# Dihydrotestosterone Binding by Cultured Human Fibroblasts

## COMPARISON OF CELLS FROM CONTROL SUBJECTS AND FROM PATIENTS WITH HEREDITARY MALE PSEUDOHERMAPHRODITISM DUE TO ANDROGEN RESISTANCE

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ABSTRACT Dihydrotestosterone binding was measured in cultured fibroblasts from 14 control subjects and from 12 patients with five different types of hereditary male pseudohermaphroditism. Two assays of binding were used—an intact monolayer assay and density gradient centrifugation of cell extracts. In the intact monolayer assay of normal cells the uptake of [$^3$H]dihydrotestosterone consisted of two components. The first was a high affinity component that exhibited saturation at approximately 1 nM dihydrotestosterone. The second was a low affinity component that was not saturable with concentrations of steroid up to 5 nM. Twice the number of high affinity binding sites were present in fibroblasts grown from genital skin (foreskin, labia majora, and scrotum) as from nongenital sites (37 vs. 14 fmol/mg protein). In the density gradient assay in 5–20% sucrose, the major peak of dihydrotestosterone binding was in the 8S region in low molarity buffer and in the 4S region in 0.5 M KCl.

High affinity binding was normal in cells from two patients with familial incomplete male pseudohermaphroditism, type 2, an autosomal recessive defect in which dihydrotestosterone formation is deficient, and in cells from a patient with male pseudohermaphroditism due to 17β-hydroxysteroid dehydrogenase deficiency, an autosomal recessive defect of testosterone synthesis. High affinity binding was low by both methods in fibroblasts from five patients with complete testicular feminization. Furthermore, binding by both methods was also low in cells from three subjects with familial incomplete male pseudohermaphroditism, type 1, a presumed X-linked recessive disorder of androgen resistance, and in fibroblasts grown from a subject with the incomplete form of testicular feminization.

The finding that dihydrotestosterone binding is abnormal in two forms of hereditary androgen resistance in addition to complete testicular feminization suggests either that these disorders are the result of allelic mutations affecting the function of the androgen-binding protein or that normal dihydrotestosterone binding requires the participation of more than one gene product.

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## INTRODUCTION

Hereditary male pseudohermaphroditism in which genetic men differentiate partly or completely as females can result from abnormalities in either of the two primary processes required for the masculinization of the male embryo, namely, mullerian duct regression or androgen-mediated virilization of the wolffian ducts, urogenital sinus, and external genitalia (1, 2). The defects in the androgen-mediated sequence can be further divided into at least two categories, namely, genetic defects in testosterone formation and abnormalities in androgen action. On endocrinological, genetic, and phenotypic grounds four distinct entities have been delineated

in which androgen production is normal and in which the entire syndrome is presumed to be the result of resistance to androgen action, namely, the complete and incomplete forms of testicular feminization and familial incomplete male pseudohermaphroditism, types 1 and 2 (1, 2).

In two of the disorders, the molecular basis of the mutation has been the subject of considerable study. For example, evidence suggests that the autosomal recessive disorder familial incomplete male pseudohermaphroditism, type 2 (pseudovaginal perineoscrotal hypospadias) may be due to a defect in the 5α-reductase enzyme that converts testosterone[1] to dihydrotestosterone in the accessory organs of male reproduction. As a result, the urogenital sinus and external genitalia of affected males differentiate in a predominantly female fashion, but virilization of the wolffian ducts, which is mediated by testosterone itself, is normal (3–6). Furthermore, in cultured fibroblasts from subjects with the complete form of testicular feminization there is a deficiency of the binding protein that is presumed to be essential to the intracellular action of androgens (7–9). As a result, all androgen-mediated processes are deficient in this disease, and development of the wolffian duct as well as the urogenital sinus and urogenital tubercle is female in character.

Nothing is known about the pathogenesis of the androgen resistance in the other disorders of androgen action. The purpose of the present manuscript is to describe studies of dihydrotestosterone binding in fibroblasts from 14 control subjects and 12 patients with hereditary male pseudohermaphroditism. Both an intact monolayer binding assay and density gradient centrifugation of extracts were utilized. Evidence was obtained that the high affinity binding of dihydrotestosterone is also abnormal in fibroblasts from patients with incomplete testicular feminization and familial incomplete male pseudohermaphroditism, type 1 (Reifenstein syndrome).

## METHODS

*Materials.* Silica Gel G-HY thin-layer chromatography sheets with plastic backs were obtained from Brinkman Instruments, Inc. (Westbury, N. Y.). Trasylol was obtained from FBA Pharmaceuticals, Inc. (New York). Eagle's minimum essential medium (catalog no. F-11), penicillin and streptomycin solution (catalog no. 619), trypsin-EDTA solution (IX), and nonessential amino acid solution (100 X) were purchased from Grand Island Biological Co. (Grand Island, N. Y.). Tissue culture flasks (75 $cm^2$, 250 ml) were from Fisher Scientific Co. (Pittsburgh, Pa.). Tissue culture plates with six 35-mm wells per plate (catalog no. FB-6) were from Linbro Chemical Co. (New Haven, Conn.), and disposable glass roller bottles (690 $cm^2$) were from Bellco Glass, Inc. (Vineland, N. J.). [1,2,4,5,6,7-$^3$H]Dihydrotestosterone, 80 Ci/mmol, was from New England Nuclear (Boston, Mass.), and nonradioactive steroids were from Steraloids, Inc. (Pawling, N. Y.). Bovine albumin powder Fraction V was obtained from Reheis Chemical Co. (Chicago, Ill.), and [$^{14}$C]acetyl-bovine serum albumin was made by the method of Siiteri et al. (10).

Fetal calf serum was from Flow Laboratories, Inc. (Rockville, Md.). Charcoal-treated serum was made by stirring fetal calf serum overnight at 4°C with 10 mg Norit A per ml serum. The mixture was centrifuged, the charcoal treatment was repeated twice, and the serum was then passed through 0.45-μm Millipore filters. Testosterone was measured on treated and untreated serum by the method of Milewich et al. (11), and estrogens were measured by the procedure of Mikhail et al. (12). The untreated serum contained 0.27 ng/ml testosterone, 335 pg/ml estradiol, and 27 pg/ml estrone, and after charcoal treatment, the respective values were $< 0.08$ ng/ml, $< 20$ pg/ml, and $< 20$ pg/ml.

*Cell culture.* The fibroblast strains used in these experiments were established from explants of genital and nongenital skin from a variety of control subjects and from patients with different forms of male pseudohermaphroditism after obtaining informed written consent from the patients or from their parents (Table I). In the case of the control subjects, the skin samples were taken from skin removed at the time of circumcision or at the time of surgery for unrelated problems. Skin samples from the patients with male pseudohermaphroditism were obtained at the time of surgery or from punch biopsy under local anesthesia.

The patient designated "incomplete testicular feminization" (strain 156) has been reported in detail (13). The patients with familial incomplete male pseudohermaphroditism, type 1 (strains 158, 327, and 333) are cases IV-32, III-38, and III-30 as described by Wilson et al. (14), and the subjects with familial incomplete male pseudohermaphroditism, type 2 (strains 1, 2, and 139) were described by Walsh et al. (3). Four of the patients with complete testicular feminization (strains 144, 147, 151, and 221) have the typical disorder; they are phenotypic females with a 46 XY karyotype, bilateral testes, male levels of plasma testosterone, scant pubic and axillary hair, female breast development, blind-ending vaginas with no mullerian or wolffian structures detected at laparotomy, and a positive family history compatible with X-linkage. Two of the patients with complete testicular feminization are sisters (strains 144 and 151). The fifth patient (strain 240) is designated as complete testicular feminization on the basis of clinical criteria only. This individual was a phenotypic female infant with an uninformative family history who was noted to have bilateral inguinal testes at birth and who has a 46 XY karotype. The diagnosis of 17β-hydroxysteroid dehydrogenase deficiency was made by Dr. Patrick C. Walsh in a 28-yr-old 46 XY individual with bilateral testes, epididymis, and vas deferens, a female introitus, and gynecomastia on the basis of elevated luteinizing hormone and

[1] *Abbreviations used in this paper:* androstanediol, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol; androstanedione, 5α-androstane-3,17-dione; androstenedione, 4-androstene-3,17-dione; androsterone, 3α-hydroxy-5α-androstan-17-one; dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; 5β-dihydrotestosterone, 17β-hydroxy-5β-androstan-3-one; testosterone, 17β-hydroxy-4-androsten-3-one; EDTA, ethylenediaminetetraacetic acid tetrasodium salt; tricine, *N*-tris(hydroxymethyl)methyl glycine; Tris, tris(hydroxymethyl)amino methane; 17β-hydroxysteroid steroid dehydrogenase, 17β-hydroxysteroid: $NADP^+$ 17-oxidoreductase (EC 1.1.1.64); 5α-reductase, NADPH: $\Delta^4$-3-ketosteroid-5α-oxidoreductase.

TABLE I
*Identification of 31 Cell Strains Obtained from 12 Patients with Male Pseudohermaphroditism and 14 Control Subjects*

| Cell strain | Site of biopsy | Diagnosis | Age |
|---|---|---|---|
| | | | *yr* |
| 144 | Labia majora | Complete testicular feminization | 15 |
| 147 | Labia majora | Complete testicular feminization | 31 |
| 151 | Labia majora | Complete testicular feminization | 14 |
| 221 | Labia majora | Complete testicular feminization | 21 |
| 156 | Labia majora | Incomplete testicular feminization | 26 |
| 1 | Labia majora | FIMP,* type 2 | 13 |
| 2 | Labia majora | FIMP, type 2 | 11 |
| 237 | Labia majora | Normal | 30 |
| 269 | Labia majora | Normal | 24 |
| 270 | Labia majora | Normal | 28 |
| 271 | Labia majora | Normal | 21 |
| 272 | Labia majora | Normal | 25 |
| 279 | Labia majora | Normal | 20 |
| 158 | Scrotum | FIMP, type 1 | 13 |
| 327 | Scrotum | FIMP, type 1 | 20 |
| 333 | Scrotum | FIMP, type 1 | 24 |
| 170 | Scrotum | Hypospadias | 8 |
| 284 | Scrotum | Normal | 3 |
| 288 | Scrotum | Normal | 23 |
| 139 | Foreskin | FIMP, type 2 | 13 |
| 183 | Foreskin | Undescended testes | 25 |
| 164 | Foreskin | Normal | 25 |
| 175 | Foreskin | Normal | 12 |
| 287 | Foreskin | Normal | 23 |
| | Nongenital skin | | |
| 240 | Arm | Complete testicular feminization | 0.1 |
| 136 | Abdominal | FIMP, type 2 | 13 |
| 210 | Arm | 17β-Hydroxysteroid dehydrogenase deficiency | 28 |
| 165 | Arm | Normal male | 25 |
| 192 | Inguinal | Normal male | 0.2 |
| 193 | Inguinal | Normal male | 0.5 |
| 273 | Arm | Normal female | 25 |

* FIMP, familial incomplete male pseudohermaphroditism.

follicle-stimulating hormone values, a plasma androstenedione of 9.70 ng/ml, and a plasma testosterone of 2.03 ng/ml.

Fibroblasts were stored in liquid nitrogen after the initial passages and were used before passage 20. Cultures were maintained in a humidified incubator at 37°C in the presence of 5% $CO_2$. Stock cultures were grown in 75-$cm^2$ flasks with 10 ml of Eagle's minimum essential medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 20 mM Tricine chloride, pH 7.4, 24 mM $NaHCO_3$, 1% (vol/vol) nonessential amino acids, and 10% (vol/vol) fetal calf serum (medium A).

*Monolayer binding.* Cells from stock flasks were dissociated with 0.05% trypsin-0.05% EDTA at 37°C and seeded (day 0) at a concentration of approximately 75,000 cells/3 ml of media in each well of the Linbro plate. On days 3 and 6 the medium was removed and replaced with the same volume of medium in which 10% charcoal-treated fetal calf serum was substituted for the regular fetal calf serum (medium B). In experiments in which growth curves were measured, cell number was not influenced by changing to charcoal-treated fetal calf serum after 3 days in regular fetal calf serum; if, however, the cells were grown in the charcoal-treated serum from day 0, cell number was about 30% less at the end of 7 days (results not shown). On day 7 the medium was removed, and the monolayers were washed twice with 1 ml of plain medium containing no serum (medium C). Monolayers were incubated in duplicate with various concentrations of [1,2,4,5,6,7-$^3$H]dihydrotestosterone in medium C with or without added 0.5 μM nonradioactive dihydrotestosterone for 45 min. The medium was removed, and the monolayers were washed 5 times at 0°C with 2 ml of 0.02 M Tris chloride containing 0.9% NaCl and 0.2% bovine albumin, pH 7.4, and twice with 2 ml of 0.9% NaCl. The monolayers were allowed to dry at room temperature. 1 ml of 0.05 N NaOH was added to each well, and the wells were stirred. 0.5 ml was removed and mixed with 10 ml of 0.4% 2,4-diphenyloxazole in xylene-Triton X-114 (3.75:1, vol/vol) for the assay of radioactivity, and protein was determined on the remaining alkaline extract with bovine serum albumin as standard (15). Binding activity was expressed as femtomoles dihydrotestosterone uptake per milligram cell protein.

*Density gradient binding.* For the density gradient studies $5 \times 10^6$ cells were seeded into roller bottles with a surface area of 690 $cm^2$ and containing 100 ml medium A. The bottles were gassed with 5% $CO_2$ in air, capped, and incubated at 37°C on a Bellco cell production roller apparatus (0.4 rpm for 24 h and 0.6 rpm thereafter). On days 3 and 6 the medium was replaced with 100 ml medium B. On day 7 the roller bottles were rinsed twice with 15 ml medium C. 50 ml of 1 nM [$^3$H]dihydrotestosterone in medium C with or without additional nonradioactive dihydrotestosterone were added to each, and the bottles were incubated for 45 min at 37°C. The medium was removed, and the cells were harvested by scraping with a rubber policeman. Cell suspensions were kept at 0°C. Cell suspensions were centrifuged at 1,500 rpm (480 *g*) for 2 min, and the pellets were resuspended in 2 ml of 0.9% NaCl. After a second centrifugation for 2 min at 1,500 rpm the pellets were resuspended in an equal vol of 20 mM Tris, 1.5 mM EDTA, pH 7.4, containing Trasylol, 5,000 U/ml, with or without 0.5 M KCl. Cell suspensions were then submitted to sonic disruption using a Biosonik III ultrasonic system (Will Scientific, Inc., Rochester, N. Y.) at 50 W power with two 5-s periods of sonication with a needle probe ($5 \times \frac{5}{32}$ inch). Sonicates were centrifuged 1 h at 100,000 *g*, and the supernate was separated. An aliquot was removed for protein determination, and 200 μl of supernate were layered on the top of 5.3 ml of a 5–20% sucrose gradient containing 10% glycerol in 20 mM Tris chloride and 1.5 mM EDTA, pH 7.4, with or without 0.5 M KCl. Osmolarity of the cytosol was measured with a 5100 vapor pressure osmometer (Wescor Inc., Logan, Utah). For some experiments the 100,000-*g* pellet was reextracted with the Tris-EDTA buffer containing 0.5 M KCl, sonicated once for 5 s, and centrifuged at 100,000 *g* for 1 h. Density gradients were centrifuged for 18 h in a SW 50.1 rotor at 50,000 rpm (250,000 *g*) at 0°C in a Beckman L5 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). 5-drop fractions were collected from the bottom of the gradient and assayed for radioactivity in 4.5 ml of 0.4% 2,4-diphenyloxazole in xylene-Triton X-114 (3.75:1, vol/vol). [$^{14}$C]Albumin was added to certain gradients as an internal marker.

*Thin-layer chromatography.* To determine the purity of the [1,2,4,5,6,7-$^3$H]dihydrotestosterone and the amount remaining unmetabolized after incubation, aliquots of medium and washed monolayers and of the 100,000-*g* supernate of the sonicated cell suspensions were extracted with 5 vol of chloroform-methanol (2:1, vol/vol). The chloroform-methanol extracts were backwashed with 1/20th volume upper

phase solvent containing chloroform-methanol-water (6:96:94, vol/vol/vol) and evaporated to dryness. The residues were dissolved in 20 μl chloroform containing 5 μg each of five nonradioactive steroids (androstanedione, androstenedione, dihydrotestosterone, testosterone, and androstanediol) and applied to 20 × 20-cm precoated plastic sheets of silica gel without gypsum. The plates were developed with one ascent of the solvent system dichloromethane: ethyl acetate: methanol (85:15:3, vol/vol/vol), air-dried, sprayed with anisaldehyde reagent (100 ml glacial acetic acid, 2 ml of concentrated $H_2SO_4$, 1 ml of *p*-anisaldehyde), and heated at 100°C for 15 min. Within each lane the zones corresponding to the five reference steroids were marked, cut with scissors, and assayed for $^3H$ in 10 ml 0.4% 2,4-diphenyloxazole in toluene-methanol (10:1, vol/vol).

## RESULTS

When normal human fibroblasts were incubated with 0.2 nM [$^3H$]dihydrotestosterone, the cellular radioactivity increased rapidly for 15 min and then approached a plateau (Fig. 1). The initial rate of uptake and the height of the apparent plateau were greater as the temperature was increased from 4 to 37°C. At this concentration of [$^3H$]dihydrotestosterone, uptake is due principally to specific high affinity binding (see Fig. 2 below). When a 400-fold excess of nonradioactive dihydrotestosterone was added to cells after prior incubation so that they had accumulated [$^3H$]dihydrotestosterone, only 28% of the radioactivity was displaced over the next 2 h (results not shown).

Uptake in normal cells as a function of dihydrotestosterone concentration could be resolved into two components, a high affinity component that was prevented by the simultaneous addition of excess nonradioactive dihydrotestosterone (60 fmol/mg protein with a half maximum saturation of 0.1 nM) and a low affinity component that did not saturate up to 5 nM and was not displaced by excess radioactive steroid (Fig. 2A). The high affinity uptake illustrated in Fig. 2A was found to be uninfluenced by plating densities between 25,000 and 75,000 cells/35-mm well or by prolonged washing up to 12 times per well (results not shown). In a given cell strain (237) the high affinity component was uninfluenced by transfer number (34, 40, 40, 42, and 34 fmol/mg protein for transfers 7, 12, 13, 15, and 17), but it was enhanced by approximately 50% when charcoal-treated fetal calf serum was substituted for the regular fetal calf serum on day 3 (results not shown). The uptake of dihydrotestosterone at low concentrations of the steroid was shown to be relatively specific in that it was largely prevented by a 20-fold excess of testosterone and dihydrotestosterone whereas other steroids tested had less effect (Table II). At the end of the 45 min incubation most of the intracellular radioactivity was recovered as unchanged dihydrotestosterone (Table III). With this assay high affinity binding was easily demonstrable in normal cell lines but was low in fibroblasts derived from labia majora and scrotum of patients with testicular feminization (Fig. 2B) and with familial incomplete male pseudohermaphroditism, type 1 (Fig. 2C).

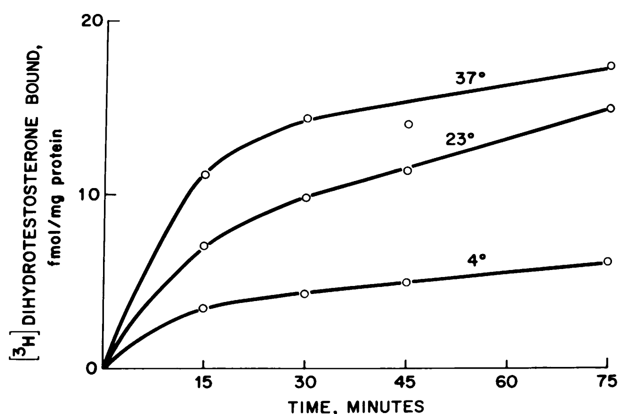


FIGURE 1 Time-course of [1,2,4,5,6,7-$^3H$]dihydrotestosterone uptake by fibroblast monolayers. Fibroblasts from control labia majora (strain 237) were grown under the standard conditions as described in the text. On day 7 the media was removed, and 1 ml of medium C containing 0.2 nM [1,2,4,5,6,7-$^3H$]dihydrotestosterone was added. The plates were incubated as indicated, and the monolayers were washed and assayed for radioactivity as described in the text. Each point represents a mean of six determinations.

TABLE II

*Effect of Various Steroids on the Uptake of [1,2,4,5,6,7-$^3H$] Dihydrotestosterone by Fibroblasts Derived from Labia Majora of a Normal Patient*

| Steroid added | Percent inhibition of [$^3H$]dihydrotestosterone uptake by added steroid — Steroid concn. . . 5 nM | 50 nM |
|---|---|---|
| Dihydrotestosterone | 77 | 82 |
| Cortisol | 3 | 13 |
| Estradiol | 26 | 69 |
| Androstanediol | 20 | 66 |
| Testosterone | 57 | 81 |
| Androstanedione | 12 | 53 |
| 5β-Dihydrotestosterone | 22 | 67 |
| Androsterone | 0 | 27 |

Fibroblasts (strain 237) were grown under the standard conditions. On day 7 the media was replaced with 1 ml of media containing 0.25 nM [1,2,4,5,6,7-$^3H$]dihydrotestosterone with or without additional steroids as indicated. The incubation and washing were performed as described in the text. In eight control wells the uptake was 1,076 ± (SD) 138 cpm/mg protein. Each value represents an average of two or three determinations.

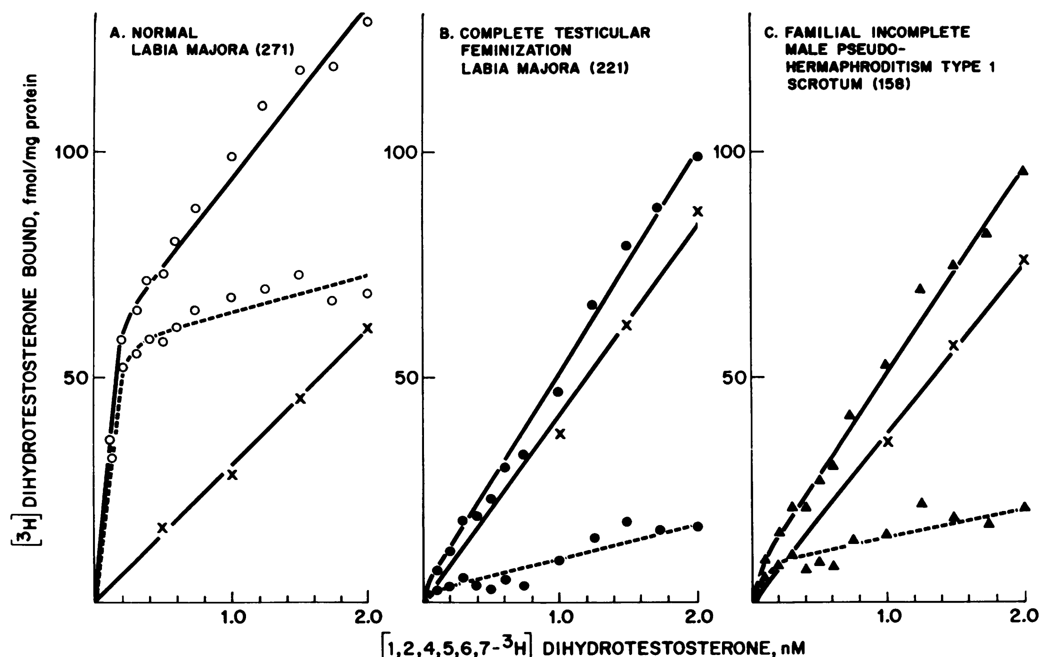


FIGURE 2 Uptake of [1,2,4,5,6,7-$^3$H]dihydrotestosterone by fibroblast monolayers as a function of dihydrotestosterone concentration. Fibroblasts from the labia majora of a control subject (strain 271)(A), from a patient with testicular feminization (strain 221)(B), and from a patient with familial incomplete male pseudohermaphroditism, type 1 (strain 158)(C) were grown under the standard conditions. On day 7, the medium was replaced with 1 ml of medium C containing [1,2,4,5,6,7-$^3$H]dihydrotestosterone that varied in concentration from 0.1 to 2 nM (○—○, ●—●, ▲—▲). In additional wells a 250-fold excess of dihydrotestosterone (0.5 $\mu$M) was added at the beginning of the incubation (×—×). The monolayers were incubated and washed as described in the text. To determine specific or displaceable binding the radioactive uptake in the presence of excess nonradioactive steroid was subtracted from the values obtained with low concentrations of dihydrotestosterone (○- - -○, ●- - -●, ▲- - -▲), and the component of specific binding was projected to the ordinate. Protein content per well averaged 0.16 mg (A), 0.15 mg (B), and 0.11 mg (C).

To characterize the uptake further, fibroblasts from a normal subject were exposed to [$^3$H]dihydrotestosterone, washed, disrupted by ultrasound, and centrifuged at 100,000 *g*, and the supernate was subjected to density gradient centrifugation in 5–20% sucrose (Fig. 3). In preliminary experiments binding in the 8S region was demonstrable only in homogenates containing about 10 mg protein/ml (results not shown), and all subsequent extracts were made in 1. 1 (vol: vol) homogenates with incubation conditions similar to those for the monolayer assay, i.e., 1 nM [1,2,4,5,6,7-$^3$H]dihydrotestosterone at 37°C for 45 min. In experiments in which the supernate was extracted after an additional 18-h incubation at 4°C, 98% of the radioactivity was recovered as unchanged dihydrotestosterone, indicating that the supernatant radioactivity did in fact represent dihydrotestosterone. When extracts of fibroblasts from normal labia majora were prepared in low ionic strength buffer that resulted in a final osmolality of 282 mosM, the major radioactivity was in a peak of approximate 8S, and a smaller peak of approximate 4S was also identified. When the 100,000-*g* pellet remaining from this preparation was reextracted in 0.5 M KCl and again centrifuged, radioactivity was recovered in the 4S but not in the 8S region. When an aliquot of the cells was extracted initially in 0.5 M KCl the only radioactive peak was in the 4S region (Fig. 3). Since KCl causes the 8S binding to change to approximate 4S binding, the subsequent comparative studies were all done by subjecting fibroblasts to extraction in low ionic and then extracting the remaining pellet in high ionic strength buffer. The assumption was made that binding demonstrable in 0.5 M KCl but not in the low ionic strength buffer probably represented androgen binding protein in membranes. Binding in both the 8S and 4S regions was prevented by excess nonradioactive dihydrotestosterone (Fig. 4). Since more than 90% of the radioactivity in the supernate in these experiments was re-

covered bound to a macromolecule and was displaced by excess nonradioactive steroid, it was concluded that the high affinity component of the uptake of [$^3$H]dihydrotestosterone by intact monolayers did in fact represent binding.

The monolayer assay was then used for a comparative study of dihydrotestosterone binding by fibroblasts from normal subjects and from patients with disorders of sexual differentiation. The intercept of the specific binding curve on the ordinate in femtomoles per milligram protein (as illustrated in Fig. 2) has been plotted in Fig. 5 for measurements in 31 fibroblast strains derived from 5 patients with complete testicular feminization, 3 patients with familial incomplete male pseudohermaphroditism, type 1, 2 patients with familial incomplete male pseudohermaphroditism, type 2, 1 patient each with incomplete testicular feminization and 17$\beta$-hydroxysteroid dehydrogenase deficiency, and 14 control subjects. The control fibroblasts include cells derived from genital skin (scrotum, labia majora, and foreskin) and from nongenital skin and includes subjects with developmental defects of the genital tract (hypospadias and cryptorchidism) as well as individuals with no known disease of the urogenital system. For purposes of this analysis labia majora and scrotum have been charted together because of their common embryological derivation. In 25 separate determinations of dihydrotestosterone binding in 9 control fibroblast strains of labia majora and scrotum, the values ranged

TABLE III
*Recovery of [$^3$H]Dihydrotestosterone from Fibroblast Monolayers*

| Fibroblast strain | Initial [$^3$H]dihydrotestosterone concentration | Percent of total radioactivity recovered in dihydrotestosterone | |
|---|---|---|---|
| | | Cells | Media |
| | *nM* | | |
| Control (237) | 0.2 | 97 | 90 |
| | 2 | 89 | 90 |
| Complete testicular feminization (221) | 0.2 | 81 | 92 |
| | 2 | 83 | 91 |
| Incomplete testicular feminization (156) | 0.2 | 82 | 92 |
| | 2 | 86 | 93 |
| Familial incomplete male pseudohermaphroditism, type 1 (158) | 0.2 | 79 | 93 |
| | 2 | 80 | 93 |

Labia majora fibroblasts were grown under the standard conditions, and at the end of 7 days the monolayers were incubated with either 0.2 or 2 nM [1,2,4,5,6,7-$^3$H]dihydrotestosterone and then washed by the standard procedure. The washed cells and the media were extracted with chloroform:methanol (2:1), and the organic phase was taken to dryness, mixed with steroid carrier, subjected to thin-layer chromatography, and assayed for radioactivity. In the zero-time control 93.8% of the radioactivity was recovered in the dihydrotestosterone area.

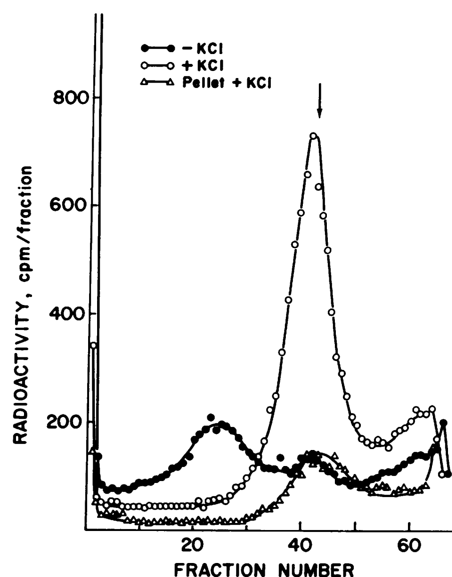


FIGURE 3 Density gradient centrifugation of fibroblast extracts after the incubation of monolayers with [1,2,4,5,6,7-$^3$H]dihydrotestosterone. Fibroblasts from control labia majora (strain 237) were grown in roller bottles and incubated with [1,2,4,5,6,7-$^3$H]dihydrotestosterone as described. After harvesting and washing, the cells were suspended in Tris-EDTA-Trasylol buffer either with or without 0.5 M KCl. After sonic disruption the extracts were centrifuged at 100,000 *g* for 1 h, and the supernate was decanted. The 100,000-*g* pellet from the low ionic strength sample was reextracted with Tris-EDTA-Trasylol containing 0.5 M KCl, sonicated, and recentrifuged at 100,000 *g* for 1 h. 200-$\mu$l samples of the various supernates (containing 10 mg protein/ml for the low ionic strength extract, 4.2 mg protein/ml for the KCl-extracted pellet, and 11.9 mg protein/ml for the sample extracted initially with KCl) were applied to 5–20% sucrose gradients containing 10% glycerol, 20 mM Tris, and 1.5 mM EDTA, pH 7.4, with or without added 0.5 M KCl. The tubes were centrifuged for 18 h at 50,000 rpm, and 5-drop fractions were collected and assayed for radioactivity. The arrow represents the position of [$^{14}$C]albumin, the internal marker for the gradient. Fraction 1 represents the bottom of the gradient.

from 20 to 75 fmol/mg protein. On an average, there was more high affinity binding in the fibroblasts derived from genital skin (44, 36, and 28 fmol/mg protein in foreskin, labia majora, and scrotum, respectively) than for fibroblasts derived from nongenital skin (14 fmol/mg protein). Furthermore, in three instances binding was compared in fibroblasts grown from genital and nongenital skin of the same individual, and in each specific binding was greater in the fibroblasts grown from genital skin (Table IV). The approximate half maximum concentrations for high affinity binding were similar in all control fibroblasts (approximately 0.1–0.2 nM). Binding was normal in the genital and nongenital cells from two patients with familial incomplete male pseudo-

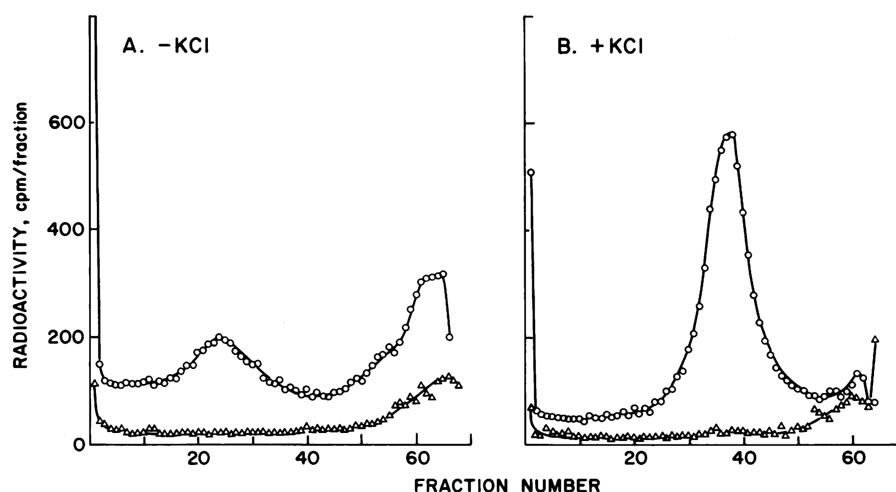


FIGURE 4 Displacement of dihydrotestosterone binding by excess nonradioactive steroid as assessed by density gradient centrifugation. Fibroblasts from control labia majora (strain 237) were grown in roller bottles, and on day 7 the media was replaced with 50 ml of media containing 1 nM [1,2,4,5,6,7-$^3$H]dihydrotestosterone with or without a 200-fold excess (0.2 $\mu$M) of nonradioactive steroid. After incubation, harvesting, and washing, the cells were resuspended in Tris-EDTA-Trasylol with or without 0.5 M KCl, and sonicates were prepared, centrifuged, and assayed for radioactivity as before. Fraction 1 represents the bottom of the gradient. ○—○, 1 nM [$^3$H]dihydrotestosterone; △—△, 0.2 $\mu$M [$^3$H]dihydrotestosterone.

hermaphroditism, type 2 and in the nongenital fibroblasts from the patient with 17$\beta$-hydroxysteroid dehydrogenase deficiency.

As expected, binding was almost undetectable in four fibroblast strains derived from labia majora and in one fibroblast strain from deltoid skin of patients with complete testicular feminization. In the fibroblasts from the patient with incomplete testicular feminization, binding in four separate experiments averaged 14 (range 9–18) fmol/mg protein, a value between the average for complete testicular feminization (4 fmol/mg protein) and that for normal labia majora (36 fmol/mg protein). Binding in the three fibroblast strains from subjects with familial male pseudohermaphroditism, type 1 ranged from a level indistinguishable from that of complete testicular feminization in one case (6 fmol/mg protein in strain 158) to values approaching that in incomplete testicular feminization in the others (an average of 13 and 11 fmol/mg protein in strains 327 and 333, respectively).

TABLE IV
*Comparison of High Affinity Dihydrotestosterone Binding in Fibroblasts Grown from Different Skin Sites from the Same Individual*

| Diagnosis | Cell strain | Site of biopsy | High affinity dihydrotestosterone binding |
|---|---|---|---|
| | | | *fmol/mg protein* |
| Normal woman | 272 | Labia majora | 26 |
| | 273 | Arm | 18 |
| Normal man | 164 | Foreskin | 48 |
| | 165 | Arm | 15 |
| Familial incomplete male pseudohermaphroditism, type 2 | 139 | Foreskin | 61 |
| | 136 | Abdomen | 17 |
| Normal man | 287 | Foreskin | 57 |
| | 288 | Scrotum | 28 |

The number of high affinity binding sites was determined as in Fig. 2, and the intercept of the specific binding curve with the ordinate has been termed "high affinity dihydrotestosterone binding."

Representative experiments from a comparative study of dihydrotestosterone binding by density gradient centrifugation are illustrated in Fig. 6. In fibroblasts from genital skin of normal subjects binding was demonstrable in the 8S region of the low ionic strength extracts and the 4S region of KCl extracts of the pellets (Fig. 6A–6C). Normal binding in the 8S and 4S regions was also demonstrated in fibroblasts from both the nongenital skin (strain 136) and foreskin (strain 139) of a patient with familial incomplete male pseudohermaphroditism, type 2 (results not shown). In a comparable preparation from the fibroblasts of a patient with complete testicular feminization virtually no binding was demonstrable in either high or low ionic strength buffers (Fig. 6D). Similar findings were obtained in fibroblasts from two other patients with complete tes-

ticular feminization (strains 147 and 144, results not shown). In the extracts of the labia majora, fibroblasts from the patient with incomplete testicular feminization binding in the 8S region was again low and not distinguishable from that in the complete form, but in the 0.5 M KCl extract of the pellet a 4S binding peak was demonstrable that was approximately half normal (Fig. 6E). In the low and high molarity extracts of scrotal fibroblasts from the patient with the type 1 form of familial incomplete male pseudohermaphroditism, binding was almost immeasurable and again could not be distinguished from that seen in the complete form of testicular feminization (Fig. 6F).

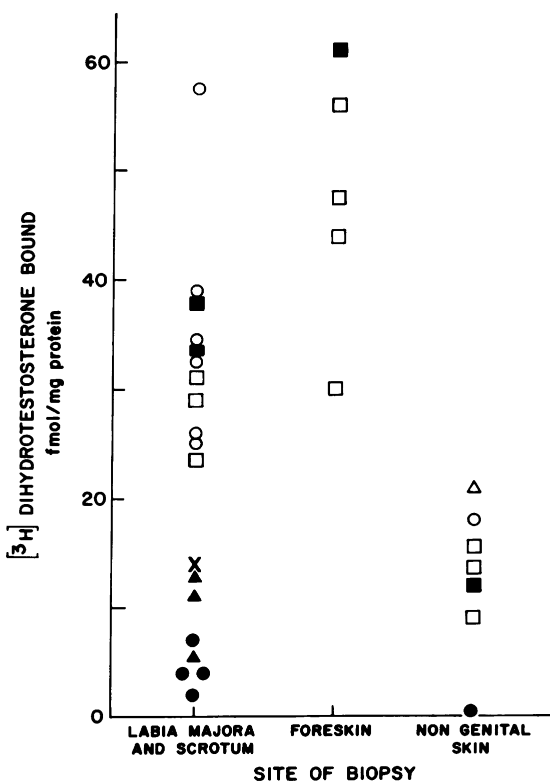


FIGURE 5 Specific binding of [1,2,4,5,6,7]dihydrotestosterone by monolayers of 29 fibroblast strains from 14 control subjects and 11 patients with hereditary male pseudohermaphroditism. Fibroblasts were grown in wells as described. On day 7, the media was replaced with media C containing 0.1–2 nM [1,2,4,5,6,7-$^3$H]dihydrotestosterone with or without 0.5 $\mu$M nonradioactive steroid. The monolayers were incubated and washed as before, and specific binding was determined as in Fig. 2. Each point represents either an individual determination (nine strains) or an average value of 2–6 measurements. □, normal male; ○, normal female; ▲, familial incomplete male pseudohermaphroditism, type 1; ■, familial incomplete male pseudohermaphroditism, type 2; ●, complete testicular feminization; ×, incomplete testicular feminization; △, 17$\beta$-hydroxysteroid dehydrogenase deficiency.

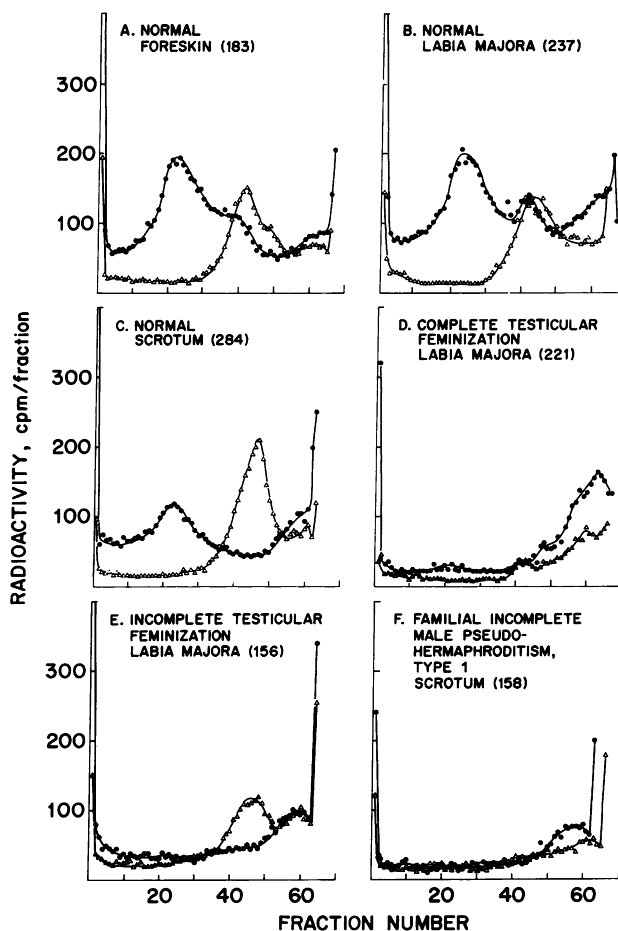


FIGURE 6 Comparison by density gradient centrifugation of dihydrotestosterone binding in fibroblasts from patients with hereditary male pseudohermaphroditism and from normal controls. Fibroblasts were grown in roller bottles, and on day 7 the media was replaced with 50 ml of media containing 1 nM [1,2,4,5,6,7-$^3$H]dihydrotestosterone. After incubation, harvesting, and washing the cells were suspended in Tris-EDTA-Trasylol buffer and sonicated. The pellet following centrifugation at 100,000 $g$ was resuspended in Tris-EDTA-Trasylol buffer containing 0.5 M KCl, and the mixture was again sonicated and centrifuged at 100,000 $g$. The supernates from both preparations (200 $\mu$l) were then applied to 5–20% sucrose density gradients containing 10% glycerol and Tris-EDTA buffer with or without 0.5 M KCl. After centrifugation at 50,000 rpm for 18 h, 5-drop fractions were collected and assayed for radioactivity. For comparative purposes, the results have been normalized to a protein content of 10 mg/ml in the low ionic strength extract and 4 mg/ml in the KCl extract of the original 100,000-$g$ pellet. ●—●, 20 mM Tris; △—△, 0.5 M KCl.

## DISCUSSION

The demonstration in the current studies that normal human fibroblasts bind dihydrotestosterone with high affinity, that the amount of binding is somewhat greater

in fibroblasts grown from genital skin than from nongenital sites, and that the high affinity binding is absent in fibroblasts from patients with complete testicular feminization is a confirmation of the previous findings of Keenan and his co-workers (7, 8). In addition, several new aspects of this process have been studied.

First, the size of the binding protein was investigated as a function of the molarity of the homogenates. In isotonic homogenates of normal cells dihydrotestosterone binding is principally in the 8S region of the gradient, whereas in high molarity buffers the major binding peak is in the 4–4.5S region. Thus, the cultured human fibroblast has an approximate 8S dihydrotestosterone-binding protein similar to the one characterized in other androgen target tissues such as rat prostate (16, 17), rat preputial gland (18), mouse kidney (19–21), and mouse submandibular gland (22).

Second, the assessment of dihydrotestosterone binding both by the monolayer assay and by density gradient centrifugation has been made in the fibroblasts from five different types of hereditary male pseudohermaphroditism in addition to complete testicular feminization. In fibroblasts from patients with complete testicular feminization high affinity binding and the 8S and 4S peaks were virtually absent. These results agree with the findings of Keenan et al. (7, 8). Since this syndrome is believed to be the result of complete resistance to androgen action both during embryogenesis and in the postnatal state as well, the absence of the binding that is believed to be essential for the movement of dihydrotestosterone into the nucleus of the cell provides a likely explanation of the pathogenesis of the androgen resistance and the male pseudohermaphroditism as well.

In fibroblasts derived from the foreskin of two patients with familial incomplete male pseudohermaphroditism, type 2, high affinity binding was in the normal range. This finding is in keeping with the concept that the fundamental defect in this disorder is deficient dihydrotestosterone formation in androgen-target tissues (3–6) and indicates that dihydrotestosterone itself does not regulate its binding protein. Dihydrotestosterone binding was also normal in fibroblasts from the arm skin of a patient with 17β-hydroxysteroid dehydrogenase deficiency in which the male pseudohermaphroditism is the result of defective testosterone synthesis during embryogenesis (23, 24). The normal values in these types of male pseudohermaphroditism, plus the normal values in fibroblasts from a subject with hypospadias and a patient with bilateral cryptorchidism, indicate that dihydrotestosterone binding is not influenced by malformations of the genital tract per se.

The results in familial incomplete male pseudohermaphroditism, type 1 are of particular interest. Like testicular feminization, this disorder is presumed to be due to a X-linked mutation, and the evidence is clear-cut in both disorders that the androgen resistance cannot be due to deficient formation of dihydrotestosterone (3, 4). Unlike testicular feminization, however, there is extreme variability in the expression of the mutant gene in that affected individuals within the same pedigree can range from almost normal males to extreme degrees of male pseudohermaphroditism (14, 25). The fact that dihydrotestosterone binding in scrotal fibroblasts from three affected individuals from one pedigree was low suggests that the androgen resistance in this disorder can also be explained as the result of abnormal dihydrotestosterone binding. Whether this mutation is allelic to that of testicular feminization or whether it involves some separate gene that regulates the binding protein is not clear.

The results in the case of incomplete testicular feminization are also of interest. The nature of the underlying mutation in this disorder is not clear in that familial occurrence has not been documented. As used here, the term describes a distinct phenotype distinguishable from testicular feminization only in that there is some evidence of androgenization of the internal and external genitalia at birth and in that partial virilization as well as feminization takes place at the time of puberty (13). The fact that both by the monolayer assay and density gradient centrifugation dihydrotestosterone binding is lower than normal suggests that androgen resistance in this disease may also result from defective dihydrotestosterone binding.

To determine whether the defects in these two forms of partial male pseudohermaphroditism are due to abnormalities in the amount of the dihydrotestosterone-binding protein or to a mutant protein that does not bind dihydrotestosterone normally, it will be necessary to develop more sensitive assays of dihydrotestosterone binding. On the basis of the evidence at hand it seems reasonable to conclude that the finding of low amounts of high affinity dihydrotestosterone binding by present methods is not diagnostic of complete testicular feminization but may be characteristic of other types of hereditary androgen resistance as well. Furthermore, the fact that dihydrotestosterone binding is abnormal in several forms of hereditary androgen resistance suggests either that these disorders are the result of allelic mutations affecting the function of the androgen-binding protein or that normal dihydrotestosterone binding requires the participation of more than one gene product.


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