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Type 10 17β-hydroxysteroid dehydrogenase expression is regulated by C/EBPβ in HepG2 cells

Mirja Rotinen, Joaquín Villar[1], Jon Celay, Ignacio Encío*

Department of Health Sciences, Universidad Pública de Navarra, Avda. Barañain, 31008 Pamplona, Spain

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ABSTRACT

17β-Hydroxysteroid dehydrogenases (HSD17Bs) are enzymes that stereospecifically reduce or oxidize a keto- or hydroxy group at C17 of the steroid scaffold, respectively. Fourteen mammalian HSD17Bs have been identified so far. We previously described that the HSD17B8 gene is regulated by C/EBPβ in the hepatocarcinoma cell line HepG2. Here, we analyze the 5′-flanking region of 14 promoters (HSD17B1–14) looking for CCAAT boxes and binding sites for CCAAT enhancer binding factors (C/EBPs). All promoters were found to have binding sites for these transcription factors except HSD17B1. Ectopic expression of C/EBPα or C/EBPβ in HepG2 cells showed that HSD17B11 expression was induced by both transcription factors while HSD17B10 expression was only induced by C/EBPβ. The first 500 bp of the 5′-flanking region of both genes contain two putative binding sites for C/EBPs. Gene reporter assays showed that C/EBPβ transactivated HSD17B10 but not HSD17B11. Additional experiments showed that several isoforms of C/EBPβ are involved in HSD17B10 regulation. Mutation of the CCAAT box located at −30/−19 induced HSD17B10 promoter activity when only LIP was expressed, while impaired LAP-induced HSD17B10 transactivation in HepG2 cells when LAP isoforms are expressed. Taken together, our findings reveal that HSD17B10 is regulated by several isoforms of C/EBPβ in HepG2 cells.

1. Introduction

17β-Hydroxysteroid dehydrogenases (HSD17Bs) are a group of enzymes that catalyze the inter-conversion of 17-ketosteroids to 17-hydroxysteroids in reproductive organs and peripheral tissues, thus regulating the biological activity of sex hormones [1]. All of them belong to the short-chain dehydrogenase family (SDR) except HSD17B5 which is an aldo-keto reductase (AKR) [2]. In humans, 14 types have been cloned so far [3]. Types 1, 3, 5 and 7 catalyze *in vivo* the reaction in the reductive direction, while types 2, 4, 8, 10, 11, 12 and 14 work in the inactivating direction. However, under *in vitro* conditions and in the presence of a substantial excess of a suitable cofactor (NADPH or NAD^+), HSD17Bs can catalyze both reactions. The enzymatic activity of HSD17B13 has not yet been tested and although HSD17B6 is listed as so in databases, it is in fact a 3(alpha → beta)-hydroxysteroid epimerase [4].

Canonical CCAAT boxes are prototypical DNA sequences that are present in many families of genes including those involved in steroidogenesis [5] or in the cholesterol metabolism [6]. It has also been described that some TATA-less promoters require intact CCAAT boxes to be activated [7]. There are different groups of proteins that are able to bind CCAAT boxes; among them is the C/EBP family. C/EBP family has 6 members (C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ) [8] that are implicated in different cellular processes. C/EBPβ regulates gene expression to control cellular proliferation, differentiation, inflammation and metabolism. Encoded by an intronless gene, C/EBPβ is expressed as several distinct protein isoforms (LAP1, LAP2 and LIP) whose expression is regulated by the differential use of several translation start sites [9]. LAP1 and LAP2 contain an N-terminal transcription activation domain, a central DNA binding domain, and a C-terminal leucine zipper domain whereas LIP lacks the transactivation domain but can dimerize and bind DNA. As a consequence, LAP1 and LAP2 are mostly transcriptional activators, whereas LIP acts as a repressor [10].

To date, several promoters of genes encoding HSD17Bs have been characterized. HSD17B1 (both gene and pseudo gene), 2, and 4 contain TATA box elements [11–13]. Interestingly, a point mutation in the TATA box of HSD17B2 gene decreased its promoter activity *in vitro* [14]. On the other hand, no TATA boxes were found in the analysis of HSD17B5, 7, 8, and 12 [15–18]. The human and

Abbreviations: HSD, hydroxysteroid dehydrogenase; C/EBP, CCAAT enhancer binding protein.

* Corresponding author. Tel.: +34 948166111; fax: +34 948270902.
E-mail address: ignacio.encio@unavarra.es (I. Encío).
[1] Current address: Section on Molecular Endocrinology, PDEGEN, NICHD, NIH, Bethesda, MD 20892, United States.

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Table 1
Sequences of sense and antisense oligonucleotide primers used for (A) expression analysis, (B) amplification of immunoprecipitated chromatin and (C) site-directed mutagenesis.

Gene	Oligonucleotides	Sequence (5′–3′)
A. Sequences of oligonucleotides used as primers in Real-Time PCR experiments		
HSD17B2	HSD17B2 (f)	GGCTGTGACCATGTTCTC
	HSD17B2 (r)	GCTGTGCTAAGATGTAGTCC
HSD17B3	HSD17B3 (f)	GTGATAACCAAGACTGCTGATGAG
	HSD17B3 (r)	GGAAGGCACCGCTGTAGAAG
HSD17B4	HSD17B4 (f)	GAGGAGAATGGTGGCTTGTTTG
	HSD17B4 (r)	TCTTGGATACTCTGAGGCTTGC
HSD17B5	HSD17B5 (f)	CGGAGTAAATTGCTAGATTTC
	HSD17B5 (r)	GACTGGGTCCTCCAAGAG
HSD17B7	HSD17B7 (f)	CGTTTGCTTCACTGCTTGG
	HSD17B7 (r)	TCTGCCTTGCTCATGTTCC
HSD17B8	HSD17B8 (f)	ACATCAGTAGCATCGTAGG
	HSD17B8 (r)	GGAGGACAGAGTTACAGC
HSD17B10	HSD17B10 (f)	TGTGGATGTAGCTGTCAACTG
	HSD17B10 (r)	CCTGGTCTGGTTCATTCTGG
HSD17B11	HSD17B11 (f)	TGCAATGACGAAGAATAACC
	HSD17B11 (r)	TTGTAAGGCAGCCAGTTC
HSD17B12	HSD17B12 (f)	ATGAGGAGTATAGGAGCAAGG
	HSD17B12 (r)	GATCAGGTATCCATTGGTTCG
GAPDH	GAPDH (f)	GGAGTCCACTGGCGTCTTC
	GAPDH (r)	ATCTTGAGGCTGTTGTCATACTTC
B. Sequences of oligonucleotides used as primers for amplification in ChIP experiments		
HSD17B5	promHSD17B5 (f)	GGCATTATCACGGCAGAAACG
	promHSD17B5 (r)	AAATGTTTGCTGCTGCTTCTCC
HSD17B10	promHSD17B10-1 (f)	ATACCTTAGCAGGCGGAATC
	promHSD17B10-1 (r)	CTCCACGGGATGGGGATG
	promHSD17B10-2 (f)	TCTCCTTGTCTGAGTGTCC
	promHSD17B10-2 (r)	CTATATGCTAGACGGTGTGC
C. Sequences of oligonucleotides used as primers for site-directed mutagenesis experiments		
HSD17B10	HSD17B10-mC1 (f)	GCCGCTTCGCCTCGGCTCCTCAACGAGCGCC
	HSD17B10-mC1 (r)	GGCGCTCGTTGAGGAGCCGAGGCGAAGCGGC
	HSD17B10-mC2 (f)	GTACTGTAATTCTCGGTATCCACCTGGACTGTGCC
	HSD17B10-mC2 (r)	GGCACAGTCCAGGTGGATACCGAGAATTACAGTAC

murine HSD17B7 promoters contain a CCAAT box located near a SREBP site [16] and it has been described that this CCAAT box binds to NF-Y transcription factor and participates in the transcriptional regulation of the gene in response to cholesterol [19]. In addition, HSD17B12 promoter also contains several NF-Y binding sites near an SREBP site. It has been suggested that this element is required for recruitment and stabilization of the SREBPs and to activate promoter activity [18]. We previously described that the region from +36 to −75 of the human HSD17B8 is enough for efficient transcription of the gene in HepG2 cells, and can thus be considered as a minimal promoter. Furthermore, this region contains two CCAAT boxes that are required for promoter activity and specific binding of C/EBPβ to these elements has been shown [17]. In addition, we described that these CCAAT boxes are essential for estradiol (E2)-induced HSD17B8 transcription. Furthermore, C/EBPβ interacts with estrogen receptor alpha (ERα) in response to E2 [20].

Here, we investigate the role of CCAAT boxes and C/EBP transcription factor family members in the regulation of HSD17Bs genes in HepG2 cells. Promoter analysis of around 500 bp of the 5′-flanking region detected CCAAT boxes and/or C/EBP binding sites in all of them except in HSD17B1. Ectopic expression of C/EBPβ increased the expression of HSD17B10 and 11. Transfection experiments and site-directed mutagenesis identified a CCAAT box required for the C/EBPβ-mediated HSD17B10 transcriptional regulation. ChIP assays and Western blot confirmed that different C/EBPβ isoforms are involved in the transcriptional regulation of HSD17B10.

2. Materials and methods

2.1. Antibodies and plasmids

C/EBPα (N-19) and C/EBPβ (Δ 198) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The C/EBPα and C/EBPβ expression vectors were a gift from Dr. Steven L. McKnight (UT Southwestern Medical Center, Dallas, TX).

2.2. Cell culture and transfection

HepG2 cells (ATCC, Manassas, VA) were cultured in DMEM-Glutamax (Invitrogen, Carlsbad, CA) containing 2 μg/mL of Plasmocin (Invivogen, San Diego, CA) and 10% foetal bovine serum (Invitrogen) at 37 °C and 5% CO_2. Media were renewed every two days and cells were subcultured at a ratio of 1:3. For RT-qPCR analysis cells were grown in 6-well plates. At 90% confluence, cells were transfected with 4 μg of the expression vector complexed with 10 μL of Lipofectamine 2000 (Invitrogen) in 500 μL of Opti-MEM medium (Invitrogen). After transfection (24 h), cells were washed with cold PBS and trypsinized for total RNA extraction with the RNAeasy Mini Kit (Qiagen, Hilden, Germany). Transfection experiments were carried out in duplicates and each experiment was repeated at least five times.

Table 2
Sequence analysis of the human HSD17Bs gene promoters. Putative CCAAT boxes and binding sites for C/EBP family found within the first 500 nucleotides of the 5′-flanking region of each gene indicating its position relative to transcriptional start site, consensus sequence and score.

Gene	Position	Binding site	Consensus sequence	Score	Motif name
HSD17B2	−369/−381	AGTTTGGAAAATA	NNTKTGGWNANNN	90	C/EBP
HSD17B3	−113/−100	GGATTATGTAAAGA	RNRTKNNGMAAKNN	86	C/EBPβ
	−245/−257	AATTTGGGAAAGT	NNTKTGGWNANNN	88	C/EBP
	−370/−358	TCTGTGGTCACTA	NNTKTGGWNANNN	86	C/EBP
HSD17B4	−268/−255	GAATGATGAAACGA	RNRTKNNGMAAKNN	83	C/EBPβ
HSD17B5	+20/+7	AGATTACAAAAACA	NNATTRCNNAANNN	94	C/EBPα
	+7/+20	TGTTTTTGTAATCT	RNRTKNNGMAAKNN	86	C/EBPβ
	−50/−37	CCATTGGTTAACCA	NNATTRCNNAANNN	86	C/EBPα
	−39/−50	GTTAACCAATGG	NNNRRCCAATSA	85	CCAAT box
	−130/−118	TCTTTGATAAGAA	NNTKTGGWNANNN	86	C/EBP
HSD17B7	+14/+3	CGTCACCAATCA	NNNRRCCAATSA	87	CCAAT box
HSD17B10	−30/−19	CTCGGCCAATCA	NNNRRCCAATSA	94	CCAAT box
	−428/−441	AGGTGGAGAAAGAG	RNRTKNNGMAAKNN	86	C/EBPβ
HSD17B11	−84/−71	CAATTGCGAAAGAC	NNNTTGCNNAANNN	87	C/EBP
	−84/−71	CAATTGCGAAAGAC	NNATTRCNNAANNN	87	C/EBPα
	−71/−84	GTCTTTCGCAATTG	NKNTTGCNYAAYNN	86	C/EBPβ
	−267/−280	AAATTGGATAAGTT	NNNTTGCNNAANNN	88	C/EBP
HSD17B12	−247/−236	TGCAGCCAATGA	NNNRRCCAATSA	94	CCAAT box
	−290/−279	ACCGGCCAATCC	NNNRRCCAATSA	86	CCAAT box
HSD17B13	+124/+136	TCGTTGGTGAAGT	NNTKTGGWNANNN	85	C/EBP
	−442/−455	GGATTGCTTCAGTC	NNNTTGCNNAANNN	85	C/EBP
HSD17B14	+71/+83	TCTGTGGGAAGAG	NNTKTGGWNANNN	85	C/EBP
	−453/−466	GTGTTGAGAAAACC	RNRTKNNGMAAKNN	85	C/EBPβ

For the determination of the activity of the promoter-reporter chimeras, cells were seeded in 24-well plates at 1.25×10^5 cells per well. After 24 h, cells were transfected with 0.8 μg of the corresponding construction and 1 ng of pRL-SV40 with 2 μL of Lipofectamine 2000 (Invitrogen) in 100 μL of Opti-MEM medium (Invitrogen). After 24 h, cells were washed with PBS 1× and lysed in 100 μL of Passive Lysis Buffer 1× (Promega, Madison, WI) for 15 min. Assays were performed on 20 μL of cell lysate with the Dual Luciferase Assay kit (Promega) and a Berthold Lumat LB 9501 luminometer (Berthold Technologies, Oak Ridge, TN). Renilla luciferase activity was used to correct the transfection efficiency. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA) using BSA as a standard.

2.3. Construction of human HSD17B10 and HSD17B11 5′-flanking region/luciferase genes

The 5′-flanking region of the human HSD17B10 gene was amplified by PCR using the primers 5′-TCCTTGTCTGAGTGTCCCAGAATCTGG-3′ (−501/−475) and 5′-TTGTCGCCGGCCACTCCACGGGATG-3′ (+6/+30) and cloned into the pCR®2.1 vector (Invitrogen). Insert was subcloned into the SacI and SmaI sites of pGL3-Basic vector (Promega) generating the construct pB10-501.

The 5′-flanking region of the human 17β-HSD11 gene was isolated by PCR using the oligonucleotides 5′-GTGATAAGCAACCTTCAACTATGA-3′ (−344/−321) and 5′-AAAGAGTAGGGGCGAGAGCAAGG-3′ (+18/−5) and cloned into a pGEM-T Easy plasmid (Promega). Insert was then obtained digesting this construct with EcoICRI and NcoI and subcloned into the SacI and NcoI sites of pGL3-Basic vector (Promega) generating the construct pB11-344.

2.4. Site-directed mutagenesis

To generate plasmids bearing mutated consensus-binding sequences for transcription factors, mutagenesis experiments were performed using the Quick Change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). The chimeric luciferase construct containing the −501/+30 region of the HSD17B10 promoter was used as a template. Oligonucleotides used were designed by the web based Quickchange Primer Design Program (http://www.stratagene.com/qcprimerdesign) and are listed in Table 1C. All mutations were confirmed by sequencing.

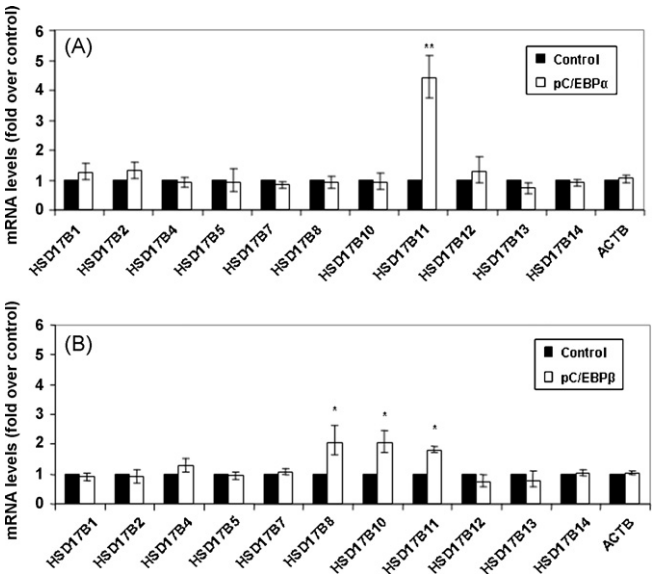

Fig. 1. C/EBPβ induces HSD17B10 and HSD17B11 expression in HepG2 cells. HepG2 cells were transfected with either a C/EBPα (A) or a C/EBPβ (B) expression vector. After 24 h, total RNA was extracted and expression of HSD17Bs genes was assessed by Real-Time PCR. HSD17B3 expression was undetectable under these conditions. Each value (mean ± SEM) was calculated from at least five independent experiments, each performed in duplicates. Asterisks represent statistically significant differences to control cells ($^*p \leq 0.05$; $^{**}p \leq 0.01$).

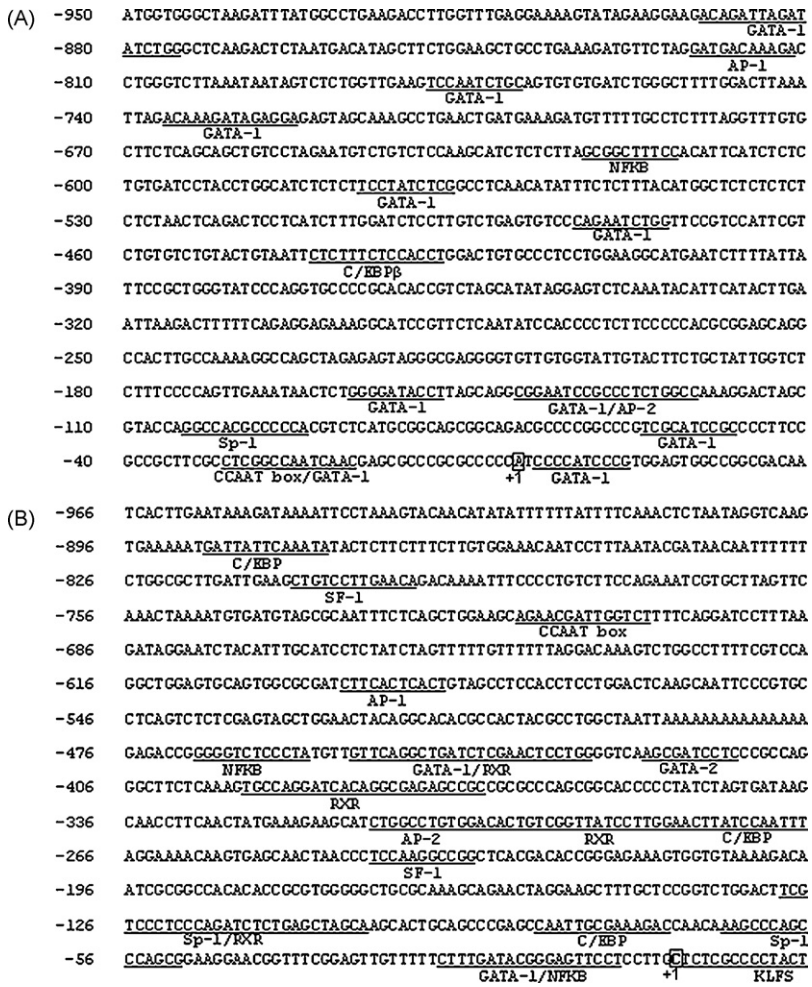

Fig. 2. Sequence analysis of HSD17B10 and HSD17B11 5′-flanking regions. (A) Sequence of the HSD17B10 5′-flanking region. (B) Sequence of the HSD17B11 5′-flanking region. Putative CCAAT boxes and transcription factor binding sites are underlined.

2.5. RT-qPCR

Three micrograms of total RNA were used for reverse transcription reaction in a final volume of 20 μL. Reactions were performed using Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) and random primers (Invitrogen) following manufacturer's instructions. One-step real-time quantitative reverse transcription-PCR was carried out using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) and detected with the Chromo-4 Real-Time PCR Detection System (MJ Research, Bio-Rad). Primers used were obtained form Sigma–Aldrich (St. Louis, MO) and are listed in Table 1A, with the exception of primers sets for human HSD17B1, HSD17B13, HSD17B14 and ACTB that were purchased from SABiosciences, Qiagen (HSD17B1 PPH21736A-200, HSD17B13 PPH15997A-200, HSD17B14 PPH08192A-200, ACTB PPH00073E-200). Values for each gene were normalized to the expression levels of GAPDH and ACTB housekeeping genes. At least five independent experiments were performed to demonstrate reproducibility.

2.6. Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation experiments were performed using the Chromatin Immunoprecipitation Kit & Shearing Kit from Active Motif (Rixensart, Belgium) according to the manufacturer's instructions. Chromatin was sheared with the sonicator Sonoplus Ultrasonic Homogenizer HD2070 (Bandelin, Berlin, Germany). Precleared chromatin was incubated overnight with the corresponding antibody and antibody–protein–DNA complexes were collected with the provided Protein G agarose beads. All oligonucleotides used as primers for the PCR were designed with the program Beacon Designer 5.1 and are listed in Table 1B.

2.7. Western blot

Methods for SDS-PAGE electrophoresis of cellular proteins, and transfer onto nitrocellulose membranes were done as previously described [20]. Briefly, control and C/EPBβ transfected HepG2 cells were lysed with RIPA buffer containing protease inhibitors (Complete Mini EDTA-free; Protease Inhibitor Cocktail Tablets, Roche Diagnostics, Mannheim, Germany). Lysates were centrifuged (13,000 × *g*, 4 °C, 15 min) and the protein content of the supernatants determined with the Bradford assay (Bio-Rad Laboratories, Inc.). Lysate aliquots (30 μg) were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were incubated with anti-C/EBPβ, washed with Tween 20 in PBS, incubated with peroxidase-conjugated secondary antibody and signal detected with a chemiluminescence-based system (ECL Plus, GE Healthcare). Immunodetection of GAPDH (L20, Santa Cruz) was used as loading control.

2.8. Statistics

Statistical differences were examined using the SPSS 16.0 for Windows (Chicago, IL). Results are expressed as mean ± SEM for five independent experiments and analyzed by Student's *t*-test.

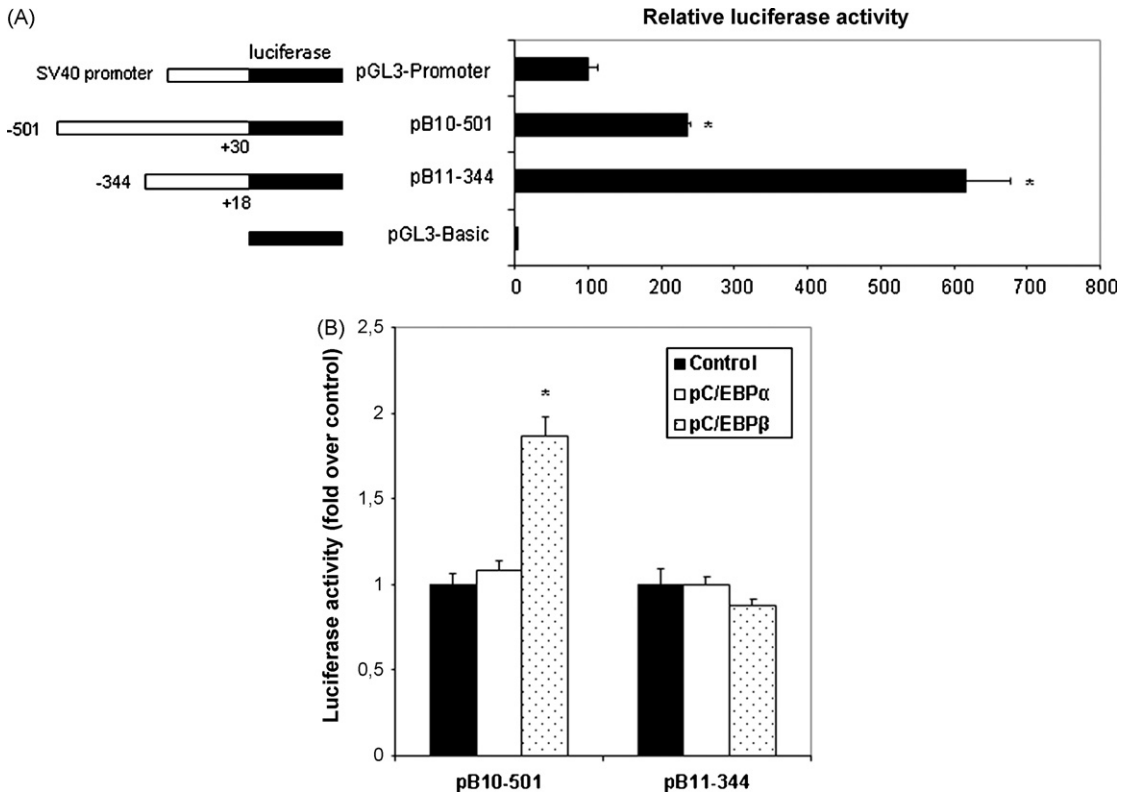

Fig. 3. Ectopic expression of C/EBPβ but not C/EBPα induces HSD17B10 transcriptional activity. (A) Transcriptional activity of the HSD17B10 and HSD17B11 promoter-luciferase chimeras. Luciferase activity is referred to the activity of the positive control pGL3-Promoter. (B) HepG2 cells were transiently transfected with 0.4 μg of the C/EBPα expression vector, C/EBPβ expression vector or the empty vector (control) and 0.4 μg of pB10-501 or pB11-344. Luciferase activity is referred to the activity of the constructs in the absence of the C/EBP expression vector. Values (mean ± SEM) were calculated from four independent experiments, each performed in triplicates. Significant differences to the reference construct ($p \leq 0.01$) are denoted by asterisks (*).

3. Results

3.1. Predictive nucleotide sequence analysis of the HSD17Bs gene promoters

To identify putative regulatory sequence elements in the human HSD17B1–14, we analyzed around 500 nucleotides of the 5′-flanking region of each gene covering its transcriptional start site. mRNA sequences and the complete genomic sequences of the genes were obtained from the NCBI database. Sequence alignment of the different HSD17Bs promoter genes showed no significant homology between these promoters (data not shown). Sequence analysis to search for transcription factors was performed with Alibaba 2.1 (http://www.gene-regulation.com/pub/programs/alibaba2) and Motif (http://www.motif.genome.jp) software based on TRANSFAC databases, and the Genomatix software (Genomatix Software, Munich, Germany). First, we searched for TATA box elements since we hypothesize that TATA-less promoters could use CCAAT boxes for transcription at basal levels. Previously, other groups described TATA box elements within the promoters of HSD17B1 (both gene and pseudo gene), 2 and 4 [11–13]. On the other hand, no TATA boxes were found in HSD17B5, 7, 8 and 12 [15,17,18,21]. We confirmed those results and also found no TATA box elements in the promoter of HSD17B10, 11, 13 and 14 (data not shown). As shown in Table 2, CCAAT boxes and binding sites for C/EBP family of transcription factors were found in all of them excluding HSD17B1 promoter. For instance, putative binding sites for C/EBP transcription factor family members were found in HSD17B2, 3, 5, 11, 13 and 14 promoters, C/EBPα binding sites were detected in HSD17B5 and 11 promoters, C/EBPβ binding sites were detected in HSD17B3, 4, 5, 10, 11 and 14 promoters and CCAAT boxes were found in HSD17B5, 7, 10 and 12 promoters. Taken together, these findings suggest that CCAAT boxes and/or C/EBP transcription factor family members binding sites might be involved in the transcriptional regulation of HSD17Bs.

3.2. C/EBPβ up-regulates HSD17B10 and 11 genes

To explore if these CCAAT boxes were involved in the regulation of these genes, we studied the effect of the ectopic expression of C/EBPα and C/EBPβ on the expression of HSD17Bs genes in HepG2 cells. After transfection of C/EBPα or C/EBPβ, cells were collected and mRNA was extracted to perform RT-qPCR. HSD17B8 expression is known to be controlled by C/EBPβ but not by C/EBPα [17]. Therefore, it was used as a control. HSD17B3 expression was undetectable under these conditions. Obtained results are shown in Fig. 1. Fig. 1A shows the effect of C/EBPα expression. We observed a 4-fold induction in the expression of HSD17B11. There were no significant changes in type 8 expression or any other genes analyzed. These results suggest that C/EBPα is involved in the regulation of HSD17B11 in HepG2 cells. Fig. 1B shows that transfection of a C/EBPβ expression vector significantly increases the expression of HSD17B10 and 11 by 2-fold. HSD17B8 expression, used as a positive control, also shows a 2-fold increase, which validates this data. This result suggests that C/EBPβ is involved in the regulation of these genes in HepG2 cells.

3.3. C/EBPβ induces HSD17B10 transcriptional activity

Regions suspected to contain human HSD17B10 and HSD17B11 promoter activity have not been analyzed so far. Computer analysis of about 1 kb of DNA sequence upstream of their respective initiation codon revealed that these regions contain no TATA box (Fig. 2).

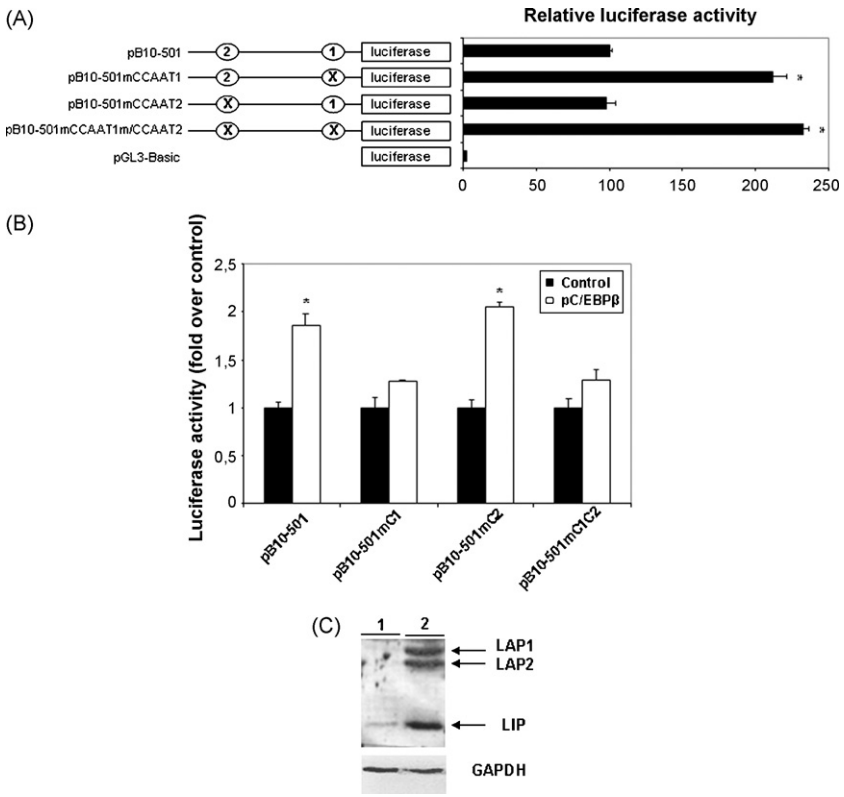

Fig. 4. The CCAAT box at −19/−30 is required for HSD17B10 transcriptional regulation by C/EBPβ isoforms. (A) Functional analysis of the CCAAT boxes detected at HSD17B10 5′-flanking sequence. Diagram of the constructs used (left) where putative CCAAT boxes are shown as ovals and referred as 1 (CCAAT box at −19) and 2 (C/EBPβ binding site at −428); mutations within the binding element are indicated by a cross. Luciferase activity is referred to the activity of the wild type pB10-501. Values (mean ± SEM) were calculated from a total of four independent experiments, each performed in triplicates. Significant differences to the reference construct are denoted by asterisks (*$p \leq 0.01$). (B) HepG2 cells were transiently co-transfected with pB10-501 mutated constructs and C/EBPβ expression vector or the empty vector. Luciferase activity is referred to the activity of the constructs in the absence of the C/EBPβ expression vector. (C) Western blot analysis of whole cell extracts obtained from HepG2 cells showing that untransfected cells (track 1) express only the isoform LIP while C/EBPβ-transfected cells (track 2) express both isoforms of LAPs and LIP. GAPDH was used as loading control.

However, putative CCAAT boxes as well as other transcription factor binding sites including Sp1, AP-1, GATA-1, NF-KB, SF-1 and RXR binding motifs, were detected (Fig. 2). To determine whether these sequences are functional promoters, 501 bp of the HSD17B10 5′-flanking region and 344 bp of the HSD17B11 5′-flanking region were cloned into pGL3-Basic vector to form pB10-501 and pB11-344, respectively. pB10-501 and pB11-344 were then transiently transfected into HepG2 cells and both of them displayed a strong transcriptional activity (Fig. 3A).

To test whether these promoter regions are involved in C/EBPs-mediated transcriptional regulation of HSD17B10 and HSD17B11, transient transfections with pB10-501 or pB11-344 and a C/EBPα or C/EBPβ expression vector were then performed in HepG2 cells. As shown in Fig. 3B, when the C/EBPβ expression vector was present pB10-501 luciferase activity increased by approximately 2-fold. However, pB11-344 luciferase activity could not be induced neither by C/EBPβ nor by C/EBPα expression vectors. These results confirm the transcriptional regulation of HSD17B10 by C/EBPβ and suggest that HSD17B11 up-regulation by C/EBPα and C/EBPβ in HepG2 cells is not mediated by its proximal promoter.

3.4. A CCAAT box is essential for the C/EBPβ mediated-stimulation of the HSD17B10 promoter

As aforementioned, HSD17B10 promoter contains a CCAAT box located at −30/−19 and a C/EBPβ binding site located at −428/−441. The role of these elements in the C/EBPβ-mediated HSD17B10 regulation was analyzed in transfection experiments in which intact and mutated constructs were employed. Obtained results are shown in Fig. 4. Surprisingly, mutation of the CCAAT box located at −30/−19 induced a 2-fold increase in the basal promoter activity (pB10-501mC1 and pB10-501mC1C2). However, no effect was observed when the C/EBPβ binding site located at −428/−441 was mutated. To further investigate the role of these motifs in the promoter activity of HSD17B10 gene, these constructs were co-transfected with C/EBPβ in HepG2 and promoter activity was measured. The transactivation observed in pB10-501 was impaired when the CCAAT box located at −30/−19 was mutated. Importantly, this transactivation was not affected by mutation of the −428/−441 C/EBPβ binding site, which confirms that this motif is not involved in the HSD17B10 promoter induction by C/EBPβ.

C/EBPβ mRNA gives rise to three different protein isoforms, the full length (38 kDa), a medium length liver activating protein LAP (35 kDa) and LIP (20 kDa). Full length and LAP isoforms contain both activation and bZIP domains and are considered to be transcriptional activators. LIP contains only the bZIP domain and acts as a dominant negative inhibitor [9]. Western blot analysis of HepG2 cells and HepG2 cells transfected with C/EBPβ expression vector showed that untrasfected HepG2 cells only expressed the 20 kDa inhibitory isoform LIP and that C/EBPβ expression vector induced the presence of the three isoforms (Fig. 4C). The fact that HepG2 cells only express the LIP form explains why mutation of CCAAT box located at −30/−19 induced the basal promoter activity and, more importantly, why C/EBPβ expression transactivated HSD17B10 promoter activity. All together, these results indicate that C/EBPβ regulates HSD17B10 expression in HepG2.

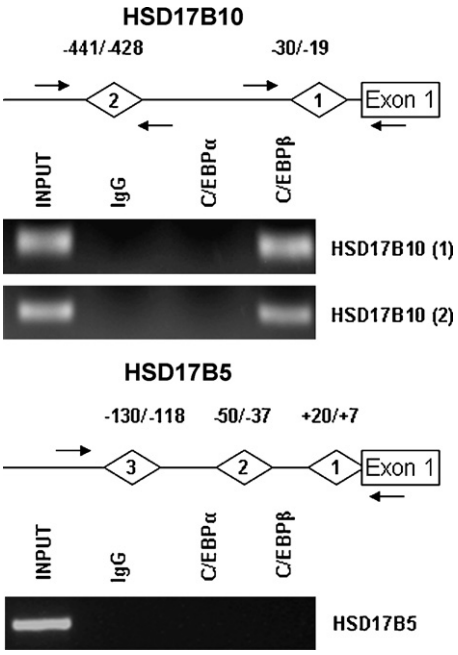

Fig. 5. C/EBPβ interacts with the HSD17B10 proximal promoter in HepG2 cells. Chromatin immunoprecipitation assays were performed in HepG2 cells using antibodies against C/EBPα, C/EBPβ or control IgG. Input tracks resulted form the amplification of soluble chromatin before immunoprecipitation. Oligonucleotides used for the PCR analysis are shown in Table 1B.

3.5. Chromatin immunoprecipitation (ChIP) assays identify recruitment of C/EBPβ in HSD17B10 in HepG2 cells

To further investigate HSD17B10 regulation by C/EBPβ, we studied if C/EBPβ is recruited by the CCAAT boxes present on its promoter. With this purpose we performed ChIP assay analysis in HepG2 cells. Since HSD17B5 does not respond to C/EBPβ (Fig. 1B) and its promoter has been described to be driven by Sp1 [15], regarding specificity of the C/EBPβ antibody HSB17B5 was used as a negative control. Obtained results are shown in Fig. 5. Recruitment of C/EBPβ was observed in both binding sites that HSD17B10 contains located at −30/−19 and −428/−441. This result confirms that C/EBPβ is involved in the transcriptional regulation of HSD17B10.

4. Discussion

In this study we analyze 500 bp of the 5′-flanking regions of HSD17B1–14 covering their transcriptional start sites, searching for CCAAT boxes and C/EBP family members binding motifs. Results show that all promoters analyzed have at least one of these motifs except HSD17B1. Ectopic expression of C/EBPβ in HepG2 cells showed increased expression of HSD17B10 and 11 (Fig. 1B) in HepG2 cells. We demonstrated that HSD17B10 and 11 5′-flanking regions are functional promoters (Fig. 3A). HSD17B10 promoter contains two putative binding sites for C/EBPβ located at −30/−19 and at −428/−441 relative to its start site (Table 2 and Fig. 2A). Transfection experiments of promoter-reporter chimeras showed that C/EBPβ increases HSD17B10 transcriptional activity by 2-fold (Fig. 3B) and site-directed mutagenesis confirmed that the CCAAT box located at −30/−19 is required for this induction (Fig. 4B). Fig. 5 shows recruitment of C/EBPβ to this binding site, which indicates that HSD17B10 expression is regulated by C/EBPβ and that this regulation is mediated by its binding to the proximal promoter of this gene.

C/EBPs belong to the bZIP class of basic domain transcription factors [22]. C/EBPβ has been implicated in a number of processes, including the inflammatory response as well as proliferation and differentiation of diverse tissues [23,24]. Importantly, C/EBPβ was shown to be a steroid-hormone regulated gene that is critical for uterine functions [25]. C/EBPβ is also involved in the transcriptional regulation of HSD11B1 [26]. HSD11B1 protein regenerates active glucocorticoids (cortisol and corticosterone) and increases intracellular glucocorticoid action in cells in which it is expressed [27]. Previously, we showed that C/EBPβ is involved in the transcriptional regulation of HSD17B8 [17]. Moreover, C/EBPβ participates in the E2-induced HSD17B8 expression. ERα interacts with C/EBPβ already bound to DNA to promote HSD17B8 transcription in response to E2 [20].

HSD17B10 has been implicated in a number of clinical conditions, from the pathogenesis of Alzheimer's disease to mild mental retardation or progressive infantile neurodegeneration [28]. HSD17B10 is a mitochondrial enzyme essential in the isoleucine breakdown pathway. Encoded by the SCHAD gene, its reduction or loss mediates mitochondrial toxicity and leads to enhanced apoptotic cell death [29]. Interestingly, HSD17B10 interacts with ERα in mitochondria, which suggests that ERα might be involved in regulating intracellular estrogens level by modulating HSD17B10 activity [30]. Even though HSD17B10 gene has been cloned [31], its promoter region has not been studied yet. Here, we show that C/EBPβ overexpression up-regulates HSD17B10 at mRNA levels (Fig. 1B) and promoter activity (Fig. 3B). We also show that different isoforms of C/EBPβ are involved in HSD17B10 regulation (Fig. 4). LIP and LAP ratio is critical in C/EBPβ-mediated gene transcription. The fact that only LIP expression was found in HepG2 cells (Fig. 4C) and that was recruited to HSD17B10 promoter (Fig. 5) explains why mutation of CCAAT box located at −30/−19 increased promoter basal activity (Fig. 4B). On the other hand, LAP expression transactivated HSD17B10, which indicates that LIP and LAP ratio is crucial for the regulation of this gene in HepG2 cells.

HSD17B11 protein converts 5α-androstan-3α, 17β-diol (3α-diol) to androsterone, suggesting it may be important in androgen metabolism [32]. Here we show evidence that proves that HSD17B11 is up-regulated by C/EBPα and C/EBPβ expression in HepG2 cells (Fig. 1A and B). HSD17B11 proximal promoter also contains two putative binding sites for C/EBP transcription factors located at −71/−84 and at −267/−280 (Table 2 and Fig. 2B). However, we also show that HSD17B11 up-regulation by C/EBPα and C/EBPβ is not mediated by its proximal promoter in HepG2 cells (Fig. 3B). Computer analysis of further sequence, up to 2 kb upstream the initiation codon revealed additional putative CCAAT boxes (−703/−713), C/EBP (−876/−888, −1187/−1200 and −1463/−1448) or NF-Y (−973/−985 and −1085/−1075) binding motifs (not shown). Additional work to test whether these motifs are involved in HSD17B11 up-regulation by C/EBPs is under progress.

In summary, ectopic expression of C/EBPβ induces HSD17B10 expression in HepG2 cells. A CCAAT box in the HSD17B10 proximal promoter, where recruitment of LIP has been detected, is required for this induction. Our results indicate that LIP and LAP C/EBPβ isoforms are involved in hepatic regulation of HSD17B10.

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