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Over-expression of Human Type I (Placental) 3β-Hydroxy-5-ene-steroid Dehydrogenase/Isomerase in Insect Cells Infected with Recombinant Baculovirus

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Human type I placental 3β-hydroxy-5-ene-steroid dehydrogenase/steroid 5 → 4-ene-isomerase (3β-HSD/isomerase) synthesizes androstenedione from fetal dehydroepiandrosterone and progesterone from pregnenolone. The full length cDNA that encodes type I 3β-HSD/isomerase was inserted into the baculovirus, *Autographa californica* multiple nucleocapsid polyhedrosis virus, and expressed in *Spodoptera fungiperda* (Sf-9) insect cells. Western blots showed that the baculovirus-infected Sf-9 cells produced an immunoreactive protein that co-migrated with purified placental 3β-HSD/isomerase. Ultracentrifugation localized the expressed enzyme activities in all the membrane-associated organelles of the Sf-9 cell (nuclear, mitochondrial and microsomal). Kinetic studies showed that the expressed enzyme has 3β-HSD and isomerase activities. The Michaelis–Menton constant is very similar for the 3β-HSD substrate, 5α-androstan-3β-ol-17-one, in the Sf-9 cell homogenate ($K_m = 17.9\ \mu M$) and placental microsomes ($K_m = 16.7\ \mu M$). The 3β-HSD activity ($V_{max} = 14.5$ nmol/min/mg) is 1.6-fold higher in the Sf-9 cell homogenate compared to placental microsomes ($V_{max} = 9.1$ nmol/min/mg). The K_m values are almost identical for the isomerase substrate, 5-androstene-3,17-dione, in the Sf-9 cell homogenate ($K_m = 14.7\ \mu M$) and placental microsomes ($K_m = 14.4\ \mu M$). The specific isomerase activity is 1.5-fold higher in the Sf-9 cells ($V_{max} = 25.7$ nmol/min/mg) relative to placenta ($V_{max} = 17.2$ nmol/min/mg). These studies show that our recombinant baculovirus system over-expresses fully active enzyme that is kinetically identical to native 3β-HSD/isomerase in human placenta.

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INTRODUCTION

Human type I placental 3β-hydroxy-5-ene-steroid dehydrogenase (3β-HSD, EC 1.1.1.145) and steroid 5 → 4-ene-isomerase (EC 5.3.3.1) copurifies as a single protein from human placenta [1, 2], as it does from other species such as rat testis [3], rat adrenal gland [4] and bovine adrenal gland [5]. In human placenta, 3β-HSD/isomerase catalyzes a two-step reaction sequence that converts pregnenolone and dehydroepiandrosterone to progesterone and androstenedione [6], respectively.

As pregnancy nears term in humans, the fetus communicates hormonally with the mother through placental 3β-HSD/isomerase because the enzyme converts fetal dehydroepiandrosterone to androstenedione. Placental aromatase and 17β-hydroxysteroid oxidoreductase [7] convert the androstenedione to estradiol-17β. This estrogen prepares the uterus for labor by stimulating the synthesis of prostaglandins [8], the production of oxytocin receptors [9] and the formation of myometrial gap junctions [10]. The absence of 3β-HSD/isomerase in the fetal zone adrenal tissue [11, 12] suggests that placental 3β-HSD/isomerase mediates estrogen production near term and thereby

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may be part of the physiologic cascade that results in labor.

The full-length cDNA for the human type I placental 3β-HSD has been isolated [13] and expressed in low yield in COS-1 [14] and HeLa [15] cells. Studies are underway in this laboratory and others [16] that analyze the structure/function of 3β-HSD/isomerase by mutations. These experiments require the production of large quantities of mutant and wild-type enzyme from a reliable over-expression system.

The baculovirus, *Autographa californica* multiple nucleocapsid polyhedrosis virus (AcMNPV), utilizes insect cells for the expression of foreign genes. The insect cell line commonly used is *Spodoptera fungiperda* (Sf-9) derived from fall armyworm ovaries. Unlike bacteria, baculovirus-infected insect cells carry out posttranslational processing of the expressed foreign protein similar to that of mammalian cells. Sf-9 cells also target and transport recombinant proteins to their native locations and assemble subunits into functional proteins. Large amounts of expressed protein can easily be obtained from suspension cultures [17, 18].

In this report, we describe the eukaryotic expression of fully active human placental 3β-HSD/isomerase in Sf-9 cells infected with recombinant baculovirus and compare the kinetics of the expressed wild-type and native placental enzymes. The development of a baculovirus over-expression system for this important steroid-metabolizing enzyme improves our ability to characterize the 3β-HSD/isomerase reaction mechanisms by molecular cloning technology.

EXPERIMENTAL

Materials

DNA modifying enzymes and Hind III λ molecular weight markers were purchased from New England Biolabs and Stratagene; Max Bac Expression System from InVitrogen Co.; TNM-FH insect medium, fetal calf serum, penicillin/streptomycin and fungizone from Washington University Tissue Support Center; trypan blue, tris and goat anti-rabbit IgG (^{125}I-labeled, 1–2 μCi) from ICN Biochemicals; acrylamide, pyridine nucleotides and EDTA from Sigma Chemical Co.; *N,N,N′,N′*-tetramethylethylenediamine, *N,N′*-methylene-bis-acrylamide, ammonium persulfate and DNA grade agarose from Bio-Rad Laboratories; Hybond-C extra supported nitrocellulose, 0.45 micron, from Amersham Corp.; 5α-androstan-3β-ol-17-one, pregnenolone, and 5-androstene-3,17-dione from Steraloids, Inc.; reagent and molecular biology grade salts from Fisher Scientific Co. Glass distilled, deionized water was used for all aqueous solutions.

Plasmid construction

The full-length 1.5 kb cDNA human 3β-hydroxysteroid dehydrogenase/Isomerase, 3β-HSD, was removed from pCMV5H3βHSD [14] by first linearizing the plasmid with EcoRI, followed by digestion with PspAI. This fragment was then inserted into the 9.6 kb plasmid pVL1392 baculovirus expression Max Bac Expression System (InVitrogen Co.) that contained the AcMNPV polyhedrin promoter, partial polyhedrin gene and a multiple cloning site to generate pVL1392-3βHSD. The construct was analyzed by restriction digest by BamHI, PvuII and double digest by SmaI and EcoRI, followed by separation via agarose electrophoresis to identify the correct placement in the expression vector.

Construction and screening of recombinant virus

Linearized AcMNPV DNA, plasmid DNA pVL1392-3βHSD and cationic liposomes were mixed. Sf-9 cells were infected with the transfection mixture for 48 h, at which time the transfection mixture was removed, stored at 4°C, and replaced with TNM-FH insect medium supplemented with 10% fetal calf serum, 10 μg/ml penicillin/streptomycin and 5 μg/ml fungizone (hereafter referred to as complete TNM-FH insect medium [19]). The infected cells were incubated for a total of 4 days postinfection to insure growth of recombinant (polyhedrin-negative) as well as wild-type (polyhedrin-positive) virus. Polyhedrin-free recombinant virus was obtained after 2 rounds of plaque purification. The supernatant was labeled as primary stock and stored at 4°C [19].

Cells and viral infections

Sf-9 cells were obtained as a gift from Drs Robert Mercer and Gustavo Blanco and maintained as described previously [19, 20]. Infections were carried out with log phase cells that showed viability of 98% (or greater) as determined by trypan blue dye exclusion. Viral stock was added to the multiplicity of infection (m.o.i.) between 5–10 and allowed to infect for 1 h and then diluted to 1.2×10^6 cells/ml with complete TNM-FH insect media. Cells were harvested 4–5 days postinfection [19].

Western blot analysis

Proteins were separated by SDS–polyacrylamide (7.5%) gel electrophoresis (SDS–PAGE) and electroblotted onto nitrocellulose membrane using Bio-Rad Trans-Blot [20]. The nitrocellulose was probed with primary antibody raised against 3β-HSD [21], followed by goat anti-rabbit IgG (^{125}I labeled, 1–2 μCi), dried and exposed overnight for autoradiography. Immunoreactive bands were quantified by densitometry utilizing an Epson ES-300C scanner interfaced with a Macintosh IIci computer. Exposures having signals within the linear range of the film were used for analysis [20].

Enzyme extraction and subcellular localization

Sf-9 cells were harvested at times described above from 100 ml suspension culture and centrifuged at 1000 *g* at 4°C. The supernatant contained the viral stock and was stored at 4°C. The cell pellet was

resuspended in 0.02 M potassium phosphate, pH 7.4, 20% glycerol (Buffer A) and washed three times. The cells were resuspended in 5 ml of Buffer A and homogenized on ice using a Brinkmann Polytron followed by probe sonication (Kontes Microultrasonic Cell Disrupter). The homogenate was centrifuged for 30 min at 1500 *g* in a Beckman model L5-65 ultracentrifuge to obtain nuclei. The supernatant was then centrifuged for 40 min at 12,000 *g* to obtain mitochondria. The resulting supernatant was centrifuged for 1 h at 110,000 *g* to obtain microsomes. The 110,000 *g* supernatant was the Sf-9 cell cytosol. Protein concentrations was determined by the method of Bradford [22].

Kinetic studies

Kinetic constants for 3β-HSD activity were determined in incubations containing 5α-androstan-3β-ol-17-one (10.0–50.0 μM, 4% final methanol content), NAD^+ (0.2 mM) and Sf-9 cell homogenate or placental microsomes (0.08 mg protein) in 0.05 M glycine-NaOH buffer, pH 9.7, at 22°C (1.0 ml total vol). Comparisons of specific 3β-HSD activity in the subcellular fractions of Sf-9 cells used the same assay with 5α-androstan-3β-ol-17-one (50.0 μM) or pregnenolone (10.0 μM) as substrate. The increase in absorbance per min at 340 nm (due to NADH formation) was measured by a Varian (Palo Alto, CA) model Cary 219 spectrophotometer to determine the 3β-HSD activity.

Kinetic constants for isomerase activity were measured in incubations containing 5-androstene-3,17-dione (5.0–40.0 μM, 4% final methanol content), NADH (0.05 mM) and Sf-9 cell homogenate or placental microsomes (0.04 mg protein) in 0.02 M potassium phosphate buffer, pH 7.4, at 22°C. Isomerase activity in the subcellular fractions of Sf-9 cells used the same assay with 5-androstene-3,17-dione (50.0 μM) as substrate. The isomerase activity was calculated from the increase in absorbance per min at 241 nm (due to androstenedione formation).

The K_m and V_{max} values of 3β-HSD and isomerase substrates were compared for wild-type enzyme expressed in Sf-9 cells and native enzyme in placental microsomes using Hanes–Woolf (S vs S/v) plots. This method of kinetic data analysis is appropriate for crude enzyme preparations because it allows the use of a broad range of evenly spaced substrate concentrations [23].

RESULTS

Western blot analysis (Fig. 1) shows that the 3β-HSD/isomerase protein is expressed in Sf-9 cells infected with the recombinant baculovirus. The expressed protein co-migrates with the purified human placental enzyme and with enzyme in placental microsomes. Uninfected Sf-9 cells do not express the enzyme protein.

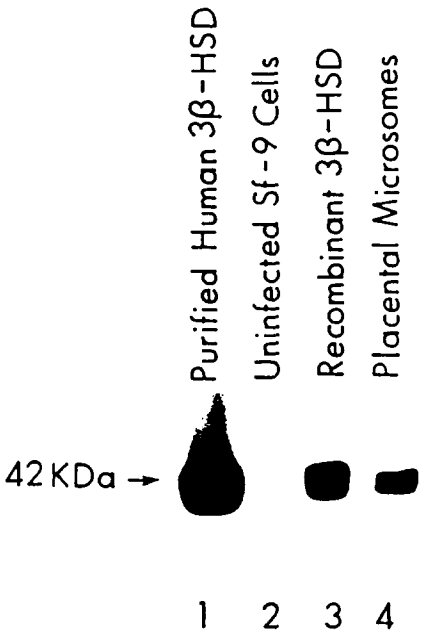

Fig. 1. Western blot showing over-expression of human type I 3β-HSD/isomerase in insect Sf-9 cells infected with recombinant baculovirus. Lane 1 contains purified 3β-HSD (2.0 μg); lane 2 contains uninfected Sf-9 cell homogenate (6.0 μg); lane 3 contains Sf-9 cell homogenate (6.0 μg) after infection with the recombinant 3β-HSD baculovirus; and lane 4 contains human placental microsomal protein (6.0 μg).

Laser densitometry measured the mass of 3β-HSD/isomerase protein in the infected Sf-9 cell homogenate by comparison to Western blots of pure enzyme of known mass. The infected Sf-9 cells produce 4.2 μg of enzyme protein/ml cell suspension (approx. 8×10^5 cells/ml) or 3.2% of the total Sf-9 homogenate protein. The same method determined that 3β-HSD/isomerase represents only 0.6% of total protein in placental microsomes.

Ultracentrifugation of baculovirus-infected Sf-9 cell homogenates localized the expressed 3β-HSD and isomerase activities in the nuclear, mitochondrial and microsomal subcellular fractions (Table 1). The cytosol

Table 1. Specific activities of the expressed 3β-HSD and isomerase among subcellular fractions of Sf-9 cells

Cell fraction	3β-HSD activity* (nmol/min/mg) Pregnenolone	5α-A-3β-ol	Isomerase activity† (nmol/min/mg) 5-A-3,17-dione
Cell homogenate	4.2	11.3	28.6
Nuclear pellet	3.9	10.0	33.0
Mitochondria	2.2	6.2	22.1
Microsomes	2.3	4.3	29.3
Cytosol	0.9	2.2	9.3

*For the 3β-HSD assay, the Sf-9 cell fraction protein (0.08 mg) was incubated with pregnenolone (10.0 μM) or 5α-androstan-3β-ol-17-one (5α-A-3β-ol, 50.0 μM) and NAD^+ (0.20 mM) in 0.05 M glycine-NaOH, pH 9.7.

†In the isomerase assay, the Sf-9 cell fraction protein (0.04 mg) was incubated with 5-androstene-3,17-dione (5-A-3,17-dione, 50.0 μM) and NADH (0.05 mM) in 0.02 M potassium phosphate buffer, pH 7.4.

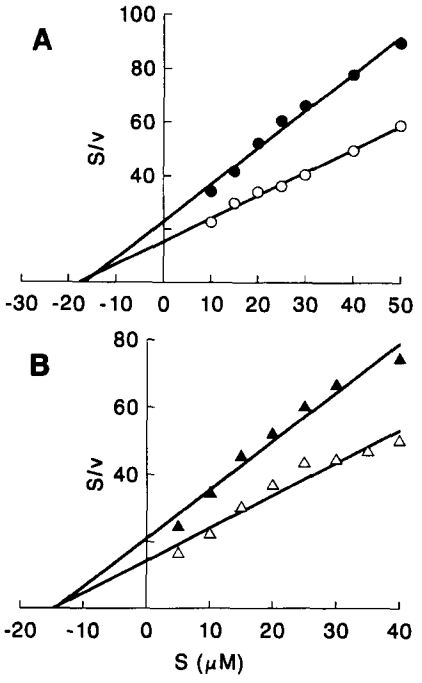

Fig. 2. Hanes–Woolf plots determine the kinetic constants for substrates of 3β-HSD and isomerase in baculovirus-infected Sf-9 cells and placental microsomes. The enzyme activities were assayed as described in the text. (A) The kinetics of 3β-HSD substrate utilization were measured using 5α-androstan-3β-ol-17-one (10.0–50.0 μM) and Sf-9 cell homogenate (○) or placental microsomes (●). (B) The kinetics of isomerase substrate utilization were measured using 5-androstene-3,17-dione (5.0–40.0 μM) and Sf-9 cell homogenate (△) or placental microsomes (▲). Substrate concentrations (S) are plotted along each abscissa. Substrate concentration divided by enzyme activity (S/v) is represented on each of the ordinates. The abscissa intercept = $-1/K_m$, and the slope = $1/V_{max}$. Each point represents the mean of two determinations.

fraction contains less than 10% of the total 3β-HSD or isomerase activity (data not shown). The 3β-HSD substrate, 5α-androstan-3β-ol-17-one (5α-A-3β-ol), produces a 2.5-fold higher 3β-HSD activity than pregnenolone, as was previously observed for purified human placental 3β-HSD activity [24]. Although it is important to show that the expressed enzyme has the physiologic placental 3β-HSD activity using pregnanolone, the higher enzyme activity with 5α-A-3β-ol permitted kinetic constants to be determined for 3β-HSD activity in these crude enzyme preparations.

Because the expressed 3β-HSD/isomerase activities are distributed throughout the Sf-9 cell membranes, kinetic constants for substrates were determined using Sf-9 homogenates and compared to those measured with placental microsomes. The Hanes–Woolf plots for the 3β-HSD activity expressed in insect cells and for the native placental enzyme intersect at the abscissa [Fig. 2(A)]. This indicates that the K_m values are very similar for 5α-A-3β-ol in the Sf-9 cell homogenate ($K_m = 17.9\ \mu M$) and placental microsomes ($K_m = 16.7\ \mu M$). The maximal specific 3β-HSD activity (V_{max} = 1/slope) is 1.6-fold higher in the Sf-9 cell homogenate ($V_{max} = 14.5$ nmol/min/mg) compared to placental microsomes ($V_{max} = 9.1$ nmol/min/mg). There is a similar relationship between the isomerase activities in the two tissues [Fig. 2(B)]. The K_m values are almost identical for 5-androsten-3,17-dione in the Sf-9 cell homogenate ($K_m = 14.7\ \mu M$) and placental microsomes ($K_m = 14.4\ \mu M$). The specific isomerase activity is 1.5-fold higher in the Sf-9 cells ($V_{max} = 25.7$ nmol/min/mg) relative to placenta ($V_{max} = 17.2$ nmol/min/mg).

DISCUSSION

The identification of catalytic amino acids that participate in an enzyme reaction mechanism is an achievable goal because of advances made in site-directed mutagenesis [25]. After affinity labeling maps the binding sites for substrates in the enzyme structure [26], probable catalytic amino acids in the identified region can be substituted by point mutation or deleted to test their function. The great promise of this technology is dependent upon having a reliable system to express the mutant protein from the modified cDNA that encodes it. This requirement can be particularly problematic when the protein of interest is membrane-bound, like human placental 3β-HSD/isomerase [6].

The expression system described in this report uses Sf-9 cells infected with recombinant baculovirus to produce substantial amounts of fully active human type I (placental) 3β-HSD/isomerase. Quantitation of the mass of the enzyme by laser densitometry of Western blots shows that 3β-HSD/isomerase represents a larger proportion of total protein in the infected Sf-9 cell homogenate than in placental microsomes. Based on the densitometry study, a 2.0 l suspension of infected Sf-9 cells produces 8.4 mg of active 3β-HSD/isomerase.

The 3β-HSD and isomerase activities are distributed among all of the membrane-associated organelles (nuclear, mitochondrial and microsomal) in the Sf-9 cells (Table 1). There is an approx. 10-fold higher isomerase than 3β-HSD activity in each organelle. This activity ratio mirrors the relationship between the 3β-HSD and isomerase activities in the single enzyme protein purified from human placenta [1, 2]. The 3β-HSD activity measured in microsomes and mitochondria of Sf-9 cells is lower than in the nuclear pellet and homogenate, but the isomerase activity is evenly distributed among all four preparations. This apparent difference may reflect the higher concentration of NADH in the metabolically active microsomes and mitochondria. NADH is a potent inhibitor of 3β-HSD activity [27], but isomerase is stimulated by NADH [28]. In addition, the enzyme protein may be bound to the nuclear membrane in a conformation that stimulates 3β-HSD activity relative to that measured in enzyme bound to other organelle-membranes. For

example, we measured an unusually high 3β-HSD activity (relative to the expected isomerase activity) in purified human placental enzyme that had been bound to artificial phospholipid vesicles (unpublished observation).

The 3β-HSD/isomerase enzyme is localized in the microsomes and mitochondria of human placenta [1, 2, 6]. Because the enzyme activities are present in the nuclear fraction as well as microsomes and mitochondria in Sf-9 cells, insect cells infected with the recombinant baculovirus target membranes for enzyme placement somewhat less precisely than placental trophoblasts normally do.

After determining the amount of enzyme present in the Sf-9 homogenate (3.2% of total protein), the calculated mass of expressed enzyme protein exhibits a specific 3β-HSD activity for pregnenolone that is similar to pure 3β-HSD activity (75 nmol/min/mg [2]) and has an expressed isomerase specific activity that equals that of pure enzyme (900 nmol/min/mg [2]) (data not shown). Thus, the expressed 3β-HSD/isomerase is fully active with little "dead" enzyme protein.

The measurement of the same K_m values for the substrates of 3β-HSD or isomerase in the Sf-9 cell homogenate and placental microsomes suggests that the expressed wild-type enzyme is very similar, if not identical, to the native placental enzyme. Co-migration of the wild-type and native enzyme in the Western blots is further evidence for identical proteins.

Our characterization of the expressed wild-type enzyme shows that the baculovirus system is an excellent method to produce mutant 3β-HSD/isomerase proteins that can be used to relate structure to function. The development of this over-expression system is a significant advance that allows the reaction mechanisms of 3β-HSD/isomerase to be determined by site-directed mutagenesis.

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REFERENCES

1. Thomas J. L., Berko E. A., Faustino A., Myers R. P. and Strickler R. C.: Human placental 3β-hydroxy-5-ene-steroid dehydrogenase and steroid 5 → 4-ene-isomerase: purification from microsomes, substrate kinetics and inhibition by product steroids. *J. Steroid Biochem.* **31** (1988) 785–793.
2. Thomas J. L., Myers R. P. and Strickler R. C.: Human placental 3β-hydroxy-5-ene-steroid dehydrogenase and steroid 5 → 4-ene-isomerase: purification from mitochondria and kinetic profiles, biophysical characterization of the purified mitochondrial and microsomal enzymes. *J. Steroid Biochem.* **33** (1989) 209–217.
3. Ishii-Ohba H., Inano H. and Tamaoki B. I.: Purification and properties of testicular 3β-hydroxy-5-ene-steroid dehydrogenase and 5-ene-4-ene-isomerase. *J. Steroid Biochem.* **25** (1986) 555–560.
4. Ishii-Ohba H., Saiki N., Inano H. and Tamaoki B. I.: Purification of rat adrenal 3β-hydroxy-5-ene-steroid dehydrogenase and 5-ene-4-ene-isomerase. *J. Steroid Biochem.* **24** (1986) 753–760.
5. Rutherfurd K. J., Chen S. and Shively J. E.: Isolation and amino acid sequence of bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase. *Biochemistry* **30** (1991) 8108–8116.
6. Ferre F., Breuiller M., Cedard L., Dechesne M. J., Saintot M., Descomps B. and Crastes de Paulet A.: Human placental Δ_5-3βhydroxysteroid dehydrogenase activity (Δ^5-3β HSDH): intracellular distribution, kinetic properties, retro-inhibition and influence of membrane delipidation. *Steroids* **246** (1975) 551–570.
7. Csapo A. I.: The "see-saw" theory of parturition. In *The Fetus and Birth* (Edited by J. Knight and M. O'Connor). Elsevier, Amsterdam, Vol. 47 (1977) pp. 159–210.
8. Schatz F. and Gurpide E.: Effects of estradiol on prostaglandin $F_{2\alpha}$ levels in primary monolayer cultures of epithelial cells from human proliferative endometrium. *Endocrinology* **113** (1983) 1274–1279.
9. Alexandrova M. and Soloff M. S.: Oxytocin receptors and parturition. I. Control of oxytocin receptor concentration in the rat myometrium at term. *Endocrinology* **106** (1980) 730–735.
10. Garfield R. E., Kannan M. S. and Daniel E. E.: Gap junction formation in myometrium: control by estrogens, progesterone and prostaglandins. *Am. J. Physiol.* **238** (1980) C81–C89.
11. Mason J. I., Ushijima K., Doody K. M., Nagai K., Naville D., Head J. R., Milewich L., Rainey W. E. and Ralph M. M.: Regulation of expression of the 3β-hydroxysteroid dehydrogenases of human placenta and fetal adrenal. *J. Steroid Biochem. Molec. Biol.* **47** (1993) 151–159.
12. Mesiano S., Coulter C. L. and Jaffe R. B.: Localization of cytochrome *P*450 cholesterol side-chain cleavage, cytochrome *P*450 17α-hydroxylase/17,20-lyase, and 3β-hydroxysteroid dehydrogenase isomerase steroidogenic enzymes in human and rhesus monkey fetal adrenal glands: reappraisal of functional zonation. *J. Clin. Endocr. Metab.* **77** (1993) 1184–1189.
13. Luu-The V., LaChance Y., Labrie C., Leblanc G., Thomas J. L., Strickler R. C. and Labrie F.: Full-length cDNA structure and deduced amino acid sequence of human 3β-hydroxy 5-ene-steroid dehydrogenase. *Molec. Endocr.* **3** (1989) 1310–1312.
14. Lorence M. C., Murry B. A., Trant J. M. and Mason J. I.: Human 3β-hydroxysteroid dehydrogenase/$\Delta^{5\rightarrow4}$ isomerase from placenta: expression in nonsteroidogenic cells of a protein that catalyzes the dehydrogenation/isomerization of C21 and C19 steroids. *Endocrinology* **126** (1990) 2493–2498.
15. Rheaume E., Lachance Y., Zhao H., Breton N., Dumont M., de Launoit Y., Trudel C., Luu-The V., Simard J. and Labrie F.: Structure and expression of a new complementary DNA encoding the almost exclusive 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase in human adrenals and gonads. *Molec. Endocr.* **5** (1991) 1147–1157.
16. Chang Y. T., Kappy M. S., Iwamoto K., Wang J., Yang X. and Pang S.: Mutations in the type II 3β-hydroxysteroid dehydrogenase gene in a patient with classic salt-wasting 3β-hydroxysteroid dehydrogenase deficiency congenital adrenal hyperplasia. *Pediatric Res.* **34** (1993) 698–700.
17. Luckow V. A.: Cloning and expression of heterologous genes in insect cells with baculovirus vectors. In *Recombinant DNA Technology and Applications* (Edited by A. Prokop, R. K. Bajpai and C. Ho). McGraw-Hill, NY (1990) pp. 97–152.
18. Miller D. W., Safer P. and Miller L. K.: An insect baculovirus host-vector system for high-level expression of foreign genes. In *Genetic Engineering* (Edited by J. K. Setlow and A. Hollander). Plenum, NY, Vol. 8 (1986) pp. 277–298.
19. Summers M. D. and Smith G. E.: A manual of methods for baculovirus and insect cell culture procedures. *Texas Agricultural Experiment Station Bulletin* **1555** (1987).
20. DeTomaso A. W., Xie Z. J., Liu G. and Mercer R. W.: Expression, targeting, and assembly of functional Na, K-ATPase polypeptides in baculovirus-infected insect cells. *J. Biol. Chem.* **268** (1993) 1470–1478.
21. Doody K. M., Carr B. R., Rainey W. E., Byrd W., Murry B. A., Strickler R. C., Thomas J. L. and Mason J. I.: 3β-Hydroxysteroid dehydrogenase/isomerase in the fetal zone and neocortex of the human fetal adrenal gland. *Endocrinology* **126** (1990) 2487–2492.
22. Bradford M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72** (1976) 248–254.

23. Segel I. H.: *Enzyme Kinetics: Behavior and analysis of rapid equilibrium and steady state enzyme systems.* John Wiley and Sons, NY (1975) pp. 208–214.
24. Milewich L., Shaw C. E., Mason J. I., Carr B. R., Blomquist D. H. and Thomas J. L.: 3β-Hydroxysteroid dehydrogenase activity in tissues of the human fetus determined with 5α-Androstane-3β,17β-diol and dehydroepiandrosterone as substrates. *J. Steroid Biochem. Molec. Biol.* **45** (1993) 525–537.
25. Kuliopulos A., Talalay P. and Mildvan A. S.: Combined effects of two mutations of catalytic residues on the ketosteroid isomerase reaction. *Biochemistry* **29** (1990) 10,271–10,280.
26. Thomas J. L., Nash W. E., Myers R. P., Crankshaw M. W. and Strickler R. C.: Affinity radiolabeling identifies peptides and amino acids associated with substrate binding in human placental 3β-hydroxy-Δ^5-steroid dehydrogenase. *J. Biol. Chem.* **268** (1993) 18,507–18,512.
27. Thomas J. L., Myers R. P., Rosik L. O. and Strickler R. C.: Affinity alkylation of human placental 3β-hydroxy-5-ene-steroid dehydrogenase and steroid 5 → 4-ene-isomerase by 2α-bromoacetoxyprogesterone: evidence for separate dehydrogenase and isomerase sites on one protein. *J. Steroid Biochem.* **36** (1990) 117–123.
28. Thomas J. L., Myers R. P. and Strickler R. C.: Analysis of coenzyme binding by human placental 3β-hydroxy-5-ene-steroid dehydrogenase and steroid 5 → 4-ene-isomerase using 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine, an affinity labeling cofactor analog. *J. Steroid Biochem. Molec. Biol.* **39** (1991) 471–477.