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Two 17β-hydroxysteroid dehydrogenases (17HSDs) of estradiol biosynthesis: 17HSD type 1 and type 7 ☆

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Abstract

Two 17β-hydroxysteroid dehydrogenases (17HSDs), type 1 and type 7, are enzymes of estradiol biosynthesis, in addition to which rodent type 1 enzymes are also able to catalyze androgens. Both of the 17HSDs are abundantly expressed in ovaries, the type 1 enzyme in granulosa cells and type 7 in luteinized cells. The expression of 17HSD7, which has also been described as a prolactin receptor-associated protein (PRAP), is particularly up-regulated in corpus luteum during the second half of rodent pregnancy. A moderate or slight signal for mouse 17HSD7/PRAP mRNA has also been demonstrated in samples of placenta and mammary gland, for example. Human, but not rodent, 17HSD1 is expressed in placenta, breast epithelium and endometrium in addition to ovaries. A cell-specific enhancer, silencer and promoter in the *hHSD17B1* gene participate in the regulation of type 1 enzyme expression. The enhancer consists of several subunits, including a retinoic acid response element, the silencer has a binding motif for GATA factors, and the proximal promoter contains adjacent and competing AP-2 and Sp binding sites.

1. Introduction

Ovary is the main source of estradiol (E2) in cycling humans and rodents. In the granulosa cells of developing follicles, cytochrome P450 aromatase (P450arom) catalyzes the aromatization of theca cell-derived androgens to estrogens, and 17β-hydroxysteroid dehydrogenase type 1 (17HSD1) converts estrone (E1) into highly active E2 [1–3]. After ovulation, follicles luteinize and transform into corpora lutea (CL), which secrete progesterone (P), E2 and peptide hormones, for example. In the case of fertilization, rat CL further mature as a result of prolactin (PRL) stimulation, and is essential for maintaining the pregnancy [4].

During pregnancy, E2 is still produced in rodent ovaries from ovarian and placental precursors, whereas in humans the placenta develops as a major source of E2. Human, but not rodent, placenta also expresses a high concentration of 17HSD1, which is evidently needed for E2 biosynthesis in human placental syncytiotrophoblasts [5]. Some rodent follicles mature and express 17HSD1 steadily throughout gestation [6], but 17HSD1 has not been detected in the CL of either cycling or pregnant rats, and its amount in ovaries

Abbreviations: CL, corpora lutea; CMV, cytomegalovirus; EGF, epidermal growth factor; EMSA, electrophoretic mobility shift assay; HEK, human embryonic kidney; 17HSD, 17β-hydroxysteroid dehydrogenase; HSD17B, 17HSD gene; LH, luteinizing hormone; P450arom, cytochrome P450 aromatase; PKA, protein kinase A; PKC, protein kinase C; PRAP, prolactin receptor associated protein; PRL, prolactin; RA, retinoic acid; RARE, RA response element; SDR, short chain dehydrogenase/reductase; A-dione, androstenedione (4-androstene-3; 17-dione); E1, estrone (3-hydroxy-1; 3,5(10)-estratrien-17-one); E2, estradiol (1; 3,5(10)-estratriene-3; 17β-diol); P, progesterone (4-pregnene-3; 20-dione); T, testosterone (17β-hydroxy-androsten-3-one).

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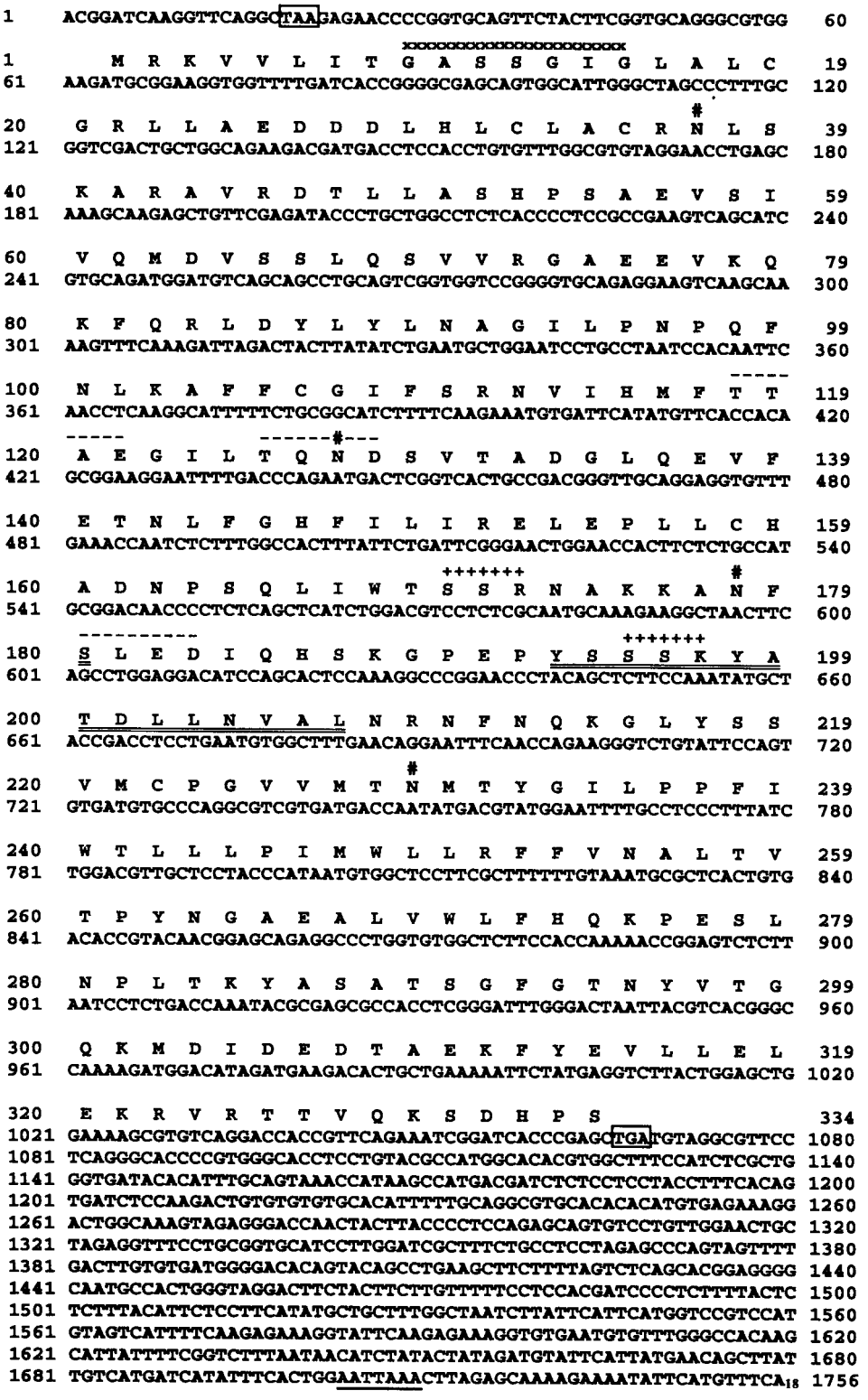

Fig. 1. Nucleotide and deduced amino acid sequence of m17HSD7. The glycine pattern and the conserved amino acids recognized as the SDR consensus sequence in the PROSITE database are shown with (xxx) and double underlining, respectively. Putative sites for N-linked glycosylation (♯), casein kinase II phosphorylation (—) and PKC phosphorylation (+ + +) are marked above the sequences. Stop codons are boxed and a potential polyadenylation signal is underlined.

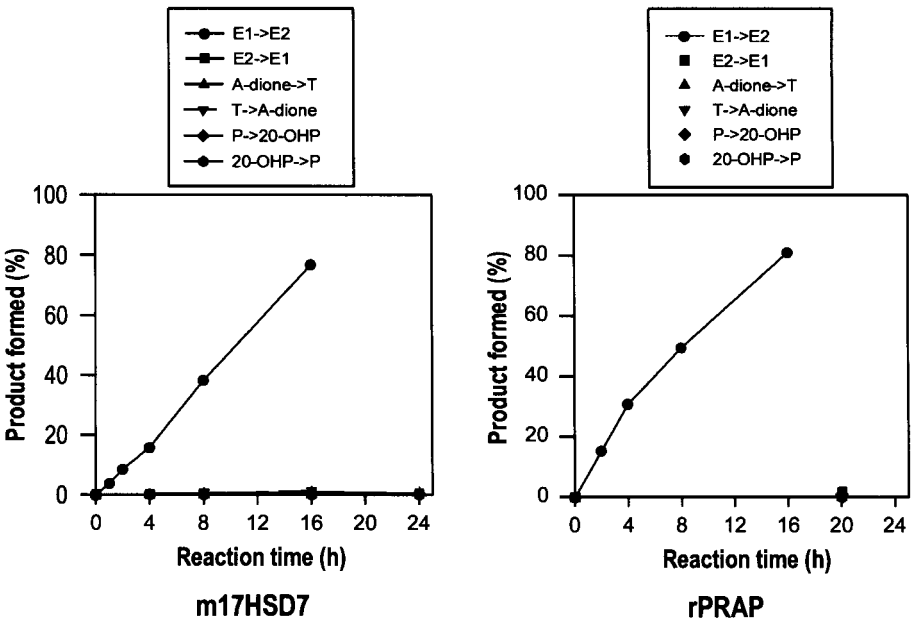

Fig. 2. Enzymatic characterization of m17HSD1 and rPRAP. HEK-392 cells grown in 6-well plates were transfected with 0.5 μg of m17HSD7.1-cDNA3.1 or 1.0 μg of rPRAP-CMV plasmid per well. 100 nM of substrate (E1, E2, A-dione, T, 20α-hydroxyprogesterone or P) was added to the culture media to determine the 17HSD and 20α-hydroxysteroid dehydrogenase activities. After the incubation periods shown in the panels, the media were collected and conversion to products was measured. The background activities observed were subtracted from the results.

does not correlate with the strongly increasing E2 production [2,6]. From mid-pregnancy until parturition, E2 is secreted mainly from CL [7]. Our results indicate that another 17HSD than the type 1 enzyme, chronologically named 17HSD type 7 (17HSD7), is responsible for the E2 biosynthesis in CL [8]. The novel cloned 17HSD is most abundantly expressed in the ovaries of pregnant animals, but is also detectable in some other tissues, such as placenta.

2. Cloning and characterization of 17HSD7

2.1. Expression cloning of m17HSD7

The HC11 cell line, which originates from the epithelial cells of the mammary gland of a pregnant mouse [9], was found to possess strong 17HSD activity. The cells effectively converted E1 to E2, but converted only poorly androstenedione (A-dione) to testosterone (T), whereas m17HSD1, for example, is known to catalyze the reduction reaction of both estrogens and androgens [10]. HC11 cells were not able to catalyze oxidative 17HSD reactions, either, and probes for known 17HSDs did not recognize any mRNAs in HC11 cell samples. In order to clone the novel type of 17HSD enzyme, an expression cDNA library was prepared from poly(A)$^+$ enriched RNA of the HC11 cell line, and a total of 600000 independent clones from the library were screened by monitoring their capability to convert E1 to E2.

Two independent clones, which included identical open reading frames, were isolated. The open reading frame encoded a peptide of 334 amino acids with a predicted molecular mass of 37317 Da, and its protein product was named m17HSD7 [8]. Similarly to the majority of 17HSDs, 17HSD7 belongs to the short-chain dehydrogenase/reductase (SDR) superfamily containing the glycine pattern (Gly9-Xaa-Xaa-Xaa-Gly13-Xaa-Gly15 in m17HSD7) and the three critical amino acid residues (Ser180, Tyr193 and Lys197) characteristic of SDR enzymes (Fig. 1) [11,12].

2.2. m17HSD7 shows 89% identity with rat prolactin receptor associated protein (PRAP) and is an enzyme of E2 biosynthesis

The identity of m17HSD7 with the other mouse 17HSD enzymes ranges within 18–28%, which is characteristic of SDR members [11]. Instead, screening of the protein databases with the m17HSD7 amino acid sequence revealed an 89% identity between m17HSD7 and a protein called Prolactin Receptor Associated Protein (PRAP) recently cloned from the rat [13]. Rat PRAP (rPRAP) is a microsomal phosphoprotein, which has been found to be associated with the short form of the PRL receptor, but whose function has remained unclear [13–15]. A comparison of the amino acid sequence of rPRAP with 17HSDs showed that it also contains the SDR consensus sequence, including the three highly conserved amino acid residues, and the glycine pattern, which are

needed for 17HSD activity [16]. On the other hand, m17HSD7 contains several putative post-translational modification sites, such as four N-glycosylation sites at positions 37, 127, 178 and 229, three casein kinase II phosphorylation sites at positions 118, 125 and 180, and two protein kinase C (PKC) phosphorylation sites at positions 170 and 195 (Fig. 1), similarly to rPRAP [13]. Characterization of the enzymatic properties of m17HSD7 and rPRAP finally demonstrated the close relationship between the proteins. m17HSD7 and reverse transcriptase-PCR generated rPRAP cDNA were expressed under a cytomegalovirus (CMV) promoter in human embryonic kidney 293 (HEK-293) cells. As shown in Fig. 2, both m17HSD7 and rPRAP catalyze exclusively the reductive reaction from E1 to E2, strongly indicating that rPRAP is the rat counterpart of 17HSD7. The enzymes will hereafter be referred to as 17HSD7/PRAP.

The substrate specificities of rodent 17HSD7/PRAP enzymes resemble that of human 17HSD1, but differ from rodent type 1 enzymes, which also catalyze androgens efficiently [10,17]. A comparison of the efficacies of m17HSD1 and m17HSD7, in turn, indicated that the 17HSD activity of the type 7 enzyme is relevant, i.e., of the same magnitude as that of 17HSD1, and thus a noteworthy role for the type 7 enzyme in E2 biosynthesis can be presented [8]. In the presence of excess NADP+, a cell homogenate enriched with 17HSD7/PRAP is also able to catalyze, to some extent, the reverse reaction from E2 to E1. The V_{max}/K_m values, 244 for E1 and 48 for E2, and particularly the results obtained in vivo, however, show that 17HSD7/PRAP evidently prefers E1 over E2 as a substrate. 17HSD7/PRAP is hence one of the reductive 17HSD enzymes, as are the types 1 and 3 [8].

2.3. 17HSD7/PRAP is abundantly, but not exclusively, expressed in CL of pregnant animals

In Northern blot analysis, mouse and rat 17HSD7/PRAP cDNA probes recognized several mRNAs 1.2–4.6 kb in size, of which the 4.6 kb (mouse) and 4.3 kb (rat) transcripts are the dominant forms [8]. Thus, there is a remarkable difference in size between the cDNAs (1.7 kb for mouse cDNA and 1.8 kb for rat cDNA) and the major mRNAs [8,13]. A possible explanation for the difference in the lengths of the cDNAs and the major mRNAs is an exceptionally long 5′-noncoding sequence, or a 3′-noncoding region including more than one polyadenylation signal. Nevertheless, the results of functional studies and an in-frame stop codon in the 5′-noncoding area confirm that the m17HSD7/PRAP cDNA contains the whole coding area.

The 4.6 kb m17HSD7/PRAP mRNA is most distinctly expressed in the ovaries and placenta, in addition to which it is detectable in samples of mammary gland, liver, kidney and testis and in HC11 cells [8]. The enzyme is expressed abundantly in the ovaries of pregnant mice. The expression of the enzyme has therefore been followed in more detail in rat ovaries during pregnancy. In agreement with the results of Duan and co-workers [15], the 4.3 kb r17HSD/PRAP mRNA has been found to be distinguishable in total RNA samples taken throughout the first part of pregnancy, on days 1 to 5, whereas on day 8 its expression is remarkably up-regulated. The expression of r17HSD/PRAP mRNA continues at a high level until close to parturition, but on day 21 the expression of 17HSD7/PRAP is again barely visible [8]. Antibodies against r17HSD/PRAP have recognized the enzyme mostly in CL samples [14,15]. In situ hybridization analyses also demonstrate the expression of 17HSD7/PRAP in CL (Nokelainen et al., unpublished data).

During rodent pregnancy, some follicles continue to mature and granulosa cells therefore secrete E2, but the biosynthesis of E2 mainly takes place in CL. In the first half of rat pregnancy, steroidogenesis in CL is stimulated by PRL and luteinizing hormone (LH), and E2 is synthesized from ovarian androgen precursors [4, and refs therein]. At mid-pregnancy, LH and pituitary PRL are down-regulated, and a luteal-placental shift takes place, as a result of which, the placenta begins to produce the androgen precursors needed for increased E2 synthesis in the ovaries. The abundant expression of 17HSD7/PRAP from day 8 onwards and during the second half of pregnancy coincides first with LH-induced E2 production from CL and then with enhanced A-dione secretion from the placenta [4], the expression of P450arom in CL [18] and rising serum E2 concentrations [7]. Altogether, the data strongly suggest that 17HSD7/PRAP is the enzyme required for E2 biosynthesis in CL. 17HSD7/PRAP is thus obviously a steroidogenic enzyme, but it is also capable of binding the short form of PRL receptor, and the potential role of 17HSD7/PRAP in PRL signaling is an interesting issue [13–15]. The E2 and PRL pathways are known to be connected at several levels. Both long and short forms of the PRL receptor are expressed in CL throughout rodent pregnancy [19], and PRL or PRL-like hormones, decidual lutropin and placental lactogen, and E2 act together on CL throughout rodent pregnancy. PRL and PRL-like hormones also maintain high concentrations of estrogen receptor in CL [4, and refs therein] and up-regulate 17HSD7/PRAP expression [15], which also demonstrates cross-talk between the E2- and PRL-signaling systems in CL.

In addition to being expressed in the ovaries, 17HSD7/PRAP is expressed in the mammary glands of pregnant mice to some extent as well as in HC11 cells, which are also derived from the epithelial cells of

the mammary gland of a pregnant animal. 17HSD7 may therefore catalyze circulating E1 to E2 locally in the mammary gland in the same way as 17HSD1 has been suggested to do in human breast epithelial cells [20 and refs therein]. The presence of 17HSD7/PRAP in mouse placenta suggests that some E2 biosynthesis may also occur in rodent placentas. In contrast to human placenta, rodent placentas have been assumed to be unable to synthesize E2 due to their lack of P450arom [21] and 17HSD1 [6,10]. 17HSD7/PRAP, however, may catalyze circulating E1 to E2 for targeted needs, whereas oxidative 17HSD2, which is abundantly expressed in rodent and human placentas, is suggested to prevent the transfer of active 17β-hydroxy forms of sex steroids between the fetus and the maternal circulation [22,23].

Based on the enzymatic character of 17HSD7/PRAP and its expression pattern, which shows developmental regulation in the ovary during gestation, we suggest that 17HSD7/PRAP is responsible for the final step in the biosynthesis of E2 in CL, whereas 17HSD1 catalyzes E1 to E2 in developing granulosa cells. Both enzymes are also expressed to some degree in mammary gland and placenta, for example, possibly participating in local biosynthesis in these tissues. Further characterization of the mechanisms regulating their expression will elucidate how their action is targeted to each cell and tissue type.

3. Regulation of 17HSD1 gene expression

Expression of 17HSD1 shows species-specific differences. Partially distinct mechanisms may also control tissue- and cell-specific regulation of the enzyme. In ovaries, 17HSD1 expression is primarily induced by follicle-stimulating hormone acting via the protein kinase A (PKA)-dependent pathway, and the extent of induction is further modulated by PKC-dependent inhibition, estrogens, androgens and autocrine/paracrine growth factors present in the ovary [2,24,25]. Luteinizing agents, in turn, cause a sharp drop in 17HSD1 expression. In human placental trophoblast-like choriocarcinoma cells, the retinoic acids (RAs) together with the epidermal growth factor (EGF) and PKA and PKC activators cause a considerable increase of 17HSD1 concentration and may consequently be involved in maintaining high 17HSD1 expression in placental syncytiotrophoblasts [26]. Several paracrine, autocrine and nutritional factors which regulate 17HSD1 expression in the placenta and/or ovaries are also available in breast tissue, as are their receptors. RAs also increase type 1 expression in the T47D breast cancer cell line, but PKA and PKC activators and EGF either decrease or have no effect on the RA-dependent increase in 17HSD1 expression [26].

3.1. Three functional elements have been identified in the hHSD17B1 gene

The human 17HSD1 gene, *hHSD17B1*, is situated in chromosome 17q21 in tandem with *hHSD17BP1*, a putative pseudogene. The *hHSD17B1* gene is transcribed into two mRNA transcripts, 1.3 and 2.3 kb in size, due to the two transcription start points situated about 1 kb apart from each other [27,28]. The function of the longer mRNA remains obscure, since its constitutive expression in several tissues and cell lines has minor association with the presence of the 17HSD1 protein [28–31]. The expression and amount of 1.3 kb mRNA, instead, largely correlates with the concentration of the 17HSD1 protein and is subject to regulation [26,29–32]. Similarly to the human gene, the rat *rHSD17B1* gene is also transcribed into two mRNAs, 1.4 and 1.7 kb in size, but they are caused by two polyadenylation signals rather than by two promoters and are thus regulated in parallel [2,33,34].

Three functional elements have been identified between the two transcription start points in *hHSD17B1*, i.e., upstream from the cap site for the shorter transcript, and they evidently affect the transcription of 1.3 kb mRNA [35]. The fragment $-78/+9$, with respect to the cap site for 1.3 kb mRNA, contains a promoter, and the region from -661 to -392 enhances the function of the promoter, whereas the action of the area between the enhancer and the promoter leads to decreased activity of the *hHSD17B1* gene [35,36].

3.2. hHSD17B1 enhancer is cell-specific and contains a retinoic acid response element

The function of the *hHSD17B1* enhancer is independent of its orientations, the promoter it has been linked with, and the distance between the promoter and the enhancer. Moreover, the enhancer acts in a cell-specific manner. It may be essential for the expression of 17HSD1 in JAR and JEG-3 choriocarcinoma cells and, consequently, for abundant placental expression of the enzyme [35]. The *hHSD17B1* enhancer is also active in certain breast cancer cell lines containing endogenous 17HSD1, but not in the cell lines which do not express endogenous 17HSD1, which further indicates the authenticity of the enhancer [35]. The *hHSD17B1* enhancer and the analogous area in the *hHSD17BP1* gene share 98% nucleotide identity, whereas the overall identity between the genes is 89%. There are thus only five dissimilar nucleotides in the *hHSD17B1* enhancer and the *hHSD17BP1* analogue, but the former increases thymidine kinase promoter activity with tenfold efficiency compared with the latter. Reporter gene analyses with mutated gene constructs indicated that two nucleotides of the five inflict

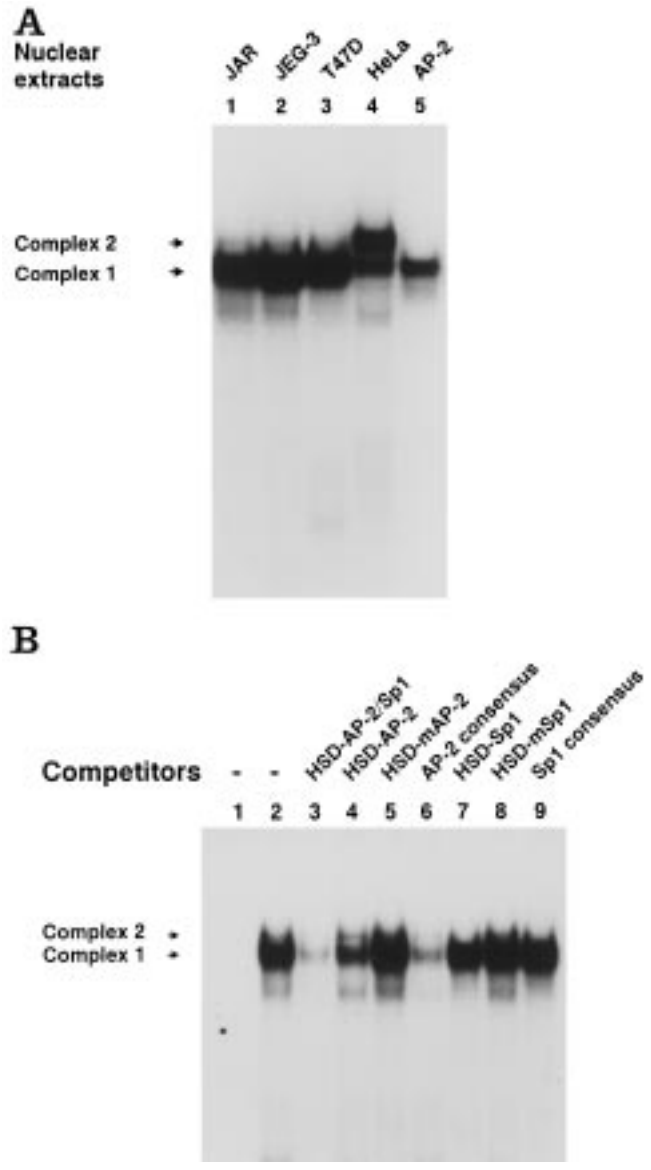

Fig. 3. EMSA for the HSD-AP-2-Sp1 fragment. (a) Interaction of the HSD-AP-2/Sp1 fragment with JAR, JEG-3, T47D and HeLa nuclear extracts and an AP-2 rich extract. 5 μg of each protein extract was incubated together with ^{32}P-labeled probe, and the complexes formed were separated in 5% polyacrylamide gel. The arrows on the left indicate the positions of the binding complexes. (b) Effect of various fragments on the binding between the HSD-AP-2/Sp1 fragment and the JAR nuclear extract. Lane 1 shows the free probe, lane 2 represents interaction of the probe with the JAR nuclear extract, and lanes 3–9 demonstrate the complexes formed when unlabeled oligonucleotide competitors were included in the binding reaction in 100-fold molar excess over the probe.

the ineffectiveness of the *hHSD17BP1* analogue (Leivonen et al., unpublished data).

The importance of the two nucleotides for the function of the *hHSD17B1* enhancer and the results of DNaseI footprinting analyses (Leivonen et al., unpublished data) and reporter gene assays with partial enhancer fragments [35] suggest that the *hHSD17B1* enhancer consists of several essential subelements. The *hHSD17B1* enhancer also contains an element for the binding of RARα/RXRα heterodimers [35]. Administration of *all-trans*-RA increases the activity of the *hHSD17B1* enhancer several-fold in T47D and JEG-3 cells [35,37], whereas the mutation of the RA response element (RARE) cancels the response to RAs, which verifies direct regulation of the *hHSD17B1* gene by RAs [35]. RAs, which have been reported to enhance 17HSD1 expression in JEG-3 choriocarcinoma and T47D breast cancer cells [26,32], hence very probably act via the identified RARE in the *hHSD17B1* enhancer.

3.3. hHSD17B1 proximal promoter contains adjacent and competing motifs for AP-2, Sp1 and Sp3

The *hHSD17B1* fragment −78/+9 generates detectable reporter gene expression in several cell lines [37], and particularly in connection with the *hHSD17B1* enhancer or the Simian virus 40 enhancer [35]. The region contains a sequence typical of a TATA box and a GC-rich area [28,38], which includes adjacent binding sites for Sp and AP-2 transcription factors [36]. The *hHSD17B1* fragment containing both Sp and AP-2 binding motifs and surrounding areas (HSD-AP-2/Sp1) makes up two major complexes with proteins in JAR, JEG-3, T47D and HeLa nuclear extracts. Complex 1 matches with the size of the AP-2 complex (Fig. 3a), and the formation of the radiolabeled complex can be reduced by an unlabelled AP-2 consensus sequence and an HSD-AP-2 fragment in electrophoretic mobility shift assay (EMSA) (Fig. 3b, [36]). Furthermore, AP-2 antibodies in the EMSA mixture retards the mobility of the complex, verifying the identification of the binding protein as AP-2 [36].

The most abundant accumulation of Complex 2 is detected when a HeLa extract, which is rich in the Sp1 factor, is used (Fig. 3a). In EMSA, formation of the labeled complex can be prevented by an unlabelled Sp consensus sequence and an HSD-Sp1 fragment, but not by its mutated counterpart (Fig. 3b). Further analyses have shown altogether three complexes, the mobility of which could have been slowed down by Sp1 and Sp3 antibodies, but not by Sp2 and Sp4 antibodies [36]. In addition, the formation of Sp1 and Sp3 complexes increases when the adjacent AP-2 motif is mutated. Finally, mutation of the Sp motif hinders the binding of proteins from the nuclear extracts to the HSD-AP-2/mSp1 fragment and decreases the *hHSD17B1* promoter activity to 30% in JEG-3 cells and to 60% in JAR cells. Binding to the Sp motif may thus have a important role in the overall functioning of the *hHSD17B1* promoter, which is in line with the observations showing that *hHSD17B1* promoter activity is low in certain cell lines expressing small

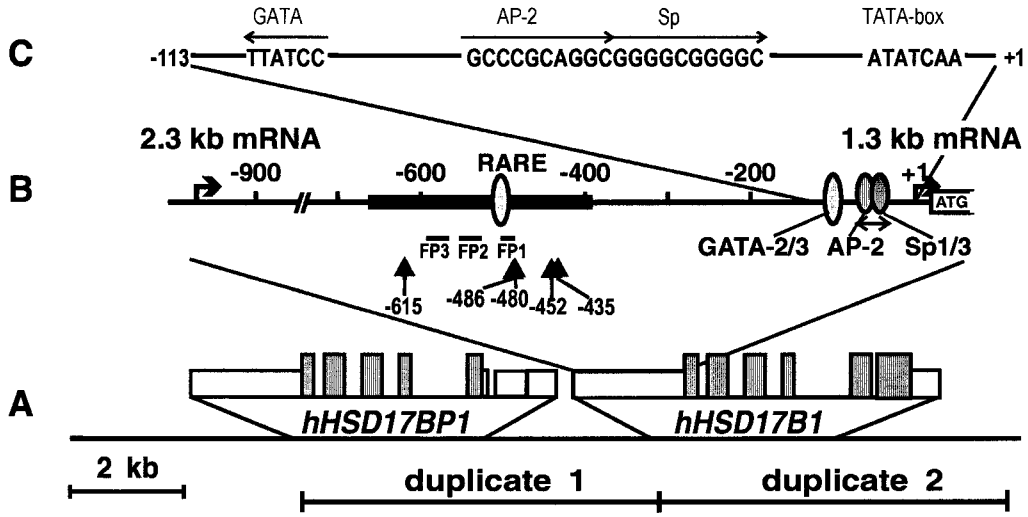

Fig. 4. Schematic presentation of the *hHSD17B1* gene and its regulatory elements. Level A: Due to the ancient gene fragment duplication, the *hHSD17B1* gene exists in tandem with the *hHSD17BP1* gene. The grey boxes demonstrate the coding area of the exons in the *hHSD17B1* gene and the analogous areas in the *hHSD17BP1* gene. The white boxes show the non-coding areas of the exons. Level B: Several regulatory elements and binding motifs have been identified between the two transcription start sites marked with arrow heads. The black box represents the *hHSD17B1* enhancer, which contains a RARE shown with an ellipse. The regions protected in DNase I footprinting analyses (FP1-3) are marked with bars, and the position of five nucleotides different in the *hHSD17B1* enhancer and the *hHSD17BP1* analogue are shown with arrows. The positions of the binding sites for the GATA, Sp and AP-2 factors are also demonstrated with ellipses. ATG is the translation initiation codon. Level C: Detailed presentation of the 5′-proximal region of the *hHSD17B1* gene and the binding motifs.

amounts of Sp1. Instead, mutation of the AP-2 element increases promoter activity to 260% in JEG-3 cells. This implies, together with the data indicating that the binding of AP-2 to its motif results in reduced binding of Sp1 and Sp3, that AP-2 can repress the function of the *hHSD17B1* promoter by preventing binding to the Sp motif [36].

Sp1 and Sp3 are widely distributed transcription factors with identical affinities to the GC-rich Sp motif [39]. Sp1 activates a large selection of cellular and viral promoters. It also interacts with several other regulatory factors and, consequently, can mediate cell- and gene-specific effects on the promoter of the target genes [40,41]. One such target is the human chorionic somatomammotropin gene, which is, similarly to *hHSD17B1*, expressed in syncytiotrophoblasts [42]. Instead, Sp3 can repress Sp1-mediated transcriptional activation by competing with Sp1 for their common binding site and is able to increase transcription in only a few cases [43,44]. The AP-2 factor, in turn, is activated by several signaling pathways, such as PKC and cAMP [45]. Further investigations may clarify the mutual interactions of Sp1, Sp3 and AP-2 and their possible role in controlling the tissue- and cell-specific transcription of the *hHSD17B1* gene.

3.4. The binding site for GATA factors is located in the hHSD17B1 silencer

Deletion of the region −391/ − 79 or −113/ − 79 from the 5′-fragments of the *hHSD17B1* gene results in elevated reporter gene expression [35,37]. Also, in connection with the SV40 enhancer, shortening of the *hHSD17B1* gene promoter from −113 to −78 leads to a significant increase in reporter gene expression in all the cell lines tested [35,37]. In combination, these results point to the presence of a silencer between the bases −392 and −78 and suggest that an essential part of it is situated between −113 and −78.

The region −103/ − 98, within the assumed silencer, contains a binding motif for GATA factors, and the transcription factors GATA-2 and, in particular, GATA-3 are able to bind to that sequence in JEG-3 cells [36]. Mutation of the motif leads to decreased binding of the GATA proteins and increased transcriptional activity, which is equal to that of constructs not containing the silencer element. GATA-2 and GATA-3 may thus repress the function of the *hHSD17B1* gene, at least in the constructs used, and the cognate motif may be an essential part of the *hHSD17B1* silencer [36]. GATA-2 and GATA-3 are expressed in placental trophoblasts and are required for the trophoblast-specific expression of placental lactogen I genes, for example [46]. GATA factors may hence be involved in limiting the function of the *hHSD17B1* gene in the placenta [36]. Altogether, at least the cell-specific enhancer containing several interacting subunits and an RARE element, a silencer element with a GATA motif and a proximal promoter region with competing Sp and AP-2 sites participate in the regulation of *hHSD17B1* gene

expression and may guide its cell- and tissue-specific expression (Fig. 4) [35,36].

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