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Deletion of steroid 5α -reductase 2 gene in male pseudohermaphroditism

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Abstract

THE conversion of testosterone into dihydrotestosterone by steroid 5α -reductase is a key reaction in androgen action, and is essential both for the formation of the male phenotype during embryogenesis and for androgen-mediated growth of tissues such as the prostate^{1,2}. Single gene defects that impair this conversion lead to pseudohermaphroditism in which 46 X, Y males have male internal urogenital tracts, but female external genitalia³. We have described the isolation of a human 5α -reductase complementary DNA from prostate⁴. Subsequent cloning and genetic studies showed that this gene (designated 5α -reductase 1) was normal in patients with 5α -reductase deficiency²⁶. We report here the isolation of a second 5α -reductase cDNA by expression cloning and the polymerase chain reaction. The biochemical and pharmacological properties of this cDNA-encoded enzyme (designated 5α -reductase 2) are consistent with it being the major isozyme in genital tissue. A deletion in this gene is present in two related individuals with male pseudohermaphroditism caused by 5α -reductase deficiency. These results verify the existence of at least two 5α -reductases in man and provide insight into a fundamental hormone-mediated event in male sexual differentiation.

To isolate cDNAs encoding additional 5α -reductases, a size-fractionated and oriented cDNA library was constructed from human prostate poly(A)⁺ messenger RNA in a pCMV expression vector.⁵ The size of cDNA pools that could be screened for expression of 5α -reductase in cultured 293 cells was determined in serial dilution transfection experiments using an expression vector containing the 5α -reductase 1 cDNA and an irrelevant cDNA library. Enzyme activity in transfected cells could be detected over background (threefold) when the 5α -reductase 1 cDNA was diluted 10⁴-fold. On the basis of this information, 200 pools of this size from the prostate cDNA library were transfected into 293 cells and assayed for expression of 5α -reductase activity as described in the legend to Fig. 1. Two pools were identified which expressed a 5α -reductase activity.

Simultaneously with the expression cloning studies, pairs of oligonucleotides derived from conserved regions of the human 5α -reductase 1 and rat cDNAs⁶, were used in polymerase chain reactions⁷ that used cDNA template reverse transcribed from human prostate RNA. One pair, derived from the region of the cDNAs encoding the carboxy terminus of the protein, generated *inter alia* a 91-base pair product whose DNA sequence was 57% identical

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to the corresponding region of the human 5 α -reductase 1 cDNA. When this product was used to screen an expressing pool of prostate cDNAs, hybridization positives were obtained at a frequency of about 1 in 10⁴. This result, combined with DNA sequence analysis of a hybridization-positive clone (see below), indicated that both approaches had identified the same cDNA.

The DNA sequence of the 2.437-kilobase cDNA insert in the expression plasmid encoded a hydrophobic protein of 254 amino acids and contained a long 3'-untranslated region (Fig. 1). The sequence of the predicted protein (5 α -reductase 2) was 50% identical to that of human 5 α -reductase 1 and 46% identical to the rat 5 α -reductase enzyme (Fig. 2). All three proteins shared almost identical hydropathy plots despite their relatively low sequence identity (not shown). A search of the data bases indicated that residues 10–85 of human 5 α -reductase 2 shared a 38% sequence identity with residues 231–305 of the tobacco chloroplast NADP-ubiquinone oxidoreductase chain 5 protein⁸, and that residues 9–72 shared a 39% identity with residues 222–281 of the *pol* polyprotein of the Cas-Br-E murine leukemia virus⁹. Surprisingly, the entire 5 α -reductase 2 protein is 28% identical to residues 264–462 of the Epstein–Barr virus terminal proteins¹⁰, suggesting the latter proteins may bind steroids or NADPH.

Cell extracts from 293 cells transfected with the expression vector containing the 5 α -reductase 2 cDNA actively reduced testosterone to dihydrotestosterone, indicating that the cDNA does encode a 5 α -reductase enzyme. The data in Fig. 3a show that 5 α -reductase 2, like the major enzyme expressed in human genital skin fibroblasts¹¹ and prostate¹², has an acidic pH optimum whereas 5 α -reductase 1 has a more basic pH optimum.

The central role of 5 α -reductase in prostate development and maintenance¹³ has led to the discovery and development of powerful inhibitors of this enzyme as potential therapeutics for the treatment of prostate disorders such as benign prostatic hyperplasia^{14,15}. The 4-azasteroid finasteride (MK-906), competitively inhibits the major human prostate 5 α -reductase enzyme^{12,26}, but is a poor inhibitor of the 5 α -reductase 1 enzyme³. Expression of the 5 α -reductase 2 cDNA in 293 cells results in the synthesis of an enzyme that is markedly inhibited by finasteride (50% inhibitory concentration IC₅₀ = 30nM), whereas expression of the 5 α -reductase 1 cDNA yields a poorly inhibited enzyme (IC₅₀ = 900nM) (Fig. 3b). More detailed kinetic experiments indicated finasteride inhibition constant (K_i) values of about 5 nM and 230 nM for the 5 α -reductase 2 and 1 enzymes, respectively (not shown).

Fibroblasts cultured from genital skin biopsies of 5 α -reductase-deficient subjects have reduced or abnormal acidic pH optimum enzyme activity^{3,16}. To determine if mutations in the gene encoding the 5 α -reductase 2 cDNA could be detected in these subjects, DNAs from multiple unrelated affected individuals were screened for gene rearrangements by Southern blotting. As a control, the same DNAs were screened with a probe from the 5 α -reductase 1 cDNA. The data of Fig. 4 indicate that a deletion in the 5 α -reductase 2 gene was found in two related pseudohermaphrodites from the Simbari Anga linguistic group in the Highlands of Papua New Guinea¹⁷ but was not present in the DNA of a normal individual from this tribe. The deletion seems to have removed a majority of the 5 α -reductase 2 gene from the affected individuals, as only a single weakly hybridizing fragment is visible on the

autoradiogram (Fig. 4). No gross rearrangements in the 5 α -reductase 2 gene were detected in affected individuals derived from 19 different pedigrees from throughout the world, indicating that, as with many other genetic diseases¹⁸, most of the mutations cannot be detected by Southern blotting.

Our results confirm the existence of two functional 5 α -reductase genes in man. The 5 α -reductase 2 cDNA and expressed enzyme described here seems to represent the major isozyme of genital tissue based on its acidic pH optima, exquisite sensitivity to finasteride, and deletion in two related individuals with 5 α -reductase deficiency. By contrast, the 5 α -reductase 1 cDNA and enzyme is expressed at much lower amounts in the prostate⁴, has a basic pH optimum, is insensitive to finasteride, and is normal in multiple 5 α -reductase deficient patients²⁶. The isolation of two 5 α -reductases raises interesting questions for future study concerning the tissue specificity and regulation of these isozymes and their roles in androgen physiology.

Male pseudohermaphroditism reflects the incomplete establishment of phenotypic sex in 46 X, Y individuals and in a majority of cases is due to mutations in the androgen receptor or 5 α -reductase³. These mutations are formally analogous to those described in nematodes and yeast that disrupt intercellular signalling systems controlling vulval development¹⁹ or mating type²⁷, respectively. Thus, the androgen receptor and 5 α -reductase are components of an intercellular signalling pathway that ultimately determines cell fate in the bipotential analage of the mammalian external genitalia. How the androgen receptor receives and transmits the inductive signal provided by dihydrotestosterone, as well as further parallels between mammalian and invertebrate sexual differentiation are topics for future study.

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120
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360
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120 130 140 150
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2400
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FIG. 1.

cDNA and amino-acid sequence of human 5α -reductase 2. Nucleotides are numbered at right. Amino acids are numbered above the protein sequence.

METHODS. An oriented and size-fractionated cDNA library was constructed in a pCMV vector⁵ from human prostate poly(A)⁺ mRNA using a kit purchased from GIBCO-BRL. The cDNA was electroporated into *Escherichia coli* HB101 cells, and pools of about 10^4 independent cDNAs were grown overnight in 10 ml cultures of superbrot media²⁰. Plasmid DNA was prepared using Quiagentip 100 columns and 5 μ g aliquots were transfected through a calcium phosphate procedure²¹ into 60 mm dishes of human embryonic kidney 293 cells (ATCC #CRL 1573). To enhance expression, 0.5 μ g of a plasmid (pVA1) containing the adenovirus VAI gene was cotransfected with the pooled cDNAs²¹. On day 2 of the transfection experiments, ^{14}C -testosterone (120 d.p.m. nmol^{-1}) was added to the medium at a final concentration of 1 μM , and conversion into dihydrotestosterone was determined 18 h later as described previously^{4,6}. A pool expressing 5α -reductase enzyme activity was subsequently screened with a probe generated by a polymerase chain reaction in which two oligonucleotides, GA(A/G)TGGTG(T/C)T(T/A)(T/C)GCN(C/T)TNGC and TTIGG(A/G)TAITC(T/C)TC(A/G)AA(T/C)TT, encoding amino acids 205 to 211 and 243 to 249, respectively, of the human and rat 5α -reductase proteins^{4,6}, were used to amplify random-primed cDNA synthesized from 0.4 μ g total human prostate RNA. The reaction conditions were those of Strathmann *et al.*²², except that 30-s incubations at 94 °C, 40 °C, and 72 °C were used in place of those described. About one hybridization-positive was found per 10^4 colonies screened from the expressing pool. The DNA sequence of one such cDNA was determined on an Applied Biosystems Model 373 fluorescence sequencer after subcloning fragments into bacteriophage M13 vectors.



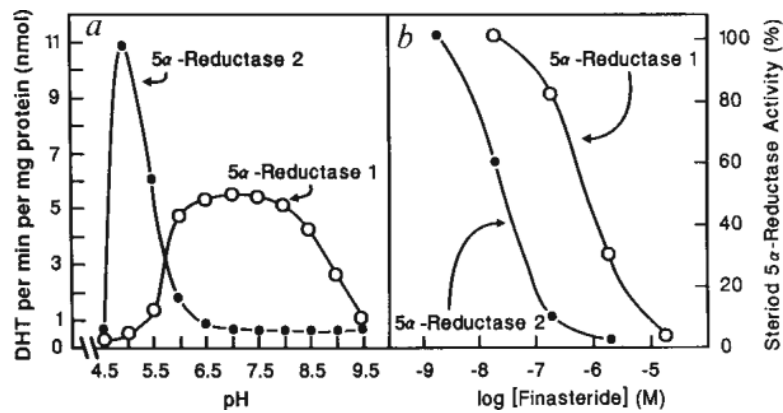
FIG. 2. Alignment of 5 α -reductase proteins. The amino-acid sequences in single-letter code of the human 5 α -reductase 2, 5 α -reductase 1 (ref. 4), and rat 5 α -reductase⁶ proteins are aligned. Identities between two or more enzymes are boxed in black. Numbers above the sequences refer to the human 5 α -reductase 2 protein.

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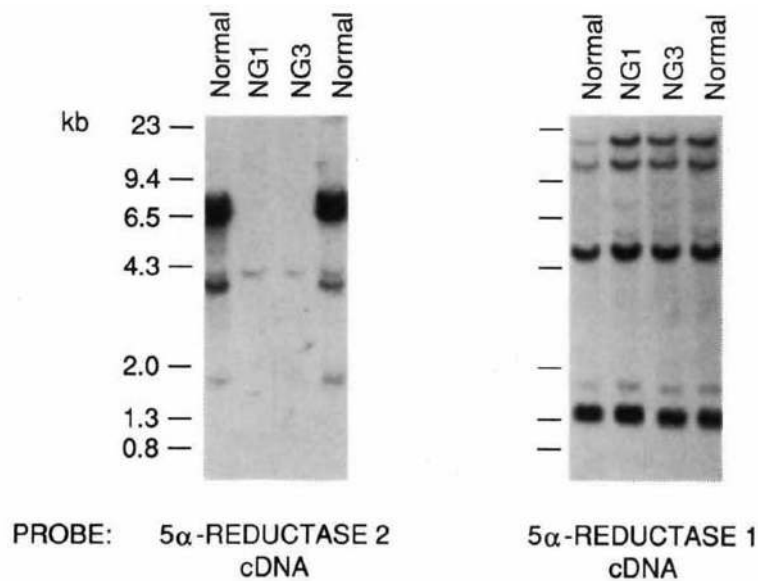
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**FIG. 3.**

Characterization of expressed 5 α -reductase isoforms, *a*, pH optima. Enzyme activity was assayed at the indicated pH in cell extracts prepared from 293 cells transfected with the 5 α -reductase 1 or 2 cDNA. Nontransfected cells express negligible amounts of enzyme activity, *b*, Inhibition by finasteride. Reductase enzyme activity obtained in extracts of transfected 293 cells in the absence of inhibitor is defined as 100%. In the presence of increasing concentrations of finasteride, progressively less 5 α -reductase enzyme activity was detected.

METHODS. An expression plasmid containing the 5 α -reductase 2 cDNA (Fig. 1) or the 5 α -reductase 1 cDNA (ref. 4) was transfected into 293 cells as described in the legend to Fig. 1. In *a*, 48 h after transfection, cell extracts were prepared as described previously⁴ and 10 μ g cell protein were assayed for 5 α -reductase enzyme activity in 0.1 M Tris-citrate buffers at the indicated pH with 10 μ M ¹⁴C-testosterone (120 d.p.m. pmol⁻¹) as substrate and 10 mM NADPH as cofactor. In *b*, 5 μ g of transfected cell protein were assayed in duplicate for 5 α -reductase activity in the presence of the indicated concentration of finasteride (MK-906)¹² (17 β -(*N*-*t*-butyl)carbamoyl-4-aza-5 α -androst-1-ene-3-one, a kind gift of G. Rasmussen, Merck, Sharp and Dohme), 4 μ M ¹⁴C-testosterone (120 d.p.m. pmol⁻¹) and 10 mM NADPH. In both panels, conversion into dihydrotestosterone was determined after 10-min incubations by thin layer chromatography as described previously⁶. Protein concentrations in cell extracts were measured by Lowry assay²³.

**FIG. 4.**

Deletion of *5α*-reductase 2 gene in subjects with *5α*-reductase deficiency. DNA isolated from two normal individuals and two related *5α*-reductase deficiency subjects from a geographically isolated tribe in the Highlands of Papua New Guinea¹⁷ was screened by Southern blotting using the indicated *5α*-reductase cDNA probes. The normal DNA analysed in the most left-hand lane was isolated from an individual from the same New Guinea tribe as the NG1 and NG3 subjects, whereas the normal DNA analysed in the right-hand lane was isolated from a caucasian American. The filter was screened with the *5α*-reductase 2 cDNA probe first, and then stripped and reprobred with the *5α*-reductase 1 cDNA probe. A deletion of all but a weakly hybridizing fragment of about 4.5 kilobases in the DNA of the affected NG1 and NG3 individuals is apparent from the autoradiogram obtained with the *5α*-reductase 2 probe. All individuals have a normal *5α*-reductase 1 gene. **METHODS.** Genomic DNA was isolated from peripheral blood samples from the indicated individuals and prepared and analysed by high-stringency Southern blotting as described previously²⁴. Aliquots of DNA (20 μg) were digested with *Hind*III before electrophoresis on agarose gels. Three single-stranded ³²P-labelled probes spanning the coding region of the *5α*-reductase 2 cDNA shown in Fig. 1 were prepared as described by Church and Gilbert²⁵ and used in hybridization. After autoradiography for 5 days at -70 °C, the filter was stripped²⁰ and reprobred with a random hexanucleotide ³²P-labelled probe²⁰ corresponding to the full-length *5α*-reductase 1 cDNA (ref. 4).