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STRUCTURE, FUNCTION AND TISSUE-SPECIFIC GENE EXPRESSION OF 3β-HYDROXYSTEROID DEHYDROGENASE/5-ENE-4-ENE ISOMERASE ENZYMES IN CLASSICAL AND PERIPHERAL INTRACRINE STEROIDOGENIC TISSUES

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Summary—The membrane-bound enzyme 3β-hydroxysteroid dehydrogenase/5-ene-4-ene isomerase (3β-HSD) catalyses an essential step in the transformation of all 5-pregnen-3β-ol and 5-androsten-3β-ol steroids into the corresponding 3-keto-4-ene-steroids, namely progesterone as well as all the precursors of androgens, estrogens, glucocorticoids and mineralocorticoids. We have recently characterized two types of human 3β-HSD cDNA clones and the corresponding genes which encode type I and II 3β-HSD isoenzymes of 372 and 371 amino acids, respectively, and share 93.5% homology. The human 3β-HSD genes containing 4 exons were assigned by *in situ* hybridization to the p11–p13 region of the short arm of chromosome 1. Human type I 3β-HSD is the almost exclusive mRNA species present in the placenta and skin while the human type II is the predominant mRNA species in the adrenals, ovaries and testes. The type I protein possesses higher 3β-HSD activity than type II. We elucidated the structures of three types of rat 3β-HSD cDNAs as well that of one type of 3β-HSD from bovine and macaque ovary λgt11 cDNA libraries, which all encode a 372 amino acid protein. The rat type I and II 3β-HSD proteins expressed in the adrenals, gonads and adipose tissue share 93.8% homology. Transient expression of human type I and II as well as rat type I and II 3β-HSD cDNAs in HeLa human cervical carcinoma cells reveals that 3β-ol dehydrogenase and 5-ene-4-ene isomerase activities reside within a single protein. These expressed 3β-HSD proteins convert 3β-hydroxy-5-ene-steroids into 3-keto-4-ene derivatives and catalyze the interconversion of 3β-hydroxy and 3-keto-5α-androstane steroids. By site-directed mutagenesis, we demonstrated that the lower activity of expressed rat type II compared to rat type I 3β-HSD is due to a change of four residues probably involved in a membrane-spanning domain. When homogenates from cells transfected with a plasmid vector containing rat type I 3β-HSD is incubated in the presence of dihydrotestosterone (DHT) using NAD^+ as co-factor, 5α-androstanedione was formed (A-dione), indicating an intrinsic androgenic 17β-hydroxysteroid dehydrogenase (17β-HSD) activity of this 3β-HSD. We cloned a third type of rat cDNA encoding a predicted type III 3β-HSD specifically expressed in the rat liver, which shares 80% similarity with the two other isoenzymes. Transient expression in human HeLa cells reveals that the type III isoenzyme does not display oxidative activity for the classical substrates of 3β-HSD. However, in common with the type I enzyme, it converts A-dione and DHT to the corresponding 3β-hydroxysteroids, thus showing an exclusive 3-ketosteroid reductase activity. When NADPH is used as co-factor, the affinity for DHT of the type III enzyme becomes 10-fold higher than that of the type I. Rat type III mRNA was below the detection limit in intact female liver. Following hypophysectomy, its concentration increased to 55% of the values measured in intact or hypophysectomized male rats, an increase which can be blocked by administration of ovine prolactin (oPRL). Treatment with oPRL for 10 days starting 15 days after hypophysectomy markedly decreased ovarian 3β-HSD mRNA accumulation accompanied by a similar decrease in 3β-HSD activity and protein levels. Treatment with the gonadotropin hCG reversed the potent inhibitory effect of oPRL on these parameters and stimulated 3β-HSD mRNA levels in ovarian interstitial cells. These data indicate that the presence of multiple 3β-HSD isoenzymes offers the possibility of tissue-specific expression and regulation of this enzymatic activity that plays an essential role in the biosynthesis of all hormonal steroids in classical as well as peripheral intracrine steroidogenic tissues.

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OUTLINE

1. INTRODUCTION

Despite its essential role in the biosynthesis of all classes of hormonal steroids, the structure of 3β-hydroxysteroid dehydrogenase/5-ene-4-ene-isomerase, hereafter called 3β-HSD, was the last one to be elucidated among the main steroidogenic enzymes [1]. This enzyme is required for the biosynthesis of progesterone, glucocorticoids, mineralocorticoids as well as androgens and estrogens (Fig. 1). 3β-HSD is found in steroidogenic tissues, namely the placenta [1–3], adrenal cortex [2, 4–6], ovary [2, 5, 7–11], and testis [2, 6, 8, 12, 13], and in several peripheral tissues, including the skin [2, 14–20], adipose tissue [2], breast [2, 12, 21], lung [22], endometrium [23], prostate [24, 25], liver [2, 8, 26], kidney [2, 8], epididymis [8] and brain [27]. The widespread distribution of 3β-HSD expression indicates that this enzyme is likely to play an important role in the intracrine [28] formation of sex steroids in a large series of peripheral target tissues. Such a high level of extragonadal formation of sex steroids is especially important in the human and some other primates whose adrenals possess the unique property of secreting large amounts of precursor sex steroids such as dehydroepiandrosterone (DHEA) and especially DHEA sulfate [28–33, and refs therein].

Congenital adrenal hyperplasia is the most frequent cause of ambiguous genitalia and adrenal insufficiency in newborn infants [34–45]. In its classical form, this autosomal recessive disease is associated with varying degrees of salt-wasting and genital ambiguity in both sexes.

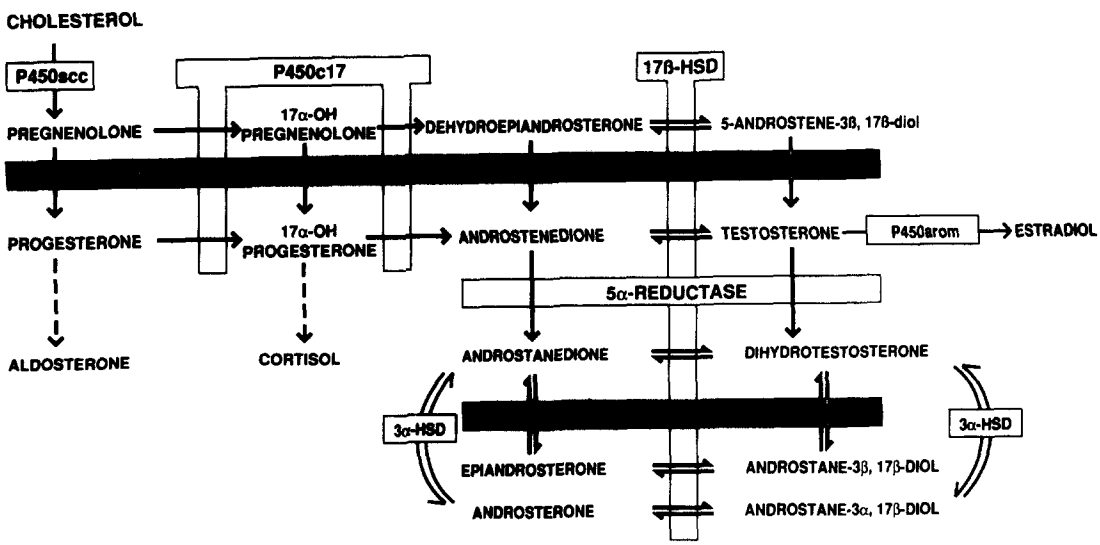

Fig. 1. Schematic representation of major steroidogenic pathways involved in the formation of PROG, the estrogens 5-androst-ene-3β-17β-diol and estradiol as well as the androgens testosterone and DHT and others indicated C-19 steroids. *P*450scc: cytochrome *P*450 side chain-cleavage enzyme: 3β-HSD: 3β-hydroxysteroid dehydrogenase/5-ene-4-ene isomerase; *P*450c17: cytochrome *P*450 17α-hydroxylase/17-20 lyase; 17β-HSD: 17β-hydroxysteroid dehydrogenase; *P*450 arom: cytochrome *P*450 aromatase.

On the other hand, signs of nonclassical 3β-HSD deficiency can vary from premature puberty and accelerated growth in children, to hirsutism, acne, temporal balding, irregular menses and infertility [35, 45–47]. The incidence of nonclassical 3β-HSD deficiency among women with signs of androgen excess as recently reviewed in the literature is about 1/6 [45]. The wide phenotypic heterogeneity of 3β-HSD deficiency syndromes and several clinical observations in these patients indicating intact peripheral and/or hepatic 3β-HSD activity suggest the existence of multiple isoenzymes and/or tissue-specific regulation of 3β-HSD gene expression.

2. HUMAN 3β-HSD ISOENZYMES AND THEIR GENES

2.1. Structure of human types I and II 3β-HSD cDNAs

Following purification of 3β-HSD from human placenta and development of antibodies against the enzyme in rabbits [3, 48], we have isolated and characterized a first cDNA type [1] and its corresponding gene [12]. These sequences have been confirmed by Lorence *et al.* [49, 50]. Northern blot analysis of RNA from human adrenals, gonads, placenta and mammary gland using human 3β-HSD cDNA as a probe identified a single 1.7 kb mRNA species [12]. However, the detection of multiple unexpected DNA fragments by Southern blot analysis of human genomic DNA [12] and the heterogeneous clinical picture in 3β-HSD deficient patients suggest the presence of multiple 3β-HSDs in the human [35]. We screened a human adrenal λgt22A cDNA library with human placental cDNA clone hp3β-HSD63 [1]. The 10 longest cDNA inserts were subcloned into the BSKS vector and sequenced by the dideoxy chain-termination method. The cDNA sequence of type II 3β-HSD includes an open reading frame of 1116 nucleotides compared to 1119 nucleotides for type I 3β-HSD cDNA. The second 3β-HSD cDNA type which corresponds to the almost exclusive mRNA species expressed in the adrenals and gonads and was chronologically designated as human type II 3β-HSD [51]. We have recently characterized the structure of the corresponding human type II 3β-HSD gene [52]. These genes were located by *in situ* hybridization at the p11–p13 region of chromosome 1 [53]. 187 residues are conserved in 10 3β-HSD sequences from 5 species indicating that this enzyme family is well conserved throughout the course of evolution (Fig. 2, Table 1).

The 3β-HSD enzyme is a membrane-bound protein located in the endoplasmic reticulum and in mitochondrial membranes [3]. Computer analysis performed according to Klein *et al.* [55] of the amino acid sequence of human type II 3β-HSD predicts two membrane-spanning segments extending from residues 73 to 90 and 286 to 304. These two transmembrane segments are also predicted using two other algorithms [56, 57], as previously reported for human type I, macaque and rat types I and III 3β-HSD proteins. In contrast, only the COOH-terminal membrane-spanning segment (~284–307; depending upon the program used) is predicted in bovine and rat type II 3β-HSD proteins [2, 8] (Fig. 3).

The sequences of mammalian 3β-HSD isoenzymes revealed no sequence similarity with 17β-hydroxysteroid dehydrogenase (17β-HSD), 11β-hydroxysteroid dehydrogenase, other dehydrogenases, cytochrome *P*450 enzymes or steroid binding proteins [58]. Moreover, a recent examination revealed a novel similarity between 3β-HSD and barley A1 protein, an NADPH-dependent reductase that converts dihydroquercetin to *cis*-leucocyanidin in barley and a bacterial cholesterol dehydrogenase, a bacterial UDP-galactose-4-epimerase as well as open reading frames in Vaccinia virus and fish lymphocystis disease virus [59]. The sequence similarity between bovine 3β-HSD and residues 3–232 of barley A1 protein indicates that these mammalian and plant enzymes may be derived from a common ancestor. The region of similarity between bovine 3β-HSD and barley dihydroflavonol reductase extends over more than half of each protein, which may be the binding site for the nucleotide cofactor.

2.2. Enzymatic characteristics of expressed human types I and II 3β-HSD isoenzymes

To verify that human types I and II 3β-HSD cDNAs encode a protein which catalyzes 3β-hydroxysteroid dehydrogenation and 5-ene-4-ene isomerization and to characterize functional differences between the two 3β-HSD types, plasmids derived from pCMV containing either type I (pCMV-type I h3β-HSD) or type II (pCMV-type II h3β-HSD) 3β-HSD cDNA inserts driven by the CMV promoter were transiently expressed in HeLa cells [51].

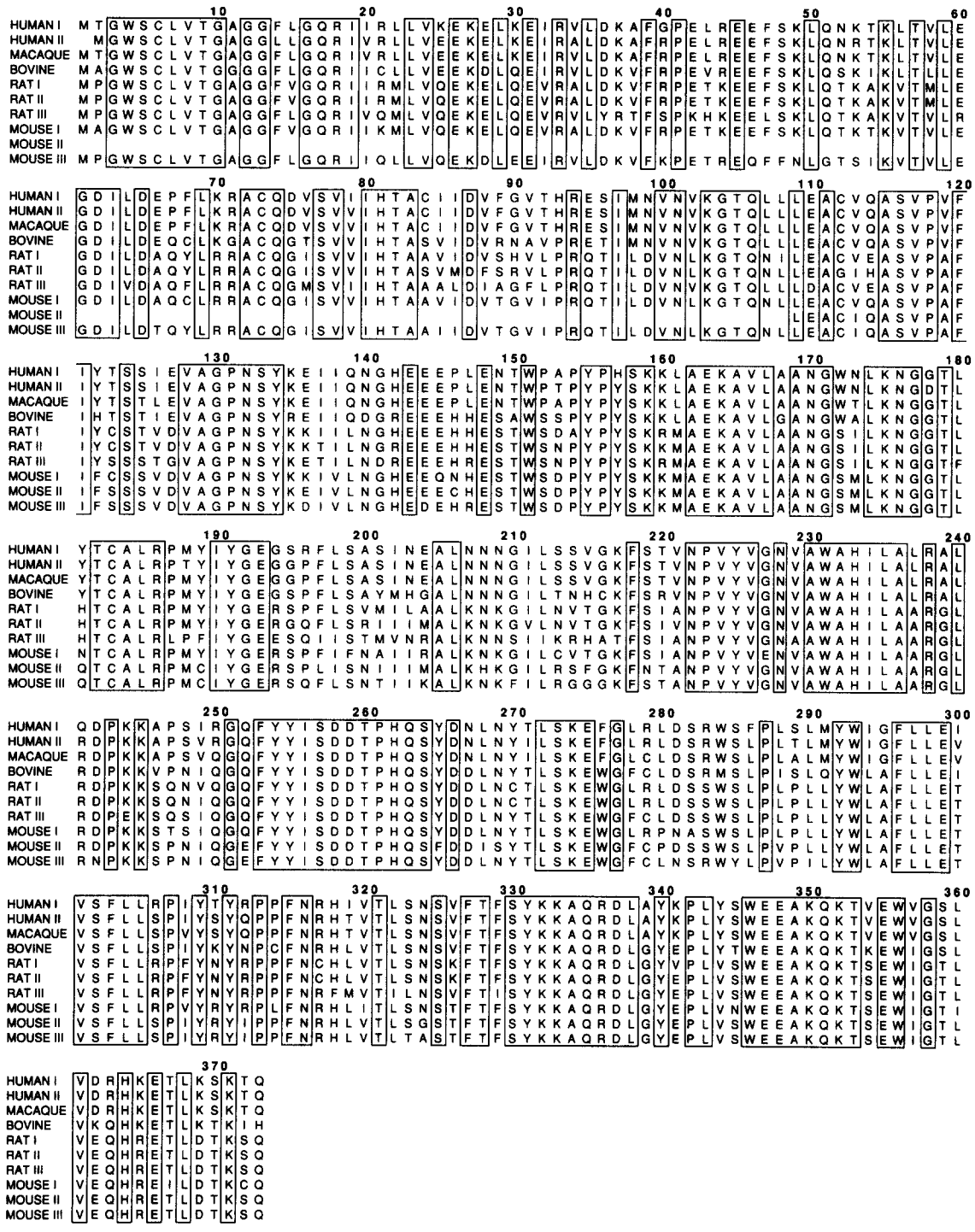

Fig. 2. Comparison of the deduced amino acid sequences of human type I [1], human type II [51] macaque [8], bovine [7, 69] rat types I, II and III [2, 26] as well as mouse types I, II and III [54] 3β-HSD proteins. Amino acid sequences are designated by the single-letter code. Amino acid residues are numbered relative to the first NH_2-terminal methionine. Residues common to all of the ten predicted sequences are boxed.

Transfection of HeLa cells with either type I or II h3β-HSD cDNA inserts resulted in the production of a single 42 kDa protein which cross-reacts with polyclonal antibodies raised against purified human placental 3β-HSD [1, 3, 48] and which co-migrates with purified human placental 3β-HSD.

In vitro incubation with homogenates from cells transfected with pCMV-type I h3β-HSD or pCMV-type II h3β-HSD in the presence of

Table 1. Percentage of homology between the deduced amino acid sequences and the corresponding nucleotide sequences of human type I [1], human type II [51], macaque [8], bovine [7] rat types I, II and III [2, 26] as well as mouse type I, II and III [54] 3β-HSD proteins

	Human I	Human II	Macaque	Bovine	Rat I	Rat II	Rat III	Mouse I	Mouse II	Mouse III
Human I		93.5	93.8	78.8	72.3	71.5	68.3	71.5	71.7	71.0
Human II	93.5		96.2	78.2	72.0	71.0	66.7	70.7	71.7	70.2
Macaque	94.4	95.2		79.6	73.1	72.0	68.0	71.5	72.5	71.2
Bovine	81.5	81.0	82.3		74.2	74.2	69.1	72.8	74.3	73.7
Rat I	78.3	77.8	79.4	77.7		93.8	80.4	87.9	83.4	82.0
Rat II	78.3	77.5	78.8	77.2	97.1		80.1	86.3	83.4	81.5
Rat III	75.3	75.4	76.6	75.3	85.7	85.6		76.6	77.4	75.5
Mouse I	77.4	76.3	78.1	75.8	91.2	91.0	83.0		85.3	83.3
Mouse II*	77.4	77.2	79.0	76.1	89.6	89.7	83.5	89.6		90.6
Mouse III	76.8	76.5	78.1	75.3	88.8	88.8	81.7	89.3	94.1	

*The data are obtained from the partial sequence of mouse II 3β-HSD. Protein homology data above the diagonal, nucleotide homology (coding region) below the diagonal.

1 mM NAD^+ and ^3H-labeled substrates showed that the type I enzyme possesses a 3β-HSD/5-ene-4-ene isomerase activity higher than type II with respective K_m values of 0.24 and 1.2 μM for pregnenolone (PREG), 0.18 and 1.6 μM for DHEA. The specific activity (V_{max}) of both types is equivalent when standardized for the estimated amount of translated proteins [51]. In incubation of cell homogenates in the presence of NADH and ^3H-labeled dihydrotestosterone (DHT), the 3β-hydroxysteroid oxidoreductase activity, as measured by the formation of 5α-androstane-3β,17β-diol is higher for type I than that of the type II 3β-HSD protein with K_m values of 0.26 and 2.7 μM, respectively. These data also show that the affinity of the human type II 3β-HSD protein is similar for the three substrates tested. Similar activity for the three substrates is also found for the type I protein. Analysis of the kinetic properties of both expressed 3β-HSD proteins reveals that the relative enzymatic activity (V_{max}/K_m) of type I is 5.9-, 4.5- and 2.8-fold higher than that of the type II 3β-HSD protein using PREG, DHEA and DHT as substrate, respectively.

2.3. Tissue-specific expression of human types I and II 3β-HSD mRNA species

To determine the tissue-specific expression of human types I and II 3β-HSD genes and the relative abundance of both types of 3β-HSD mRNA populations, we performed a ribonuclease protection assay which offers the opportunity to discriminate accurately a few base pair mismatches occurring after annealing of types I or II cRNA probes to the expected 3β-HSD types I or II mRNA species. Using specific cRNA probes, the type II 3β-HSD mRNA population is the almost exclusive species detectable in the human adrenal gland, testis and ovary, as revealed by the presence of the expected full-length (220 nucleotides) protected fragment using the specific type II cRNA probe as well as by the occurrence of the expected small fragments (about 98 and 64 nucleotides) when the type I cRNA probe was used [Fig. 4(A)]. The presence of type I 3β-HSD protected mRNA fragments in testis and ovary mRNA was detected after a longer time exposure. However, even with

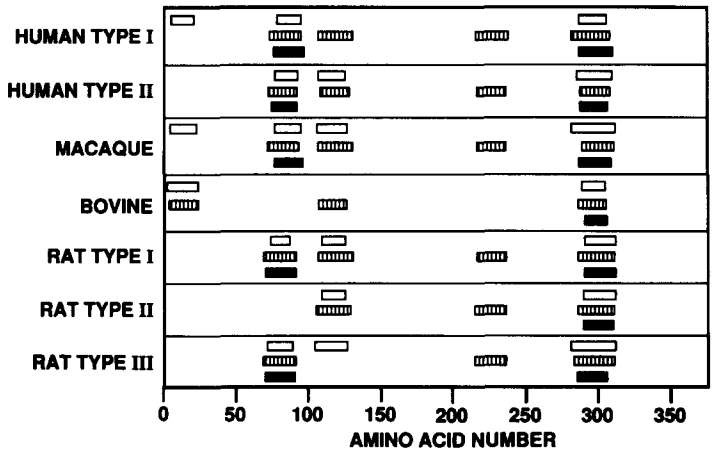

Fig. 3. Comparison of the predicted membrane-spanning domains of deduced human type I and II, macaque, bovine as well as rat types I, II and III 3β-HSD proteins according to Rao and Argos [56; open boxes], Eisenberg *et al.* [57; hatched boxes] and Klein *et al.* [55; closed boxes].

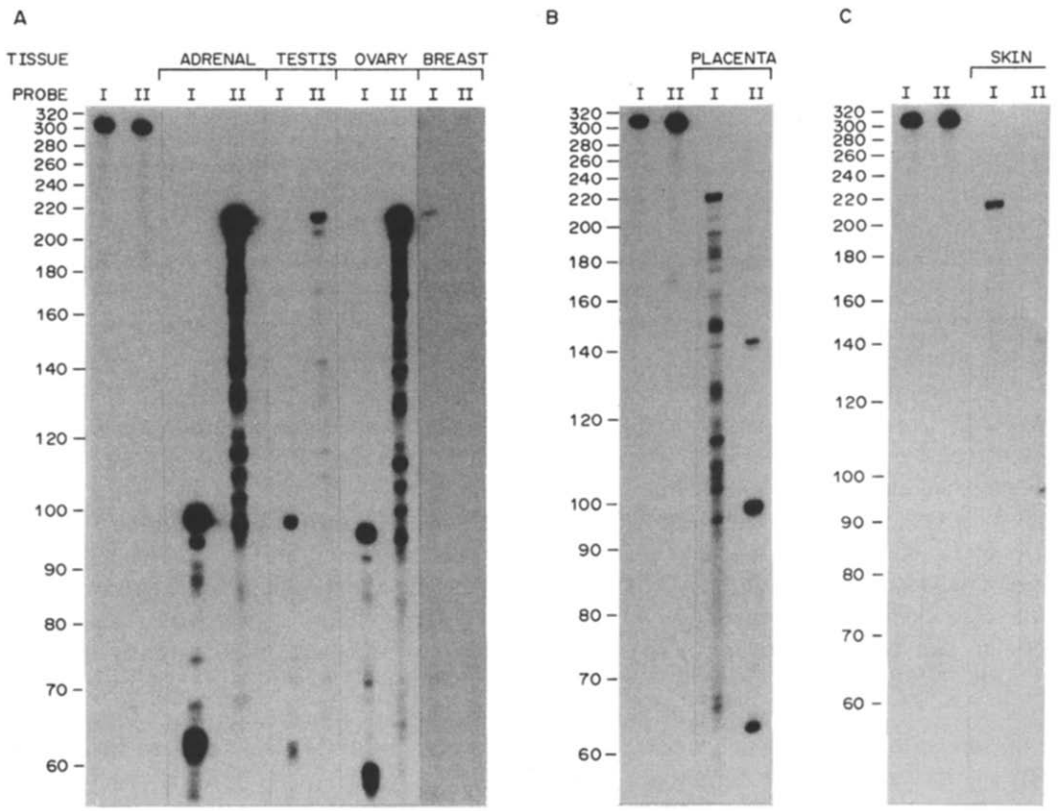

Fig. 4. Ribonuclease protection analysis of the distribution of human types I and II 3β-HSD mRNAs in classical steroidogenic as well as peripheral tissues in the human. Samples of total RNA from the human adrenal (3 μg), ovary (10 μg), placenta (20 μg), skin (from the breast region) (20 μg) or poly (A)$^+$ for the testis (10 μg) or breast (normal mammary gland) (10 μg) were hybridized to the type I or II cRNA probes for 14 h at 37°C, and digested with ribonuclease A and T1. The protected fragments were resolved on 6% polyacrylamide-7M urea sequencing gels. With either probe (315 nucleotides), the longest protected fragment (220 nucleotides) which included nucleotides +688 to +909 for human type I 3β-HSD and +685 to +904 for human type II 3β-HSD corresponds to the homologous RNA species protected by the cRNA probe. Lanes corresponding to the adrenal and placenta were exposed to X-Ray film for 14 h while those from testis, breast and ovary were exposed for 7 days, while the skin sample was exposed for 9 days. Lanes corresponding to adrenal, ovary and placenta are overexposed in order to further demonstrate the type I- or II-specific mRNA expression in these tissues (figure taken from Ref. [51]).

overexposed autoradiographs, it was not possible to detect human type I 3β-HSD mRNA in total RNA from either human adrenal or human type II 3β-HSD mRNA in human placenta [51]. The human type I 3β-HSD mRNA population corresponds to the sole detectable species in human placenta and skin under the experimental conditions used [Fig. 4(B and C)]. In addition, ribonuclease protection assay analysis of mammary gland RNA showed that the type I 3β-HSD mRNA is the predominant species while some type II 3β-HSD mRNA population could be detected on the original autoradiograph [Fig. 4(A)]. This study was performed using total RNA from placenta, adrenal, ovary and skin while poly A$^+$ RNA from testis and mammary gland tissue was required to detect a hybridization signal.

2.4. Structure of human types I and II 3β-HSD genes

α-^{32}P-labeled human placental 3β-HSD cDNA (hp3β-HSD63) was used to screen a human leucocyte genomic DNA library constructed in the λ-EMBL3 phage vector. The human 3β-HSD genes corresponding to the human cDNAs type I and II contain 4 exons and 3 introns within a total length of 7.8 kbp (Fig. 5). The first exon of both genes contains 53 nucleotides in the 5′-noncoding region. Exon II contains 85 or 89 nucleotides in the 5′-noncoding region of the type I and II genes, respectively, the nucleotide sequence of the first 48

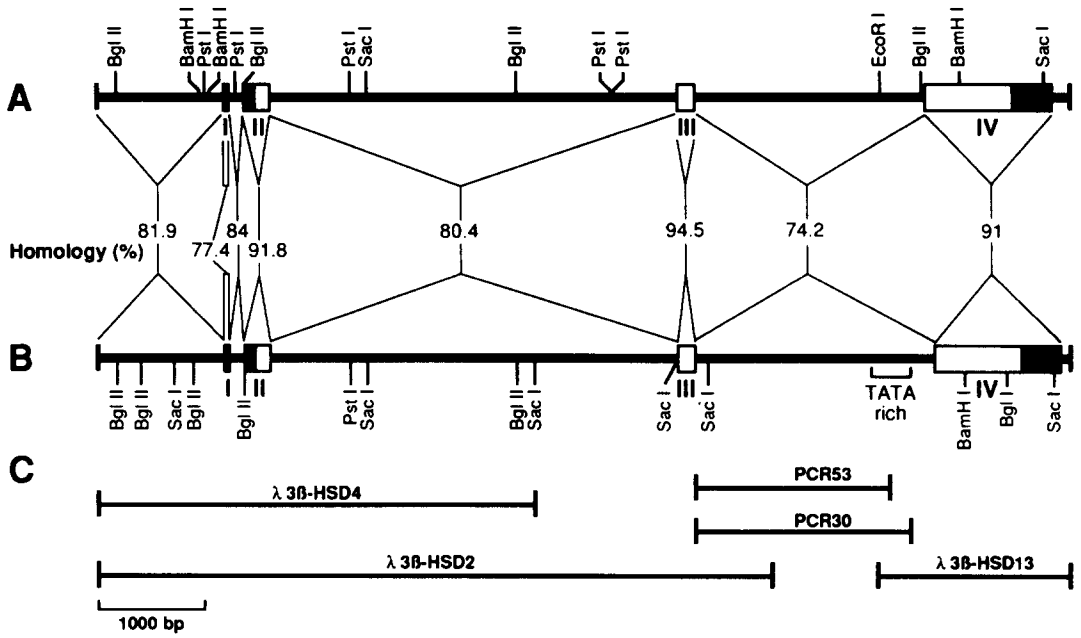

Fig. 5. Structure, restriction map and % of homology of human type I (A) and type II (B) 3β-HSD genes. Exons are represented by boxes and numbered from I to IV. Black box areas correspond to noncoding regions while open box areas indicate coding regions. Intron and flanking regions are represented by solid lines. The scale bar represents 1000 bp. Panel C: Identification and sizes of genomic DNA fragments isolated from λ-EMBL-3 recombinant clones (type II gene). Adapted from Lachance *et al.* [52].

amino acids of type I 3β-HSD or of the first 47 residues of type II 3β-HSD and the first nucleotide of the following *Lys* codon. Exon III contains the last 2 nucleotides of the *Lys* codon, encodes the 53 following residues and the first nucleotide of the following *Gly* codon. In fact, exon IV contains the last two nucleotides of the *Gly* codon and the nucleotides encoding the following 269 amino acids, the stop codon as well as 417 and 416 nucleotides in the 3′-untranslated region for the type I and II genes, respectively. Comparison of the nucleotide sequences of the two genes indicates that they share 77.4, 91.8, 94.5, 91.0, 84.0, 80.4 and 74.2 homology in exons I, II, III and IV as well as in introns I, II and III, respectively. The 1250 nucleotides in the 5′ flanking region share 81.9% homology. The coding region of the human 3β-HSD gene type I and the previously reported cDNA sequence are identical except for two nucleotide substitutions at codons 338 and 367 [12] which has recently been shown to be due to polymorphism [60, 61].

2.5. Discussion

Type II 3β-HSD mRNA species is detected by RNase protection in the adrenal, ovary and testis. In contrast, the type I 3β-HSD mRNA population is the almost exclusive mRNA species revealed in the placenta and skin, and is the predominant species in mammary gland tissue. The higher K_m value of type II 3β-HSD mainly expressed in steroidogenic tissues could be related to the higher levels of endogenous substrates present in these classical steroidogenic tissues. The approximately 10-fold higher affinity of type I 3β-HSD, which is preferentially expressed in peripheral intracrine tissues, such as the skin, could greatly facilitate steroid formation from relatively low concentrations of substrates usually present in these tissues. The physiological importance of 3β-HSD gene expression in the skin is supported by the observation that DHEA can stimulate sebaceous gland secretion in humans [62], following its conversion into the potent androgens testosterone and DHT [18], thus indicating, in addition, the presence of 17β-HSD and 5α-reductase activity in human skin.

The elucidation of the structure of human type II 3β-HSD, which represents the almost exclusive isoenzyme expressed in the adrenals and gonads should provide the necessary tools for the characterization of the molecular basis of 3β-HSD deficiencies [34–45, 110, and refs therein]. Such studies should also provide useful information on the heterogeneity of the related disease, while offering the opportunity of an

earlier diagnosis and a possible improvement of the therapeutical approaches.

The finding of the expression of the same 3β-HSD isoenzyme in human adrenals and gonads is in agreement with clinical observations of the impairment of steroidogenesis in these tissues in patients suffering from 3β-HSD deficiencies [35, 40, 41, 43–45]. However, the observations that both type I and II 3β-HSD possess an equivalent affinity for C-21 (PREG) and C-19 (DHEA and DHT) steroids argues against the existence of 3β-HSD isoenzymes having a different substrate-specificity, as previously suggested to explain the clinical observations in a 3β-HSD deficient patient [38].

The novel 3β-HSD isoenzyme has been chronologically designated as type II by reference to our previously characterized human type I 3β-HSD obtained from a placental cDNA library, without any relationships with rat types I and II which are both expressed in the adrenals and gonads [2]. The human type II 3β-HSD differs from the macaque ovarian 3β-HSD by only 13 residues, while 23 different residues distinguish the human types II and I 3β-HSD proteins. The human type II 3β-HSD and the macaque 3β-HSD genes may have evolved from a common ancestral gene, while the human type I gene may have evolved from another member of this gene family, which has diverged after a duplication which took place earlier in evolution.

The tissue-specific expression of the gene for the two human types of 3β-HSD, especially the specific or predominant expression of type I 3β-HSD in the mammary gland and skin is in agreement with the evidence for intact peripheral (extraadrenal and extragonadal) 3β-HSD activity in patients with classical congenital adrenal 3β-HSD deficiency as well as in the late-onset form of the disease [34–36, 40–45].

The existence of multiple 3β-HSD mRNAs offers many possibilities for the regulation of tissue- and substrate-specific expression of this enzyme. The enzyme purified from human placenta [1, 48, 63] possesses 3β-hydroxysteroid dehydrogenase as well as 5-ene-4-ene isomerase activities for both pregnene and androstene substrates. A similar co-purification of 3β-hydroxysteroid dehydrogenase and 5-ene-4-ene isomerase activities was also observed in other mammalian species, including rat adrenals [64], rat testis [65], ovine adrenals [66], bovine ovaries [67], and bovine adrenals [68, 69]. Expression of human and rat 3β-HSD cDNAs in nonsteroidogenic cells has shown that a single 42-kDa protein contains both 3β-oldehydrogenase and 5-ene-4-ene isomerase activities [2, 12, 51, 70]. We have recently provided evidence for distinct dehydrogenase and isomerase sites within a single 3β-HSD protein [71].

3. RAT 3β-HSD ISOENZYME FAMILY

Since rat is the best known model for studies of endocrine regulation, the availability of rat 3β-HSD probes would make possible detailed investigation of the tissue-specific expression and regulation of 3β-HSD in steroidogenic as well as in peripheral tissues. For this reason, we have used the human type I 3β-HSD cDNA to first screen a rat ovary λgt11 cDNA library.

3.1. Structure of rat types I and II 3β-HSD cDNAs

The nucleotide sequences of both types of rat 3β-HSD cDNAs and the deduced amino acid sequences has been characterized [2, 72]. The first in-frame ATG codon in each sequence is designated as position 1 and is preceded by an in-frame terminator codon TGA located 120 nucleotides upstream. The sequences of both type I and II cDNAs have an open reading frame of 1119 nucleotides with only 33 bp differences between the two sequences. The 5′-untranslated sequences of rat type I and II cDNAs contain 171 and 152 bp, respectively, with only two differences in their overlapping region. The 3′-untranslated regions of rat type I and II cDNAs contain 355 and 369 bp, respectively. There are eight nonidentical nucleotides in the 3′-untranslated regions in addition to an insertion of 14 additional nucleotides at position 1440 in type I cDNA. These sequences have been independently confirmed [73, 78].

The deduced amino acid sequences of rat types I and II 3β-HSD have only 23 nonidentical residues (Fig. 1, Table 1). These sequences share approx. 78% similarity with that of human, macaque and bovine 3β-HSD, while both 3β-HSD types share about 90% similarity with the sequence of mouse 3β-HSD [1, 2, 7, 51, 54, 72] (Table 1). RNase protection analysis using specific mouse cRNA probes indicates that mouse type I mRNA is detected in the adrenals and gonads, while type II and III mRNA species are detected in liver and kidney [54].

Table 2. Kinetic properties of wild type rat type I and II 3β-HSDs as well as of chimeric I-MSD and II + MSD proteins

Expressed protein	K_m (μM)		Relative V_{max}		Relative specificity (relative V_{max}/K_m)	
	PREG	DHEA	PREG	DHEA	PREG	DHEA
Wild type rat type I 3β-HSD	0.74	0.68	100	100	135	147
Chimeric I-MSD	11.7	11.0	5.5	7.60	0.47	0.69
Wild type rat type II 3β-HSD	14.3	12.9	30.8	40.8	2.1	3.16
Chimeric II + MSD	0.36	0.40	28.0	42.7	77.8	106.7

Kinetic parameters were determined using Lineweaver–Burk plot ($1/v$ vs $1/[S]$). Relative V_{max} values were calculated assuming the V_{max} for the wild type rat type I enzyme equal to 100. The specific activity values for the wild type I 3β-HSD using PREG or DHEA as substrates were 5.1 and 4.1 nmol/min/mg protein, respectively (table taken from Ref. [70]).

3.2. Enzymatic characteristics of expressed rat types I and II 3β-HSD isoenzymes

Plasmids derived from pCMV which contained either the type I or the type II 3β-HSD cDNA inserts driven by the CMV promoter were transiently expressed in HeLa human cervical carcinoma cells in order to verify that both proteins were functional. Transfection of HeLa cells with either type I or II 3β-HSD cDNA inserts resulted in the production of a single 42-kDa protein which co-migrated with purified human placental 3β-HSD and which cross-reacted with polyclonal antibodies raised against purified human 3β-HSD [2, 70].

Transfection of HeLa cells with either pCMV-type I 3β-HSD or pCMV-type II 3β-HSD, imparted 3β-HSD activity as evidenced by the conversion of [^{14}C]PREG and [^{14}C]DHEA to [^{14}C]progesterone (PROG) and [^{14}C]Δ^4-androstenedione (Δ^4-dione), respectively. The magnitude of the conversion was proportional to the amount of 3β-HSD cDNA-containing vector transfected [2]. The pCMV-type II 3β-HSD protein demonstrated a much lower conversion rate of PREG and DHEA as compared with pCMV-type I 3β-HSD, despite comparable transfection efficiencies, as ascertained by co-transfection of a growth hormone-synthesizing plasmid, and equivalent amounts of 3β-HSD protein translated in HeLa cells transfected with either plasmid [2]. Cells transfected with the pCMV vector alone did not display 3β-HSD activity [2].

In vitro incubation with homogenates from cells transfected with pCMV-type I-3β-HSD or pCMV-type II-3β-HSD in the presence of 1 mM NAD^+ and ^{3}H-labeled PREG showed that type I had a 3β-HSD/5-ene-4-ene isomerase relative specificity, as determined by relative V_{max}/K_m ratio (relative V_{max} values were calculated assuming that the rate for wild type I is equal to 100), 64 times higher than type II with corresponding K_m values of 0.74 μM (type I) and 14.3 μM (type II), with V_{max} values of 5.1 and 1.57 nmol of progesterone formed from PREG/min/mg protein for the type I and II proteins, respectively (Table 2). The much higher relative specificity of rat type I 3β-HSD compared to type II 3β-HSD was confirmed using DHEA as labeled substrate with respective K_m values of 0.68 and 12.9 μM, while their V_{max} values were 4.10 and 1.67 nmol androstenedione formed/min/mg protein, respectively. The present data thus indicate that the lower activity of type II 3β-HSD results primarily from an approx. 95% decrease in affinity for both substrates, while the V_{max} value is only decreased by 60 to 70%.

We next investigated structural differences which could explain the much higher activity of type I 3β-HSD. As predicted by computer analysis [55], there is a potential membrane-spanning domain (MSD) common to the deduced rat types I and II as well as human, macaque and bovine 3β-HSD protein sequences, between residues 287 to 303 (Fig. 3) [2, 7, 8, 26, 51]. Analysis of types I and II rat 3β-HSDs indicates that the change of residues 83, 85, 87 and 89 in the rat type II 3β-HSD protein prevents the formation of another potential MSD present in the rat type I enzyme between residues 75 and 91 as well as in human and macaque 3β-HSD proteins [2, 8]. In order to characterize the functional significance of MSD 75–91 in type I 3β-HSD, we have compared the kinetic properties of wild type rat types I and II 3β-HSD proteins with those of a chimeric type I protein lacking this MSD (I-MSD) and of a chimeric type II protein having gained this putative MSD (II + MSD). The chimeric proteins I-MSD and II + MSD were obtained by site-directed mutagenesis [70].

In homogenate preparations from HeLa cells transfected with pCMV-I-MSD or pCMV-II + MSD. The expressed chimeric I-MSD protein, which is predicted to lack the MSD at position 75–91, showed a markedly reduced affinity for PREG and DHEA with K_m values of 11.7 and 11 μM, respectively, while its relative specificity was decreased to 0.35 and 0.47% compared to type I when PREG and DHEA were used, respectively. Thus removal of this putative MSD markedly affects the specific activity as well as the affinity of the enzyme (Table 2) [70].

The expressed protein II + MSD had an affinity for PREG and DHEA comparable to that of wild type rat type I with K_m values of 0.36 and 0.40 μM, respectively [70]. The introduction of a putative MSD in type II 3β-HSD by site-directed mutagenesis increased the relative specificity by 36- and 34-fold, respectively, using PREG and DHEA as substrates. The relative specificity of the chimeric II + MSD protein became 58% for PREG and 73% for DHEA compared to the wild type I 3β-HSD (Table 2). The V_{max} of the protein encoded by pCMV-II + MSD is almost identical to the wild type rat type II at 1.43 and 1.75 nmol/min/mg protein for PREG and DHEA, as substrates, respectively [70].

These data provide strong evidence supporting the crucial role of the predicted MSD between residues 75 to 91 for the enzymatic specificity of rat type I 3β-HSD. It is thus likely that the absence of this putative MSD in type II 3β-HSD explains its much lower activity.

3.3. Tissue-specific expression of rat types I and II 3β-HSD mRNAs

In view of our finding of two cDNAs encoding rat 3β-HSD and the impossibility of distinguishing the corresponding mRNAs by conventional RNA blot analysis, we studied the distribution of 3β-HSD mRNAs in rat tissues using the sensitive RNase protection assay. Using the cRNA probes specific for types I and II 3β-HSD mRNAs, both mRNAs are detected in the rat ovary, testis and adrenal [2]. Both mRNAs species were also detected in female rat adipose tissue whereas none were detected in male fat under the experimental conditions used. Only type I mRNA could be detected in both male and female kidney poly(A)$^+$ RNA [2].

The existence of two 3β-HSD mRNAs offers many possibilities for the regulation of tissue-specific expression of this enzyme. As is the case for the human isozymes, both the rat type I and the type II 3β-HSD cDNAs encode functional 3β-HSD proteins, which have both 3β-ol-dehydrogenase and 5-ene-4-ene isomerase activities.

3.4. Androgenic 17β-HSD activity of rat type I 3β-HSD

It has been reported that some purified preparations of oxidoreductase enzymes show dual steroidogenic activities [74, 75]. Since the previous data on dual enzymatic activities could be due to lack of purity of the preparations, transient expression of a recombinant enzyme in nonsteroidogenic mammalian cells should permit the examination of such multiple enzymatic activities.

An 8-h incubation of protein homogenate from HeLa cells transfected with the pCMV vector containing the rat type I or II 3β-HSD insert in the presence of NADH as co-factor led to the conversion of DHT into its metabolite 3β-diol [76]. Type I 3β-HSD is much more active than the type II enzyme, in agreement with our previous observation for the classical substrates of 3β-HSD [2, 70].

Somewhat unexpectedly, we then observed that homogenate obtained from cells transfected with the pCMV-type I 3β-HSD in the presence of the oxidative form of the cofactor, i.e. NAD^+, converts DHT into a highly polar metabolite [76]. This metabolite has been shown by HPLC to correspond to the 17β-oxidative form of DHT, namely 5α-androstanedione (A-dione), thus demonstrating that the expressed enzyme possesses 17β-HSD activity. Endogenous 17β-HSD activity in HeLa cells transfected with pCMV alone is very low [76]. After a 12-h incubation in the presence of 1 mM NAD^+, homogenate from cells transfected with pCMV-type I 3β-HSD converts about 65% of DHT into A-dione.

In contrast to the type I 3β-HSD protein, type II 3β-HSD, as well as chimeric I-MSD and II + MSD proteins, do not possess significant androgenic 17β-HSD activity [76]. Finding that the chimeric II + MSD 3β-HSD protein is devoid of 17β-HSD activity strongly suggests that the lack of such 17β-HSD enzymatic activity for the type II 3β-HSD isoenzymes is not due to the absence of a MSD between residues 75 to 91, but rather to another structural difference resulting from one or several of the 19 other

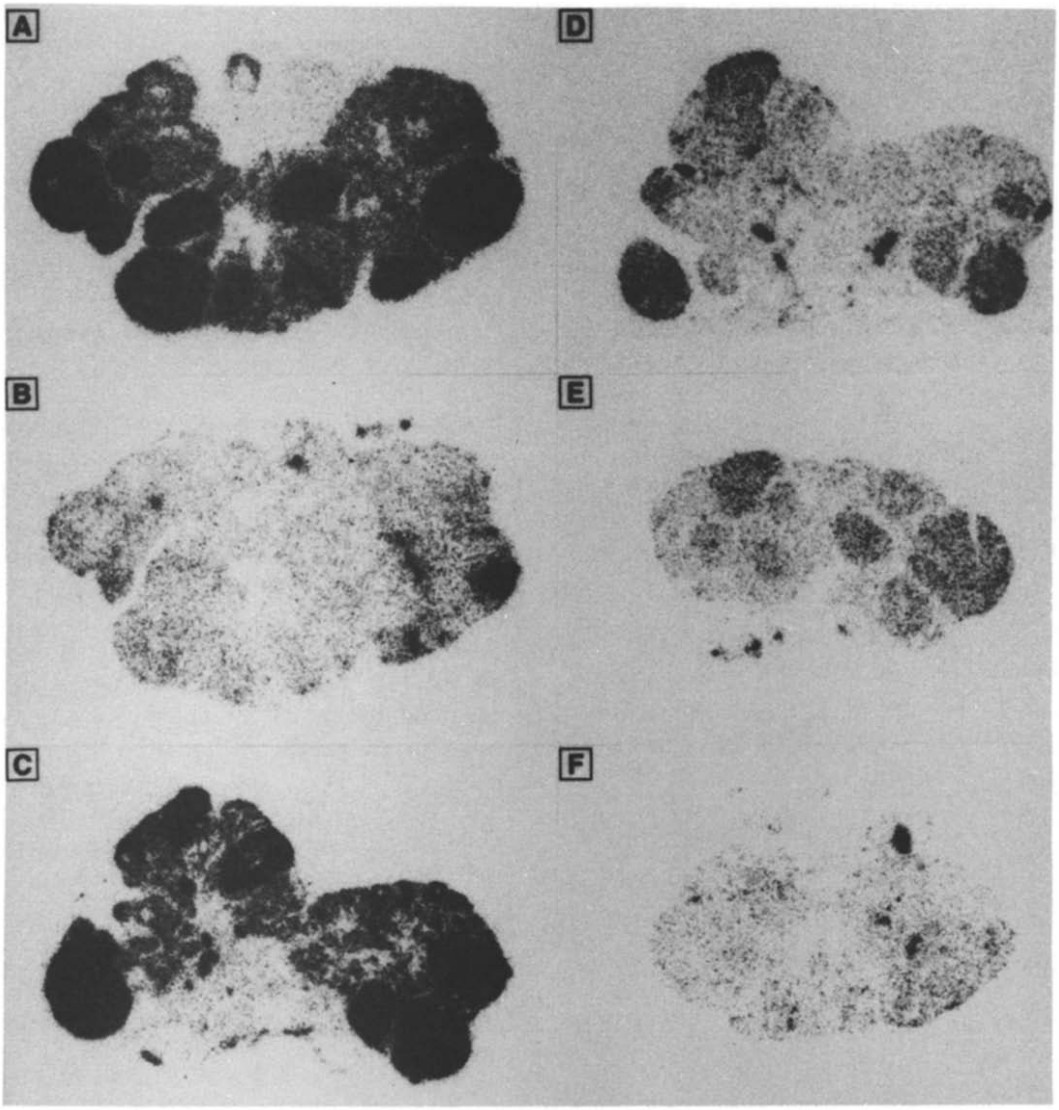

Fig. 6. *In situ* hybridization of ovarian sections with ^{35}S-labeled type I- and II-specific oligonucleotide probes. Demonsrtration of the hybridization of the 3β-HSD type I probe to ovarian cell mRNA. (A) ^{35}S-labeled type I antisense probe; (B) displacement by 500-fold excess cold (unlabeled) type I probe; and (C) competition with 500-fold excess cold type II probe. Demonstration of the hybridization of the 3β-HSD type II probe to ovarian cell mRNA; (D) ^{35}S-labeled type II antisense probe; (E) competition with 500-fold excess cold type I probe; and (F) displacement by 500-fold excess cold type II probe (figure taken from Ref. [2]).

amino acid changes observed between the type I and II enzymes [76].

The K_m value for the transformation of DHT into 3β-diol, $4.02 \pm 0.67\ \mu$M by the expressed type I 3β-HSD is in the same range as that obtained with the human type II 3β-HSD ($K_m = 2.7\ \mu$M) [51] but higher than the K_m obtained with the human placental type I 3β-HSD [51, 49]. When NAD^+ is used as co-factor, the androgenic 17β-HSD activity of rat type I 3β-HSD shows a K_m of $7.97 \pm 2.18\ \mu$M. As measured in HeLa cell homogenate, the V_{max} of 3β-HSD activity of expressed type I 3β-HSD is much higher than that of 17β-HSD activity (24.9 ± 1.18 vs 1.67 ± 0.13 nmol/min/mg) [76].

However, when the same enzymatic assays are performed in unbroken JEG-3 human choriocarcinoma cells transfected with the rat type I 3β-HSD, 17β-HSD activity is clearly predominant over 3β-HSD activity. Cells transfected with rat type I 3β-HSD metabolize DHT exclusively into A-dione, while no 3β-diol formation can be detected over basal values obtained in cells transfected with the pCMV plasmid [76]. A-dione formation rose above 50% after 12 h of incubation in cells transfected

with the pCMV-type I 3β-HSD plasmid. The low 3β-HSD activity of the type I enzyme observed in living cells is probably due to the low concentration of the reductive co-factor NADH compared to the oxidative co-factor NAD^+. These findings suggest that 17β-HSD activity, although "secondary" in homogenate of transfected cells in the presence of NADH, could well play a major role in the metabolism of DHT in the NAD^+-rich environment of living cells. The 17β-HSD activity of rat type I 3β-HSD seems to be specific to 5α-androstane steroids, since other classical substrates of 17β-HSD, namely DHEA, Δ^5-diol, testosterone, Δ^4-dione, 17β-estradiol or estrone, were not transformed into their respective metabolites in homogenates of HeLa cells transfected with the pCMV-type I 3β-HSD plasmid in the presence of appropriate co-factors [76].

3.5. Tissue-specific expression of 3β-HSD in rat tissues

We examined the cellular distribution of the two 3β-HSD mRNA types in sections of rat ovary by *in situ* hybridization using ^{35}S-5′ end-labeled 24-mer type-specific oligonucleotide probes [2, 72]. Both mRNA types I [Fig. 6(A)] and II [Fig. 6(D)] are most abundant in corpora lutea which are active in progesterone biosynthesis. The weaker hybridization signals seen by light microscopy examination indicate lesser amounts of 3β-HSD mRNA in interstitial and thecal cells. Sense oligonucleotide probes did not hybridize to ovarian mRNA. The specificity of the oligonucleotide probes was demonstrated by the inability of the unlabeled type II 3β-HSD oligonucleotide to displace ^{35}S-type I probe [Fig. 6(C)] and vice versa [Fig. 6(E)]. On the other hand, unlabeled type I [Fig. 6(B)] and type II [Fig. 6(F)] probes completely displaced their corresponding labeled counterparts. The predominance of type I mRNA species in the ovary as suggested by *in situ* hybridization was confirmed by S1 nuclease analysis [72].

3β-HSD enzymatic activity was measured in homogenates from 14 tissues using both PREG and DHEA as precursors. The highest levels of 3β-HSD activity in male rats were found in the adrenal glands and testes, while in female animals, the adrenal glands and ovaries showed the highest levels of enzymatic activity [2]. Appreciable levels of 3β-HSD activity were also found in the livers of male and female rats. In female animals, mammary, adipose, and uterine tissues converted significant amounts of 3β-hydroxy-5-ene steroids into 3-keto-4-ene steroids. In male rats, appreciable levels of 3β-HSD activity were also measured in the kidney, skin, prostate, adipose tissue, seminal vesicles, lung, heart, thymus, brain, and spleen. Some tissues (testis, liver, skin and prostate in males and the mammary gland, uterus, and adipose tissue in females) appeared to favor the conversion of [4-^{14}C]DHEA into [4-^{14}C]Δ^4-dione over the conversion of [4-^{14}C]PREG into [4-^{14}C]PROG. Conversely, the female liver and male adipose tissue favor pregnene 3β-HSD activity over androstene 3β-HSD activity [2].

The presence of 3β-HSD mRNAs in a wide variety of rat tissues such as adipose tissue and kidney, as well as the demonstration of 3β-HSD activity in hormone-sensitive organs such as the breast, uterus, ventral prostate, and seminal vesicle, indicate that these tissues may form active sex steroids from circulating 3β-hydroxy 5-ene steroid precursors. We previously demonstrated that the administration of DHEA to castrated rats caused a marked increase in ventral prostate weight accompanied by increased prostatic DHT and androgen-dependent mRNA levels [77]. Considering their relatively large size, the skin and adipose tissue are likely to be important sites of extragonadal sex steroid formation.

3.6. Structure and expression of rat 3-keto steroid reductase (type III 3β-HSD)

Following screening of a male rat liver λgt11 cDNA library, a third type of cDNA encoding a new member of the rat 3β-HSD family was isolated [26]. Rat type III cDNA has an open reading frame of 1119 nucleotides which displays 85.7% similarity with that of rat type I or II 3β-HSD cDNA. The predicted rat type III enzyme has an M_r of 42080 and contains 372 amino acids which shares 80% similarity with type I and II 3β-HSD proteins (Fig. 2, Table 1). Type III cDNA contains a 736 bp 3′ untranslated region before the poly(A) tail while the corresponding region in type I and II cDNAs contains 355 and 369 bp, respectively [2, 26].

In agreement with a longer 3′-untranslated region in type III mRNA, RNA blot analysis shows a 2.1 kb mRNA band in male liver and a 1.7 kb mRNA, which corresponds to type I and II 3β-HSD mRNA in the adrenal and testis. No signal was detected in poly(A)$^+$ RNA from intact female liver, while a strong hybridization signal was detected in total RNA from female

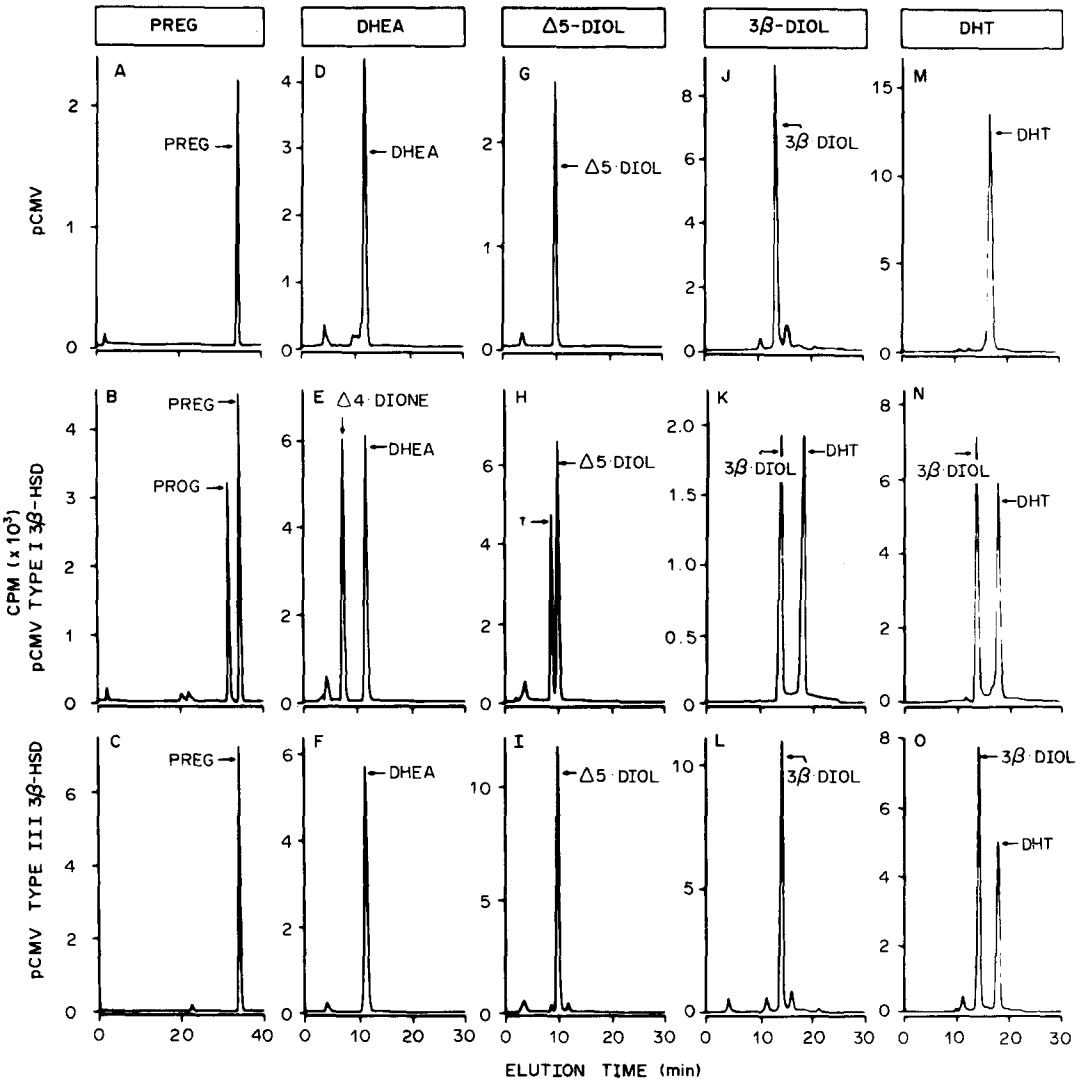

Fig. 7. Enzymatic activity of expressed rat type I 3β-HSD isoenzyme and the liver-specific type III isoform analyzed by HPLC. pCMV-type I 3β-HSD and pCMV-type III 3β-HSD recombinant plasmids were expressed in HeLa mammalian cells using the calcium phosphate precipitation procedure, while mock transfections were carried out with the pCMV alone. 10 μg of protein from HeLa cells transfected with pCMV (A, D, G, J, M) or pCMV-type III 3β-HSD (C, F, I, L, O) or 2 μg of protein from cells transfected with pCMV-type I 3β-HSD (B, E, H, K, N) were incubated for 8 h at 37°C in the presence of 1 mM NAD^+ and 300 nM tritiated PREG (A, B, C), DHEA (D, E, F), Δ^5-diol (G, H, I) or 3β-diol (J, K, L) or in the presence of 1 mM NADH and 300 nM DHT (M, N, O) (figure taken from Ref. [79]).

liver obtained from animals hypophysectomized 24 days earlier [26].

Using cRNA probes specific for type I, II or III mRNAs, both type I and II mRNAs can be detected in the rat adrenal and ovary but not in the male liver, while the type III species is detected specifically in the male liver [26]. The original finding of an apparently pituitary hormone-induced repression of gene expression of a liver-specific member of the rat 3β-HSD family is in agreement with the well recognized masculinization of rat liver enzyme activity following hypophysectomy in female rats.

The pCMV-type I 3β-HSD expressed enzyme shows high 3β-HSD/5-ene-4-ene isomerase activity illustrated by the conversion of PREG into PROG [Fig. 7(B)], DHEA into Δ^4-dione [Fig. 7(E)], and Δ^5-diol into T [Fig. 7(H)]. Furthermore, the type I isoenzyme also displays oxidoreductase activity, since it converts the 3-keto steroid 5α-DHT into 3β-diol [Fig. 7(N)] as well as the 3β-diol into DHT [Fig. 7(K)]. These data are in close agreement with our previous studies on the specificity of human and rat 3β-HSD enzymes [2, 51, 70]. However, somewhat surprisingly, incubation

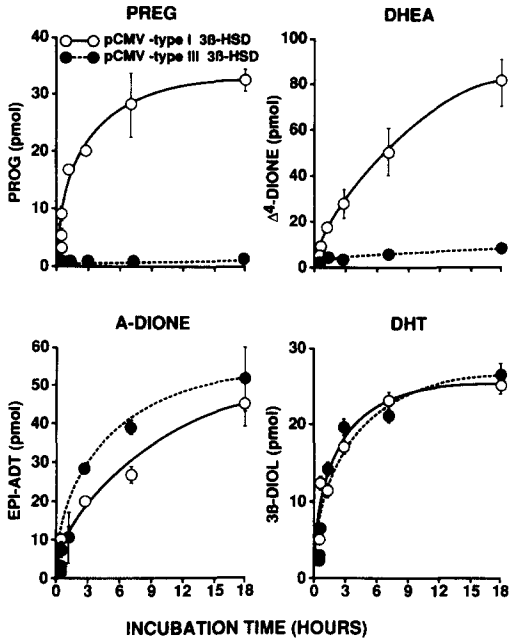

Fig. 8. Time course of the enzymatic activity of homogenates from cells transfected with pCMV-type III 3β-HSD plasmid (10 μg of protein) or pCMV-type I 3β-HSD plasmid (2 μg of protein). The incubation was performed for the indicated time periods in the presence of 300 nM tritiated PREG or DHEA supplemented with 1 mM NAD^+, or in the presence of 300 nM tritiated DHT or A-dione supplemented with 1 mM NADH. Steroids were separated by TLC and the amounts of products formed by the isoenzymes of the 3β-HSD family, i.e. PROG for PREG, Δ^4-dione for DHEA, 3β-diol for DHT, and 5α-epiandrosterone (EPI-ADT) for A-dione, were calculated after subtraction of the very low intrinsic 3β-HSD activity of HeLa control cells (figure taken from Ref. [79]).

with homogenate from cells transfected with the pCMV-type III 3β-HSD in the presence of 1 mM NAD^+ and ^{3}H-labeled PREG [Fig. 7(C)], DHEA [Fig. 7(F)], Δ^5-diol [Fig. 7(I)] and 3β-diol [Fig. 7(L)] shows that the type III enzyme lacks 3β-HSD activity, while it can efficiently reduce DHT into 3β-diol [Fig. 7(O)] [79]. No significant conversion of [^{3}H]PREG was observed after 8 h of incubation with protein homogenate from HeLa cells transfected with pCMV alone [Fig. 7(A)] while only minimal transformation of [^{3}H]DHEA [Fig. 7(D)], [^{3}H]Δ^5-diol [Fig. 7(G)], [^{3}H]3β-diol [Fig. 7(J)] and [^{3}H]DHT [Fig. 7(M)] could be detected. The endogenous activity of the corresponding steroid metabolizing enzymes in control HeLa cells [79] is extremely low.

PREG or DHEA were not metabolized to a significant degree by the type III isoenzyme while these two hydroxy-5-ene steroids were efficiently converted into PROG and Δ^4-dione, respectively, by the type I isoform (Fig. 8). In contrast, incubation of the same homogenates from cells transfected with pCMV-type I 3β-HSD and pCMV-type III 3β-HSD in the presence of 1 mM NADH and the oxidative forms of two well-known androstane substrates of 3β-HSD, i.e. A-dione and DHT, clearly show comparable enzymatic activity for both types I and III isoenzymes (Fig. 8). Thus, among the

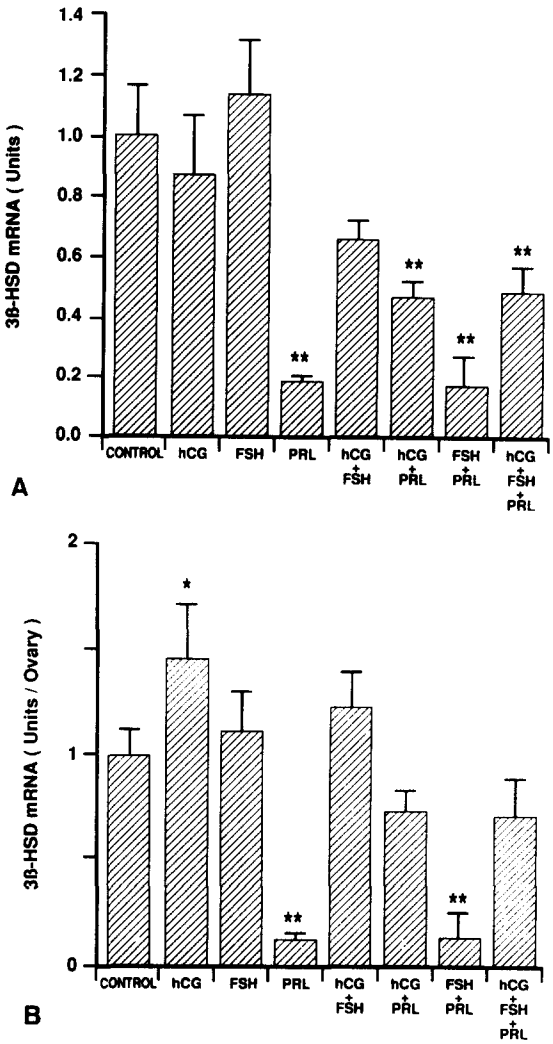

Fig. 9. Modulation of rat ovary 3β-HSD mRNA levels (A) and total ovarian 3β HSD mRNA content (B) by gonadotropins and PRL. Adult female rats hypophysectomized 15 days previously received b.i.d. injections of hCG (10 IU), oFSH (0.5 μg), or oPRL (1.0 mg), singly or in combination, for 10 days. RNA was extracted from individual whole ovaries and blotted onto nylon membranes. Ovarian 3β HSD mRNA levels were measured by dot blot hybridization using the ^{32}P-random primer-labeled full-length rat ovary 3β-HSD cDNA (ro 3β HSD56). The amounts of ovarian 3β HSD mRNA were calculated relative to those levels observed in hypophysectomized rat ovaries using RNA from intact ovaries as standard and expressed in units (A), or corrected for ovarian weight and expressed in units/ovary (B). Data are expressed as means ± SEM ($n = 6$–7) and statistically significant variations were determined by Duncan–Kramer. *, $P < 0.05$; **, $P < 0.01$ vs hypophysectomized control (figure taken from Ref. [10]).

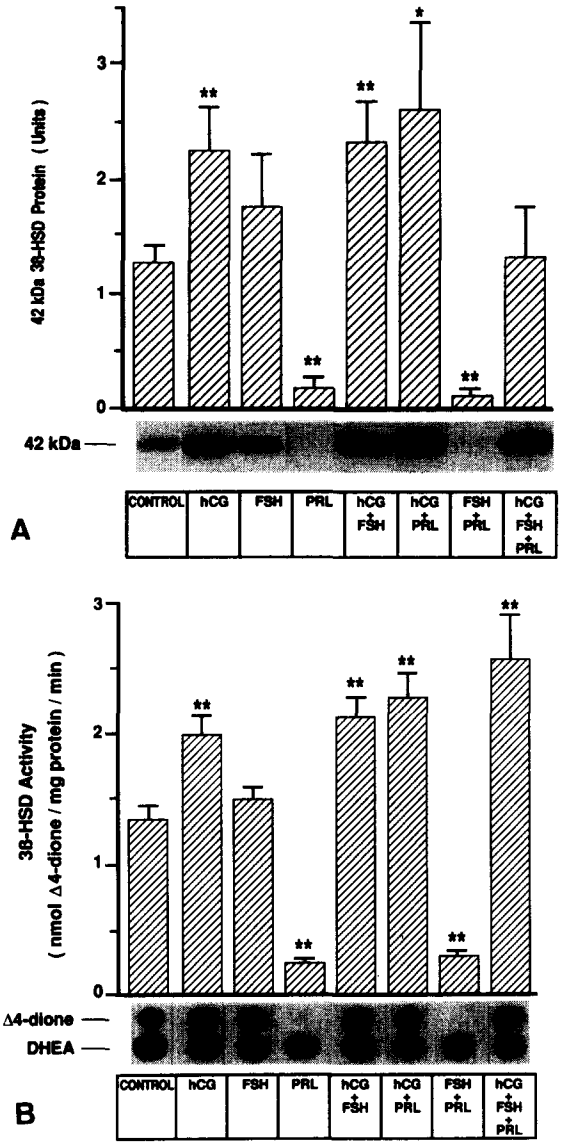

Fig. 10. Modulation of rat ovary 3β-HSD protein levels (A) and 3β-HSD activity (B) by treatment of hypophysectomized rats with hCG, oFSH, or oPRL as indicated in the legend to Fig. 9. Panel A, the 42-kDa ovarian 3β-HSD protein was revealed by sequential hybridization to rabbit antibodies directed against human placental 3β-HSD and ^{125}I-labeled goat antirabbit immunoglobulin G as depicted in the accompanying autoradiograph. Panel B, ovarian 3β-HSD activity was assayed by measuring the rate of formation of [4-^{14}C]Δ^4-dione from [4-^{14}C]DHEA. Total ovarian homogenates were incubated with 1 μM [4-^{14}C]DHEA and 10 μM cold DHEA. After separation on TLC plates, the radioactivity was measured in a scintillation counter. The autoradiograph accompanying the histogram shows the separation of [^{14}C]Δ^4-dione and [^{14}C]DHEA by TLC.

different 3β-HSD types so-far characterized, including the human type I and II [12, 51, 80], rat type I and II [2, 70] and bovine (Y. de Launoit, J. Simard and F. Labrie, unpublished data) 3β-HSDs, only the rat liver-specific type III isoform is unable to convert 3β-hydroxy-5-ene steroids into the corresponding 3-keto-4-ene steroids.

In order to further characterize the enzymatic kinetic properties of the male liver-specific type III isoenzyme, we compared the K_m values of the type I and III 3β-HSD expressed enzymes. The amount of type I and III 42-kDa proteins expressed in HeLa cells transfected with pCMV-type I 3β-HSD and pCMV-type III 3β-HSD plasmids, respectively, was evaluated by immunoblot analysis and quantification with an image analyzer [79]. The K_m values obtained with homogenates from cells transfected with pCMV-type I 3β-HSD and pCMV-type III 3β-HSDs are similar (5.05 and 6.16 μM) when DHT is used as substrate and NADH is used as co-factor. These values are in the same range as those for the human type II 3β-HSD protein ($K_m = 2.7$ μM) [51]. The rat type I 3β-HSD has a relative specificity (relative V_{max}/K_m) 17-fold higher than that of the rat type III enzyme [79].

The K_m for the type I 3β-HSD with DHT as substrate and NADPH as co-factor is 1.18 μM. This value is in the same range as that obtained with NADH as co-factor. However, the affinity of the type III enzyme for DHT is much higher with NADPH is used as co-factor with a K_m value of 0.12 μM as compared to 6.16 μM than NADH as co-factor, with a relative specificity 18.1-fold higher with NADPH than with NADH for the type III isoform. When NADPH is used as co-factor, comparable relative V_{max} values were observed for both types. These data show that NADPH is the preferred co-factor for the 3-ketosteroid reductase activity of the type III enzyme. The well recognized 5α-reductase substrates Δ^4-dione and testosterone were not reduced by the expressed rat type III enzyme [79].

These data indicate that the lack of oxidation and isomerization of 3β-hydroxy-5-ene steroid precursors into 3-keto-4-ene steroids by the rat type III enzyme is not due to the absence of isomerase activity, but they indicate that the oxidative activity is also completely absent. The rat type III enzyme does not significantly convert 3β-diol into DHT during an 8-h incubation [Fig. 7(L)]. Even after a longer incubation for 24 h, the expressed type III enzyme did not significantly convert 3β-diol into DHT in the presence of NAD^+ or $NADP^+$. In contrast, type I 3β-HSD incubated in the presence of NAD^+ led to a 25% conversion within 8 h. This activity was not observed in the presence of $NADP^+$ [79].

4. REGULATION OF 3β-HSD EXPRESSION AND ACTIVITY IN RAT TISSUES

4.1. Regulation of 3β-HSD expression and activity in the rat ovary

We studied the effects of the gonadotropins human CG (hCG)/LH and FSH, as well as that of prolactin (PRL), on rat ovarian 3β-HSD mRNA levels, protein content and enzymatic activity in hypophysectomized rats (Figs 9 and 10) [10].

Whereas hCG alone had no effect on the steady-state levels of whole ovarian 3β-HSD mRNA, ovine PRL (oPRL) exerted a potent inhibitory effect (Fig. 9). Simultaneous treatment with hCG partially reversed the inhibitory effect of oPRL, increasing 3β-HSD mRNA levels. Ovine FSH, on the other hand, had no significant effect when administered alone or in combination with hCG, oPRL or hCG + oPRL. Since hCG and oPRL exerted significant effects on ovarian weight, when we compared 3β-HSD mRNA content following correction for relative changes in total ovarian weight, hCG was seen to cause a 45% increase whereas oPRL exerted an 88% inhibition of total ovarian 3β-HSD mRNA content as compared to the values found in control hypophysectomized rat ovaries.

The potent inhibitory effect of oPRL on ovarian 3β-HSD mRNA content was also observed on the levels of 3β-HSD protein [Fig. 10(A)] and 3β-HSD activity [Fig. 10(B)]. In fact, ovarian 3β-HSD protein content and enzymatic activity varied in a manner almost superimposable to 3β-HSD mRNA content (Fig. 9).

Since measurement of ovarian 3β-HSD mRNA by dot blot hybridization reflects overall changes in ovarian 3β-HSD mRNA, sections of fixed ovaries were hybridized with the same cDNA probe to examine the cellular distribution of 3β-HSD mRNA levels.

The corpora lutea found in the ovaries of rats hypophysectomized 25 days previously still express large quantities of 3β-HSD mRNA as indicated by the intense labeling of all corpora lutea while the interstitial cells are very weakly labeled [10]. Treatment with oPRL alone caused a reduction in ovarian volume mainly related to a decrease in the size of the corpora lutea which also show markedly reduced levels of 3β-HSD mRNA [10]. oFSH and hCG alone do not appear to affect the intensity of the labeling of the corpora lutea. However, hCG treatment increases the size of the interstitial glands and their content of 3β-HSD mRNA [10].

While oPRL can be clearly seen to inhibit corpora lutea-associated 3β-HSD mRNA in hCG- and hCG + oFSH-treated rats, it appears less effective in blocking the stimulatory effect of hCG on the ovarian interstitial cells. It thus seems that the inhibitory effect of oPRL on 3β-HSD mRNA accumulation is exerted principally in luteal cells. Measurements of the intensities of the *in situ* hybridization signals relating corpora lutea 3β-HSD mRNA to total ovarian 3β-HSD mRNA, confirm that the decrease in whole ovarian 3β-HSD mRNA content observed by dot blot hybridization largely reflects an effect on luteal cells [10].

The demonstration that PRL exerts a potent inhibitory effect on 3β-HSD expression and activity in the ovaries of hypophysectomized rats confirms and extends previous data concerning the luteolytic effect of PRL under these circumstances. The PRL-induced decrease in 3β-HSD activity translates into a marked inhibition of circulating PROG levels as well as decreased uterine weight which reflects reduced estrogen secretion [10]. On the other hand, while hCG reverses the inhibitory effect of PRL, the same treatment stimulates 3β-HSD expression and activity in interstitial cells.

In situ hybridization has proven to be a valuable tool in discriminating between the responses of specific cell populations to hCG and PRL. The high levels of basal 3β-HSD expression and activity in the corpora lutea of hypophysectomized rats masked the variations in 3β-HSD mRNA occurring in interstitial cells, such that measurements of 3β-HSD mRNA concentration in whole ovaries, even when corrected for β-actin, did not accurately reflect the heterogenous responses to hCG and PRL. *In situ* hybridization, however, clearly indicated that interstitial cells and corpora lutea responded differently to hCG and PRL.

LH acts selectively on thecal cells to increase the synthesis of androgens, which are converted into estrogens by the aromatase system in granulosa and luteal cells [81–83]. The observation of a somewhat smaller effect of PRL (in the presence of hCG) on 3β-HSD protein in interstitial vs luteal cells could suggest a preferential stimulatory effect of hCG on interstitial androgen-producing cells [84]. In fact, the present data obtained by *in situ* hybridization appear to indicate that the inhibitory effect of PRL and the stimulatory effect of hCG are exerted primarily on luteal and interstitial cells, respectively.

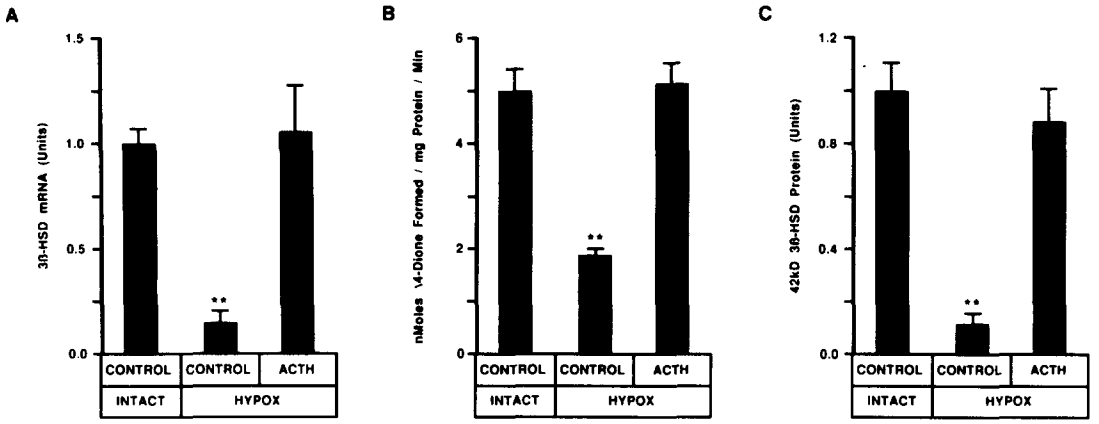

Fig. 11. Effects of hypophysectomy (HYPOX) and treatment with ACTH (10 IU) on female rat adrenal 3β-HSD mRNA levels (A), enzymatic activity (B) and specific protein content (C). Intact or hypophysectomized (15 days before) adult female rats received twice daily injections of vehicle (intact control and HYPOX control) or ACTH for 9 days. Data are expressed as means ± SEM ($n = 6$–7). $^{**}P < 0.01$ (figure taken from Ref. [97]).

The molecular mechanisms whereby PRL induces luteolysis and inhibits corpora lutea 3β-HSD expression are unknown. hCG and PRL exert opposite effects on 3β-HSD expression, indicating that the 3β-HSD gene(s) probably contain(s) elements responsive to the intracellular mediators of both hormones.

In luteinized porcine granulosa cells, gonadotropins as well as agents increasing intracellular cAMP accumulation [cholera toxin, forskolin, $(Bu)_2$cAMP] increased 3β-HSD mRNA levels [85, 86]. Activation of the protein kinase C pathway induces cAMP accumulation but leads to a marked inhibition of the stimulatory effect of hCG, LH, forskolin, and cholera toxin on 3β-HSD mRNA levels in luteinized porcine granulosa cells in culture [86].

Since it is well known that PRL exerts inhibitory effects on LH secretion [87], the present data indicate that PRL, in addition to its inhibitory effect at the hypothalamo–pituitary level, can also act directly on ovarian cells to suppress steroidogenic enzyme gene expression, thus providing an additional explanation for the antifertility effects of hyperprolactinemia in women [88, 89].

The balance between the actions of PRL, LH and FSH is finely modulated to control the expression of the steroidogenic enzymes in the ovary. For example, although PRL inhibits expression of aromatase in the rat ovary [90], aromatase activity is elevated in aging rats despite elevated circulating levels of PRL [91]. Low doses of LH/hCG can stimulate follicular development in pregnant rats where plasma PRL concentrations are high [92].

While much progress has been made towards a greater understanding of the regulation of the expression of other steroidogenic enzyme genes in the rat ovary, the recent availability of 3β-HSD cDNA probes permits detailed studies on the control of this crucial enzyme, thus offering the possibility of obtaining a more complete overall picture of the regulation of ovarian steroidogenesis. The close correlation observed between 3β-HSD mRNA, protein content, and activity levels indicate that the modulation of 3β-HSD activity by both hCG and PRL is largely, if not exclusively, related to parallel changes in 3β-HSD gene expression and/or 3β-HSD mRNA stability.

4.2. Regulation of 3β-HSD expression and activity in the rat adrenal

In the human adrenal cortex, 3β-HSD is necessary for the formation of the glucocorticoid cortisol and the mineralocorticoid aldosterone as well as DHEA and its sulfate DHEA-S, which act as precursors for the formation of androgens and estrogens in peripheral tissues [28, 93–95]. In the rat, corticosterone is the main glucocorticoid secreted by the adrenals. Thus, in the rat adrenocortical cell, following transformation of cholesterol into pregnenolone by the cholesterol side chain cleavage enzyme (*P*450scc), 3β-HSD converts PREG into PROG which is transformed into 11-deoxycorticosterone by steroid 21 hydroxylase (*P*450c21) and then into the glucocorticoid corticosterone

by the action of 11β-hydroxylase (11β-OHase) [94, 96].

Since no information was yet available on the control of expression of 3β-HSD in rat adrenals, we studied the regulation of expression and activity of 3β-HSD following treatment with adrenocorticotropin (ACTH) and corticosterone in the adrenals of intact rats of both sexes as well as the effect of ACTH in hypophysectomized female animals.

In intact male rats, ACTH stimulated 3β-HSD mRNA levels by 50% above the control value, while corticosterone caused a 58% decrease in the value of this parameter [97]. When corticosterone was administered in combination with ACTH, the individual effect of each treatment was neutralized. In contrast, in the intact female rat adrenal, 3β-HSD mRNA were unchanged by such treatments [97]. On the other hand, when the changes in adrenal size induced by each treatment were taken into account, a significant stimulatory effect of ACTH was observed in both male and female rats (56 and 32%, respectively), while corticosterone caused a 74% inhibition of 3β-HSD mRNA total content in males and a 39% decrease in females [97].

We next studied the effect of hypophysectomy on adrenal 3β-HSD mRNA levels [Fig. 11(A)], enzymatic activity [Fig. 11(B)] and protein content [Fig. 11(C)] in female rat adrenals. The results obtained on each parameter are almost superimposable. Hypophysectomy caused a marked decrease in the value of each parameter: 85% on mRNA levels, 62% on 3β-HSD activity and 88% on 3β-HSD protein content. Administration of ACTH to hypophysectomized female rats stimulated the value of each parameter to those found in intact animals. Since two types of 3β-HSD mRNAs are expressed in the rat adrenal [2], we wanted to assess the possibility of a differential regulation of the expression of the corresponding genes by ACTH and corticosterone. Type I mRNA is much more abundant than type II mRNA in the rat adrenal [2]. RNase protection analysis fo 2 μg total RNA from hypophysectomized female adrenals showed that ACTH treatment caused a marked and similar increase in the level of both 3β-HSD mRNA types compared to hypophysectomized vehicle-treated female rats [97].

These findings demonstrate the potent stimulatory effect of ACTH on the expression of the genes encoding 3β-HSD in the rat adrenal. The close parallelism observed between adrenal 3β-HSD mRNA levels, protein content and enzymatic activity suggests that the potent stimulatory effect of ACTH on corticosterone secretion can, at least partly, be explained by increased 3β-HSD gene expression and/or 3β-HSD mRNA stability. Moreover, the close correlation observed between these parameters suggests that 3β-HSD gene expression and/or 3β-HSD mRNA stability play an important role in the regulation of adrenocortical steroidogenesis.

5. CONCLUSIONS

The adrenals of humans and some other primates secrete large amounts of adrenal steroids, especially DHEA-S, which are metabolized into active androgens and estrogens in peripheral mammalian tissues [28–33, 98]. It is estimated that 40% of total androgens in men are synthesized in peripheral intracrine tissues from inactive adrenal precursors while in women, peripheral estrogen formation is even more important [28–33, 77, 98].

The formation of active sex steroids from the inactive adrenal precursors DHEA, DHEA-S and/or androstenedione locally in the same cells where synthesis takes place without release in the extracellular space has been recently described as intracrine activity [28]. Intracrine activity represents an economical system which requires minimal amounts of hormone to exert maximal function. In classical endocrine systems, large amounts of hormones are needed with only a small fraction used for regulation while the rest is degraded. Since steroid receptors have high affinity, it is likely that the active androgens and estrogens synthesized intracellularly through intracrine activity bind to their specific receptors with minimal loss of concentration or time.

The major importance of steroid biosynthesis by peripheral tissues is clearly indicated by the widespread distribution of expression of key steroidogenic enzymes, namely, 3β-HSD, 17β-HSD, 5α-reductase and aromatase in human tissues including skin, prostate, breast, endometrium, adipose tissue, lung and liver [21–25, 28, 98–111, and refs therein].

In summary, the recent characterization of the structure of the cDNAs and/or genes encoding these enzymes should greatly facilitate detailed *in vivo* as well as *in vitro* studies on the regulation of the expression of these crucial enzymes in peripheral tissues and thus facilitate

studies on the regulation of sex steroids formation and action in both normal and cancerous tissues.

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