Molecular Genetics of Steroid 5α-Reductase 2 Deficiency

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Abstract

Two isozymes of steroid 5α-reductase encoded by separate loci catalyze the conversion of testosterone to dihydrotestosterone. Inherited defects in the type 2 isozyme lead to male pseudohermaphroditism in which affected males have a normal internal urogenital tract but external genitalia resembling those of a female. The 5α-reductase type 2 gene (gene symbol *SRD5A2*) was cloned and shown to contain five exons and four introns. The gene was localized to chromosome 2 band p23 by somatic cell hybrid mapping and chromosomal in situ hybridization. Molecular analysis of the *SRD5A2* gene resulted in the identification of 18 mutations in 11 homozygotes, 6 compound heterozygotes, and 4 inferred compound heterozygotes from 23 families with 5α-reductase deficiency. 6 apparent recurrent mutations were detected in 19 different ethnic backgrounds. In two patients, the catalytic efficiency of the mutant enzymes correlated with the severity of the disease. The high proportion of compound heterozygotes suggests that the carrier frequency of mutations in the 5α-reductase type 2 gene may be higher than previously thought. (*J. Clin. Invest.* 1992. 799–809.) Key words: androgen metabolism • dihydrotestosterone • human mutations • sexual differentiation • male pseudohermaphroditism

Introduction

The establishment of male phenotypic sex in man is a complex process mediated by two types of hormones from the fetal testes. The peptide hormone müllerian inhibiting substance is responsible for regression of the müllerian ducts, and steroidal androgens mediate development of the male urogenital tract and of the male external genitalia (reviewed in 1, 2).

The androgen-dependent development of male genitalia can be further divided into events requiring testosterone and those requiring dihydrotestosterone (2). Testosterone induces the virilization of the Wolffian ducts into the seminal vesicles, vasa deferentia, and epididymides, and also serves as the prohormone for the synthesis of dihydrotestosterone in a reaction catalyzed by membrane-bound steroid 5α-reductase (5α-reductase) enzymes (2, 3). Dihydrotestosterone in turn induces the formation of the external genitalia, urethra, and prostate (2). Although they mediate separate embryonic developmental programs, the two steroids act through the same androgen receptor protein (4).

The two-androgen model of male phenotypic sexual differentiation was initially formulated on the basis of studies of dihydrotestosterone formation in the anlage of the external genitalia (5). This hypothesis received strong support from the study of an autosomal recessive disorder (5α-reductase deficiency) that results from impairment of the conversion of testosterone to dihydrotestosterone (6, 7). The disorder is caused by mutations in a 5α-reductase enzyme with an acidic pH optimum (8). At birth, affected males usually exhibit genital ambiguity characterized by normal Wolffian duct derivatives but abnormal external genitalia and a rudimentary prostate (4). The external genitalia in affected subjects exhibit phenotypic variation and can range from almost normal female structures (pseudovaginal perineoscrotal hypospadias), the usual finding (4, 6, 7, 9), to a clear cut male phenotype with hypospadias (10). The genetic basis for this heterogeneity is not known, but biochemical analyses of fibroblasts and tissues from patients have revealed several different classes of mutations that affect 5α-reductase activity (4).

Complementary cDNAs have been isolated for two human 5α-reductase isozymes (designated types 1 and 2) with distinct biochemical, genetic, and pharmacological properties (11, 12). Genetic analyses excluded the type 1 locus as the responsible gene in eight unrelated families with the disorder (13), and a deletion in the type 2 gene was found in affected subjects from a small tribe in New Guinea (12).

Here, we analyze the molecular genetics and biochemistry of 5α-reductase deficiency in a cohort of subjects from 25 families from various parts of the world. The results demonstrate that mutations in the type 2 gene account for this disorder, and they confirm the previously observed genetic heterogeneity. Over 40% of the affected individuals were compound heterozygotes, suggesting that the carrier frequency of 5α-reductase 2 mutations in the population may be higher than previously thought. Biochemical analysis of two substitution mutations revealed determinants of both testosterone substrate and NADPH cofactor binding, as well as insight into the unusual in vitro acidic pH optimum characteristic of the type 2 isozyme.

Methods

Subjects. As indicated in Table I, 14 of the subjects have been described in the literature (6, 7, 9, 10, 12–24). The diagnosis of 5α-reductase

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deficiency was established in the other individuals by physical examination, measurements of ratios of plasma testosterone to dihydrotestosterone, pedigree analysis, studies of 5α-reductase in cultured skin fibroblasts, and karyotype.

5R2-São Paulo-1 was a 17-yr-old 46, XY individual with microphallus, perineoscrotal hypospadias, a vaginal pouch, and bilateral cryptorchidism. His parents were first cousins once removed; no other members of the family are affected. He was raised as a female until age 15 when he began to virilize and changed gender role to male. No breast tissue was palpable. Plasma testosterone was 386 ng/dl, and plasma dihydrotestosterone was 4 ng/dl.

5R2-São Paulo-2 was raised as a female until age 3 and then assigned male gender. When assessed at age 6 he had microphallus, perineoscrotal hypospadias, a vaginal pouch, and testes palpable in the inguinal canals bilaterally. His parents are first cousins, and a brother and three other relatives are similarly affected. After an hCG stimulation test, the plasma testosterone was 292 ng/dl and dihydrotestosterone was 6.6 ng/dl.

5R2-Pakistan, a patient of Dr. J. J. Van Wyk of The University of North Carolina, Chapel Hill, NC, was born in Pakistan and was evaluated in infancy because of microphallus, perineoscrotal hypospadias, and left cryptorchidism. He was raised as a male. The parents are first cousins; no other family members are affected. The karyotype was 46,XY with a t(11;20)(q21;p11.2) balanced translocation. At age 19 plasma testosterone was 603 ng/dl, and dihydrotestosterone was 17 ng/dl.

5R2-Chicago-3, a patient of Dr. Paul Wong of Presbyterian-St. Luke's Hospital, Chicago, was born in Pakistan and raised as a male. When examined in Chicago at age 9.5 he had microphallus (3 cm) with perineoscrotal hypospadias and testes palpable in the groin. The karyotype was 46,XY. There was no known consanguinity, but one sibling had an identical phenotype. After administration of hCG plasma testosterone was 227 ng/dl and dihydrotestosterone was 8 ng/dl.

5R2-São Paulo-3 was raised as a female but changed gender role behavior to male at age 30. When evaluated at age 31 he had microphallus, perineoscrotal hypospadias, a vaginal pouch, and right sided cryptorchidism. No gynecomastia was present. His parents are first cousins, and he has two affected siblings. Plasma testosterone was 575 ng/dl, and plasma dihydrotestosterone was 10 ng/dl.

5R2-São Paulo-4 was also raised as a female and changed gender role behavior to male at age 14. When evaluated at age 29, he had microphallus with perineoscrotal hypospadias, a vaginal pouch, and testes in the inguinal canals. There is no consanguinity, but two siblings are affected. Plasma testosterone was 666 ng/dl, and plasma dihydrotestosterone was 24 ng/dl.

5R2-Irvine, a patient of Dr. Ann K. Kershnar of the Children's Hospital, Orange, CA, was raised as a female and evaluated at age 17 because of the development of clitoromegaly. There was no consanguinity, and the family history was uninformative. The karyotype was 46,XY, and bilateral testes and epididymis were identified at exploratory laparotomy.

5R2-Chicago-2, a patient of Dr. Robert L. Rosenfeld, University of Chicago, Chicago, IL, was evaluated as a newborn because of ambiguous genitalia, namely, microphallus with a perineal urethra and a chordee, no vaginal pouch, and testes palpable in the labia. There was no consanguinity, and the family history was uninformative. An hCG stimulation test was done at 42 mo of age; plasma testosterone was 717 ng/dl, and plasma dihydrotestosterone was 19 ng/dl. The decision was made to raise the subject as a female, and the testes were removed.

5R2-New York-2, a patient of Dr. Deborah Vine, Mount Sinai School of Medicine, New York, was born in Jordan and reared as a female but was told at an early age that she was a genetic male. At age 28, she was evaluated in New York and found to have a 3-cm phallus with pseudovaginal perineoscrotal hypospadias, unfused labia, a blind ending vaginal pouch, and one testes was palpable in the left labia majora and the other was palpable in the right inguinal canal. The karyotype was 46,XY, and the diagnosis was established by measuring ratios of urinary 5α to 5β androgenic and other steroid metabolites in urine (20).

5R2-New York-3 was identified at birth and raised as a female. At age 5, during inguinal exploration for a hernia, a left testes was found with epididymus and a spermatic cord. No uterus or fallopian tubes were found. At age 13.5, she was placed on estrogen replacement therapy with good breast development. She had anxiety about her sexual identity and other psychological problems throughout adolescence and into adulthood. On physical examination at age 27, she had a 2.5-cm clitoral-like phallus, a female urethra, a blind-ending vaginal pouch, and slight posterior fusion of the labia. The ratio of etiocholanolone to androsterone in urine was 3.4 (normal 0.93±0.37).

5R2-Los Angeles-3, a 13-yr-old girl, was evaluated by Dr. Barbara Lippe, University of California, Los Angeles, for failure to feminize and development of clitoromegaly and a deepened voice. On physical examination, she had a female introitus and a shallow vagina, and testes were palpable bilaterally in the groin. The parents are first cousins once removed, but the family history was otherwise unremarkable. The karyotype was 46,XY. Plasma testosterone was 810 ng/dl and plasma dihydrotestosterone was 35 ng/dl. The decision was made to continue to raise her as a female, and the testes were removed. The epididymis and vas deferens were male in character.

Measurement of 5α-reductase activity in cultured skin fibroblasts. 5α-reductase activity was measured at pH 5.5 in extracts of fibroblasts cultured from genital skin (labia majora, foreskin, scrotum) as before (23). In 14 subjects, the activity was undetectable (0.2 pmol/h per mg protein or less). In five instances (5R2-Pakistan, 5R2-Austria, 5R2-New York-1, 5R2-London-2, and 5R2-Los Angeles-1) activity was measurable but was shown to be qualitatively abnormal on the basis of studies of enzyme stability and assessment of apparent K_ms for NADPH and/or testosterone (4). In one case, (5R2-New Haven) activity measured at different times in the same fibroblast strain was highly variable, but the diagnosis was established on endocrine and phenotypic grounds (10).

Isolation of 5α-reductase 2 gene. Bacteriophage λ clones harboring inserts corresponding to the 5α-reductase 2 gene were isolated by high stringency hybridization screening (25) using ^{32}P-radiolabeled probes (26) spanning the full length cDNA (12). Hybridization-positive plaques were purified through multiple rounds of screening. Bacteriophage DNA was prepared from plate lysates (27), and human genomic DNA inserts were released by digestion with the restriction enzyme SalI before cloning into plasmid vectors (pBluescript, Stratagene Inc., La Jolla, CA). The intron-exon structure of the gene was determined by restriction enzyme mapping, Southern blotting, and DNA sequence analysis using oligonucleotide primers derived from the cDNA sequence (12).

Genomic DNA isolation. Fibroblasts biopsied from affected or normal individuals were grown to confluency in 15-cm dishes before genomic DNA isolation. White blood cells were obtained from blood withdrawn in the presence of EDTA. Total cellular DNA was isolated with nucleic acid extractor (model 340A; Applied Biosystems, Inc., Foster City, CA) and stored in a buffer containing 10 mM Tris-Cl, pH 8.0, and 1 mM EDTA. DNA concentration was determined by fluorimetry using bisbenzimide.

Polymerase chain reactions. Individual exons of the 5α-reductase 2 gene were amplified via the polymerase chain reaction (PCR)[1] using thermostable DNA polymerases (28). The locations and names of the exon-specific pairs of oligonucleotides are described in Fig. 1 and Table II. Exon DNA to be analyzed for the presence of single strand conformation-dependent DNA polymorphisms (SSCPs) was amplified in a 20-μl reaction containing 0.1 μg genomic DNA, 1.75 μM each oligonucleotide primer, 50 μM each of the four deoxynucleoside triphosphates, 1.5% (vol/vol) glycerol, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-Cl,

1. *Abbreviations used in this paper:* PCR, polymerase chain reaction; SSCP, single strand conformation-dependent DNA polymorphism.

pH 8.3 (at 22°C), 10 μCi [α-^{32}P]dCTP, and 3 U of thermostable DNA polymerase. All exons were amplified using a thermocycler program of 35 cycles of 1 min/94°C, 1 min/68°C, and a final cycle of 1 min/94°C, 3 min/68°C.

Exon DNA to be sequenced was amplified in a 50-μl reaction containing 1.2 μg genomic DNA, 20 μM each oligonucleotide primer, 100 μM each of the four deoxynucleoside triphosphates, 1.5% (vol/vol) glycerol, 50 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-Cl, pH 8.3, and 3 U of thermostable polymerase. Thermocycler conditions were those described above. The amount of amplified DNA product in a given reaction was estimated by agarose gel electrophoresis.

SSCP Analysis. Conformation-dependent DNA polymorphisms were scored for by modifications of the procedures of Orita et al. (29, 30). Neutral polyacrylamide gels containing 5.4% (wt/vol) acrylamide-bisacrylamide (29:1), 90 mM Tris-borate, pH 8.3, 4 mM EDTA, 0.05% (wt/vol) NH_4SO4, and 0.01% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine were used to separate DNA conformers. Glycerol was included at 10% (vol/vol) in some gels. Exon DNA (4.5 μl) amplified and radiolabeled as described above was diluted into 25 μl of 0.1% (wt/vol) SDS, 10 mM EDTA. 2 μl of the diluted DNA was then added to 2 μl of formamide loading buffer (95% formamide (vol/vol), 50 mM EDTA, 0.05% (wt/vol) each of xylene cyanol and bromophenol blue), incubated at 100°C for 6 min, and then quick cooled on ice. 1 μl of this sample was electrophoresed at 250 V for 14 h at 22°C (plus glycerol gels) or 4°C (minus glycerol gels). A nondenatured sample consisting of 0.14 μl of the original amplification reaction diluted into 2 μl of sucrose buffer (60% sucrose [wt/vol], 50 mM EDTA, 0.05% [wt/vol] each of xylene cyanol and bromophenol blue) was loaded in an adjacent lane to determine the position of migration of the double-stranded exon DNA fragment.

Polymerase chain reaction sequencing. Exon DNA amplified in the absence of radiolabeled deoxynucleoside triphosphates was purified on Centricon-100 columns (Amicon Corp., Danvers, MA) using a protocol supplied by the manufacturer. Aliquots corresponding to 3–10% of the purified DNA were subjected to PCR sequencing (31) in the presence of dideoxynucleoside triphosphates using ^{32}P-radiolabeled oligonucleotide primers corresponding to those used to originally amplify the DNA. A thermocycler program consisting of 20 cycles of 30 s/95°C, 30 s/55°C, 30 s/70°C, followed by 10 cycles of 1 min/95°C, 1 min/70°C was employed. Sequencing reactions were electrophoresed on denaturing polyacrylamide gels, dried, and exposed to Kodak XAR-5 film.

Site-directed mutagenesis. Oligonucleotide-directed mutagenesis of an expressible 5α-reductase type 2 cDNA (12) was carried out as described by Zoller and Smith (32) using uracil-containing bacteriophage M13 templates (33). Mutagenic oligonucleotide primers were 20 bases in length. The desired mutant was identified by direct DNA sequence analysis of single-stranded bacteriophage M13 DNA and reconstructed into the starting pCMV expression vector (34) by standard methods of genetic engineering (25).

Expression analysis in transfected 293 cells. Normal and mutant cDNAs were transfected into human embryonic 293 cells (CRL 1573; American Type Culture Collection, Rockville, MD) as described previously (12). Assay of enzyme activity in whole cells, preparation and assay of cell lysates, and determination of pH optima were carried out as described previously (11, 12). Immunoblotting of transfected cell lysates was carried out as per Thigpen and Russell (35), except that a rabbit antipeptide antiserum specific for the human 5α-reductase 2 isozyme was employed.

Chromosome mapping. Southern blot analysis of normal human and hamster controls and 14 human X Chinese hamster somatic cell hybrids derived from six independent fusion experiments (36) was carried out with a 250-bp MluI-BamHI fragment from the coding region of the human 5α-reductase 2 cDNA as described previously (37).

Chromosomal in situ hybridization was carried out on PHA-stimulated peripheral blood lymphocyte cultures synchronized as described by Yunis (38) except that the methotrexate block was released by a 5-h treatment with BrdU (0.1 mM final concentration). Slides were made immediately after harvest and stored desicated at −20°C.

A plasmid (pR5), containing a 20-kb human genomic DNA insert spanning exons 2–5 of the *SRD5A2* gene, was used as a probe after biotin-dUTP labeling via nick translation (kit from Boehringer Mannheim Corp., Indianapolis, IN). Hybridization was carried out as described previously (37). Probe concentration was 67 ng/μl, and 200 ng/μl each of human placental and salmon sperm DNA were used as competitor. After 48 h hybridization at 37°C, a biotin/avidin/FITC detection system was used (39) with one round of amplification. Chromosomes were counter-stained with either propidium iodide or (4,6-diamino-2-phenylindole [DAPI]), both at a final concentration of 160 ng/ml. A Zeiss Axiophot microscope equipped with epifluorescence and with a cooled CCD camera (Photometrics PM512)/Macintosh computer system was used with imaging software (GeneJoin®) supplied by Tim Rand (Yale University). Photographs were taken on Kodacolor Gold ASA 100 or 400 print film. Signals were counted as specific only when the FITC signal(s) were observed lying side-by-side on both chromatids of a chromosome. All single signals were judged to represent random hybridization.

Results

5α-Reductase deficient subjects. Table I summarizes the important clinical, geographical, enzymatic, and genetic features for the 5α-reductase deficiency subjects analyzed. Affected individuals from 25 families representing 19 ethnic groups were studied. The diagnosis of 5α-reductase deficiency was based on clinical findings, family studies, endocrine criteria, and in 21 families on analysis of 5α-reductase activity in cultured skin fibroblasts. The subjects described in Table I represent over half (~ 55%) of those reported in the literature to date with 5α-reductase deficiency.

Characterization of normal 5α-reductase 2 gene. With the exception of the gross deletion reported previously in the New Guinea subjects (12), Southern blot analysis of genomic DNA isolated from affected individuals described in Table I did not reveal any major rearrangements in the 5α-reductase 2 gene (data not shown). To detect point mutations, a partial structure for the normal gene was established (Fig. 1). A single hybridization-positive clone was isolated that spanned the 3'-end of the gene after screening three genomic DNA libraries. Characterization of the cloned genomic fragment revealed the presence of four exons and four introns. No DNA sequence differences were found between the exons and the cDNA, and the positions at which the introns interrupted the coding region of the gene were identical to those for the type 1 gene (40). From these data, we inferred that the type 2 gene was composed of five exons and four introns (Fig. 1).

The DNA sequences at the boundaries of exons 2 through 5

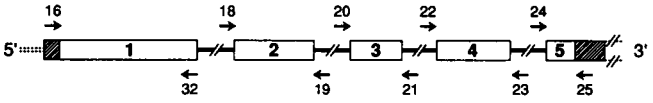

Figure 1. Structure of the 5α-reductase type 2 gene and location of oligonucleotide primers. The five exons of the type 2 gene are shown as numbered boxes and are drawn to size. Thick black lines connecting exons represent cloned introns of the gene. Dashed lines indicate uncloned 5'- and 3'-flanking sequences. The sizes of the introns have not been determined. The locations, numbers, and orientations of oligonucleotide primers used in polymerase chain reactions are indicated above and below the gene. Introns 1, 2, 3, and 4 interrupt the gene at sequences encoding amino acids 94, 149, 183, and 233, respectively.

Table I. Subjects with Male Pseudohermaphroditism Due to Steriod 5α-Reductase 2 Deficiency

Family designation	Cell strain	Ethnic group	Consanguinity/ positive family history	5α-Reductase activity in genital skin fibroblasts	Molecular lesion				Reference
					Type	Location	Mutation	Comment	
				*n = 1–100 pmol/h per mg protein**					
Class 1: Homozygotes:									
5R2-New Guinea‡	848, 849, 850	New Guinean	Yes/Yes	<0.2	Deletion (>20 kb)	5′FR-3′FR	Deletion of all exons	Deletion not sequenced	12–14
5R2-Dominican Republic‡	40, 41, 42, 338, 506	Dominican Republic	Yes/Yes	<0.2	Missense	Exon 5	C → T, R246W	CG Dinucleotide, altered NADPH K_m	7, 13, 15, 16
5R2-São Paulo-1	—	White Brazilian	Yes/No	ND§	Missense	Exon 5	C → T, R246W	CG Dinucleotide, altered NADPH K_m	This study
5R2 São Paulo-2	—	Creole Brazilian	Yes/Yes	ND	Nonsense	Exon 4	C → T, R227‖	CG Dinucleotide	This study
5R2-Pakistan	904	Pakistani	Yes/No	1.8; unstable¶	Missense	Exon 5	G → A, R246Q	CG Dinucleotide	13, this study
5R2-Chicago-3	537, 538	Pakistani	No/Yes	0.2	Missense	Exon 5	G → A, R246Q	CG Dinucleotide	This study
5R2-New Haven	728, 729, 759, 760, 828, 829	Greek American	No/No	variable	Missense	Exon 4	G → A, G196S	CG Dinucleotide, altered NADPH K_m	10
5R2-Louisiana	196	Creole American	Yes/Yes	<0.2	Missense	Exon 2	A → G, Q126R		17 (Subject 2)
5R2-Los Angeles-2	632	Vietnamese	No/Yes	<0.2	Missense	Exon 1	G → A, G34R	CG Dinucleotide, altered NADPH K_m	9 (Subject 5)
5R2-London-1	215, 216, 272, 379, 380, 381	Pakistani	Yes/Yes	<0.2	Splice Junction	Exon 4/ Intron 4	G → T		18 (Subject AA)
5R2-Chicago-1	26, 739	Mexican American	No/Yes	<0.2	Nonsense	Exon 4	C → T, R227**	CG Dinucleotide	9 (Subject 1)
5R2-Phoenix	426, 427	Native American	No/No	<0.2	Missense	Exon 4	T → C, L224P	No SSCP	9 (Subject 2)
5R2-São Paulo-3	—	Black Brazilian	Yes/Yes	ND	Missense	Exon 3	G → A, G183S		This study
Class 2A: Compound heterozygotes:									
5R2-Austria	667	Austrian	No/Yes	1.6; unstable	Missense	Exon 4	C → A, A207D		9 (Subject 3), 13, 19
					Missense	Exon 5	G → A, R246Q	CG Dinucleotide	
5R2-São Paulo-4	—	Creole Brazilian	No/Yes	ND	Missense	Exon 2	A → G, Q126R		This study
					Missense	Exon 3	A → T, D164V		
5R2-Irvine	231, 232	Mexican American	No/No	<0.2	Missense	Exon 1	G → A, G34R	CG Dinucleotide, altered testosterone K_m	This study
					Missense	Exon 2	G → A, G115D		
5R2-New York-2	445	Jordanian	No/No	<0.2	Missense	Exon 1	T → A, L55Q		20 (Subject 29)
					Missense	Exon 1	A → G, Q56R		
5R2-New York-1	106, 163	Sicilian	No/No	0.6; unstable	Missense	Exon 1	G → C, G34R		13, 21
					Missense	Exon 3	G → C, R171S		

5R2-London-2	490	Maltese	No/No	0.6; unstable	Deletion 2 bp Missense	Exon 2 Exon 3	Deletion of TC at nucleotide 359; G → C, R171S		13, 18 (Subject MM)
Class 2B: Heterozygotes:									
5R2-Los Angeles-1	70, 71, 73, 74	Black American	No/Yes	3.0; unstable	Missense	Exon 5	G → A, R246Q	CG Dinucleotide	13, 22, 23
5R2-Dallas	65, 66, 121, 129, 139	Black American	No/Yes	<0.2	Missense	Exon 4	A → G, H231R		6, 13
5R2-Chicago-2	318, 394, 395, 418	White American	No/No	0.38	Missense	Exon 4	A → G, H231R		This study
5R2-New York-3	352	Russian American	No/No	0.2	Missense	Exon 4	G → C, E197D		This study
Class 3: No abnormality identified:									
5R2-Los Angeles-3	526	Latvian American	Yes/No	<0.2	?‡	?	?	Exon sequences normal	13 and this study
5R2-London-3	325, 326	Cypriot	No/Yes	<0.2	?	?	?	Exon sequences normal	18 (Subject CP), 24

* Normal range = 1–100 pmol/h per mg protein as described in ref. 23. † Previously described, included for comparison. § ND, not done. ‖ **, Termination codon. ¶ unstable, enzyme activity unstable at elevated temperatures. ‡ ?, no mutation found. FR, flanking region.

were used to design pairs of oligonucleotides (Fig. 1, Table II) for use in the PCR. To amplify exon 1, a 5′ oligonucleotide (h5a2-16) was synthesized based on the sequence of the 5′-untranslated region of the cDNA and a 3′ oligonucleotide (h5a2-32) was derived from the cDNA sequence at the predicted boundary of intron 1 (Fig. 1). A thermocycler program was identified empirically to amplify all exons of the gene. DNA fragments of the expected size were detected with each primer pair, including those used to amplify exon 1 (data not shown). The latter result indicates that the gene structure shown in Fig. 1 is correct and that no additional introns are present in the coding region.

SSCP analysis of 5α-reductase 2 gene. In most subjects, mutations in the 5α-reductase 2 gene were detected by SSCP analyses (Fig. 2). SSCPs were detected in one or more exons of almost all affected individuals. The differences in migration of the single stranded DNAs caused by the sequence changes ranged from the dramatic (e.g., exon 4, 5R2-São Paulo-2) to the subtle (e.g., exon 3, 5R2-New York-1 and 5R2-London-2). In most cases it was possible to determine the presence of homozygosity or compound heterozygosity (Fig. 2). In one instance, a point mutation in the 5α-reductase 2 gene was not detected by this technique but was identified by DNA sequencing (see below). The inheritance of a given SSCP coincided with the disease in several families, and this method has been used to diagnose carrier status in individuals of previously unknown genotype (data not shown).

DNA sequence of mutations. The DNA sequences of the putative mutations detected by SSCP analysis were determined by PCR sequencing (Table I). In toto, the 19 mutations not previously described included 16 amino acid substitutions, a splice junction alteration, a nonsense codon, and a small deletion. The L224P mutation, present in subject 5R2-Phoenix (Table I), was detected only by DNA sequencing. Mutations of only one allele were detected in four individuals (5R2-Los Angeles-1, 5R2-Dallas, 5R2-Chicago-2, and 5R2-New York-3). We have tentatively classified these latter subjects as heterozygotes. No mutations were detected in two subjects (Los Angeles-3, London-3, Table I). For reasons discussed below, we believe that 5α-reductase deficiency in both the heterozygotes and the no abnormality identified group is due to mutations that map outside of the exon and immediate flanking intron sequences examined here.

The locations of the mutations in the gene and their consequences for the 5α-reductase 2 enzyme are summarized in Fig. 3. Each of the substitution mutations alters an amino acid that is conserved among the sequenced 5α-reductase enzymes (12). This finding suggests that the changes are in fact the cause of the disease rather than the result of random DNA polymorphisms. In agreement with this suggestion, no affected individual had more than two of the mutations shown in Fig. 3, and none of the mutations has been detected in multiple normal individuals (> 31) screened to date (data not shown). Identical mutations are present in different ethnic groups (Table I, Fig. 4) suggesting the possibility of mutational hotspots in the gene. A complete description of the Dominican Republic mutation has been reported elsewhere (Thigpen, A. E., D. L. Davis, T. Gautier, J. Imperato-McGinley, and D. W. Russell, submitted for publication). This mutation is described here for comparison purposes.

5α-Reductase mutations and clinical expression. To gain insight into correlations between a particular mutation and the

severity of the disease (i.e., degree of femaleness in 46,XY individuals) and structure-function relationships in the enzyme, two mutations were produced and analyzed in an expressible cDNA for 5α-reductase 2 (Table III, Fig. 5). The test cases represent the extremes of disease manifestation; an arginine substitution for glycine at position 34 (present in homozygous form in 5R2-Los Angeles-2, Table I) resulted in a female phenotype with only minor virilization at expected puberty (9). The second mutation, a serine substitution for a glycine at position 196 (present in homozygous form in 5R2-New Haven, Table I) produced a predominantly male phenotype (10).

The mutant cDNAs and a normal control were transfected into cultured human embryonic 293 cells and assayed in whole cells and cell lysates for 5α-reductase activity. As shown in Table III, the G196S mutation reduced enzyme activity in whole cells to 8.3% of normal, while the G34R mutation reduced activity to ~ 1.7% of normal.

Immunoblotting of lysates from the transfected cells indicated that essentially equal amounts of 5α-reductase protein were produced from the transfected cDNAs (Fig. 5 *A*), suggesting that the mutations did not affect the expression or the stability of the enzyme. The G196S mutation reduced the affinity of the enzyme for NADPH but did not alter the apparent K_m for testosterone (Table III). In contrast, the G34R mutation drastically reduced the affinity of the mutant protein for testosterone but did not affect the NADPH K_m.

The two substitution mutations changed the pH activity profiles of the mutant enzymes (Fig. 5 *B*). The normal 5α-reductase 2 enzyme shows a characteristic narrow acidic pH optimum when assayed in transfected cell lysates (*top panel*). The G196S mutation (*middle panel*) shows a slightly more basic pH optimum (5.2 vs. 4.9 for normal). The G34R enzyme shows a different pH profile with a broad peak of activity in the neutral to basic range (*bottom panel*). This pH activity profile is similar to that of the type 1 isozyme of 5α-reductase (12, 13). In experiments not shown, identical results with respect to pH optima were obtained when the cDNAs were transfected into simian kidney COS cells, and the subcellular localization of the mutant proteins was normal as judged by immunocytochemistry.

Chromosomal localization of the 5α-reductase 2 gene. The chromosomal localization of the disease gene was determined by in situ hybridization analysis of human lymphocyte prometaphase chromosomes and by Southern blot analysis of somatic cell hybrid panels. As shown in Fig. 6, a genomic probe spanning exons 2–5 of the type 2 gene (symbol *SRD5A2*) hybridized to band 2p23 on the short arm of chromosome 2. Of 20 metaphases scored, 16 had specific signal on both chromatids of both chromosome 2 homologues at band 2p23, while two metaphases had specific signal on only one chromosome 2 and the other two metaphases had no specific signal. The identification of the stained chromosomes was possible due to an R-banding pattern caused by the incorporation of BrdU after cell synchronization. There was no cross-hybridization detectable between the *SRD5A2* gene probe used here and the sites containing the *SRD5A1* gene or the *SRD5AP1* pseudogene (40). In somatic cell hybrid panels, a 250-bp cDNA probe detected human-specific BglII (8.8 kb) and PstI (14 and 3.5 kb) DNA fragments in all hybrids retaining human chromosome 2. There were at least two discordant hybrids for all other human chromosomes (data not shown).

Discussion

The current studies describe a molecular genetic analysis of the 5α-reductase type 2 gene in a worldwide cohort of subjects with male pseudohermaphroditism. 20 different mutations have been identified in the 5α-reductase type 2 gene on the short arm of chromosome 2. Over half of the affected individuals studied were true homozygotes whereas 40% were either compound heterozygotes or inferred compound heterozygotes. Identical mutations were found in individuals with widely differing geographical and ethnic backgrounds, suggesting the presence of mutational hotspots in the gene. Biochemical analysis of two mutations representing opposite poles of disease manifestation (i.e., feminization of external genitalia versus predominantly male development), suggested a correlation between clinical expression and severity of the impairment of enzyme function.

Mutations in the 5α-reductase type 2 gene were present in all but two individuals (5R2-Los Angeles-3, 5R2-London-3). The failure to find mutations in these patients could be due to clinical misdiagnosis, locus heterogeneity in 5α-reductase deficiency, *trans*-acting mutations, or mutations mapping outside the coding regions of the type 2 gene. Of these, we favor the latter explanation inasmuch as the present screen was confined to the exon and immediately flanking intron regions of the gene. In addition, a sequence encoding eight amino acids at the

Table II. Sequence and Locations of Oligonucleotides in 5α-Reductase 2 Gene Used for Polymerase Chain Reactions

Oligonucleotide	Location	Amplification target	Sequence 5′ → 3
h5a2-16	5′-Untranslated, Exon 1	Exon 1	CATCTAGAGAGCGTCCGCAGCGGCCACCGGCGAGG
h5a2-32	Exon 1	Exon 1	CGAAGCTTCACTGTGGAAGTAATGTACGCAGAAGA
h5a2-18	Intron 1	Exon 2	CAGGATCCGAACAGTGAATCCTAACCTTTCCTCCC
h5a2-19	Intron 2	Exon 2	CGAAGCTTCATTGTTAGCTGGGAAGTAGGTGAGAA
h5a2-20	Intron 2	Exon 3	CAGGATCCGATGTGAAAAAAGCACCACAATCTGGA
h5a2-21	Intron 3	Exon 3	CGAAGCTTCAGCTCCAGGGAAGAGTGAGAGTCTGG
h5a2-22	Intron 3	Exon 4	CAGGATCCGATGCAATGATTGACCTTCCGATTCTT
h5a2-23	Intron 4	Exon 4	CGAAGCTTCAGTTTGGAGAAGAAGAAAGCTACGT
h5a2-24	Intron 4	Exon 5	CAGGATCCGATCAGCCACTGCTCCATTATATTTA
h5a2-25	3′-Untranslated, Exon 5	Exon 5	CGAAGCTTCATTGACAGTTTTCATCAGCATTGTGG

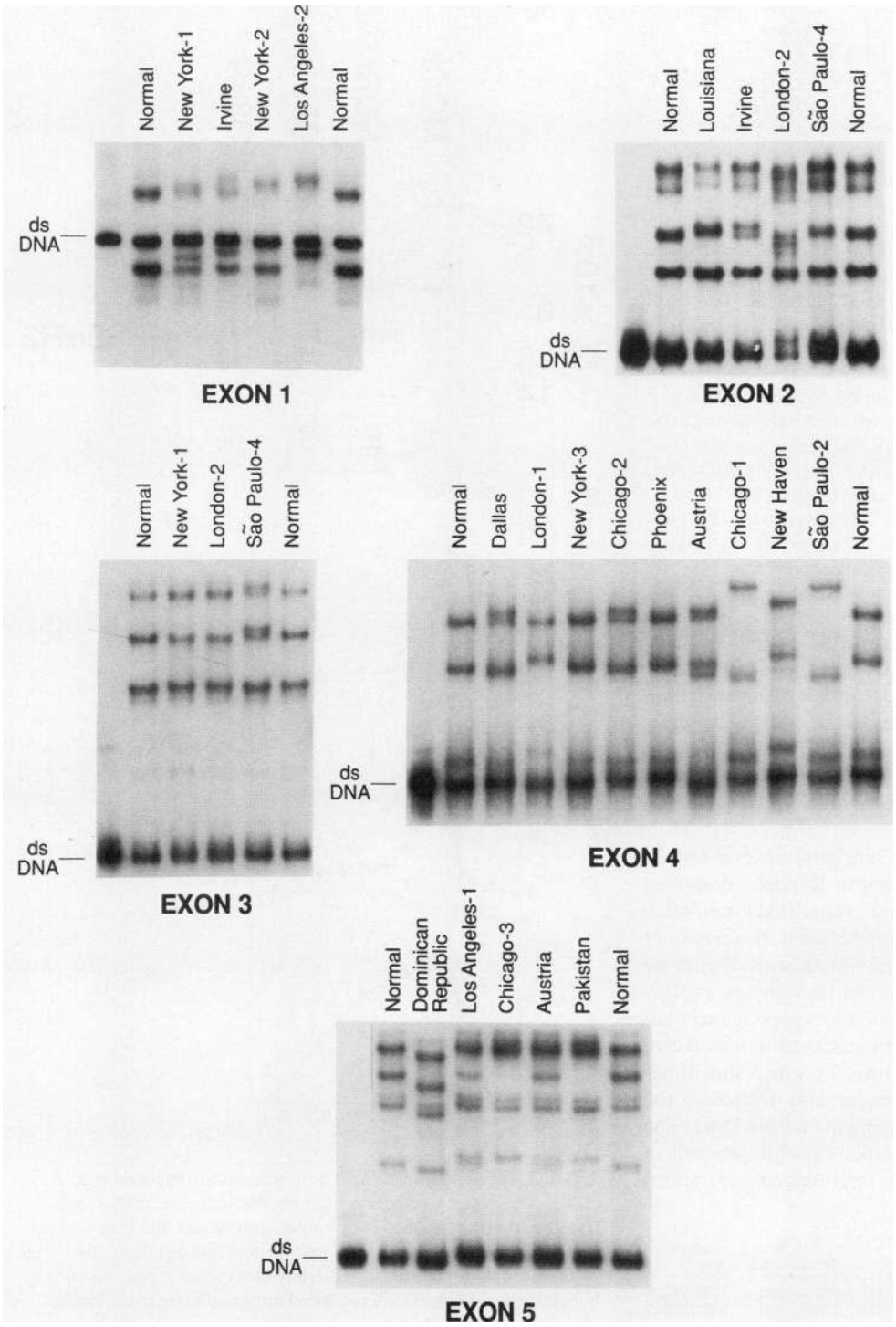

Figure 2. Detection of mutations in 5α-reductase type 2 gene by SSCP analysis. Individual exons were amplified from genomic DNA extracted from the indicated subject and screened for SSCPs. Exon 1 DNA was cleaved with BamHI before analysis to better resolve SSCPs in this 337-bp exon. Only the larger BamHI fragment (244 bp) is shown in the autoradiogram. All other exons were analyzed without restriction enzyme digestion. The migrational positions of the double stranded exon DNAs are indicated on the left of the autoradiograms. Subject New York-2 has two mutations in the exon 1 DNA fragment analyzed here. The relative contributions of these two mutations to the detected SSCP are not known.

3′-end of exon 1 was not screened due to the location of the oligonucleotide used to amplify this exon (Fig. 1). Clinical misdiagnosis is unlikely as these patients met all the criteria of 5α-reductase deficiency and have been thoroughly characterized at the biochemical and endocrine levels. In fact, the 5R2-London-3 family has been studied by two different groups (18, 24). The existence of the type 1 isozyme of 5α-reductase renders genetic heterogeneity a formal possibility; however, in the case of 5R2-Los Angeles-3 the type 1 gene was excluded as the disease locus (13).

Different mutations at some human loci can confer either a dominant or recessive inheritance pattern depending on the structure of the protein affected and the nature of the lesion (41). If 5α-reductase were to function as a multimer, then the single mutations found in the four individuals classified as heterozygotes in Table I might compromise the activity of a normal allele and lead to the disease. This type of mutation would be expected to manifest itself in carrier individuals, i.e., in a dominant fashion. The family of subject 5R2-Dallas has been studied by us, and no evidence for the disease was adduced in

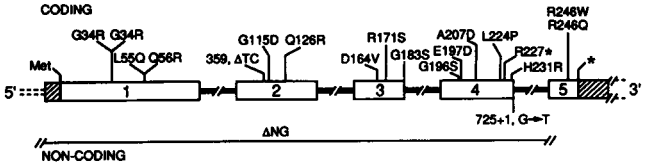

Figure 3. Mutations in 5α-reductase type 2 gene and protein. A schematic diagram of the 5α-reductase type 2 gene is shown together with the locations of 20 different mutations. The single letter amino acid code is used. All substitution mutations occur in amino acids that are conserved amongst the sequenced 5α-reductase proteins (12). Other mutations either delete a large (ΔNG) or small (359, ΔTC) portion of the gene, alter conserved splice junction signals (725 + 1, G → T), or cause premature termination (R227*). Amino acid numbers refer to those of the normal 5α-reductase type 2 protein (12). Other numbers refer to the normal cDNA and indicate the position at which a given mutation occurred (e.g., 359, ΔTC indicates a deletion of a TC dinucleotide at position 359 of the cDNA). Two different mutations (GGG → *A*GG and GGG → *C*GG) result in the G34R substitution. The ΔNG mutation was described previously (12) and the R246W mutation in the Dominican Republic individuals is extensively characterized in Thigpen, A. E., et al. (submitted for publication). They are included here for comparison and completeness.

63 known relatives spanning three generations (6). Furthermore, the father of subject 5R2-Dallas is a carrier of the H231R mutation found in both this individual and an affected sibling, a finding that rules out dominant inheritance or the occurrence of a spontaneous mutation. In subject 5R2-Dallas and the other inferred compound heterozygotes it seems likely that a more exhaustive study of the 5α-reductase type 2 gene will reveal a second mutant allele.

In two subjects analyzed at the biochemical level and in affected individuals from the Dominican Republic described in another study (Thigpen, A. E., et al., submitted for publication), there appears to be a correlation between the severity of manifestations and the severity of the impairment of enzyme function. Thus, the G196S mutation identified in a boy (5R2-New Haven) with a predominant male phenotype reduces enzyme activity to ~ 8% of normal when assayed in transfected whole cells (Table III). The mechanism by which this mutation decreases enzyme activity is presumably related to the ~ 15 fold decrease in affinity of the enzyme for NADPH. The effect of this mutation on the catalytic activity of the enzyme in vivo is difficult to estimate because intracellular concentrations

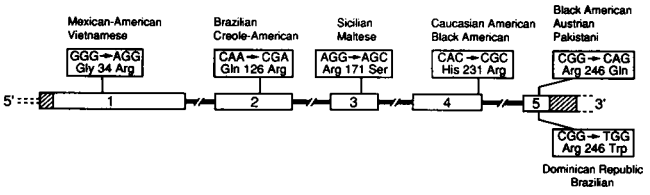

Figure 4. Apparent recurrent 5α-reductase type 2 mutations. A schematic diagram of the 5α-reductase type 2 gene is shown. The location of six mutations that occur in individuals of different ethnic backgrounds is indicated above and below the gene structure. These mutations are designated apparent recurrent because DNA polymorphisms have not yet been identified that would allow detailed haplotype analysis and subsequent determination of whether a given lesion occurred on two different genetic backgrounds, or represents two descendants from a common ancestor.

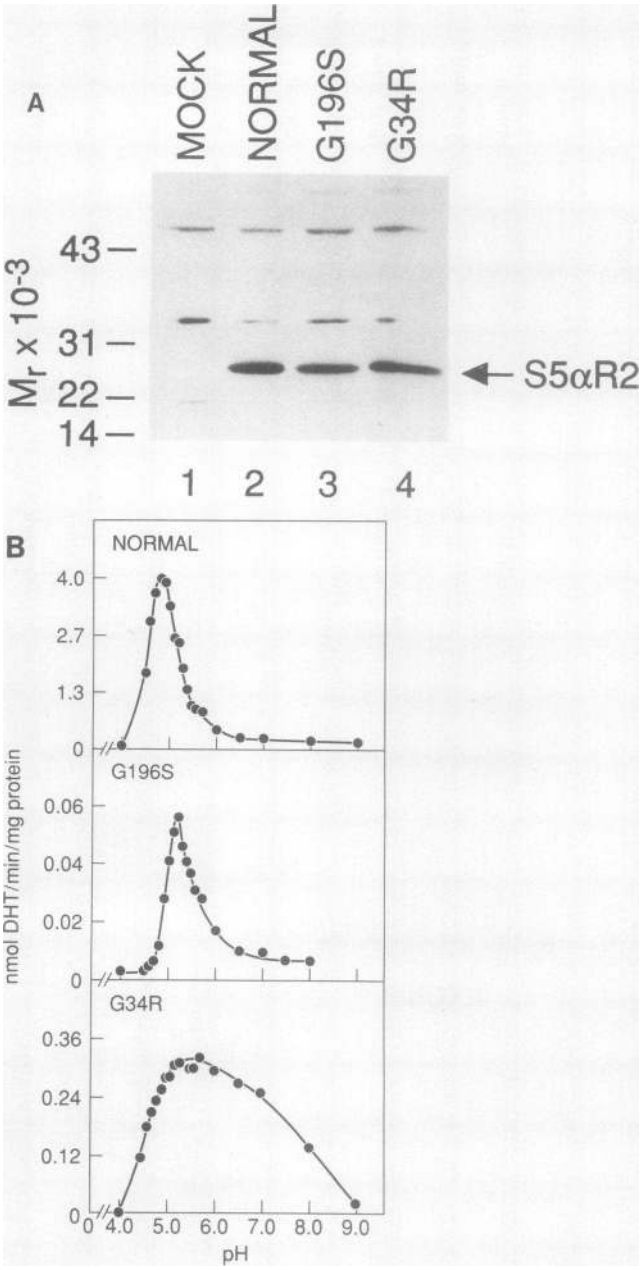

Figure 5. Expression of normal and mutant 5α-reductase type 2 cDNAs. Expression vectors containing the indicated cDNAs were transfected into monolayers of human embryonal 293 cells and assayed in cell lysates for 5α-reductase protein and enzyme activity. In *A*, an immunoblot of transfected cell lysates (60 μg protein) is shown indicating that each cDNA produced approximately equal steady-state levels of 5α-reductase protein. Lane *1*, mock transfected cells; lanes *2*, *3*, and *4*, cells transfected with the normal, G196S, and G34R cDNAs, respectively. The migrational positions of proteins of known molecular weight are shown on the left of the luminogram. In *B*, the pH optima of the normal and mutant 5α-reductase enzymes is shown. Aliquots of the normal enzyme (11 μg), the G196S enzyme (55 μg), and the G34R enzyme (42 μg) were assayed for 10–30 min at 37°C in the presence of 5 μM testosterone and 10 mM NADPH at the indicated pHs. The normal enzyme shows a narrow acidic pH optimum centered around 4.9 (*upper panel*). The G196S mutation (*middle panel*) drastically reduces overall enzyme activity relative to normal (compare ordinate scales) and increases the pH optimum slightly. The G34R substitution (*bottom panel*) reduces enzyme activity and dramatically shifts the pH optimum.

Table III. Biochemical Characterization Two 5α-Reductase Type 2 Mutations

	Normal*	G196S*	G34R*
Activity in whole cells‡	69.2%	5.7%	1.2%
pH optimum	4.8–4.9	5.1–5.2	5.2–6.0
V_{max} (nmol dihydrotestosterone/ per mg protein)	2.0–5.0	0.05–0.07	0.4–0.6
K_m Testosterone	0.5–1.0 μM	0.5–1.0 μM	10–12 μM
K_m NADPH	8–13 μM	150–180 μM	8–15 μM

* Values are the averages determined in at least two experiments carried out on different days with different cells or cell lysates. ‡ Expressed as percent conversion of [^{14}C]testosterone (1 μM) to dihydrotestosterone by whole cells transfected with the indicated cDNA in a 30-min incubation at 37°C in a cell culture incubator.

of NADPH vary between 50 and 400 μM (42). It is therefore conceivable that the level of 5α-reductase activity in the anlage of the external genitalia in this subject was sufficient to induce partial virilization.

In contrast, the G34R mutation found in subject 5R2-Los Angeles-2 reduces enzyme activity in whole cells to a greater extent and does so by reducing the affinity of the enzyme for testosterone substrate some 15-fold (Table III). The intracellular testosterone concentration is not known but is assumed to be near that of the serum in the fetus (~ 50 nmol/liter). At this substrate concentration, the G34R enzyme is essentially inactive, thus giving rise to the female phenotype seen in subject 5R2-Los Angeles-2. The finding that the G34R mutation in the type 2 isozyme alters the substrate K_m agrees well with a study (35) that mapped determinants of a competitive inhibitor to residues 26–29 of the type 1 isozyme of 5α-reductase. Together these results suggest that the substrate binding domain of 5α-reductase is encoded at least in part by residues encoded by exon 1.

Both G34R and G196S mutations shift the pH optimum of the 5α-reductase enzyme (Fig. 5 *B*). A narrow acidic pH optimum distinguishes the type 2 from the type 1 isozyme, which has a broad neutral to basic pH optimum. The biochemical basis for the acidic pH optimum of the type 2 enzyme is unknown, but this feature is also present in the rat type 2 enzyme (Normington, K., and D. W. Russell, unpublished observations) and is observed in multiple mammalian cell lines in which a type 2 human cDNA has been expressed (Thigpen, A. E., and D. W. Russell, unpublished observations). These data and the finding that the two substitution mutations shift the pH optimum, suggest that the acid requirement is an inherent feature of the enzyme and not a consequence of an inhibitor in the cell lysate or a cell type–specific membrane environment.

The acidic pH optimum may represent an artifact associated with cell lysis or reflect localization of the type 2 enzyme within an acidic subcellular compartment. The G34R and G196S mutations appear to alter the conformation of the membrane-bound enzyme in such a manner that the requirement for an acid environment is reduced. The acidic pH optimum of the normal enzyme may thus reflect an activation requirement. The study of additional naturally occurring and synthetic mutations in the type 2 enzyme may shed further light on this interesting feature.

The two 5α-reductase genes are nonsyntenic: the type 1 gene (symbol *SRD5A1*) is located in band p15 on the short arm of chromosome 5 (40), and the type 2 gene (symbol *SRD5A2*) is located in band p23 on the short arm of chromosome 2 (Fig. 6). The different chromosomal locations of these genes is consistent with their divergence at the DNA sequence

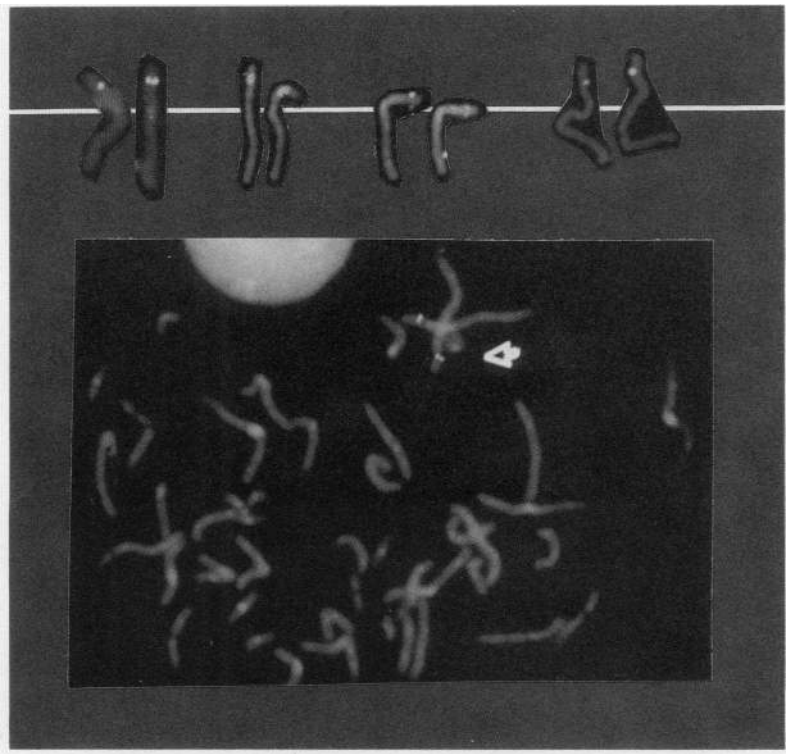

Figure 6. In situ hybridization of 20 kb genomic 5α-reductase type 2 probe to human metaphase chromosomes. (*Top*) Pairs of chromosome 2 with centromeres placed on white line and specific hybridization signals at band 2p23. (*Bottom*) Representative metaphase with signals on both chromosomes 2 that are overlapping (arrow). CCD images were obtained with the appropriate filter sets on an Axiophot microscope, of chromosomes stained with DAPI or propidium iodide and of hybridization signals generated by FITC-avidin bound to biotin-labeled 5α-reductase 2 gene probe. The images were pseudocolored and superimposed with the GeneJoin® program.

level (50% identity). *SRD5A1* and *SRD5A2* have similar gene architectures (5 exons/4 introns) and the positions at which introns interrupt the genes are identical between them (Fig. 1; ref. 40). These findings suggest that they arose as a consequence of an ancient duplication event and subsequently evolved separate physiological roles. The function of the type 2 gene is defined by 5α-reductase deficiency, while the role of the type 1 gene in adrogen physiology has not yet been elucidated.

5α-Reductase deficiency leading to male pseudohermaphroditism is a rare autosomal recessive disorder whose frequency has not been directly measured. The finding that almost half of the subjects analyzed here are known or presumed compound heterozygotes suggests that the carrier frequency of mutations in the type 2 gene may be quite high. The reasons behind and the effects of a putative high carrier frequency of mutations in the 5α-reductase type 2 gene are not known. The apparent recurrent mutations identified here (Fig. 4) suggest the possibility of mutational hotspots (43) in the gene that might lead to an increased carrier frequency. Alternatively, there may be an as yet unidentified selective advantage to heterozygous carriers.

Mutations leading to subtle abnormalities in the enzyme may underlie some forms of commonly encountered urogenital birth defect in males such as hypospadias or microphallus. Along these same lines, regulatory defects in the type 1 or type 2 genes may underlie a wide variety of androgen-dependent disorders such as male pattern baldness, acne, hirsutism, and benign or cancerous growth of the prostate. The availability of DNA probes for both genes should allow the future definition of the genetic role of 5α-reductase in these diseases.

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