, Misawa *et al.* 2003). As shown in our study, this is also the case for human and murine HSD17B7. Using supershift assays, we could show that SREBP-2 binds to the predicted binding site in the human and murine promoters. Due to lack of specificity of the used SREBP-1 antibody, which recognizes both SREBP-1a, and SREBP-1c, we could not distinguish which isoform binds to the promoters. However, according to the described preference of SREBP-1c for genes involved in fatty acid synthesis, it seems likely that SREBP-1a is responsible for the supershifted band shown in Fig. 7.

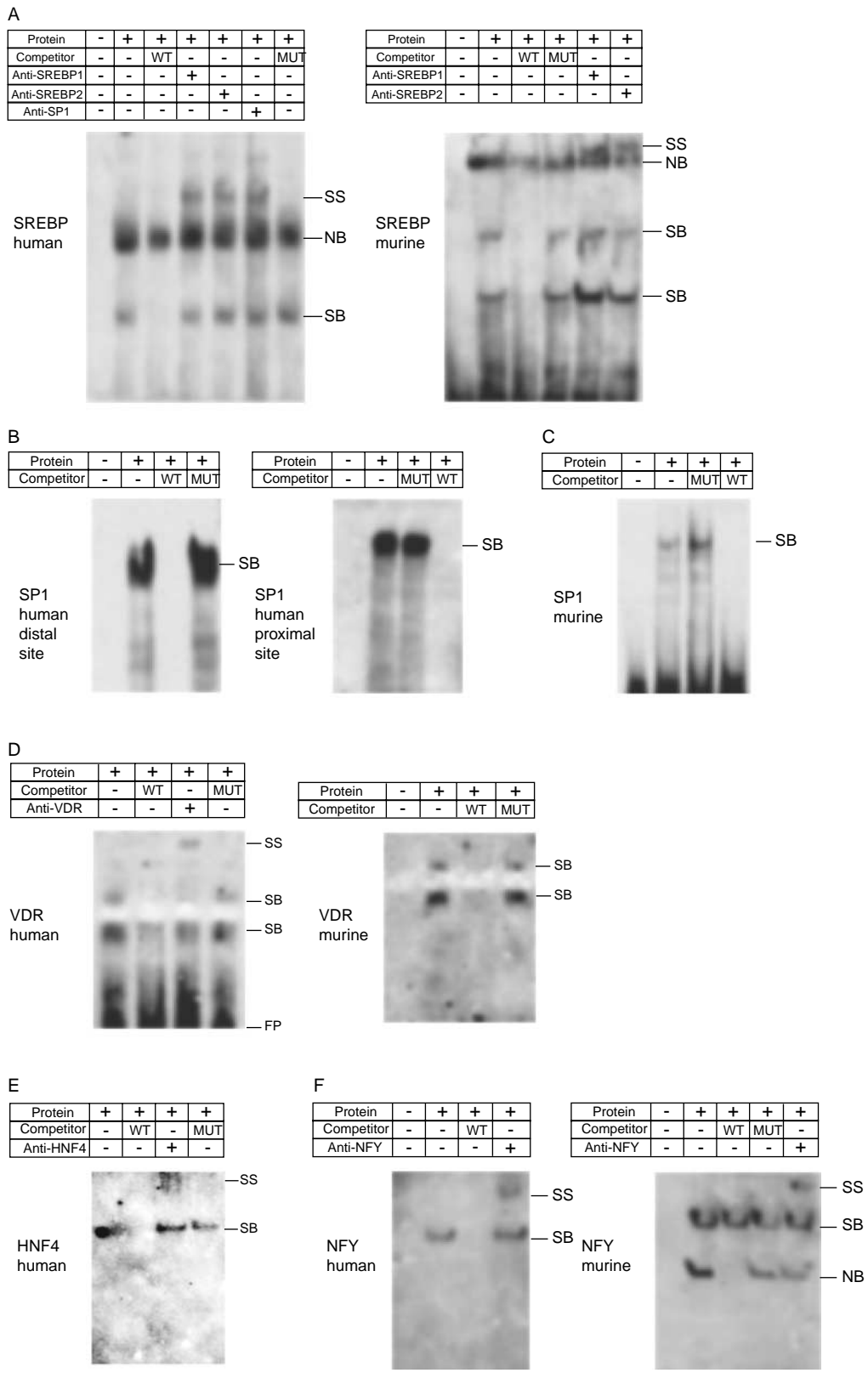


Table 2 Sequences of nucleotides used for EMSA studies

Name	Sequence (5'–3')
HHNF4	ACCAGGATTAAGATCAGGCGC
hSP1 distal	CACGCAGCTCCGCCCTCGGCATC
HVDR	CCCTCGGCCTATCTACCCGAC
HSREBP	TCACGCAGCTCCGCCCTCGGCCTATCTACCCGACGCCGTCTC
HNF-Y distal	TGGCGGACTGAGATTGGAGGGATCAG
hSP1 proximal	GCAACGGGAGGCGGGGCGTGGCCGTA
HNF-Y proximal	CCGACTCTGATTGGTGACGGG
mHNF4	CACTCAGAACAAGCCCCCGCCG
mSP1	CACGTGGCTCCGCCCTCGGCATC
MVDR	CCCTCGGCCTATCTACCTCA
MSREBP	TCACGTGGCTCCGCCCTCGGCCTATCTACCTACCCGGTCT
MNF-Y distal	CGAGTCGCGATTGGCGGGCCTAACG
MNF-Y proximal	CCGAGCCTTGATTGGTCGAGGTG

Estradiol does not induce transcriptional activity of HSD17B7 promoters

Due to the bioinformatic results pointing to a role in cholesterol biosynthesis and the previously described dual functionality of HSD17B7 in steroidogenesis (conversion of estrone to estradiol, *Nokelainen et al. 2000*) as well as in cholesterol biosynthesis (conversion of zymosterone to zymosterol, *Marijanovic et al. 2003*), we previously examined the effects of cholesterol and estradiol on the transcriptional activity of the HSD17B7 promoters (*Ohnesorg & Adamski 2006*). In our study, estradiol had no significant effect on luciferase activity in Hepa 1–6 and HepG2 cells.

Cholesterol regulation of HSD17B7 expression

The examined and verified TFs play different roles in the regulation of HSD17B7. Mutations of the SREBP adjacent to the SP1 and NF-Y-binding sites showed weaker induction effects due to cholesterol depletion than the mutation of the SREBP site itself. Double mutation of both SP1 and NF-Y-binding sites abolishes the induction effect almost completely, whereas double mutations of either SP1 or NF-Y and SREBP, although exhibiting stronger effects compared with single mutations, still show a significant increase in luciferase activity by inducing growth condition.

Another interesting point is the novel involvement of the VDR/RXR heterodimer in transcriptional

regulation of HSD17B7. Whereas VDR/RXR is known to play a role in cholesterol catabolism (*Makishima 2005*, *Wehmeier et al. 2005*), to our knowledge, no report addressing the involvement of this TF in the regulation of any gene of cholesterol biosynthesis has been published so far. The only study in this area concerns *Insig-2*, a negative regulator of the SREBP-activating cascade (*Lee et al. 2005*). The authors of this study could show that the VDR/RXR heterodimer enhances expression of *Insig-2* in preadipocytes, thus downregulating many components of the cholesterol biosynthesis pathway. In contrast to these findings, the mutagenesis of the VDR/RXR-binding site had negative regulatory effects on the transcriptional activity of both the human and murine HSD17B7 promoters, under basal and also under inducing growth conditions. Using nuclear extracts of HepG2 and Hepa 1–6 cells, we were not able to prove binding of VDR/RXR to the corresponding binding sites; however, EMSA performed with recombinant VDR protein without its ligand vitamin D₃ (and also lacking RXR) showed detectable bands (*Fig. 7*). Therefore, the role of the VDR/RXR heterodimer, or VDR alone without its ligand, in transcriptional regulation of HSD17B7 remains unclear. Due to the position of the VDR/RXR-binding site which overlaps the SREBP-binding site, one could, speculate that this might enable the transcriptional machinery to react via the same enhancer region on two different stimuli (both cholesterol and vitamin D₃) are synthesized from an

Figure 7 EMSA analyses of human and murine HSD17B7 promoters. Nuclear extracts of HepG2 (human) and Hepa 1–6 (murine) were incubated with biotin-labeled probes containing putative TF sites as indicated in *Table 2*. For analysis of SP1 and VDR-binding sites, recombinant human SP1 and murine VDR proteins were used respectively. As indicated for each figure, unlabeled wild type or mutated competitors were used in 300-fold molar excess. To confirm the identity of proteins, we added antibodies specific to the corresponding TFs. Analysis was performed as described in 'Materials and methods'. FP, free probe; SB, specific binding; NB, non-specific binding; SS, supershift; WT, wild type; MUT, mutant. (A), EMSA for SREBP-1 and SREBP-2. For human promoter (left side), additionally SP1-specific antibody was used, mouse promoter is on the right side; (B), EMSA for both human SP1-binding sites (distal left side, proximal right side); (C), EMSA for murine SP1-binding site; (D), EMSA for VDR-binding sites (human left side, murine right side); (E), EMSA for human HNF4-binding site; (F), EMSA for distal NF-Y-binding sites (human left side, murine right side).

identical precursor, 7-dehydrocholesterol) under various conditions or in different tissues. Further experiments addressing these questions might lead to interesting new insights into the functions of vitamin D and its receptor.

Unfortunately, we were not able to identify the TF-binding site to the proximal NF-Y-binding site; gel-shift analysis with the corresponding probe revealed a weak signal (not shown). However, the addition of a NF-Y-specific antibody did not lead to a detectable supershifted band. Therefore, a TF other than NF-Y seems to act as a transcriptional silencer.

Another TF required but not essential for cholesterol-dependent regulation of human HSD17B7 is HNF4. In contrast to all other identified TFs, HNF4 is predominantly expressed not only in liver, but also in kidney and pancreas. Besides HSD17B7, there are some further genes of postsqualene cholesterol biosynthesis regulated by HNF4. A study addressing transcriptional regulation of the *SI* gene (*SI* catalyzes the step after HSD17B7 in this pathway) identified HNF4 as an important factor of cholesterol-dependent regulation (Misawa *et al.* 2003). Due to the weak effect of mutation of HNF4-binding site in the human HSD17B7 promoter it seems likely that HNF4 is used for transcriptional fine-tuning only. At least, HNF4 does not seem to be the only reason for the much higher induction levels in HepG2 cells compared with HEK-293 and HeLa cells.

Species differences

The comparison of human and murine HSD17B7 promoters shows striking similarities as well as some differences. Like the human, the murine promoter contains functional binding sites for SREBP, VDR/RXR, and NF-Y. However, only one SP1-binding site was detected. On the other hand, two additional putative binding sites for the progesterone receptor and SF1/LRH-1 are present in the murine promoter. Although we did not prove functionality of these sites, their presence alone might point to an involvement of murine HSD17B7 in steroidogenesis. Almost all genes of steroidogenesis are regulated by SF1 and/or LRH-1 (see, for a review, Val *et al.* (2003)). Further experiments in this direction should be carried out using steroidogenic cell lines.

Closing remarks

Our study aimed to identify TFs responsible for cholesterol or steroid-dependent regulation of both the human and murine HSD17B7. Overall, the high similarity of promoter regions in human, mouse, and rat leads us to the conclusion that HSD17B7 is involved in the same metabolic pathway in these species. Novel pattern of TF-binding sites known for genes of

cholesterol biosynthesis confirms the involvement of HSD17B7 in this process. However, general steroidogenesis remains a possible target of HSD17B7.

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