Identification and Characterization of the G15D Mutation Found in a Male Patient with 3β-Hydroxysteroid Dehydrogenase (3β-HSD) Deficiency: Alteration of the Putative NAD-Binding Domain of Type II 3β-HSD†

Eric Rhéaume,‡,§ Rocio Sanchez,‡ Farida Mébarki,|| Ève Gagnon,‡ Jean-Claude Carel,⊥ Jean-Louis Chaussain,⊥ Yves Morel,|| Fernand Labrie,‡ and Jacques Simard*,‡

MRC Group in Molecular Endocrinology, CHUL Research Center and Laval University, Québec, Canada G1V 4G2, INSERM U329 and Department of Pediatrics, Université de Lyon and Hôpital Debrousse, Lyon Cedex 05, France, and Hôpital Saint-Vincent de Paul, Paris, France

Received August 9, 1994; Revised Manuscript Received November 29, 1994®

ABSTRACT: We report the detection of a homozygous G to A mutation converting codon Gly^{15} into Asp^{15} in the type II 3β-hydroxysteroid dehydrogenase/$\Delta^5-\Delta^4$-isomerase (3β-HSD) gene in a male pseudohermaphrodite born from consanguineous parents and suffering from severe salt-losing 3β-HSD deficiency. To investigate further the potential involvement of residue 15 in the βαβ dinucleotide-binding fold, we have studied the effect of substituting Gly^{15} for Ala^{15}. We assessed the effect of the G15D and G15A missense mutations on enzymatic activity by analyzing mutant enzymes generated by site-directed mutagenesis of type II 3β-HSD cDNA after their transient expression in COS-1 cells. In intact transfected cells, after a 2-h incubation, the percentage of conversion of [^{3}H]pregnenolone (PREG) into [^{3}H]progesterone (PROG) was 35% and 50% for the G15A and native type II 3β-HSD enzymes, respectively, whereas no detectable activity was observed in cells expressing the G15D protein. This finding is in agreement with the severity of the disease in the homozygote G15D index case. On the other hand, in homogenates from cells transfected with the normal pCMV-type II 3β-HSD plasmid or with the mutated pCMV-G15D or pCMV-G15A plasmid, the K_m values for PREG were 0.72 μM, 3.2 μM, and 3.4 μM, respectively, when incubated for 1 h in the presence of excess (1 mM) NAD^+. Moreover, the expressed G15D and G15A proteins had decreased affinities for NAD^+ with K_m values of 113 μM and 148 μM, respectively, compared with 22 μM for normal type II 3β-HSD. The marked difference in activity of the G15D and G15A proteins expressed in intact cells compared with their similar level of activity in homogenates remains to be elucidated. It could be suggested that, in addition to decreasing apparent affinity for both the substrate and cofactor, the G15D mutation may alter the proper intracellular localization of this integral membrane protein or that its association with intact membranes *in vivo* may exert some strain, preventing the adoption of its final maximally efficient conformation.

The bifunctional enzyme 3β-hydroxysteroid dehydrogenase (EC 1.1.1.145)/$\Delta^5-\Delta^4$-isomerase (EC 5.3.3.1), hereafter called 3β-HSD,[1] catalyzes NAD-mediated dehydrogenation and isomerization of Δ^5-3β-hydroxysteroids into the corresponding Δ^4-3-ketosteroids (Labrie et al., 1992; Mason, 1993). The membrane-bound 3β-HSD enzyme catalyzes the conversion of pregnenolone (PREG), 17-hydroxypregnenolone, dehydroepiandrosterone (DHEA), and 5-androstene-3β,-17β-diol into progesterone (PROG), 17-hydroxyprogesterone, 4-androstenedione (Δ^4-DIONE), and testosterone, respectively (Figure 1). Thus, 3β-HSD enzymatic activity is essential for the biosynthesis of all classes of active steroid hormones, namely, PROG, mineralocorticoids, glucocorticoids, and the androgens and estrogens.

We recently demonstrated that classical 3β-HSD deficiency with or without salt loss is due to point mutation(s) in the type II 3β-HSD gene (Rhéaume et al., 1992; Sanchez et al., 1994b,c; Simard et al., 1993b, 1994). Classical 3β-HSD deficiency causes a blockade in adrenal and gonadal steroidogenesis that results in either salt-wasting or non-salt-losing congenital adrenal hyperplasia (CAH) and subvirilization in genetic males, whereas normal or slightly virilized genitalia are observed in genetic females (Bongiovanni, 1962; Zachmann et al., 1970; Kenny et al., 1971; Parks et al., 1971; Schneider et al., 1975; Pang et al., 1983).

In humans, two different 3β-HSD isoenzymes are expressed in a tissue-specific way. The two types of human 3β-HSD cDNAs and their corresponding genes were recently characterized (Luu-The et al., 1989; Lachance et al., 1990, 1991, 1992; Lorence et al., 1990: Rhéaume et al., 1991). Both genes consist of four exons and three introns, with 93.5% homology within their coding regions (Lachance et

† This work was supported by the Medical Research Council (MRC) of Canada, Endorecherche, and Hospices Civils de Lyon, INSERM U329, the "Projet national de recherche clinique" (PNRC). E.R. is the recipient of an MRC Studentship, J.S. is the recipient of an MRC Schorlarship. R.S. is the recipient of a studentship from the Ministère de l'Enseignement Supérieur et de la Science of Québec, and F.M. is the recipient of a studentship from the Ministère de la Recherche et de la Technologie de France.

* To whom correspondence should be addressed. Telephone: (418) 654-2296. Fax: (418) 654-2735.

‡ MRC Group in Molecular Endocrinology.

§ Present address: Laboratory of Immunology, Clinical Research Institute of Montreal, Montreal, Canada H2W 1R7.

|| Université de Lyon and Hôpital Debrousse.

⊥ Hôpital Saint-Vincent de Paul.

® Abstract published in *Advance ACS Abstracts,* February 15, 1995.

[1] Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase/$\Delta^5-\Delta^4$-isomerase; PREG, pregnenolone; PROG, progesterone; DHEA, dehydroepiandrosterone; Δ^4-DIONE, 4-androstenedione.

0006-2960/95/0434-2893$09.00/0

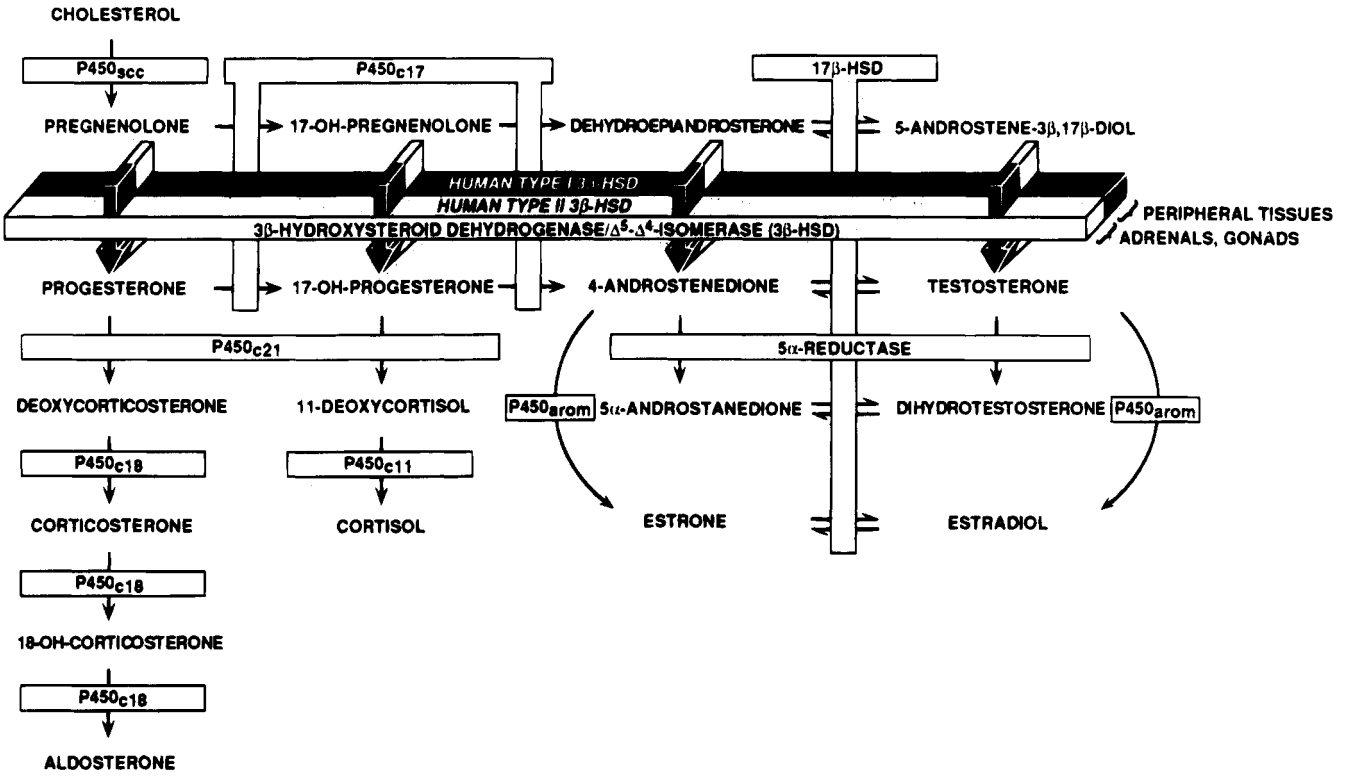

FIGURE 1: Major steroid hormone biosynthesis pathways in human. This is a composite scheme; not all of these steps are possible in a given steroidogenic tissue. P450s are cytochrome P450 enzymes. $P450_{scc}$ is an enzyme mediating 20α-hydroxylation, 22-hydroxylation, and scission of C20−C22 carbon bond; this enzyme is termed cholesterol side-chain cleavage enzyme. In human, the tissue-specific expression of type I and type II 3β-HSD isoenzymes is responsible for 3β-HSD activity. P450c21, 21-hydroxylase. $P450_{c17}$ catalyzes both 17α-hydroxylase and 17−20-lyase activities. $P450_{c11}$, 11β-hydroxylase. $P450_{c18}$ mediates 11β-hydroxylation and further reactions involved in the biosynthesis of aldosterone. 17β-HSD, 17β-hydroxysteroid dehydrogenase isoenzymes, also termed 17-ketosteroid reductase. $P450_{arom}$, aromatase.

al., 1990, 1991, 1992). The type I gene encodes the predominant 3β-HSD isoenzyme expressed in the placenta, skin, and mammary gland, whereas the type II gene is the almost exclusive 3β-HSD gene expressed in the adrenals and gonads (Rhéaume et al., 1991).

The 3β-HSD cDNAs from different species and of different isoforms were recently characterized (Luu-The et al., 1989; Zhao et al., 1989, 1990, 1991; Bain et al., 1991; Rhéaume et al., 1991; Clarke et al., 1993; Simard et al., 1991a, 1993a). These 3β-HSDs prefer NAD^+ to $NADP^+$ as cofactor for dehydrogenation of Δ^5-3β-hydroxysteroids, except for rat liver type III and mouse kidney type IV 3β-HSDs, which both prefer NADPH as cofactor for their almost exclusive 3-ketosteroid reductase activity (de Launoit et al., 1992; Clarke et al., 1993; Sanchez et al. 1994a). Sequence analysis suggests that the bovine 3β-HSD enzyme possesses a typical βαβ dinucleotide-binding fold with a "glycine-rich" sequence GXGXXG, similar to the motif found in many dehydrogenases, for example, alcohol dehydrogenases, lactate dehydrogenases, and glyceraldehyde dehydrogenases (Scrutton et al., 1990; Rutherfurd et al., 1991). This βαβ ADP-binding fold is responsible for hydrogen bonding of the adenine ribose of the NAD molecule (Baker et al., 1992). By comparison, a similar motif, GAGGXXG, is present at the N-terminal region of all other mammalian 3β-HSDs characterized so far.

Little is known about the structure−function relationships of the 3β-HSD family of enzymes. However, affinity radiolabeling of purified bovine adrenal 3β-HSD with 5′-[*p*-(fluorosulfonyl)benzoyl][*adenine*-8-^{14}C]adenosine identified the presence of two cysteine residues at or near the putative NAD-binding site found in peptides I^{18} to K^{26} and E^{274} to R^{282} (Rutherfurd et al., 1991). More recently, affinity radiolabeling of human type I 3β-HSD with 2α-bromo[2′-^{14}C]acetoxyprogesterone identified two tryptic peptides, peptides Lys^{175} and Arg^{250}, comprising amino acids Asn^{176} to Arg^{186} and Gly^{251} to Lys^{274}, respectively, that should contain residues involved in the putative substrate-binding domain (Thomas et al., 1993). These two last regions are highly conserved in human type II 3β-HSD and other members of this family of enzymes (Labrie et al., 1992, 1994) and might be involved in the substrate-binding domain of most members of the 3β-HSD family of enzymes.

This report describes the detection of the novel missense mutation G15D found in the homozygous state in a patient with severe salt-losing 3β-HSD deficiency (Gendrel et al., 1979) and the *in vitro* characterization of the kinetic properties of mutant G15D protein generated by site-directed mutagenesis. We also describe the functional characterization of another mutant protein, G15A, which possesses an Ala instead of a Gly at position 15. This amino acid change is expected to be conservative because both amino acids are small and hydrophobic.

PATIENT AND EXPERIMENTAL PROCEDURES

Patient. The male patient of this study was described as patient #2 in a previous study reporting his clinical case and steroid analysis (Gendrel et al., 1979). Briefly, the case study was reported as the second boy of a family; the parents are first cousins. At birth, perineal hypospadias with bifid

scrotum was noticed and the XY karyotype established. Salt-wasting occurred at the age of one and a half months. Dynamic testing with human chorionic gonadotropin gave an understimulation of testosterone levels. DHEA levels were elevated for his age, and the DHEA/Δ^4-DIONE ratio, at 4.1, was elevated.

Selective PCR Amplification of Type II 3β-HSD Gene Fragments. Selective amplification of type II 3β-HSD gene fragments was performed as described (Rhéaume et al., 1992). Thus, three different primer pairs that span the four exons and the first intron were used for amplification of the coding region and the exon−intron splicing junction boundaries of the type II 3β-HSD gene.

Direct Sequencing of PCR Products. Single-stranded DNA was produced as previously described (Rhéaume et al., 1992; Simard et al., 1993) from both strands, and the resulting PCR-DNA fragments were completely sequenced in both orientations by the dideoxy method using limiting PCR primers or internal 3β-HSD sequence-specific primers with the T7 sequencing kit (Pharmacia LKB Biotechnology).

Site-Directed Mutagenesis. The technique of site-directed mutagenesis by DNA overlap-extension PCR (Higuchi et al., 1988) was used to incorporate the G15D and the G15A mutations into type II 3β-HSD cDNA inserted into pCMV expression vector (kindly provided by Dr. Michael Mattews, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) using a pCMV-specific primer upstream of the polylinker site of this expression plasmid vector, whose sequence is 5′-GCGGTAGGCGTGTACGGTGGGAGGTC-3′, and type II 3β-HSD-specific downstream primer II.8824d (Rhéaume et al., 1992) with the overlapping downstream and upstream primers 5′-GATCCTCTGA(T or **G**)CCAGAAGCCC-3′ and 5′-GGGCTTCTGG(A or **C**)TCAGAGGATC-3′, respectively, which carry the G15D (underlined nucleotides) or the G15A (bold nucleotides) mutations. The first two PCR products obtained for each different mutation (G15D and G15A) after amplification of plasmid ha 3β-HSD 34 (pCMV-type II 3β-HSD) with the previously described set of primers were then used as overlapping fragments in combination with the pCMV-specific upstream primer and primer II.8824d to obtain third PCR products containing the G15D or the G15A mutation flanked by *Bal*I and *Aat*II sites. These sites were used to replace the *Bal*I and *Aat*II DNA fragments of plasmid ha 3β-HSD 80 (Rhéaume et al., 1991) by the corresponding codon 15 mutant fragments. The *Bgl*II DNA fragments of the recombinant plasmids containing the mutated cDNAs were then used to replace the *Bgl*II DNA fragment of pCMV-type II 3β-HSD (Rhéaume et al., 1991). The DNA sequence of the newly introduced fragments in pCMV-G15D and pCMV-G15A was confirmed by double-stranded DNA sequence analysis.

Expression of Type II 3β-HSD, Mutated G15D, and G15A cDNAs. Transient expression of wild-type human type II 3β-HSD and mutated G15D and G15A cDNAs was done using the experimental conditions previously described (Rhéaume et al., 1991; Sanchez et al., 1994a−c; Simard et al., 1993a,b). The previously described pCMV-type II 3β-HSD (Rhéaume et al., 1991) and the above-described pCMV-G15D and pCMV-G15A recombinant plasmids were amplified and subsequently purified by poly(ethylene glycol) precipitation and cesium chloride−ethidium bromide density gradient ultracentrifugations. Expression of the plasmids was carried out in COS-1 simian virus 40-transformed kidney cells of an African Green monkey or in 293 human adenovirus type 5-transformed human primary embryonal kidney cells (American Type Culture Collection, Rockville, MD) by the transfection method of calcium phosphate precipitation. Mock transfections were carried out with the pCMV plasmid expression vector alone, whereas transfection efficiency was monitored by cotransfecting the tested plasmids with the control pXGH5 plasmid that constitutively expresses growth hormone (GH), that is secreted into the culture medium. To ascertain the amount of translated wild-type human type II 3β-HSD and mutant recombinant G15D and G15A proteins in transfected cells, proteins were size-separated by electrophoresis on a SDS/5−15% polyacrylamide gel and transferred to nitrocellulose filters. Immunoblot analyses were made on a PhosphorImager Model 400E (Molecular Dynamics, Sunnyvale, CA), and corrections for the amount of 3β-HSD enzyme in assays of 3β-HSD enzymatic activity were performed as described (Simard et al., 1993).

Assay of 3β-HSD Enzymatic Activity. To determine 3β-HSD activity in intact COS-1-, or 293-transfected cells, 3×10^5 cells were plated in Falcon 6-well plastic culture plates (10 cm^2). Media were changed 24 h later, and the incubations were continued for 3 h before transfection. Forty-eight hours after transfection, 100 μL of medium was collected and assayed for GH by radioimmunoassay to estimate the efficiency of transfection, whereas to assay 3β-HSD activity, 30 nM [7-^{3}H]PREG (23.5 Ci/mmol; NEN) was added to the medium at a final concentration of 1% v/v ethanol. After the indicated times, media were collected and steroids extracted by adding 4 volumes of diethyl ether. The organic phase was then evaporated and separated on thin-layer chromatography (TLC) plates as previously described (Rhéaume et al., 1991; Sanchez et al., 1994a−c; Simard et al., 1993a,b).

To determine 3β-HSD enzymatic activity in cell homogenates, COS-1-transfected cells were harvested by scraping with a rubber policeman and resuspended at a concentration of 10×10^6 cells/mL in 50 mM phosphate-buffered saline (PBS), pH 7.4. Cells were then lysed by sonication. To measure 3β-HSD activity for the time course of formation of [^{3}H]PROG from [^{3}H]PREG, 5, 23, and 14 μg of protein from homogenates of type II 3β-HSD-, pCMV-G15D-, and pCMV-G15A-transfected cells, respectively, were incubated for the indicated times at 37 °C in presence of 30 nM [7-^{3}H]PREG labeled steroid substrate in 100 mM of KH_2PO_4, pH 7.2, containing NAD^+ in excess (1 mM). To determine the K_m for PREG, we used 13 different concentrations of the substrate, varying from 0.055 to 100 μM. To measure the K_m for NAD^+ of normal type II 3β-HSD and mutant 3β-HSD enzymes, 40, 50, and 40 μg of protein homogenates of type II 3β-HSD-, pCMV-G15D-, and pCMV-G15A-transfected cells were incubated for 1 h at 37 °C in the presence of 30 μM of a mixture of [7-^{3}H]PREG and unlabeled PREG substrate, 1000:1, respectively, in 100 mM KH_2PO_4, pH 7.2, and at least 10 different concentrations of NAD^+, ranging between 0.1 μM and 4 mM. The enzymatic reactions were stopped by adding 4 volumes of diethyl ether and chilling the incubation mixture in a dry ice−ethanol bath. Steroids were analyzed by TLC as previously described (Rhéaume et al., 1991; Simard et al., 1993a,b).

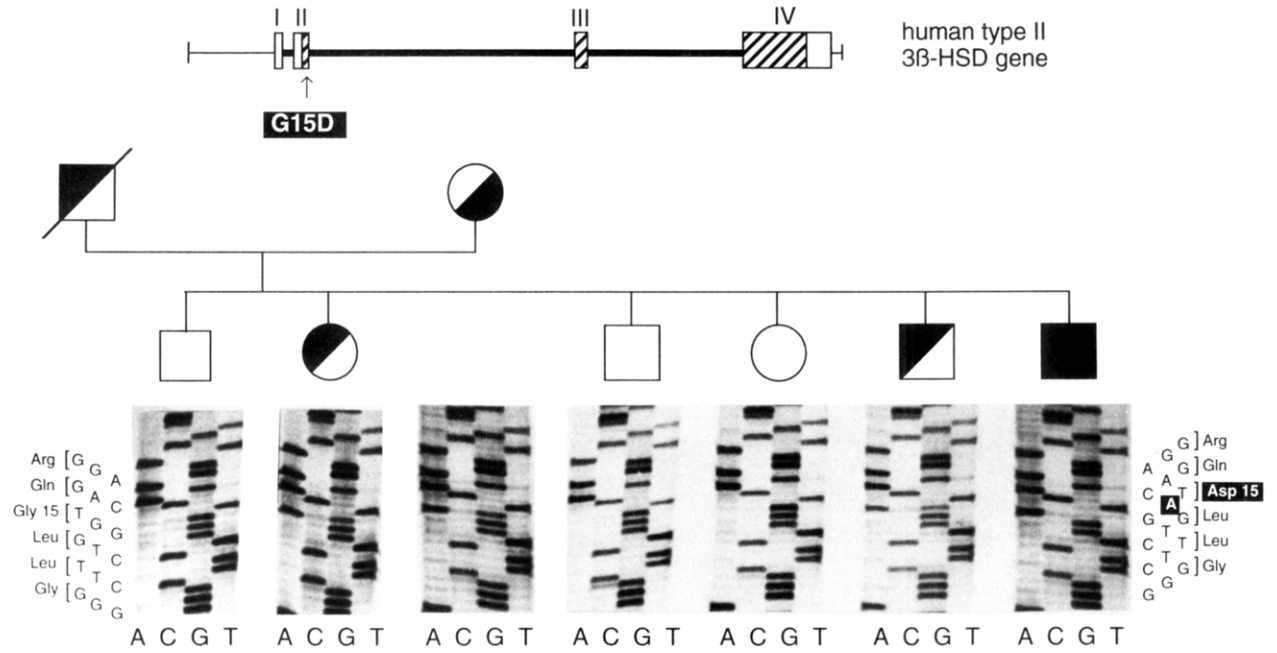

FIGURE 2: Mutational analysis of the index case family. A schematic representation illustrates the position of the point mutation G15D in the exon II of the human type II 3β-HSD gene. A pedigree of the index case's family illustrates that the affected male patient is homozygous for the G15D mutation and that the mother is heterozygous for the G15D mutation; the father is assumed to have carried the same mutation in the heterozygous state. Partial nucleotide sequence of exon II of the type II 3β-HSD gene shows the point mutation in the members of the index case's family. Sequences were determined by direct sequencing of asymmetric PCR products. Note that both G and A are present as second nucleotides at codon 15 in the mother's, one sister's, and one brother's type II 3β-HSD gene sequence, indicating heterozygosity.

The K_m and V_{max} values were calculated using the ENZFITTER software (Biosoft, Cambridge, England) after normalization of the amounts of translated mutant recombinant G15D and G15A proteins to equivalent normal human type II 3β-HSD protein expression in each cell homogenate preparation.

RESULTS

Identification of the Homozygous G15D Mutation in the Index Case's Type II 3β-HSD Gene. To identify the molecular lesion(s) in the patient's type II 3β-HSD gene responsible for the congenital adrenal hyperplasia due to a salt-losing form of classical 3β-HSD deficiency in this patient, we determined the nucleotide sequence of the whole coding region and exon−intron splicing boundaries of the type II 3β-HSD gene using a set of three primer pairs for selective PCR amplification (Rhéaume et al., 1992). Sequencing of exon II of the type II 3β-HSD gene revealed the presence of a G to A transition in both alleles of the index case, thus converting codon 15 (GGT) encoding Gly into GAT encoding Asp (Figure 2). This mutation was the only one found in the regions covered by the different PCR primer pairs and that were sequenced in both orientations. Moreover, the G15D mutation was found in the heterozygous state in the mother, in one brother, as well as in one sister, whereas two brothers and one sister did not carry the mutation; the deceased father is presumed to have carried the G15D mutation in the heterozygous state (Figure 2).

Expression of the Mutated Recombinant G15D and G15A Proteins. To assess the influence of the point mutation G15D on type II 3β-HSD enzymatic activity, we compared the activity of normal type II 3β-HSD with that of mutant type II 3β-HSD G15D generated by site-directed mutagenesis. This mutation was suspected to occur in the glycine-rich turn

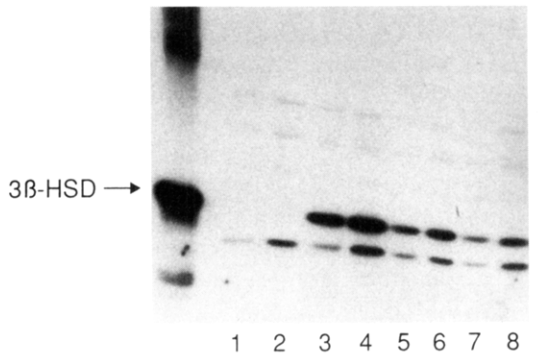

FIGURE 3: Immunoblot analysis of expressed 3β-HSD proteins encoded by wild-type human type II 3β-HSD cDNA and mutant recombinant G15D and G15A cDNAs, generated by site-directed mutagenesis, performed using antiserum raised in rabbits against purified human placental type I 3β-HSD. Homogenates from COS-1 cells transfected with control pCMV alone (lane 1, 50 μg; lane 2, 100 μg), pCMV-type II 3β-HSD (lane 3, 50 μg; lane 4, 100 μg), pCMV-G15A (lane 5, 50 μg; lane 6, 100 μg), and pCMV-G15D (lane 7, 50 μg; lane 8, 100 μg) were separated on SDS/5−15% polyacrylamide gel and quantitated with a Model 440E PhosphorImager. Purified human placental 3β-HSD protein was used as a positive control (left lane).

of the dinucleotide-binding fold of the enzyme, and we wanted to better characterize this putative functional domain. We decided to generate a more conservative amino acid change and chose to replace Gly[15] by Ala at position 15. A "GXXGXXA" motif similar to the Gly-rich turn motif of the $\beta\alpha\beta$ dinucleotide-binding fold occurring in wild-type pig cytoplasmic malate dehydrogenase was generated (Birktoft et al., 1989; Baker et al., 1992). As illustrated in Figure 3, immunoblot analysis and quantification on a PhosphorImager Model 400E show that the amounts of the mutant recombinant 3β-HSD G15A (lanes 5 and 6) and G15D (lanes 7 and 8) expressed proteins were about 36% and 22% compared to normal type II 3β-HSD (lanes 3 and 4)

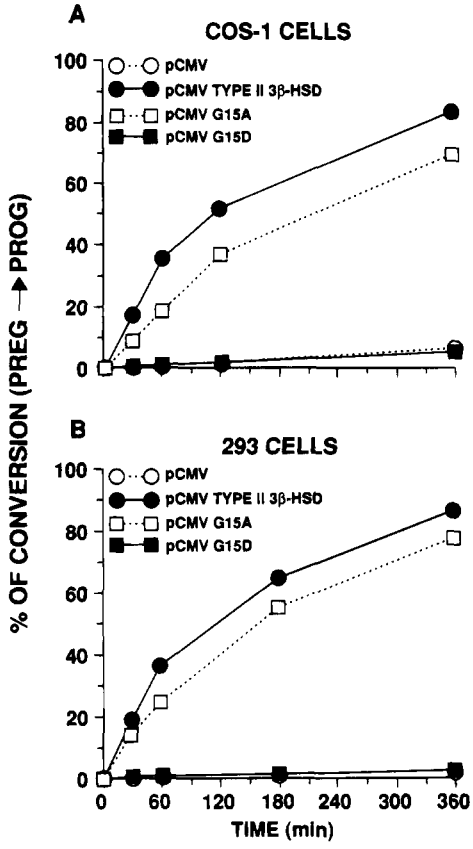

FIGURE 4: Comparison of the time course of enzymatic conversion of [^{3}H]PREG into [^{3}H]PROG in intact COS-1 cells (A) and 293 cells (B) transfected with 10 μg of pCMV, pCMV-type II 3β-HSD, pCMV-G15D, or pCMV-G15A plasmid. The results are presented in percentages as the means ± SEM of the free steroid metabolites present in culture medium as percentage of total free steroid. When SEM overlaps with the symbol used, only the symbol is illustrated.

following transient expression in COS-1 cells. Moreover, no significant endogenous immunoreactive 3β-HSD protein was detected in nontransfected COS-1 cells (lanes 1 and 2), although an unknown anti-3β-HSD immunoreactive protein can be detected in nontransfected COS-1 cells. The left lane contains purified human placental 3β-HSD protein, used as a positive control.

Activity of the Mutated Recombinant G15D and G15A Proteins. We first investigated the time course of formation of [^{3}H]PROG from [^{3}H]PREG in intact COS-1 and 293 cells transfected with pCMV, pCMV-type II 3β-HSD, pCMV-G15D, or pCMV-G15A plasmid. As illustrated in Figure 4A, in COS-1 cells transfected with pCMV-type II 3β-HSD, the percentages of [^{3}H]PROG formed after 30, 60, 120, and 360 min of incubation with [^{3}H]PREG were 17.0, 36.7, 50, and 77.2 (above pCMV-transfected cells values, used as negative control), respectively, whereas in COS-1 cells transfected with pCMV-G15A, the respective percentages of [^{3}H]PROG formed were 8.7, 18, 35, and 63 (above control values). In COS-1 cells transfected with pCMV-G15D, no conversion of [^{3}H]PROG from [^{3}H]PREG (above control values) was observed. Very similar results were obtained with the human adenovirus type 5-transformed human primary embryonal kidney cell line 293. As illustrated in Figure 4B, in 293 cells transfected with pCMV type II 3β-HSD, the percentages of [^{3}H]PROG formed after 30, 60, 240, and 360 min of incubation with [^{3}H]PREG were 18.7, 36, 63, and 84.7 (above control values), respectively, whereas

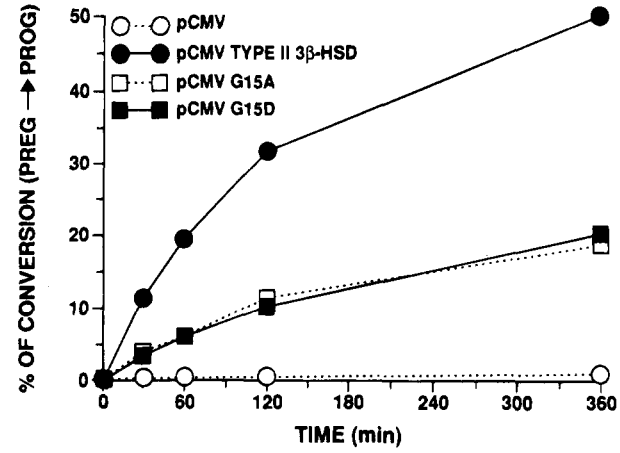

FIGURE 5: Comparison of the time course of enzymatic conversion of [^{3}H]PREG into [^{3}H]PROG in COS-1 cell homogenates from cells transfected with 10 μg of pCMV, pCMV-type II 3β-HSD, pCMV-G15D, or pCMV-G15A plasmid. The results are presented in percentages as the means ± SEM. When SEM overlaps with the symbol used, only the symbol is illustrated.

in COS-1 cells transfected with pCMV-G15A, the respective percentages of [^{3}H]PROG formed were 13.8, 23.8, 53.5, and 75.0 (above control values). Moreover, in 293 cells transfected with pCMV G15D, no significant 3β-HSD activity was detectable (Figure 4B).

To investigate if the change in 3β-HSD activity of mutant G15D and G15A proteins observed in intact transfected cells in culture can also be found when measured by classic approaches using cell homogenate preparations, we next compared the time course of enzymatic activity of normal type II 3β-HSD with that of the mutant G15D and G15A proteins (Figure 5). *In vitro* incubation of equivalent amounts of 3β-HSD immunoreactive protein, relative to normal type II 3β-HSD, in homogenates from COS-1 cells transfected with pCMV-G15D and pCMV-G15A in the presence of 1 mM NAD^+ showed lower but significant 3β-HSD activity compared with normal type II 3β-HSD activity. In COS-1 cells transfected with pCMV type II 3β-HSD, the percentages of conversion of [^{3}H]PREG into [^{3}H]PROG after 30, 60, 120, and 360 min of incubation were 11.2, 19.1, 31.3, and 49.0 (above control values), whereas in homogenates from COS-1 cells transfected with pCMV G15A, the respective percentages of [^{3}H]PROG formed were 3.5, 5.5, 10.6, and 18.1 (above control values). It was interesting that, in homogenates from COS-1 cells transfected with G15D, the respective percentages of [^{3}H]PROG formed were 3.4, 5.7, 10.1, and 19.5 (above control values). We also performed a time course of conversion of [^{3}H]PREG into [^{3}H]PROG in the presence of 5 mM $NADP^+$ with homogenates from COS-1-transfected cells expressing normal type II 3β-HSD, the mutant G15D, or the G15A protein, but no significant conversion was achieved even after 6 h of incubation for any of these enzymes (data not shown).

Kinetic Analysis of the Mutant G15D and G15A Proteins. To gain more information about the enzymatic characteristics of the mutant G15D and G15A proteins, we investigated their kinetic properties using cell homogenate preparations. The expressed mutant G15D protein has an apparent affinity for PREG that is different from that of the normal type II 3β-HSD enzyme, with K_m values of 3.2 ± 0.3 and 0.72 ± 0.05 μM, respectively, and V_{max} values of 0.60 ± 0.04 and 0.50 ± 0.03 nmol/(min·mg of total protein) normalized for 3β-

Table 1: Kinetic Parameters of Native Type II 3β-HSD and Mutant G15D and G15A Proteins

protein	K_m^{PREG} (μM)	V_{max}^{PREG} (nmol/min·mg)	K_m^{NAD} (μM)	V_{max}^{NAD} (nmol/min·mg)	$V_{max}^{PREG}/K_m^{PREG}$ [1/(min·(mg/mL))]	V_{max}^{NAD}/K_m^{NAD} [1/(min·(mg/mL))]
native	0.72 ± 0.05	0.50 ± 0.03	22 ± 2	1.20 ± 0.04	690 ± 70	55 ± 6
G15D	3.2 ± 0.3	0.60 ± 0.04	113 ± 8	1.45 ± 0.04	190 ± 30	13 ± 6
G15A	3.4 ± 0.5	0.67 ± 0.08	148 ± 18	1.39 ± 0.07	200 ± 30	9 ± 6

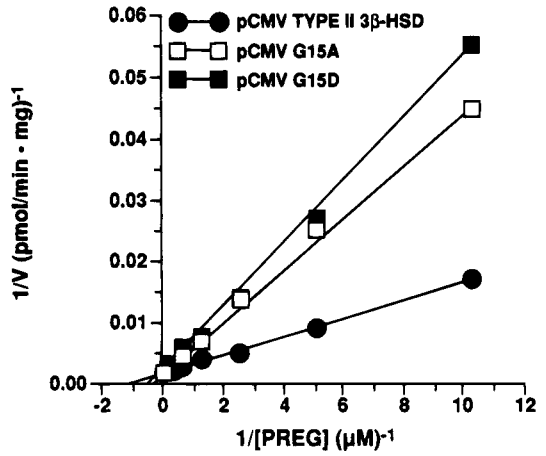

FIGURE 6: Comparison of the kinetic properties of the expressed wild-type human type II and mutant G15D and G15A 3β-HSD proteins using varying concentrations of PREG and fixed concentrations of NAD^+ (1 mM). Data are displayed as Lineweaver–Burk plots. The enzymatic reaction was performed for 60 min at 37 °C using 5, 20, or 40 μg of homogenate proteins from COS-1 cells transfected with pCMV-type II 3β-HSD (closed circles), pCMV-G15D (closed squares), or pCMV-G15A (open squares) plasmids, respectively. The amounts of translated normal type II and mutant recombinant G15D 3β-HSD proteins, as measured by immunoblot analysis, were corrected in each corresponding cell homogenate preparation. The percentage of total conversion was less than 17% under the experimental conditions used. The results are presented as the mean ± SEM (n = 3). When SEM overlaps with the symbol used, only the symbol is illustrated.

HSD immunoreactivity, respectively (Figure 6; Table 1). Furthermore, the K_m and V_{max} values for the expressed mutant G15A protein are 3.4 ± 0.05 μM and 0.67 ± 0.08 nmol/(min·mg), respectively. As measured by the first-order rate constant $V_{max}^{PREG}/K_m^{PREG}$, the present findings indicate that, under these experimental conditions, the mutant recombinant G15D and G15A enzymes possess about 27.5% and 29.0%, respectively, of the activity catalyzed by the normal type II 3β-HSD using PREG as substrate in the presence of excess (1 mM) NAD^+ concentration.

Because it was predicted that G15D and G15A mutations would affect the Gly-rich turn motif of the putative $\beta\alpha\beta$ dinucleotide-binding fold of the type II 3β-HSD enzyme, we studied the affinity of these enzymes for their cofactor, NAD^+, in the presence of excess (30 μM) PREG. As indicated in Table 1, the expressed mutant G15D protein has an apparent affinity for NAD^+ that is quite different from that of the native type II 3β-HSD enzyme, with K_m values of 113 ± 8 and 22 ± 2 μM, respectively, and similar V_{max} values of 1.45 ± 0.04 and 1.20 ± 0.04 nmol/(min·mg of total protein) normalized for 3β-HSD immunoreactivity, respectively, whereas the K_m and V_{max} values for the expressed mutant G15A protein were 148 ± 18 μM and 1.39 ± 0.07 nmol/(min·mg), respectively. Thus, the specificity for NAD^+, as measured by the first-order rate constant V_{max}^{NAD}/K_m^{NAD} (Table 1), indicates that the mutant G15D and G15A enzymes possess about 23.6% and 17.1%, respectively, of the activity catalyzed by the normal type II 3β-HSD using NAD^+ as cofactor in the presence of an excess (30 μM) of the C21-steroid PREG substrate. In the absence of precise information concerning the enzymatic mechanism of the reactions catalyzed by human type II 3β-HSD, the estimated overall *in vitro* catalytic efficiencies can be approximated by the expression $((V_{max}^{PREG} + V_{max}^{NAD})/2)/(K_m^{PREG} \times K_m^{NAD})$, which is similar to the expression used to calculate the overall efficiency of malate dehydrogenase's mutants, which is known to catalyze its reaction according to an ordered BiBi mechanism (Nishiyama et al., 1993). Moreover, one of the only mammalian 3β-HSDs to be mechanistically studied, the bovine adrenal 3β-HSD, was suggested to catalyze its reaction via an ordered BiBi mechanism (Brandt & Levy, 1989). Using this approximation, the overall efficiency of the mutant G15D and G15A enzymes would be 5.2% and 3.7%, respectively, relative to the overall efficiency of the normal human type II 3β-HSD enzyme with NAD^+ as cofactor and PREG as substrate.

DISCUSSION

The present study reports the detection and describes the functional characterization of a novel missense mutation in both type II 3β-HSD alleles in a patient with a severe salt-losing form of classical 3β-HSD deficiency (Gendrel et al., 1979). This mutation, G15D, was suspected to occur in the NAD-binding domain of the type II 3β-HSD enzyme.

The mutation G15D is a conversion of Gly to Asp at codon 15. This Gly at position 15 is located within a highly conserved Gly-X-X-Gly-X-X-Gly fingerprint (where X is any amino acid) found in all members of the 3β-HSD superfamily that comprises mammalian 3β-HSDs (Labrie et al., 1992, 1994; Mason, 1993), vaccinia virus open reading frame SalF7L (Moore & Smith, 1992a,b), and *Nocardia* cholesterol dehydrogenase [the last two possessing 3β-HSD activity (Moore & Smith, 1992a; Sanchez et al., 1993)], and some enzymes involved in the metabolism of sugar nucleotides like *Escherichia coli* UDP-galactose 4-epimerase (Baker & Blasco, 1992). This Gly-X-X-Gly-X-X-Gly fingerprint is similar to the common Gly-X-Gly-X-X-Gly conserved sequence of most NAD(H) binding enzymes (Wierenga et al., 1985; Scrutton et al., 1990; Baker et al., 1992). In these enzymes, a $\beta\alpha\beta$-fold is centered around the highly conserved Gly-rich fingerprint, which constitutes a tight turn at the end of the first strand of β-sheet and marks the beginning of the subsequent dinucleotide binding α-helix (Wierenga et al., 1986). This α-helix's dipole interacts with the negatively charged pyrophosphate moieties of the dinucleotide, either NAD(P) or FAD(P). The third Gly of the fingerprint, which corresponds to Gly at codon 15 of type II 3β-HSD, seems to provide space for a close interaction between the β-strands and the α-helix (Scrutton et al., 1990). It might be expected that insertion of the bulkier and negatively charged Asp residue would disrupt the geometry of the putative NAD-binding site.

It is of interest to note that a role in NAD^+ binding was suggested for a cysteine at position 21 of the bovine adrenal 3β-HSD enzyme, because preincubation with NAD^+ appeared to protect the enzyme against inhibition by a thiol-modifying agent reacting at the NAD^+-binding site (Rutherfurd et al., 1991). Although this Cys is not conserved within the 3β-HSD family, a similarity in the 3D structure possibly exists. Combined with the fact that a Gly-rich motif proximal to Cys^{21} is highly conserved, it is suggested that this region of type II 3β-HSD may be the NAD^+-binding domain. The present study was intended to try to confirm this hypothesis by studying the enzymatic characteristics of mutant enzymes of the Gly^{15} position.

To better evaluate the implication of Gly^{15} in the NAD^+-binding domain, we investigated the effect of a more conservative substitution than G15D for this position and thus made the replacement of Gly^{15} with the second smallest amino acid after Gly, Ala. Moreover, the presence of an Ala in place of the fingerprint's third Gly is a common feature for NADP-binding enzymes (Wierenga et al., 1985; Scrutton et al., 1990). Recently, Baker et al. suggested the existence of two types of hydrogen-bonding network, independent of coenzyme specificity and determined by the sequence pattern of the Gly-rich turn of the fingerprint (Baker et al., 1992). Of the structurally known dinucleotide binding enzymes, only pig cytoplasmic malate dehydrogenase (porcine cMDH) and *E. coli* UDP-galactose 4-epimerase do not conform to those two patterns. In *E. coli* UDP-galactose 4-epimerase, the Gly-rich turn has a sequence GGSGYIG. This uncommon fingerprint, different in that two residues occur between the first two Gly instead of the more usual one, is shared with the Gly-X-X-Gly-X-X-Gly pattern of the mammalian family of 3β-HSD enzymes.

The mutant G15D and G15A type II 3β-HSD enzymes share very similar kinetics in homogenates from transfected cells. Both possess slightly elevated K_m values for PREG of 3.2 and 3.3 μM, respectively, compared with 0.72 μM for the normal type II 3β-HSD enzyme, and elevated K_m values for NAD^+ of 113 and 148 μM, respectively, compared with 22 μM for normal type II 3β-HSD enzyme, which is almost identical to the reported value of 21.63 μM for 3β-HSD from homogenates from human adrenal microsomes (Yates & Deshpande, 1974). Although we should have obtained more similar V_{max}^{PREG} and V_{max}^{NAD} values for each enzyme, the results we obtained (that is, a relatively lower V_{max}^{PREG} than the V_{max}^{NAD} value) are relatively similar to those reported for human type I 3β-HSD enzyme using slightly different conditions (Thomas et al., 1989).

Given the very similar results for G15D and G15A mutant enzymes in homogenates, the difference between the results of the time course of formation of PROG from PREG in intact COS-1 and 293 cells transfected with G15D compared with those obtained with the G15A mutant recombinant plasmid remains to be explained. In intact cells, only the G15A mutant is effective in the conversion of PREG into PROG; the G15D mutant is inactive. The 3β-HSD enzyme is a membrane-bound protein that is present mostly in smooth endoplasmic reticulum and to some extent in mitochondria (Yates & Deshpande, 1974; Chapman & Sauer, 1979; Thomas et al., 1989). Evidence has been recently reported suggesting that the active site of 3β-HSD in the smooth endoplasmic reticulum *in vivo* faces the cytoplasm and has free access to cytoplasmic NAD^+, whereas the active site of mitochondrial 3β-HSD faces the matrix space (m side of the inner membrane) (Sauer et al., 1994). The marked difference in activity of G15D and G15A proteins in intact cells compared with their similar activity in homogenate preparations could be related to an alteration of the proper intracellular localization of this integral membrane protein by the G15D mutation, thus affecting the activity of the G15D enzyme more severely *in vivo* than *in vitro*. Thus, the already much higher value of K_m for NAD^+ may be an underestimation of the *in vivo* effect of the mutation G15D.

The presence of an Ala in place of the third Gly in the Gly-rich fingerprint of many $NADP^+$ binding enzymes suggested that this residue could facilitate binding of $NADP^+$ over NAD^+ (Scrutton et al., 1990). However, our work with the G15A mutant shows that the mutation caused a decreased overall catalytic efficiency with NAD^+ as cofactor, but that it had no or little effect on the efficiency with $NADP^+$ as cofactor as suggested by the absence of conversion with either the normal type II enzyme or the G15A mutant enzyme in the time course of conversion of PREG into PROG in the presence of 5 mM $NADP^+$; thus, the mutant retained the original preference for NAD^+ over $NADP^+$. Other residues may be involved in the specificity of NAD^+ versus $NADP^+$ recognition, for example, a conserved negatively charged Asp residue predicted to occur at the C-terminus of the second β-strand of the putative βαβ unit binding of NAD of 3β-HSDs. This residue is conserved in most members of the mammalian 3β-HSD family except in rat type III and mouse type IV, two isoenzymes exhibiting preference for NADPH over NADH in their almost exclusive peculiar 3-ketosteroid reductase activity (de Launoit et al., 1992; Clarke et al., 1993; Sanchez et al., 1994a).

The patient described here would have been predicted to have a mutation causing a severe decrease in type II 3β-HSD enzymatic activity because of the early salt-wasting syndrome that occurred when the patient was aged only one and a half months (Gendrel et al., 1979). Such early occurrence is usually associated with the most severe form of 3β-HSD deficiency. For comparison, in other patients with salt-wasting 3β-HSD deficiency, either destructive nonsense or frameshift mutations (Rhéaume et al., 1992; Chang et al., 1993, Simard et al., 1993b, 1994) or missense mutations that completely (Simard et al., 1993b) or almost completely (Sanchez et al., 1994b) inactivate the type II 3β-HSD enzyme were found. Moreover, a mutation found in a patient with non-salt-losing 3β-HSD deficiency caused an approximately 90% decrease in type II 3β-HSD activity (Simard et al., 1993b). Thus, the homozygous G15D mutation with its estimated *in vitro* maximal overall efficiency of 5.2% would predict a borderline salt-wasting 3β-HSD deficiency unless the activity of the G15D enzyme *in vivo* is much lower than what is estimated from its homogenate activity and kinetic parameters. The latter hypothesis is supported by the observation of the absence of 3β-HSD activity in the G15D-transfected intact COS-1 and 293 cells (Figure 4). By analogy, it is interesting to note that an equivalent mutation, G23D, affecting the third Gly of the Gly-rich pattern of the βαβ dinucleotide fold of human dihydropteridine reductase (DHPR), was found in a patient with DHPR deficiency (Dianzani et al., 1993; Smooker et al., 1993). This mutation was reported to completely inactivate the DHPR protein (Smooker et al., 1993), although the conditions of expression and of enzymatic assays were

different. For example, it was not reported by Smooker et al. that they tried using NADH concentrations higher than 100 μM in their *in vitro* assays of DHPR activity, which may have limited the sensitivity of their assays in this peculiar case (Smooker et al., 1993).

ACKNOWLEDGMENT

We wish to thank the patient and his family for their much appreciated cooperation and generosity.

REFERENCES

Bain, P. A., Yoo, M., Clarke, T., Hammond, S. H., & Payne, A. H. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8870−8874.
Baker, M. E., & Blasco, R. (1992) *FEBS Lett. 301*, 89−93.
Baker, P. J., Britton, K. L., Rice, D. W., Rob, A., & Stillman, T. J. (1992) *J. Mol. Biol. 228*, 662−671.
Birktoft, J. J., Rhodes, G., & Banaszak, L. J. (1989) *Biochemistry 28*, 6065−6081.
Bongiovanni, A. (1962) *J. Clin. Invest. 41*, 2086−2092.
Brandt, M., & Levy, M. A. (1989) *Biochemistry 28*, 140−148.
Chang, Y. T., Kappy, M. S., Iwamoto, K., Wang, J., Yang, X., & Pang, S. (1993) *Pediatr. Res. 34*, 698−700.
Chapman, J. C., & Sauer, L. A. (1979) *J. Biol. Chem. 254*, 6624−6630.
Clarke, T. R., Bain, P. A., Greco, T. L., & Payne, A. (1993) *Mol. Endocrinol. 7*, 1569−1578.
de Launoit, Y., Zhao, H. F., Bélanger, A., Labrie, F., & Simard, J. (1992) *J. Biol. Chem. 267*, 4513−4517.
Dianzani, I., Howells, D. W., Ponzone, A., Saleeba, J. A., Smooker, P. M., & Cotton, R. G. H. (1993) *J. Med. Genet. 30*, 465−469.
Gendrel, D., Chaussain, J. L., Roger, M., & Job, J. C. (1979) *Arch. Fr. Pédiatr. 36*, 647−655.
Higuchi, R., Krummel, B., & Saiki, R. K. (1988) *Nucleic Acids Res. 15*, 7351−7367.
Kenny, F. M., Reynolds, J. W., & Green, O. C. (1971) *Pediatrics 48*, 756−765.
Labrie, F., Simard, J., Luu-The, V., Bélanger, A., & Pelletier, G. (1992) *J. Steroid Biochem. Mol. Biol. 43*, 805−826.
Labrie, F., Simard, J., Luu-The, V., Pelletier, G., Belghmi, K., & Bélanger, A. (1994) in *Hormones, enzymes and receptors. Baillière's clinical endocrinology and metabolism* (Sheppard, M. C., & Stewart, P. M., Eds.) pp 451−474, Baillière Tindall Ltd., London.
Lachance, Y., Luu-The, V., Labrie, C., Simard, J., Dumont, M., de Launoit, Y., Guérin, S., Leblanc, G., & Labrie, F. (1990) *J. Biol. Chem. 265*, 20469−20475.
Lachance, Y., Luu-The, V., Verreault, H., Dumont, M., Rhéaume, E., Leblanc, G., & Labrie, F. (1991) *DNA Cell Biol. 10*, 701−711.
Lachance, Y., Luu-The, V., Labrie, C., Simard, J., Dumont, M., de Launoit, Y., Guérin, S., Leblanc, G., & Labrie, F. (1992) *J. Biol. Chem. 267*, 3551.
Lorence, M. C., Murry, B. A., Trant, J. M., & Mason, J. I. (1990) *Endocrinology 126*, 2493−2498.
Luu-The, V., Lachance, Y., Labrie, C., Leblanc, G., Thomas, J. L., Strickler, R. C., & Labrie, F. (1989) *Mol. Endocrinol. 3*, 1310−1312.
Mason, J. I. (1993) *Trends Endocrinol. Metab. 4*, 199−203.
Moore, J. B., & Smith, G. L. (1992a) *EMBO J. 11*, 1973−1980.
Moore, J. B., & Smith, G. L. (1992b) *EMBO J. 11*, 3490.
Nishiyama, M., Birktoft, J. J., & Beppu, T. (1993) *J. Biol. Chem. 268*, 4656−4660.
Pang, S., Levine, L. S., Stoner, E., Opitz, J. M., Pollack, M. S., Dupont, B., & New, M. I. (1983) *J. Clin. Endocrinol. Metab. 56*, 808−818.
Parks, G. A., Bermudez, J. A., Anast, C. S., Bongiovanni, A. M., & New, M. I. (1971) *J. Clin. Endocrinol. 33*, 269−278.
Rhéaume, E., Lachance, Y., Zhao, H. F., Breton, N., Dumont, M., de Launoit, Y., Trudel, C., Luu-The, V., Simard, J., & Labrie, F. (1991) *Mol. Endocrinol. 5*, 1147−1157.
Rhéaume, E., Simard, J., Morel, Y., Mébarki, F., Zachmann, M., Forest, M. G., New, M. I., & Labrie, F. (1992) *Nature Genet. 1*, 239−245.
Rutherfurd, K. J., Chen, S. A., & Shively, J. E. (1991) *Biochemistry 30*, 8116−8123.
Sanchez, R., Monté, D., & de Launoit, Y. (1993) in *Proceedings of the 75th Annual Meeting of the Endocrine Society*, pp 433, #1529, Las Vegas, The Endocrine Society Press.
Sanchez, R., de Launoit, Y., Durocher, F., Bélanger, A., Labrie, F., Simard , J. (1994a) *Mol Cell. Endocrinol. 103*, 29−38.
Sanchez, R., Mébarki, F., Rhéaume, E., Laflamme, N., Forest, M. G., Bey-Omard, F., David, M., Morel, Y., Labrie, F., & Simard, J. (1994b) *Hum. Mol. Genet. 3*, 1639−1645.
Sanchez, R., Rhéaume, E., Laflamme, N., Rosenfield, R. L., Labrie, F., & Simard, J. (1994c) *J. Clin. Endocrinol. Metab. 78*, 561−567.
Sauer, L. A., Chapman, J. C., & Dauchy, R. T. (1994) *Endocrinology 134*, 751−759.
Schneider, G., Genel, M., Bongiovanni, A. M., Goldman, A. S., & Rosenfield, R. L. (1975) *J. Clin. Invest. 55*, 681−690.
Scrutton, N. S., Berry, A., & Perham, R. N. (1990) *Nature 343*, 38−43.
Simard, J., Melner, M. H., Breton, N., Low, K. G., Zhao, H. F., Periman, L. M., & Labrie, F. (1991a) *Mol. Cell. Endocrinol. 75*, 101−110.
Simard, J., de Launoit, Y., & Labrie, F. (1991b) *J. Biol. Chem. 266*, 14842−14845.
Simard, J., Couët, J., Durocher, F., Labrie, Y., Sanchez, R., Breton, N., Turgeon, C., & Labrie, F. (1993a) *J. Biol. Chem. 268*, 19659−19668.
Simard, J., Rhéaume, E., Sanchez, R., Laflamme, N., de Launoit, Y., Luu-The, V., van Seters, A. P., Gordon, R. D., Bettendorf, M., Heinrich, U., Moshang, T., New, M. I., & Labrie, F. (1993b) *Mol. Endocrinol. 7*, 716−728.
Simard, J., Rhéaume, E., Leblanc, J. F., Wallis, S. C., Joplin, J. F., Gilbey, S., Allanson, J., Mettler, G., Bettendorf, M., Heinrich, U. E., & Labrie, F. (1994) *Hum. Mol. Genet. 3*, 327−330.
Smooker, P. M., Howells, D. W., & Cotton, R. G. H. (1993) *Biochemistry 32*, 6443−6449.
Thomas, J. L., Myers, R. P., & Strickler, R. C. (1989) *J. Steroid Biochem. 33*, 209−217.
Thomas, J. L., Nash, W. E., Myers, R. P., Crankshaw, M. W., & Strickler, R. C. (1993) *J. Biol. Chem. 268*, 18507−18512.
Wierenga, R. K., De Maeyer, M. C. H., & Hol, W. G. J. (1985) *Biochemistry 24*, 1346−1357.
Wierenga, R. K., Terpsa, P., & Hol, W. G. J. (1986) *J. Mol. Biol. 187*, 101−107.
Yates, J., & Deshpande, N. (1974) *J. Endocrinol. 60*, 27−35.
Zachmann, M., Völlmin, J. A., Mürset, G., Curtius, H. C., & Prader, A. (1970) *J. Clin. Endocrinol. Metab. 30*, 719−726.
Zhao, H. F., Simard, J., Labrie, C., Breton, N., Rhéaume, E., Luu-The, V., & Labrie, F. (1989) *FEBS Lett. 259*, 153−157.
Zhao, H. F., Rhéaume, E., Trudel, C., Couet, J., Labrie, F., & Simard, J. (1990) *Endocrinology 127*, 3237−3239.
Zhao, H. F., Labrie, C., Simard, J., de Launoit, Y., Trudel, C., Martel, C., Rhéaume, E., Dupont, E., Luu-The, V., Pelletier, G., & Labrie, F. (1991) *J. Biol. Chem. 266*, 583−593.

BI9418333