Molecular Basis of Congenital Adrenal Hyperplasia due to 3β-Hydroxysteroid Dehydrogenase Deficiency

Jacques Simard*, Eric Rhéaume†, Rocio Sanchez‡, Nathalie Laflamme, Yvan de Launoit§, Van Luu-The, Arnoud P. van Seters, Richard D. Gordon, Markus Bettendorf, Udo Heinrich, Thomas Moshang, Maria I. New, and Fernand Labrie

Medical Research Council Group in Molecular Endocrinology (J.S., E.R., R.S., N.L., Y.d.L., V.L.-T., F.L.)
CHUL Research Center and Laval University
Québec, G1V 4G2, Canada

Department of Endocrinology and Metabolic Diseases (A.P.v.S.)
University Hospital Leiden
2333 AA Leiden, The Netherlands

Department of Medicine (R.D.G.)
University of Queensland and Greenslopes Hospital
Brisbane 4120, Queensland, Australia

Kinderklinik (M.B., U.H.)
Abteilung Pädiatrische Endokrinologie
Ruprecht-Karls-Universität Heidelberg
6900 Heidelberg, Germany

Division of Endocrinology (T.M.)
Children's Hospital of Philadelphia and
University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania 19104

Department of Pediatrics (M.I.N.)
Division of Pediatric Endocrinology
The New York Hospital-Cornell Medical Center
New York, New York 10021

Congenital adrenal hyperplasia is the most frequent cause of adrenal insufficiency and ambiguous genitalia in newborn children. In contrast to congenital adrenal hyperplasia due to 21-hydroxylase and 11β-hydroxylase deficiencies, which impair steroid formation in the adrenal cortex, exclusively, classical 3β-hydroxysteroid dehydrogenase (3β-HSD) deficiency affects steroid biosynthesis in the gonads as well as in the adrenals. The structures of the highly homologous type I and II 3β-HSD genes have been analyzed in three male pseudohermaphrodite 3β-HSD deficient patients from unrelated families in order to elucidate the molecular basis of classical 3β-HSD deficiency from patients exhibiting various degrees of severity of salt losing. The nucleotide sequence of DNA fragments generated by selective polymerase chain reaction amplification that span the four exons, the exon-intron boundaries, as well as the 5′-flanking region of each of the two 3β-HSD genes have been determined in the three male patients. The five point mutations characterized were all detected in the type II 3β-HSD gene, which is the gene predominantly expressed in the adrenals and gonads, while no mutation was detected in the type I 3β-HSD gene, predominantly expressed in the placenta and peripheral tissues. The two male patients suffering from severe salt-losing 3β-HSD deficiency are compound heterozygotes, one bearing the frame-shift mutation 186/insC/187 and the missense mutation Y253N, while the other bears the nonsense mutation W171X and the missense mutation E142K. The influence of the detected missense mutations on enzymatic activity was assessed by *in vitro* expression analysis of mutant recombinant enzymes generated by site-directed mutagenesis in heterologous mammalian cells. Recombinant mutant

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type II 3β-HSD enzymes carrying Y253N or E142K substitutions exhibit no detectable activity. On the other hand, the nonsalt-losing patient is homozygous for the missense mutation A245P. This mutation decreases 3β-HSD activity by approximately 90%. The present findings, describing the first missense mutations in the human type II 3β-HSD gene, provide unique information on the structure-activity relationships of the 3β-HSD superfamily. Moreover, the present findings provide a molecular explanation for the enzymatic heterogeneity responsible for the severe salt-losing form to the clinically inapparent salt-wasting form of classical 3β-HSD deficiency. The impairment of steroid formation in both the adrenal and gonadal tissues of patients suffering from classical 3β-HSD deficiency thus results from mutation(s) in the type II 3β-HSD gene, causing various levels of impairment of enzymatic activity and, consequently, varying clinical severity of the disease. On the other hand, the finding of a normal type I 3β-HSD gene provides the basis for the well recognized intact peripheral intracrine steroidogenesis in these patients. (Molecular Endocrinology 7: 716–728, 1993)

INTRODUCTION

Steroid hormones play a crucial role in the differentiation, development, growth, and physiological function of most human tissues (1). Of special importance is membrane-bound 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD), which catalyses the conversion of Δ^5-3β-hydroxysteroids, namely pregnenolone, 17-OH pregnenolone, dehydroepiandrosterone, and androst-5-ene-3β,17β-diol, into the corresponding Δ^4-3-ketosteroids, progesterone, 17-OH-progesterone, Δ^4-androstenedione, and testosterone, respectively. This enzyme is in fact required for the formation of all classes of steroid hormones, namely progesterone, mineralocorticoids, and glucocorticoids, as well as androgens and estrogens (2–7).

Classical 3β-HSD deficiency is responsible for a severe form of congenital adrenal hyperplasia (CAH) with impaired steroidogenesis in both the adrenals and gonads (8–16), while 21-hydroxylase and 11β-hydroxylase deficiencies are characterized by defective steroid formation limited to the adrenal cortex. Newborns affected by 3β-HSD deficiency exhibit signs and symptoms of adrenal insufficiency of varying degree, which may be fatal if not diagnosed and treated early (8–17), although cases of nonsalt-wasting 3β-HSD deficiency have also been described (10, 18–23). In male newborns, the 3β-HSD defect in the testis leads to pseudohermaphroditism with incomplete masculinization of the external genitalia, while affected females show normal sexual differentiation or mild virilization (8–23). An elevated ratio of Δ^5- to Δ^4-steroids is considered to be the best biological parameter for the diagnosis of 3β-HSD deficiency (15, 17). It is well recognized, however, that 17-OH-progesterone and Δ^4-androstenedione plasma levels and other Δ^4-steroids are frequently elevated in 3β-HSD-deficient patients (9–11, 13–15, 17, 22, 23). Such observations suggest the expression of functional 3β-HSD in peripheral tissues, which is responsible for the extraadrenal and extragonadal conversion of Δ^5-hydroxysteroid precursors into the corresponding Δ^4-3-ketosteroids.

The structures of two types of human 3β-HSD cDNA clones encoding the type I and type II 3β-HSD isoenzymes, respectively, have been recently characterized (2, 4, 6). The complete nucleotide sequence of the corresponding genes shows that both consist of four exons and three introns included within a DNA fragment of approximately 7.8 kilobases (3, 5, 7) and assigned to chromosome 1p13 (24). These data provide the necessary tools to elucidate the molecular basis of classical 3β-HSD deficiency and will most likely provide useful information on the structure-activity relationships of the 3β-HSD superfamily (1–7, 25–33).

RESULTS

In order to identify the molecular lesions in the type I and/or type II 3β-HSD genes responsible for the syndrome of classical 3β-HSD deficiency in patients from three unrelated families, we determined the nucleotide sequence of the whole coding region, exon-intron splicing boundaries, as well as 5′-flanking region including the putative promoter and the 3′-noncoding region including the polyadenylation site of each of the two genes, using two sets of five primer pairs for selective polymerase chain reaction (PCR) amplification (Fig. 1). All the amplified PCR fragments of both 3β-HSD genes from the three male pseudohermaphrodite patients had the expected length, thus suggesting the absence of relatively small gross abnormalities within these fragments. Moreover, the complete sequence of the PCR products obtained from the type I 3β-HSD gene from the three patients was identical to that of the corresponding known regions in the normal gene (3, 5), thus providing no evidence suggesting a genetic alteration affecting type I mRNA processing or a structural change in the type I 3β-HSD isoenzyme in these three patients.

Selective PCR amplification of the type II 3β-HSD gene from the index case of family 1 (34, 35) revealed the presence of a maternal frame-shift mutation in exon IV of one allele (Fig. 2). This point mutation, designated 186/insC/187, results from insertion of a single C between codons 186 and 187, thus leading to a predicted truncated protein of 202 amino acids (including the first Met) (Fig. 2). The frame-shift mutation was also confirmed by manual sequencing after subcloning of PCR products obtained with the primer pair II.7866u-II8824d into Bluescript KS II plasmid. In addition, this index case carries a paternal missense point mutation in the other type II 3β-HSD allele. This mutation, designated Y253N,

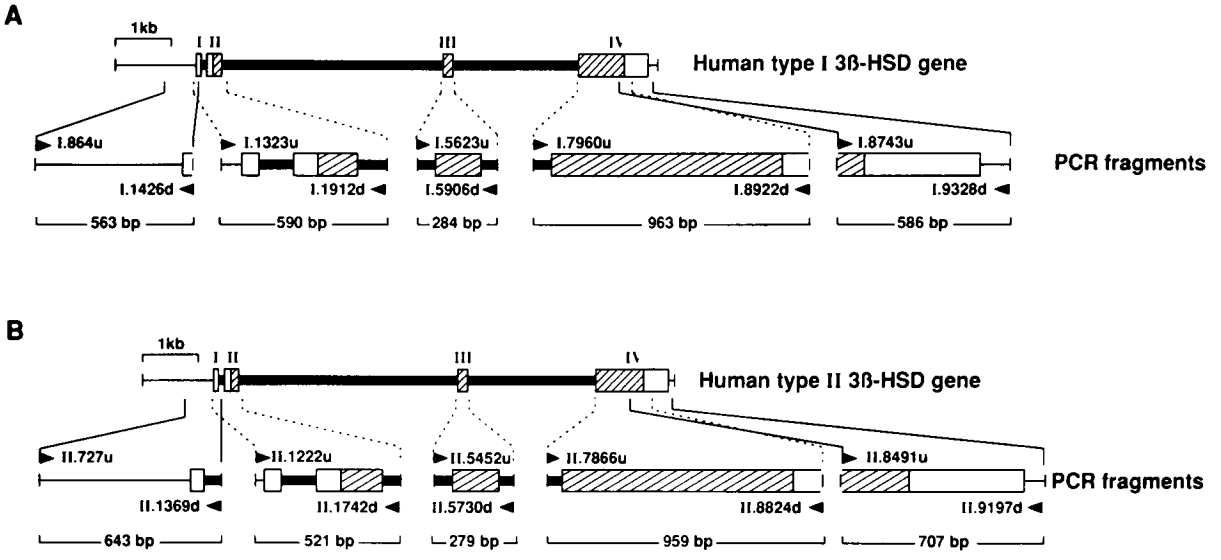

Fig. 1. Schematic Representation of the Human Type I (A) and Type II (B) 3β-HSD Genes and the Selective PCR Amplification Strategy of Their Coding Regions, Exon-Intron Splicing Boundaries, as Well as 5′- and 3′-Noncoding Regions

Exons I, II, III, and IV of the type I and II 3β-HSD genes are represented by *boxes* in which *hatched lines* demarcate the coding regions, while *open boxes* represent the noncoding regions. Introns are represented by *black bold lines*. The location and directionality of the oligonucleotide primer binding sites used for PCR amplification are shown by *numbered arrowheads*. The numbers indicate the binding site position of the 5′-nucleotide of each 25-mer primer in the type I or in the type II 3β-HSD gene; u and d indicate upstream or downstream primers, respectively.

is caused by a T to A transversion converting codon 253 (TAT) encoding Tyr into AAT encoding Asn (Fig. 2). The unaffected brother is heterozygous for the maternal 186/insC/187 mutation (Fig. 2A).

Elucidation of the sequence of the type II 3β-HSD gene from the patient of family 2 (17) indicates the presence of an inherited paternal nonsense W171X mutation caused by a G to A transition, thus changing codon 171 (TGG) encoding Trp to a TAG stop codon, and of a maternal missense E142K point mutation due to a G to A transition converting codon 142 (GAA) encoding Glu into AAA encoding Lys (Fig. 3).

As shown in Fig. 4, the proband of family 3 (36) is homozygous for the missense point mutation A245P changing codon 245 (GCC) encoding Ala to CCC encoding Pro in his type II 3β-HSD gene, while both parents and his unaffected sister are heterozygous for the same mutation. It is of interest to note that the A245P as well as the E142K and Y253N point mutations were not found in more than 40 unrelated individuals, indicating that these single nucleotide changes do not correspond to polymorphisms present in the normal population.

In order to assess the influence of point mutations Y253N, E142K, and A245P on 3β-HSD activity, we then compared the activity of wild type II 3β-HSD to that of mutants type II 3β-HSD Y253N, E142K, and A245P generated by site-directed mutagenesis. As illustrated in Fig. 5A, immunoblot analysis shows that the amount of mutant recombinant 3β-HSD Y253N, E142K, and A245P proteins is similar to that of the wild type type II 3β-HSD after transient expression in COS-1 monkey kidney cells. We first investigated the time course of formation of [^{3}H]progesterone (PROG) from [^{3}H]pregnenolone (PREG) in intact COS-1 cells in culture transfected with pCMV (cytomegalovirus), pCMV type II 3β-HSD, pCMV-Y253N, pCMV-E142K, or pCMV-A245P. As illustrated in Fig. 5B, in cells transfected with pCMV-Y253N or pCMV-E142K, no significant conversion of [^{3}H]PREG into [^{3}H]PROG could be detected over basal values obtained in cells transfected with the pCMV plasmid, thus suggesting the lack of detectable activity of these mutant recombinant proteins. Furthermore, it can be seen in Fig. 5B that in cells transfected with pCMV type II 3β-HSD, the percent of [^{3}H]PROG formed after 15, 30, 60, 120, and 360 min of incubation was 6.5, 7.0, 21.4, 32.3, and 59.6 (above basal values), respectively. It is noteworthy that in cells transfected with pCMV-A245P, the percent of [^{3}H]PROG formed after 60, 120, and 360 min was 1.1, 2.4, and 4.0 (above basal values), respectively, which is significantly higher ($P < 0.01$) than that measured in control pCMV-transfected cells, showing 6.7–10.2% of the enzymatic activity catalyzed by wild type human type II 3β-HSD.

In order to ascertain that the change of 3β-HSD activity of the three mutant recombinant proteins demonstrated in intact transfected cells in culture is also observed when measured by classical approaches using transfected cell homogenates (3, 4, 6, 26, 31), we next compared the time course of enzymatic activity of wild type II 3β-HSD to that of the three mutant proteins. *In vitro* incubation of homogenates from COS-1 cells transfected with pCMV type II 3β-HSD in the presence of 1 mM NAD^+ showed high 3β-HSD/Δ^5-Δ^4 isomerase activity as revealed by the rapid and almost complete

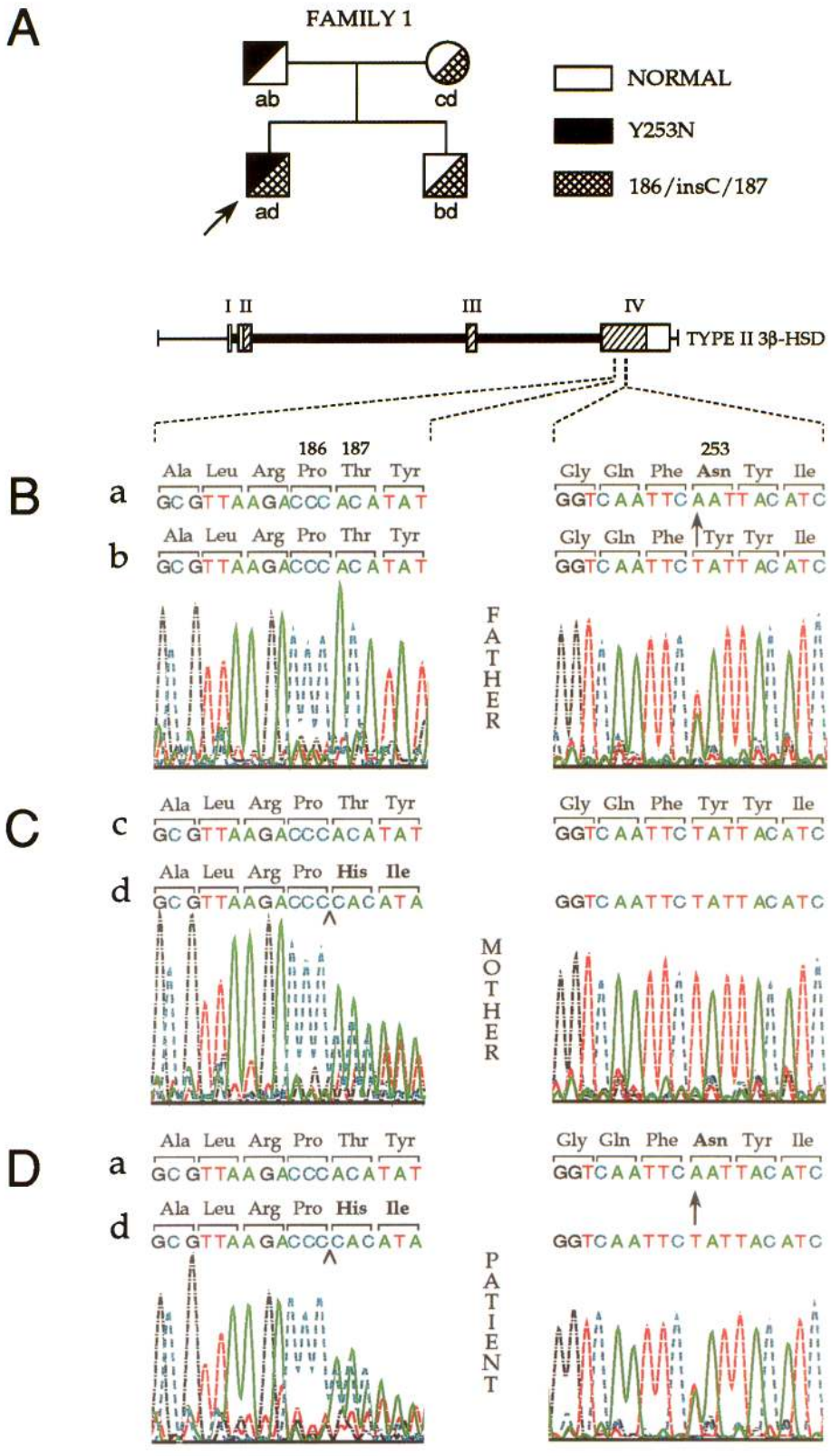

Fig. 2. Mutational Analysis of Family 1

A, Pedigree of family 1. The compound heterozygote-affected male patient inherited both the maternal 186/insC/187 mutation (*half-hatched symbol*) and the paternal missense Y253N mutation of the type II 3β-HSD gene. The brother of the index case, on the other hand, is heterozygote for the frame-shift mutation. Partial nucleotide sequences of the sense strand of exon IV of the type II 3β-HSD gene and the corresponding deduced amino acid sequence of each allele from the father (B), mother (C), and patient (D). The duplication of nucleotide sequences observed in the the mother's and patient's type II 3β-HSD gene starting after codon 186 results from insertion of a C in one chromosome, thus indicating heterozygosity. Note that the frame-shift 186/insC/187 mutation leads to a predicted truncated protein of 202 amino acids (including the first Met), thus explaining the absence of encoded amino acid between codons 250 and 255 on allele d of the 3β-HSD genes.

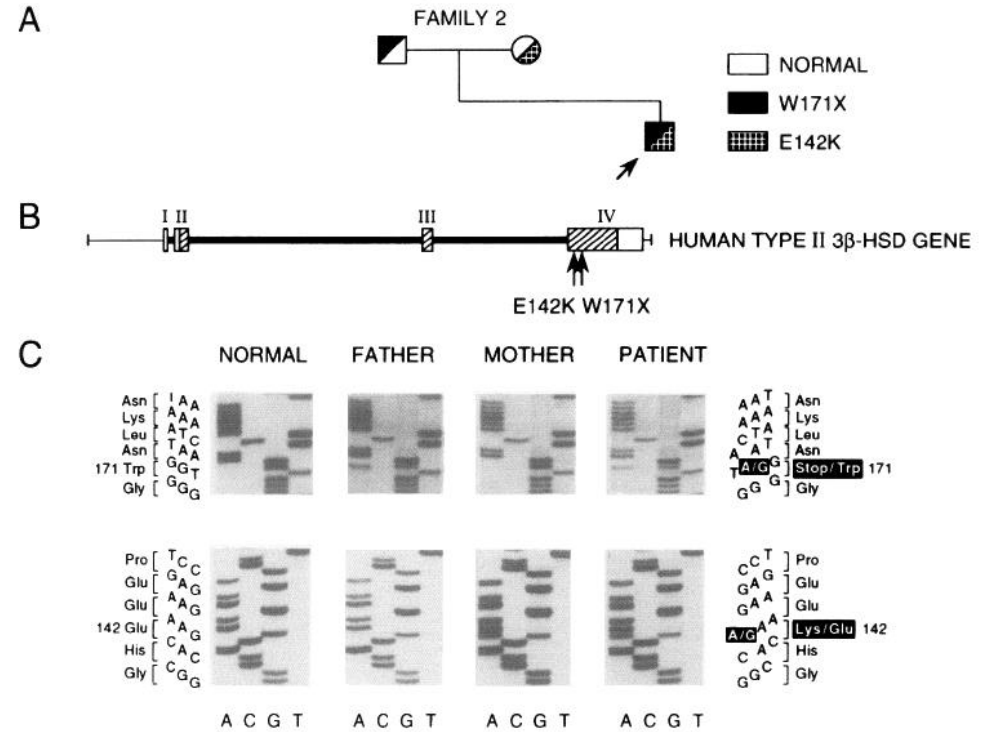

Fig. 3. Mutational Analysis of Family 2

A, Pedigree of family 2 illustrating that the compound heterozygote-affected male patient has inherited both the paternal nonsense W171X mutation (*half-solid symbol*) and the maternal missense E142K mutation (*half-hatched symbol*). B, Schematic representation illustrating the position of the point mutations W171X and E142K in the exon 4 of the human type II 3β-HSD gene. C, Partial nucleotide sequence of the sense strand of exon IV of the type II 3β-HSD gene showing the point mutations in the members of family 2 and in a normal type II 3β-HSD gene.

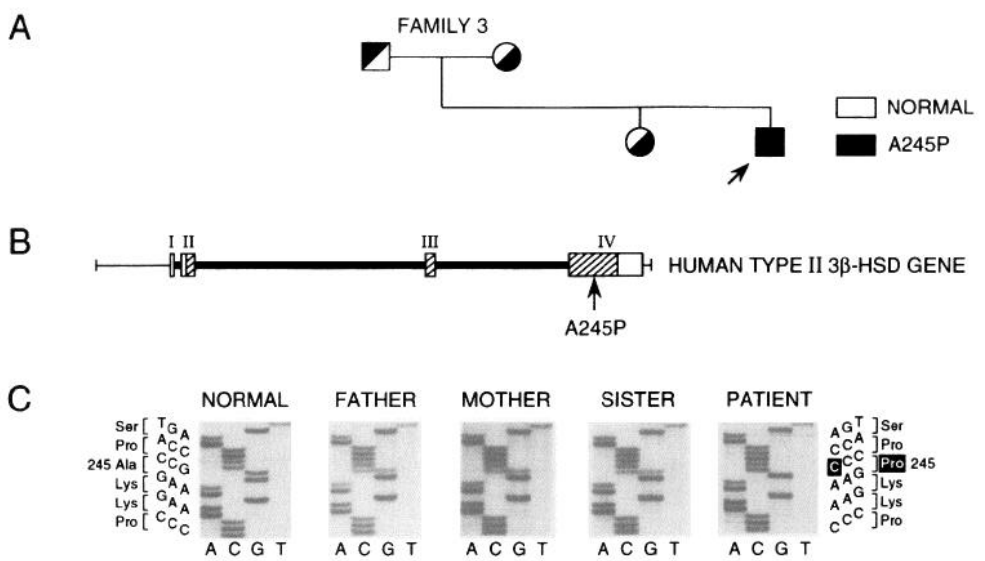

Fig. 4. Mutational Analysis of Family 3

A, Partial pedigree of family 3 illustrating that the homozygous-affected male patient has inherited both the paternal and maternal missense mutation A245P (*half-solid symbol*). B, Schematic representation illustrating the position of the point mutation A245P in the exon 4 of the human type II 3β-HSD gene. C, Partial nucleotide sequence of the sense strand of exon IV of the type II 3β-HSD gene showing the point mutations in the members of family 3. Sequences were determined by direct sequencing of asymmetric PCR products by manual sequencing. Note that both G and C are present as first nucleotides at codon 245 in the father's, mother's, and sister's type II 3β-HSD gene sequence, indicating heterozygosity.

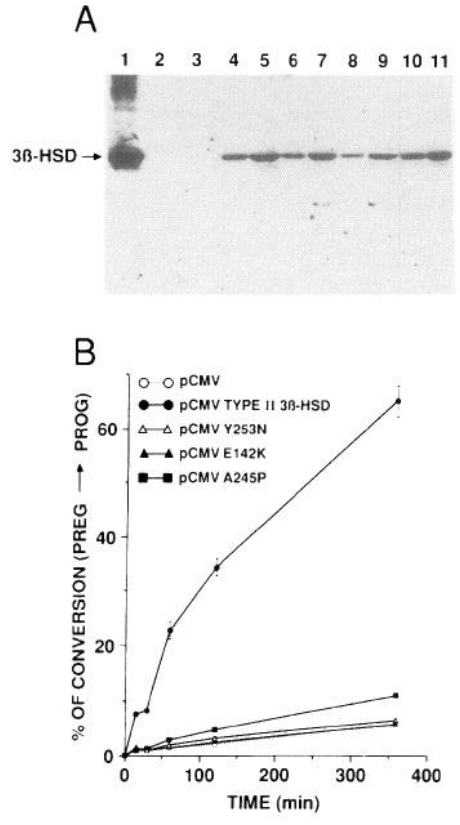

Fig. 5. Transient Expression of Wild Type Type II-3β-HSD and Recombinant Mutant 3β-HSD Y253N, E142K, and A245P Enzymes in Mammalian Cells

A, Immunoblot analysis of expressed 3β-HSD proteins encoded by wild type human type II 3β-HSD cDNA and mutant recombinant Y253N, E142K, or A245P cDNAs, generated by site-directed mutagenesis, was performed using antiserum raised in rabbits against purified human placental type I 3β-HSD. Homogenates from COS-1 monkey kidney transformed cells transfected with control pCMV alone (lane 2, 50 μg; lane 3, 100 μg), pCMV type II-3β-HSD (lane 4, 50 μg; lane 5, 100 μg), pCMV-Y253N (lane 6, 50 μg; lane 7, 100 μg), pCMV-A245P (lane 8, 50 μg; lane 9, 100 μg), and pCMV-E142K (lane 10, 50 μg; lane 11, 100 μg) were separated on 5–15% polyacrylamide gels and quantitated with a model 440E phosphorimager (Molecular Dynamics, Sunnyvale, CA). Purified human placental 3β-HSD protein was used as a positive control (lane 1). B, Comparison of the time course of enzymatic conversion of [^{3}H]PREG into [^{3}H]PROG in intact COS-1 cells in culture transfected with 10 μg pCMV, pCMV type II 3β-HSD, pCMV Y253N, pCMV E142K, or pCMV A245P plasmid with that of human wild type type II 3β-HSD. The results are presented in percent as the means ± SEM of the free steroid metabolites present in culture medium as percent of total free steroid. When SEM overlaps with the symbol used, only the symbol is illustrated.

conversion of [^{3}H]PREG into [^{3}H]PROG as well as [^{3}H] dehydroepiandrosterone (DHEA) into [^{3}H]Δ^4-androstenedione (Δ^4-dione), while no significant transformation was observed in cells transfected with the control pCMV plasmid (Fig. 6, A, B, and C). On the other hand, incubation with homogenates from cells transfected with pCMV-Y253N (Fig. 6A), pCMV-E142K (Fig. 6B), or pCMV-A245P (Fig. 6C) indicates the absence of significant 3β-HSD/Δ^5-Δ^4-isomerase activity of the three mutant recombinant proteins up to 360 min of incubation with the C-21 Δ^5-steroid PREG as well as the C-19 Δ^5-steroid DHEA used as substrates. We have also demonstrated that the lack of oxidation and isomerization of these Δ^5-hydroxysteroids into Δ^4-3-ketosteroids by these mutant proteins is not due to the isolated absence of isomerase activity but also involves oxidative activity, since these mutant proteins were unable to catalyze the oxidation of 5α-androstane-3β,17β-diol into 5α-androstane-17β-ol-3-one (dihydrotestosterone) in contrast to the highly efficient conversion catalyzed by wild type II-3β-HSD (data not shown).

Knowing of a previously reported nonsalt-losing CAH case secondary to 21-hydroxylase deficiency (37) in which a significant 21α-hydroxylase activity was detectable in intact transfected cells in culture but not in cell homogenates in the absence of glycerol, while such activity was detectable in the presence of glycerol, a stabilizing agent for several membrane-bound enzymes, we next analyzed the enzymatic activity of the mutant A245P enzyme compared with that of the wild type type II 3β-HSD using the cell homogenates prepared in absence (Fig. 7, A and C) or in presence (Fig. 7, B and D) of 20% glycerol. As illustrated in Fig. 7, A and C, the activity of the mutant A245P protein was not detectable in the absence of glycerol, in agreement with the data of Fig. 6C. On the other hand, it can be seen in Fig. 7B that in the presence of glycerol, the mutant A245P protein was able to catalyze the conversion of [^{3}H]PREG into [^{3}H]PROG above basal values ($P < 0.01$), at 9.8–12.0% of the activity of the wild type enzyme. Closely similar data were obtained when using [^{3}H]DHEA as substrate (Fig. 7D). As expected, *in vitro* incubation of homogenates from cells transfected with pCMV-Y253N or pCMV-E142K in the presence of 20% glycerol confirmed the lack of detectable 3β-HSD activity of these mutant recombinant proteins (data not shown).

In order to gain more detailed information about the mutant A245P protein, we next investigated its kinetic properties using the cell homogenate preparations in the presence of 20% glycerol under conditions where the amount of translated 42-kilodalton A245P protein, as measured by immunoblot analysis, was equivalent to that of wild type type II 3β-HSD protein. It is of interest to see in Fig. 8, A and B, that the expressed mutant A245P protein had an affinity for PREG comparable to that of the wild type type II 3β-HSD enzyme, with Michaelis-Menten constant (K_m) values of 4.64 ± 0.60 and 2.63 ± 0.32 μM, respectively, while the maximum velocity (V_{max}) values were 13.02 ± 1.0 and 62.06 ± 4.1 pmol/min·μg total protein, respectively. As illustrated in Fig. 8, C and D, the expressed mutant A245P protein also had an affinity for DHEA similar to that of the wild type type II 3β-HSD enzyme, with K_m values of 2.22 ± 0.53 and 1.57 ± 0.32 μM, respectively, while the V_{max} values, were 5.9 ± 0.6 and 31.9 ± 2.4 pmol/

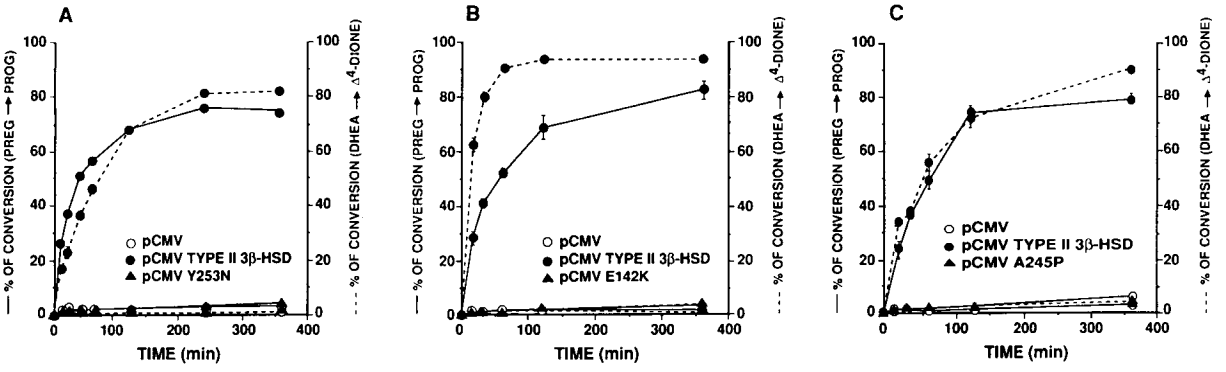

Fig. 6. Comparison of the Time Course of Enzymatic Conversion of [^{3}H]PREG into [^{3}H]PROG (*Solid Line*) or [^{3}H]DHEA into [3H] Δ^4DIONE (*Dashed Line*) in COS-1 Cell Homogenates Prepared as Previously Described (6, 26, 28, 31; see *Patients and Methods*) from Cells Transfected with 40 μg pCMV, pCMV type II 3β-HSD, pCMV Y253N, pCMV E142K, or pCMV A245P Plasmid

The results are presented in percent as the means ± SEM of the free steroid metabolites present in culture medium as percent of total free steroid. When SEM overlaps with the symbol used, only the symbol is illustrated.

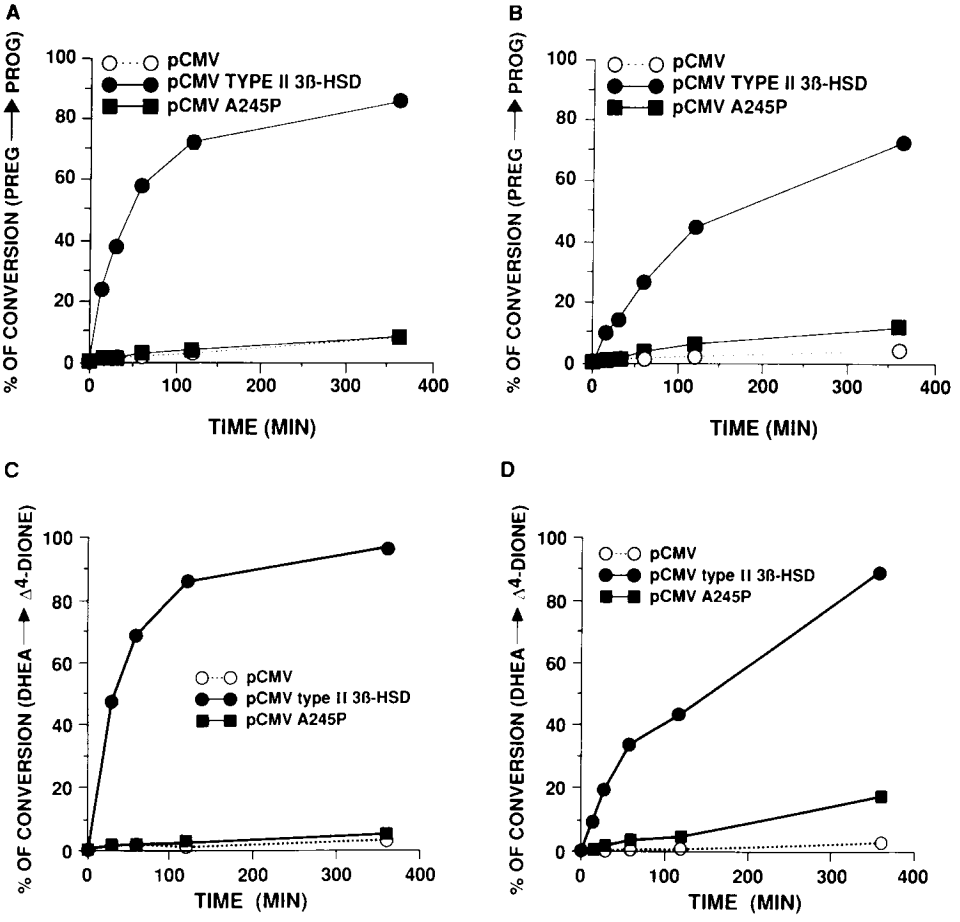

Fig. 7. Comparison of the Time Course of the Expressed Human Wild Type Type II and Mutant A245P 3β-HSD Proteins

Time course of enzymatic conversion of [^{3}H]PREG into [^{3}H]PROG (A and B) or [^{3}H]DHEA into [^{3}H]Δ^4-dione (C and D) in homogenates from COS-1 cells transfected with 40 μg pCMV, pCMV type II 3β-HSD, or pCMV A245P plasmid prepared in the absence (A and C) or presence (B and D) of 20% glycerol. The results are presented as the mean ± SEM (n = 3). When SEM overlaps with the symbol used, only the symbol is illustrated.

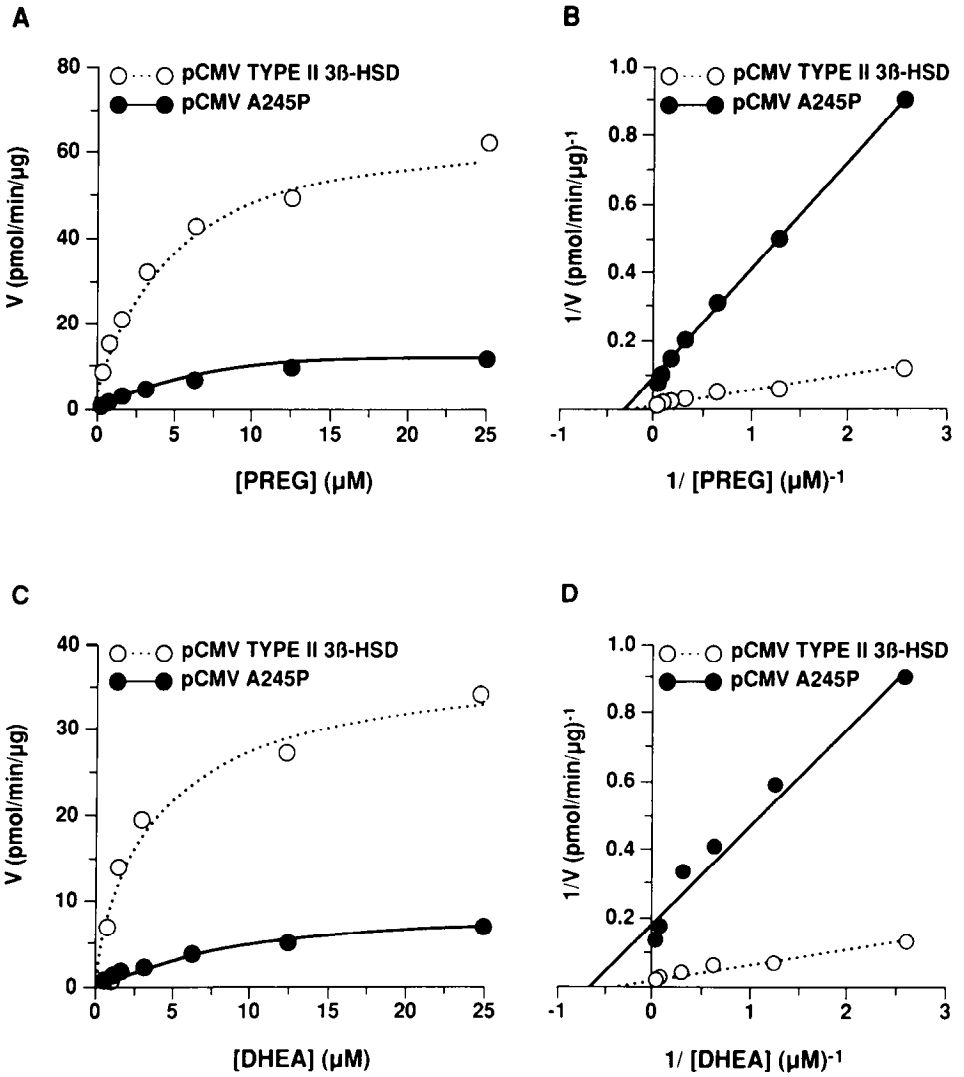

Fig. 8. Comparison of the Kinetic Properties of the Expressed Human Wild Type Type II and Mutant A245P 3β-HSD Proteins

The enzymatic reaction was performed during 120 min at 37 C using 50 µg or 300 µg homogenate protein prepared in the presence of 20% glycerol from COS-1 cells transfected with pCMV-type II 3β-HSD (○) or pCMV-245P (●) plasmids, respectively. The percent of total conversion was less than 12% under the experimental conditions used. A and C, K_m plot of V (pmol/min·µg normalized total protein) against [PREG] (A) or [DHEA] (C) in micromolar concentrations. B and D, Data are displayed as Lineweaver-Burk plots. The amount of translated wild type type II and mutant recombinant A245P 3β-HSD proteins were equivalent in each corresponding cell homogenate preparation as measured by immunoblot analysis. The results are presented as the mean ± SEM (n = 3). When SEM overlaps with the symbol used, only the symbol is illustrated.

min·µg total protein, respectively. As measured by the first-order rate constant V_{max}/K_m, the present findings thus indicate that the mutant recombinant A245P enzyme possesses about 11.9 and 13.1% of the activity catalyzed by the wild type type II 3β-HSD using PREG or DHEA as substrates, respectively.

DISCUSSION

The present report describes the molecular basis of classical 3β-HSD deficiency in three male pseudohermaphrodites affected by this monogenic autosomal recessive syndrome of CAH accompanied by varying degrees of salt losing. In two patients with a severe form of CAH associated with salt wasting, the disorder results from point mutations completely abolishing the activity of the 3β-HSD enzyme encoded by the type II 3β-HSD gene, which is the gene type primarily expressed in the adrenal, ovary, and testis (6). In addition, the present study demonstrates for the first time that the nonsalt-losing form of classical 3β-HSD deficiency seen in the patient of family 3 results from an homozygous missense mutation in the type II 3β-HSD gene that causes a approximately 88% loss of enzymatic activity, thus leaving sufficient enzymatic activity to prevent salt wasting. No mutation was detected in the

type I 3β-HSD gene, which is specifically expressed in the placenta and in peripheral intracrine tissues such as the skin and mammary gland (6), thus providing an explanation for intact peripheral intracrine steroidogenesis in these patients.

In addition to providing a molecular explanation for the clinical and biological observations of classical 3β-HSD deficiency with or without the symptoms of salt loss, the present findings provide the first information on the structure-function relationships of the 3β-HSD isoenzyme family by demonstrating the crucial role of residues Glu^{142}, Tyr^{253}, and Ala^{245} for 3β-HSD catalytic activity. The importance of the Glu^{142} and Tyr^{253} amino acid residues for 3β-HSD activity is well supported by the observation that these two amino acids are conserved in all 3β-HSD isoenzymes characterized thus far in the rat, mouse, bovine, macaque, and human (Fig. 9) (1–6, 25–31) as well as in two other members of the 3β-HSD superfamily, namely the bacterial *Nocardia* cholesterol dehydrogenase (32) and the Vaccinia virus open reading frame SalF7L, encoding a protein possessing 3β-HSD/Δ^5-Δ^4 isomerase activity (32, 33)..

Since Ala^{245} is not conserved in the 3β-HSD superfamily, it is conceivable that the A245P mutation generating Pro^{245} causes a turn in the polypeptide chain generating a structural change in the protein, which could be responsible for the decrease in activity of this mutant type II 3β-HSD enzyme. The finding of no detectable 3β-HSD activity in cell homogenates in the absence of glycerol (Figs. 6C and 7, A and C), while significant enzymatic activity could be measured in intact transfected cells (Fig. 5B) as well as in cell homogenates prepared in the presence of glycerol, a known stabilizing agent (Figs, 7, B and D, and 8), could suggest that this mutation weakens the association of the mutant A245P enzyme with proper intracellular membranes. In fact, the absence of salt wasting in this patient could well be explained by the weak but measurable 3β-HSD activity, which may permit the formation of mineralocorticoids in sufficient quantities to avoid severe salt wasting, in analogy with some nonsalt-losing CAH cases secondary to 21-hydroxylase deficiency (37–39).

The expected severe impairment of testosterone biosynthesis by the fetal testis bearing mutated alleles of the type II 3β-HSD gene provides an explanation for the pseudohermaphroditism observed in these three male patients. In fact, testicular 3β-HSD activity starts to rise at the end of the second month of pregnancy, and androgens are known to be required before 12 weeks of gestation for penile development and urethral fold fusion (40–43). On the other hand, the presence of subnormal to normal Δ^4-steroid plasma levels in 3β-HSD-deficient patients (8, 9, 11, 12, 16, 17, 19, 23, 35) can be explained by the present findings of a normal peripheral type I 3β-HSD isoenzyme that is exposed to elevated circulating levels of substrates resulting from the deficient conversion of 3β-hydroxysteroids in the affected adrenals and testes.

The observation of an important peripheral conversion of Δ^5-hydroxysteroids into Δ^4-steroids in 3β-HSD-deficient patients is in agreement with the recent evidence that a large proportion of androgens (about 40%) in men arise from extraadrenal and extragonadal steroidogenesis (see Ref. 44 for review). It should be mentioned that although type I 3β-HSD transcripts were not detectable by ribonuclease protection assay in the normal adult testis (6), Δ^4-steroids could also originate, to an unknown extent, from gonadal type I 3β-HSD activity. In fact, since this enzyme possesses an approximately 5-fold higher activity than the type II isoenzyme (6), an increase in testicular type I isoenzyme

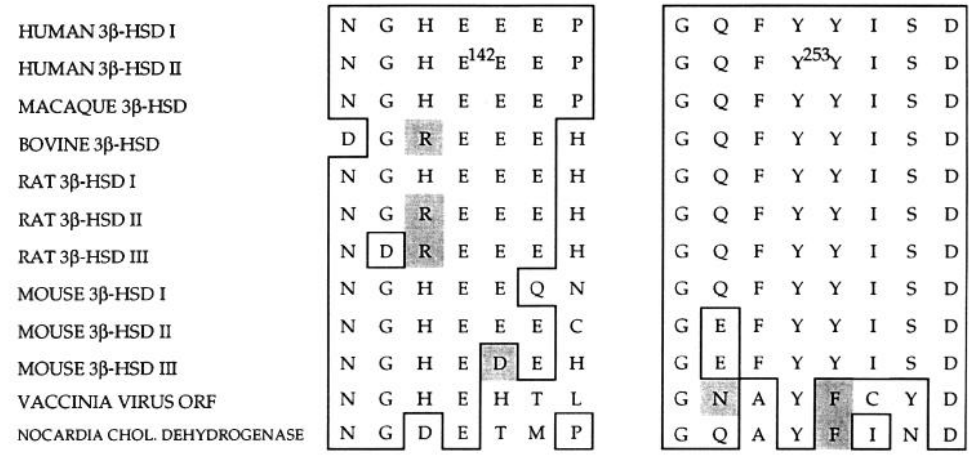

Fig. 9. Comparison of the Amino Acid Sequences of Mammalian 3β-HSD Isoenzymes as Well as Alignment of Bacterial *Nocardia* Cholesterol Dehydrogenase and of the Vaccinia Virus Open Reading Frame SalF7L Flanking Glu^{142} and Tyr^{253} in Human Type II 3β-HSD (2, 6, 25–33)

Amino acid residues are designated by *single letter codes*. Residues common to human type II 3β-HSD are *boxed*, while respective conservative residues are *shadowed*. Note that the different types of 3β-HSD in different species have been designated in chronological order of their respective elucidation.

activity induced by excess gonadotropin secretion, resulting from activation of the feedback mechanisms by low circulating androgen levels, could well lead to significant androgen biosynthesis in the affected testis during later life.

The demonstration of the W171X mutation leading to a predicted truncated protein of 169 amino acids (excluding the first Met) instead of the normal 371 residues of the type II 3β-HSD protein (6, 7) is of interest, since we recently characterized the same nonsense mutation in three patients from two other unrelated pedigrees (45). We first described a homozygous W171X mutation in two classical 3β-HSD deficient females showing severe salt wasting accompanied by a marked blockade of ovarian and adrenal steroidogenesis in the two related families previously described by Zachmann *et al.* (13, 14, 46). Furthermore, elucidation of the molecular basis of the classical 3β-HSD deficiency in the male patient previously described by Parks *et al.* (11) reveals that this index case is a compound heterozygote being heterozygous for the W171X mutation while bearing the same frame-shift mutation 186/insC/187 in his other type II 3β-HSD allele. The present findings, taken together with these recent observations (45), suggest the relative high frequency of these two mutations as being responsible for classical 3β-HSD deficiency.

While leading to a better understanding of the phenotypic heterogeneity of severe and mild forms of this autosomal recessive disorder, further characterization of the molecular basis of CAH due to 3β-HSD deficiency should also provide important information concerning the structure-function relationships of the 3β-HSD superfamily.

PATIENTS AND METHODS

Patients

The present study analyzes the structure of the types I and II 3β-HSD genes in three male (46 XY) patients from three unrelated families affected by the classical form of 3β-HSD deficiency (17, 34–36). The index case of the Dutch family 1, born in 1963, suffered from severe salt loss soon after birth and presented with urethral diverticula and hypospadias (34, 35). Several standard biological studies performed during the last 10 yr clearly demonstrated blockade of adrenal 3β-HSD activity as reflected by markedly elevated basal and ACTH-stimulated Δ^5-/Δ^4-steroid ratios (34, 35; our unpublished observations). The index case of American family 2, born in 1985, was diagnosed during the first week of life as suffering from classical 3β-HSD deficiency illustrated by an electrolyte imbalance, a grade IV perineal hypospadias, a bifid scrotum, as well as elevated basal and human CG-stimulated Δ^5-/Δ^4- steroid ratios (17). The family history of the index case of family 3, a 4-year-old boy, indicates that both parents originated from the same village in Eastern Anatolia, Turkey, and that out of 7 children, 4 died in early infancy at 3, 3, 12, and 42 months from undetermined causes (36). The index case has been diagnosed as a classical form of 3β-HSD deficiency, characterized by a grade III scrotal hypospadias, a bifid scrotum associated with marked elevated basal and ACTH-stimulated Δ^5-/Δ^4-steroid ratios, and with serum electrolytes as well as plasma renin activity that were low to within the normal range when tested on several occasions (36). For example, during the first week of life the plasma renin-angiotensin activity was 8.2 ng angiotensin/ml·h. The urinary excretion of aldosterone and tetrahydroaldosterone was low (1.04 μg/24 h and 2.74 μg/24 h, respectively) during the second week of life, while excretion of these steroids was within the normal ranges (4.08 μg/24 h and 18.5 μg/24 h, respectively) during the 11th month. During the 22nd month, the basal plasma aldosterone level was 21.4 ng/ml.

Selective PCR Amplification of Type I or Type II 3β-HSD Gene Fragments

Selective amplification of type I or type II 3β-HSD gene fragments was achieved using primer pairs with 3′-end nucleotides that only match the complementary bases of the specific gene type to be amplified (47). Five different primer pairs were used for the amplification of the coding region, the exon-intron splicing junction boundaries, the putative promoter region, as well as the polyadenylation site of each gene, as shown in Fig. 1. The sequences of the primers are as follows: I.864u, 5′-ACCCAGGGCTCTCCAGGGGCAAATG-3′; I.1426d, 5′-AAAGCTGGACAGAAGAGTGGACGTA-3′; I.1323u, 5′-GAGATCAAAGTGATAAGGGTTGGGC-3′; I.1912d, 5′-GCTTAGATTTGGCTACAAATTTCAA-3′; I.5623u, 5′-TATCAGAAAACTTCTCAGCCAGATA-3′; I.5906d, 5′-TTCTTTGTGATCCTTAATTCCCCAC-3′; I.7960u, 5′-GTGGTTGGCACCTCTTAGGGATATA-3′; I.8922d, 5′-GTGCCCTTGTCACTTTCTGTATGAG-3; pr; I.8743u, 5′-CTGGGAGGAAGCCAAGCAGAAAACG-3;pr; I.9328d, 5′-CTACTCTTCTAAATTGGAGATGGCA; II.727u, 5′-CAGAGCTCTCCAGGGAAAAATTGCA3′; II.1369d, 5′-TTTACAAAAATTCCATGACCCCACA-3′; II.1222u, 5′-GCATAAAGCTCCAGTCCTTCCTCCA-3′; II.1742d, 5′-TTGCTAGACAAGGTCAACCTCCCCA-3′; II.5452u, 5′-TATCAGAAAACTTCCCAGCCAGATC-3′; II.5730d, 5′-TCTGATCCTCATTTAACCAACTTGT-3′; II.7866u, 5′-TGGGATATTTCCTGACACTGTCATC-3;pr; II.8824d, 5′-AGGACCTGGGCTTGTGCCCCTGTTG-3′; II.8491u, 5′-GGAAGTAGTGAGCTTCCTACTCAGC-3′; II.9197d, 5′-ATGGTGATAGTTGGAAATGAAAGGA-3′. Numbers indicate the location of the 5′-nucleotide of each primer in type I (GenBank accession number M38180) or in type II (GenBank accession number M77144) 3β-HSD gene; u stands for upstream primer; and d stands for downstream primer.

PCRs, using the indicated sets of primers, were performed as previously described (45) in a 100-μl vol containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.0 mM $MgCl_2$, 50 μM deoxy-NTP, 0.25 μM of each primer, and 0.25 μg genomic DNA extracted from blood leukocytes of the three patients and their relatives. The reaction mixture was covered with mineral oil, and DNA templates were denatured at 100 C for 10 min and then cooled down to 72 C ("hot start"; 48) before the addition of 2.5 U Taq polymerase (Perkin-Elmer Cetus, Norwalk CT). The reactions were carried out using a Perkin-Elmer Cetus thermal cycler (model 480) with a temperature cycle consisting of 1 min of denaturation at 95 C, 1 min of annealing at 60 C, and 1.5 min of extension at 72 C. After 30 cycles, primers were removed by selective DNA precipitation. The PCR products (1/30) were then subjected to a subsequent 40-cycle asymmetric amplification under the same conditions, except for the use of 3 nM limiting primer. After selective precipitation, one-third of the asymmetric PCR products were then used for each sequencing reaction.

Direct Sequencing of PCR Products

Single-strand DNA was produced from both strands, and the PCR DNA fragments were completely sequenced in both orientations by manual and/or automated methods as previously described (45). Manual sequencing was performed by the dideoxy method using the limiting PCR primer or internal sequence-specific primers with the T7 sequencing kit (Phar-

macia LKB Biotechnologies, Piscataway, NJ). Automated direct sequencing was performed using an Applied Biosystems (Foster City, CA) automated DNA sequencer model 373A with fluorescent dye-labeled 3β-HSD gene sequence-specific primers and a sequenase dye-primer sequencing kit (using Mn^{2+} and pyrophosphatase; U.S. Biochemical Corp., Cleveland, OH, and Applied Biosystems Inc.).

Site-Directed Mutagenesis

PCR fragments obtained from patients' genomic DNA bearing the Y253N or the A245P mutation, amplified with the primer pair II.7866u and II.8824d, were digested with *Bam*HI, thus generating 596-base pair fragments (nucleotides 627–1224 of the corresponding cDNA) that include the desired mutations. These fragments were substituted to the *Bam*HI-*Not*I fragment of plasmid ha3β-HSD 27 (6). The *Bgl*II fragments of the recombinant mutated cDNAs were then used to replace the *Bgl*II fragment of pCMV-type II 3β-HSD. The technique of site-directed mutagenesis by overlap extension PCR was used to generate the E142K mutation using an upstream pCMV-specific primer, 5′-GCGGTAGGCGTGTACGGTGGGACCTC-3′, and primer II.8824d, with the overlapping primers 5′-AGGCTCTTCTTTGTGGCCGTTC-3′ and 5′-GAACGGCCACAAAGAAGAGCCT-3′, respectively, which contain the E142K mutation. The first two PCR products obtained were then used as overlapping fragments in conjunction with the pCMV primer and primer II.8824d to obtain a third PCR product containing the E142K mutation flanked by *Apa*I sites. These sites were used to replace the *Apa*I fragment of pCMV-type II 3β-HSD by the corresponding codon 142 mutant fragment. The DNA sequences of the newly introduced fragments in pCMV-Y253N, pCMV-A245P, and pCMV-E142K were confirmed by double-strand DNA sequence analysis.

Expression of Type II 3β-HSD and Mutated Y253N, E142K, and A245P cDNAs

Transient expression of wild type human type II 3β-HSD and mutated Y253N, E142N, and A245P cDNAs was performed using the experimental conditions previously described (6). The previously described pCMV-type II 3β-HSD (6), as well as the above-described pCMV-Y253N, pCMV-E142K, and pCMV-A245P recombinant plasmids, were amplified and subsequently purified by two cesium chloride-ethidium bromide density gradient ultracentrifugations. Expression of the plasmids was carried out in the COS-1 SV40-transformed African green monkey kidney cells (American Type Culture Collection, Rockville, MD) by the transfection method using calcium phosphate precipitation (6, 26, 28, 31). Mock transfections were carried out with pCMV alone, while transfection efficiency was monitored by cotransfecting the tested plasmids with the control pXGH5 plasmid that constitutively expresses GH, which is secreted into the culture medium. In order to ascertain the amount of translated wild type human type II 3β-HSD and mutated recombinant Y253N, E142K, and A245P proteins in transfected cells, proteins were size-separated on a 5–15% polyacrylamide gel and transferred to nitrocellulose filters, and immunoblot analyses were performed as described (6, 26, 28, 31).

Assay of 3β-HSD Enzymatic Activity

In order to determine 3β-HSD enzymatic activity in cell homogenate, COS-1 transfected cells were harvested by scraping with a rubber policeman and resuspended at a concentration of 10×10^6 cells/ml in buffer containing 50 mM PBS, pH 7.4. Cells were then submitted to three freeze/thaw cycles. To measure 3β-HSD activity, 150 μg protein from cell homogenates were incubated for the indicated time intervals at 37 C in the presence of [^{3}H]labeled steroid substrates in 50 mM Tris buffer (pH 7.5) containing 1 mM NAD^+ in the presence of 8 nM [1,2,6,7-^{3}H] DHEA (86.6 Ci/mmol; New England Nuclear, Boston, MA) or 32 nM [7-^{3}H] PREG (23.5 Ci/mmol; New England Nuclear). The enzymatic reaction was stopped by adding 4 vol ether/diethyl and chilling the incubation mixture in a dry ice-ethanol bath. Steroids were analyzed by TLC and/or HPLC using a System Gold unit (Beckman Istruments, Palo Alto, CA), as previously described (6, 26, 28, 31).

To determine 3β-HSD activity in intact transfected cells, 3×10^5 cells were plated in Falcon (Oxnard, CA) six-well plastic culture plates (10 cm^2). Media were changed 24 h later, and the incubations were continued for 3 h before performing transfection. Forty-eight hours after transfection, 100 μl medium were collected and assayed for GH by RIA. Thirty nanomolar [7-^{3}H] PREG (23.5 Ci/mmol; New England Nuclear) was added in the medium at a final concentration of 1% (vol/vol) ethanol. After the indicated time periods, media were collected, and steroids were extracted by adding 4 vol diethylether. The organic phase was then evaporated and separated on TLC plates as previously described (6, 26, 28, 31).

To investigate the effects of glycerol on the enzymatic activity of wild type type II 3β-HSD and mutant recombinant 3β-HSD enzymes, the enzymatic assays were performed as previously described above, except they were carried out as described (37) using 100 μg protein from sonicated transfected COS-1 cells in 100 mM KH_2PO_4, pH 7.2, with the indicated concentrations of [7-^{3}H] PREG in the presence or absence of 20% glycerol (vol/vol) and 1 mM NAD^+ at 37 C for the indicated time periods. K_m and V_{max} values were calculated using ENZFITTER software (Biosoft, Cambridge, UK) as previously described (31) after normalization for the amount of translated wild type type II 3β-HSD and mutant recombinant A245P protein in each cell homogenate preparation.

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Address requests for reprints to: Prof. Fernand Labrie, Medical Research Council Group in Molecular Endocrinology, CHUL Research Center, 2705 Laurier Boulevard, Québec, G1V 4G2, Canada.

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§ Held an MRC Fellowship and is now a Senior Research Associate of the Fonds National de la Recherche Scientifique of Belgium.

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