Familial Incomplete Male Pseudohermaphroditism Associated with Impaired Nuclear Androgen Retention

STUDIES IN CULTURED SKIN FIBROBLASTS

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ABSTRACT The androgen resistance syndromes are generally felt to be due to quantitative or qualitative abnormalities of the androgen receptor. Some patients with testicular feminization have no demonstrable fibroblast cytosol androgen binding, whereas others have androgen binding in cultured fibroblasts that is thermolabile or fails to be stabilized by sodium molybdate. I describe here familial incomplete testicular feminization associated with reduced nuclear androgen retention. Fibroblasts, cultured from pubic skin biopsies of two phenotypic female 46XY siblings, were assayed for whole cell and nuclear uptake of [^{3}H]dihydrotestosterone in dispersed, intact cells. Whole cell binding of [^{3}H]dihydrotestosterone at 22°C in the patients' fibroblasts was in the normal range. However, no high affinity, saturable binding of [^{3}H]dihydrotestosterone was demonstrable in crude nuclear pellets prepared from the patients' fibroblasts incubated at 37°C with the hormone. Incubating the patients' cells with [^{3}H]methyltrienolone or examining the nuclear uptake of [^{3}H]dihydrotestosterone in these cells at 22°C did not alter these findings. Although cytosol from the patients' cells revealed a quantitatively diminished 8S peak for [^{3}H]dihydrotestosterone after centrifugation on sodium molybdate-containing sucrose gradients, there was no peak of ^{3}H in the 4S region from 0.3 M KCl nuclear extracts of the patients' cells after they had been incubated with [^{3}H]dihydrotestosterone at 37°C.

Although whole cell binding studies at 37°C showed minimally diminished androgen binding in the patients' cells compared with binding at 22°C, Griffin (1979. *J. Clin. Invest.* **64:** 1624–1631.) has demonstrated thermolability of the androgen receptors in fibroblasts also cultured from these patients. The observations with intact cells coupled with the diminished cytosol 8S peak of [^{3}H]dihydrotestosterone on sucrose gradients indicate that these patients have cytosol androgen receptors that are qualitatively abnormal physicochemically, the physiologic consequence of which is failure of nuclear androgen localization. Thus, although the underlying defect in the pathogenesis of the androgen resistance in these patients appears to reside in the androgen receptor, the crucial biologic manifestation of the molecular lesion is impaired nuclear androgen retention. These experiments, therefore, suggest that assessment of nuclear [^{3}H]dihydrotestosterone uptake is an effective indicator of the functional integrity of the androgen receptor system in patients with various forms of androgen insensitivity and provides additional insights to those obtained by thermolability or cytosol sucrose gradient studies.

INTRODUCTION

Male pseudohermaphroditism can occur either as a result of inadequate intrauterine androgen secretion or ineffective androgen action (1). Among the conditions that occur as a consequence of insufficient androgen production there have been cases reported with deficiencies for each of the five enzymes involved in testosterone biosynthesis (2). In a similar fashion, defects in androgen action could be expected to occur at any one of the links in the chain of steps involved in this complex intracellular process at the target tissues (3). The predominant data available suggest that

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the syndromes of androgen resistance (also called androgen insensitivity) in humans result most often from quantitative or qualitative abnormalities of the androgen receptors within the target tissues (4–8). The androgen binding in cells from patients with qualitatively abnormal receptors has been shown to be thermolabile and may not be stabilized normally by sodium molybdate (8, 9). There are, however, some patients with either complete testicular feminization or familial incomplete male pseudohermaphroditism, type 1, who seem to have normal cellular androgen receptors (5, 6, 10–12). In these receptor-positive variants, the other, more distal sites in the chain of androgen action (such as transfer of the hormone-receptor complex from the cytoplasm to the nucleus, hormone-receptor complex binding to the chromatin, and the steps involved in gene expression), are theoretically vulnerable for generation of these phenotypes, and have been implicated as being the source of the observed androgen resistance (13). However, experimental evidence in support of these possibilities has been lacking.

This report describes studies in cultured skin fibroblasts obtained from two male pseudohermaphrodite siblings who have previously been shown to have qualitatively abnormal androgen receptors (8, 9). The studies reported on here indicate that these patients' cells are unable to accumulate androgen in the nucleus despite the fact that androgen receptor binding in whole, intact cells is measurable and, under certain circumstances, in the normal range.

METHODS

Materials. Collagenase (Type I from *Clostridium histolyticum*), Tricine (*N*-Tris-[hydroxymethyl]methyl glycine), and heat denatured calf thymus DNA, were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (mycoplasma and virus screened) was obtained from Gibco Laboratories, Grand Island Biological Co. (Grand Island, NY). Tris (Tris[hydroxymethyl]aminomethane) was obtained from Bethesda Research Laboratories, Inc. (Rockville, MD). [1,2,4,5,6,7-^{3}H(*N*)]Dihydrotestosterone ([^{3}H]DHT),[1] sp act 123-139 Ci/mmol [17α-methyl-^{3}H]methyltrienolone ([^{3}H]R1881), sp act 87 Ci/mmol, [*methyl*-^{14}C]bovine serum albumin ([^{14}C]BSA), sp act 9.9 Ci/mg, and liquid scintillation fluid (Aquasol) were purchased from New England Nuclear (Boston, MA). Insulin (Iletin, U-100) was from Eli Lilly and Co. (Indianapolis, IN). Tissue culture flasks (75 cm^2 and 150 cm^2) were purchased from Costar, Data Packaging, Inc. (Cambridge, MA), Falcon Labware, Div. Becton, Dickinson Co. (Oxnard, CA) and Corning Glass Works (Corning, NY). Petri dishes, 60 mm in diam, were also from Falcon Labware. Nonradioactive methyltrienolone was a gift from Dr. J. P. Raynaud, Roussel UCLAF (Romainville, France). All tissue culture media, trypsin-EDTA solutions, and phosphate-buffered saline (PBS) were prepared and supplied by the National Institutes of Health Media Unit.

Cell culture. The clinical description of the patients whose fibroblasts were analyzed has been reported previously (14) and a more detailed presentation of their physical, histologic, and hormonal characteristics is in preparation. Briefly, 17- (D.W.) and 15- (W.W.) yr-old 46 XY siblings with phenotypic female features presented for evaluation of primary amenorrhea and decreased sexual hair (Tanner stage I-II pubic hair). Breast development was minimal (Tanner Stage II-III). The external genitalia showed a normal clitoris and no evidence of labioscrotal fusion, typical of patients with complete testicular feminization (CTF). When bilateral orchiectomy was performed, epididymes and vasa deferentia were noted in both patients, as is seen in patients with incomplete testicular feminization, but a uterus and fallopian tubes were absent. Before gonadectomy serum testosterone was 215 ng/dl (D.W.) and 256 ng/dl (W.W.).

Fibroblast strains were established in this laboratory from the patients and the normal male controls either from mons pubis punch biopsies (4-mm diam) or from genital skin specimens obtained at surgery. The specimens were finely minced in 60-mm petri dishes containing 4–5 ml of improved Eagle's minimal essential medium supplemented with 8% fetal calf serum; 10^{-7} M insulin; glutamine, 0.584 g/liter; collagenase, 2 mg/ml; penicillin, 100 U/ml; and streptomycin, 100 μg/ml; medium A. This medium was freshly prepared for each sample and filtered through a Swinnex-13 Millipore filter (Millipore Corp., Bedford, MA) immediately prior to use. After mincing the mixtures were transferred to 25-cm^2 tissue culture flasks and maintained at 37°C in the presence of 5% CO_2 in a humidified incubator. After 24 h, the medium was changed to a growth medium (medium B) consisting of medium A without collagenase. When the cells became confluent (usually within 1–3 wk) they were detached from the flasks with 0.05% trypsin-0.02% EDTA in saline at 37°C and passed serially into 75- and 150-cm^2 flasks. MCF-7 human breast cancer cells (provided by Dr. M. E. Lippman of the National Cancer Institute) were grown in a similar fashion. These cells have been shown to have androgen receptors with essentially the same binding properties as cultured human skin fibroblasts (15) and were used where indicated as controls.

Whole cell androgen binding assay. For routine assay, fibroblasts or MCF-7 cells were grown to confluence in four or five 150-cm^2 tissue culture flasks. With fibroblasts this usually required 4–6 wk from the time of the initial seeding of the cell line. 2 d before assay the medium was changed to medium B, which lacks fetal calf serum (medium C). This was repeated again 24 h before the assay. The remainder of the procedure was as previously described (15). The results were calculated with the aid of a computer (16). Binding capacity was expressed as sites/cell. In some experiments, [^{3}H]methyltrienolone was substituted for [^{3}H]DHT.

Nuclear binding of [^{3}H]DHT. The nuclear uptake of [^{3}H]DHT was examined using a modification of the method of Tsai and Samuels (17). Cells were prepared and incubated with [^{3}H]DHT as in the whole cell assay except that binding was standardly performed at 37°C for 45 min. After incubation the cells were collected by centrifugation, resuspended in 2 ml of PBS, and recentrifuged. The cell pellets were suspended in ice-cold 2 ml buffer A (0.25 M sucrose, 1.1 mM $MgCl_2$, 20 mM Tris, pH 7.85, and 0.5% Triton X-

[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; CTF, complete testicular feminization; DCC, dextran-coated charcoal; DHT, dihydrotestosterone; FIMP, familial incomplete male pseudohermaphroditism; PBS, phosphate-buffered saline; PRR, post-receptor resistance; R1881, methyltrienolone.

100) and then centrifuged at 1,000 *g* for 3 min to collect the nuclei. The crude nuclear pellet was resuspended in 2 ml of the same buffer and recentrifuged. Binding was assessed and calculated as for the whole cells, based on a cell count determined before the addition of buffer A.

Sucrose density gradient analysis. To prepare cytosol, cells that had been placed in medium C 24 h before were harvested with 0.01% Trypsin-0.02% EDTA in PBS, washed with PBS, suspended in buffer B (10 mM Tris-HCl, pH 7.4, 10 mM Na molybdate, 1.5 mM EDTA, 0.5 mM dithiothreitol), and homogenized with 40 strokes of a Dounce homogenizer (Kontes Co., Vineland, NJ). Cytosol was obtained by centrifugation of the homogenate at 100,000 *g* for 1 h at 4°C, and incubated with 5 nM [^{3}H]DHT for 3 h at 4°C. Nonspecific binding was assessed by the addition of 10^{-6} M R1881. Pellets of dextran-coated charcoal (DCC) were obtained by centrifugation of 0.5 ml of DCC suspension (0.25 g/100 ml Norit A, 0.0025 g/100 ml dextran in 0.01 M Tris-HCl, pH 7.4) at 1,000 *g* for 10 min; then 0.5 ml of the incubation mixtures were added, vortexed, and allowed to stand 10 min at 4°C to remove unbound [^{3}H]DHT. Following centrifugation for 10 min at 1,000 *g* at 4°C, 0.35 ml of the supernatant were collected from the bottom of the gradient and the radioactivity determined after the addition of 0.5 ml H_2O and 5 ml Aquasol. [^{14}C]BSA was added to each gradient as an internal marker. Protein was measured by the method of Lowry et al. (18).

Nuclear extracts of cells incubated with 5 mM [^{3}H]DHT in the presence or absence of 10^{-6} M R1881 for 45 min at 37°C were also prepared and analyzed by sucrose gradients. Fibroblasts were harvested, dispersed in media, incubated intact as above for estimation of nuclear androgen binding, and were washed twice with PBS. After centrifugation the cells were suspended in buffer B and homogenized. A crude pellet, obtained by centrifugation of the homogenate at 2,000 *g* for 10 min at 4°C, was then resuspended in 0.5 ml buffer B supplemented with 0.3 M KCl and homogenized over 60 min at 4°C. This mixture was added to a DCC pellet and subjected to centrifugation as above. 0.35 ml of the resulting supernatant and [^{14}C]BSA were applied to 5–20% sucrose gradients in buffer B and 0.3 M KCl and centrifuged for 18 h at 0°C, at 250,000 *g*. Fractions were collected and counted for radioactivity as described above.

RESULTS

Whole cell and nuclear androgen uptake in intact cells. The uptake of [^{3}H]DHT into dispersed, intact fibroblasts at 22°C has been shown to be a high affinity, saturable process that reaches equilibrium by ~1 h (15). As is shown in Fig. 1, equilibrium for specific uptake of [^{3}H]DHT in crude nuclear pellets prepared from dispersed, cultured skin fibroblasts is achieved between 30 and 60 min of incubation at 37°C. This agrees well with the results of previously reported studies (11, 19). The results of whole cell and nuclear uptake studies with [^{3}H]DHT using one of these patients' pubic skin fibroblasts (D.W.) and fibroblasts from a normal male are shown in Fig. 2. The data in panel A demonstrate that both whole cell and nuclear uptake of [^{3}H]DHT into normal cells are saturable processes. Scatchard analysis of these data, shown in Fig. 2 panel B, shows the binding to be to

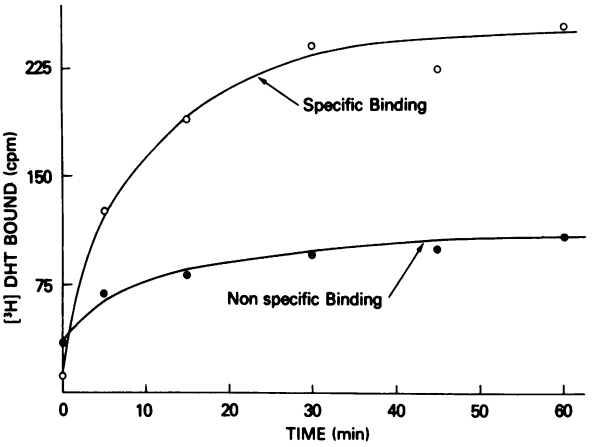

FIGURE 1 Time course of [^{3}H]DHT nuclear binding in dispersed human foreskin fibroblasts. Fibroblasts were grown to confluence, exposed to serum-free medium for 48 h, and dispersed with trypsin in the standard manner. Cells (350,000/tube) were incubated at 37°C for varying times with 1.5×10^{-9} M [^{3}H]DHT in the presence or absence of 10^{-6} M unlabeled methyltrienolone. After incubation the cells were collected by centrifugation and washed twice with PBS. Nuclear uptake was determined by exposing the washed cells to an isotonic buffer containing 0.5% Triton-X, centrifugation to collect the crude nuclear pellets, resuspension in PBS, and ethanol extraction. Specific binding is plotted in open circles; nonspecific binding, closed circles.

a single class of sites with high affinity in both the whole cells and extracted nuclei. In contrast to the results with fibroblasts from a normal male, panel C of Fig. 2 shows whole cell binding of [^{3}H]DHT with the older sibling's fibroblasts to be normal, while no high affinity, saturable binding is apparent in the nuclei obtained from these fibroblasts. Scatchard analyses of these data, shown in Fig. 2 panel D, indicate a single class of receptors for the intact cells similar to the representative normal, but no evidence of a single class of nuclear sites. Fig. 3 summarizes the whole cell and nuclear uptake data from both patients compared to similar data obtained with fibroblasts from 26 normal males in the left-hand column and from five patients with CTF in the right-hand column. The patients' whole cell [^{3}H]DHT uptake at 22°C was indistinguishable from the normal males (i.e., in the normal range), whereas the nuclear uptake of [^{3}H]DHT in the patients' fibroblasts at 37°C was undetectable, similar to that for the cells from patients with CTF in whom no saturable [^{3}H]DHT binding was demonstrable in either whole cells or nuclei.

As can be seen in Table I, substituting the nonmetabolizeable synthetic androgen [^{3}H]R1881 for [^{3}H]DHT or increasing the temperature from 22° to 37°C did not significantly alter the whole cell androgen uptake in the patients' fibroblasts (see experiments 1, 3, 4, and

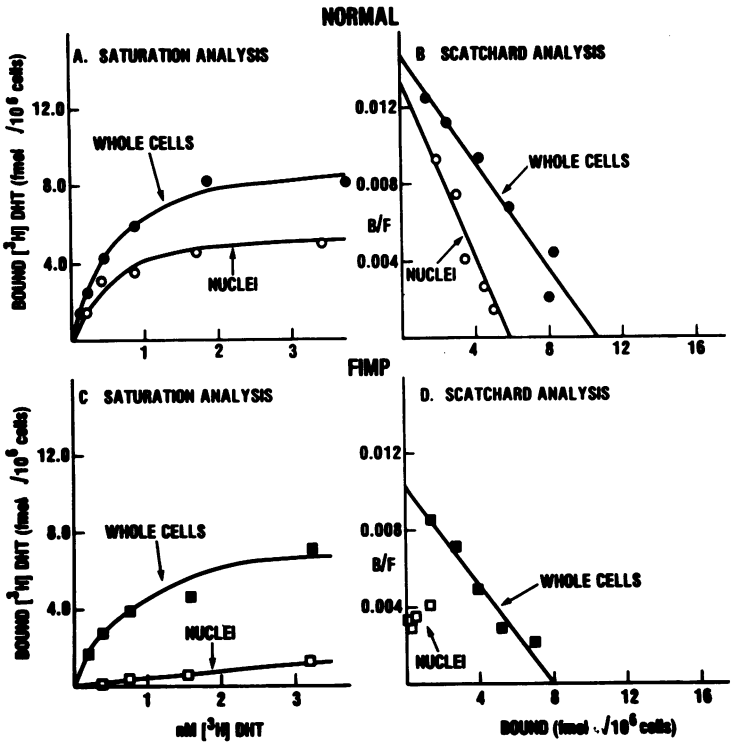

FIGURE 2 Whole cell and nuclear uptake of [^{3}H]DHT in dispersed human skin fibroblasts. Pubic skin fibroblasts from a normal male (A) and from patient D.W. (C) were grown and assayed for whole cell uptake of [^{3}H]DHT by incubation for 60 min at 22°C and for nuclear uptake of [^{3}H]DHT by incubation for 45 min at 37°C as described in Fig. 1. Specific binding of [^{3}H]DHT is plotted as a function of DHT concentration. Scatchard analysis of these data are plotted in panels B and D. The data are normalized to cell number that was determined in a hemocytometer. Circles refer to normal fibroblasts, squares refer to patient D.W. Filled in symbols designate whole cell uptake; open symbols designate nuclear uptake.

6). However, it should be noted that compared with control cells in experiments 3, 4, and 6, the amount of whole cell [^{3}H]DHT binding in the patient's cells was decreased. During the course of the incubation of fibroblasts with [^{3}H]DHT there was no significant metabolism of the ligand at either temperature (8, 15). On the other hand, decreasing the temperature of the incubation with [^{3}H]DHT to 22°C, while leading to slightly lower nuclear uptake in normal fibroblasts and MCF-7 human breast cancer cells, did not result in detectable uptake of [^{3}H]DHT into the nuclei of one of the patients' fibroblasts. The defect in the nuclear uptake of [^{3}H]DHT in these patients' cells was not altered by lowering the incubation temperature. As mentioned above, there is a suggestion of decreased binding in the patient's cells relative to the MCF-7 controls both at 22 and 37°C. However, as shown in Table II, which provides data on the variability of the androgen binding by the MCF-7 cells, the precision of the assay is such that it is difficult to make any statement about quantitative changes in binding at one temperature vs. the other due to the interassay variability.

Sucrose gradient analysis of cellular extracts. When cytosol was prepared from one of the patient's fibroblasts in a sodium molybdate-containing buffer, incubated with [^{3}H]DHT at 4°C and subjected to centrifugation on a sucrose gradient, a peak of radioactivity is demonstrable in the 8S region that is abolished in the presence of excess unlabeled methyltrienolone (Fig. 4). However, when compared with cytosol from a normal fibroblast strain (normalized for protein content), this peak was quantitatively markedly reduced, ~25% the area of the normal control.

As a means of verifying the results of the nuclear uptake studies with dispersed, intact cells, sucrose gradient analysis was also performed on 0.3 M KCl extracts of nuclei prepared from dispersed cells that had been incubated with [^{3}H]DHT at 37°C (Fig. 5). In contrast to an easily identifiable peak of radioactivity in the 4S region of the gradient for the nuclear extract of the normal cells that was eliminated by coincubation

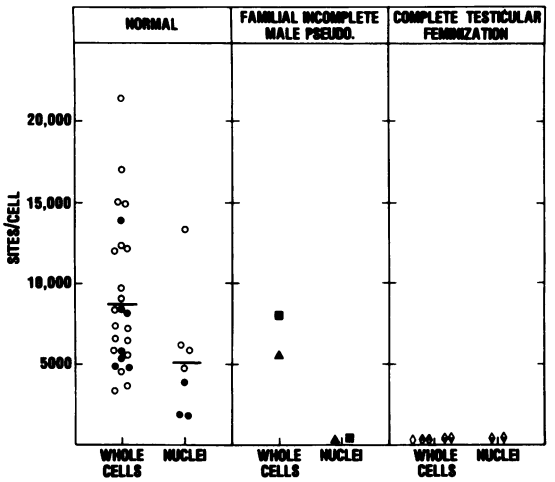

FIGURE 3 Scattergram of binding capacities for whole cell and nuclear binding of [^{3}H]DHT in dispersed human fibroblasts from 26 normal males, 2 siblings with familial incomplete male pseudohermaphroditism, and 5 patients with CTF. Fibroblasts were grown and assayed for [^{3}H]DHT uptake into whole cells and nuclei as described in Fig. 2. The binding capacity was calculated by Scatchard analysis. Each point represents an individual determination (26 normal strains) or the average of two determinations in the seven androgen resistance strains. Circles designate normal males; square (patient D.W.), triangle (patient W.W.) and diamonds (◇, ◆, ◆) five patients from three kindreds with CTF. Open circles designate genital skin strains and closed symbols represent pubic skin strains.

of the cells with excess unlabeled methyltrienolone (panel A), there was no peak of ^{3}H in the 4S region for the nuclear extracts prepared from either patient's fibroblasts (panel B). Although other workers have indicated that 0.3 M KCl may not completely extract out all the radioactivity incorporated into the nucleus (20, 21) it is unlikely that using a higher salt containing solution to extract the nuclei would have significantly altered the results of the gradient analysis, since the amount of specific total radioactivity incorporated into the nuclei of the patients' cells is so low (Fig. 1 and Table I).

DISCUSSION

The biologic action of the steroid hormones (and the calciferols) is generally agreed to be initiated at the target tissues by intracellular receptor proteins in the cytosol, which bind the active form of the hormone, are "activated" to a form that can enter the nucleus, and then transport the ligand to the nucleus (22). Once inside the nucleus, the hormone-receptor complex is thought to bind to critical "acceptor" sites on the chromatin, following which specific gene transcription and messenger RNA translation result in new protein synthesis and the ultimate hormonal response.

Although there is an abundance of biochemical data to support this overall scheme for all the steroids and 1,25-dihydroxycholecalciferol, studies from genetic errors or tissue culture-induced mutations have been few and thus far have supported the validity of this model for the mechanism of action for the glucocorticoids (23–25), androgens (13), and more recently with estrogens (26) and calciferols (27, 28). The data presented in this paper demonstrate, that fibroblasts cultured from two siblings with male pseudohermaphroditism are unable to accumulate [^{3}H]DHT within their nuclei despite the presence of intracellular androgen receptors. The characteristics of the nuclear uptake assay used in this study, which shows ~40–50% of the binding sites found in the intact, whole cell, are in accord with published data (11, 19). Also, the whole cell assay gives androgen binding characteristics for fibroblasts (15) and the MCF-7 cells that are also in good agreement with the results of others (21). Moreover, the results of the sucrose gradient analysis on nuclear extracts shown in Fig. 5 clearly corroborate the validity of the results of the nuclear uptake assay with intact, dispersed cells.

Prior to this report, studies with cultured fibroblasts have revealed androgen resistance variants that are receptor-negative, receptor-deficient, and receptor-defective (receptors that are thermolabile or are not stabilized by sodium molybdate) as well as receptor positive (4–13, 24–31). Others have examined nuclear [^{3}H]DHT uptake in fibroblasts from several patients with androgen resistance whose fibroblast whole cell [^{3}H]DHT uptake was apparently normal (10, 11, 20, 30). In no case was the nuclear uptake of [^{3}H]DHT absent. Such patients, on the basis of whole cell and nuclear [^{3}H]DHT binding studies, would, therefore, be classified as having postreceptor resistance (PRR). Interestingly, Brown and his colleagues (32) have recently shown that one family, earlier found to have receptor-positive androgen resistance (10), have qualitatively abnormal androgen receptors as judged by thermolability and rapid hormone dissociation (32). These receptors were capable of conversion to a 3-4S form by exposure to high salt, indicating that a qualitative receptor abnormality does not necessarily preclude "activation" and nuclear binding. While the current studies were in progress, Medina et al. (33) have demonstrated decreased, but not absent, nuclear uptake of [^{3}H]DHT in fibroblasts from a single patient with incomplete testicular feminization also presumably due to a receptor defect. From mixing experiments with cytosol and nuclear extracts from normal fibroblasts these workers concluded that the defect was due to diminished cytosol DHT binding that was then responsible for decreased cytosol-DHT receptor complex migration into the nucleus and subsequent di-

TABLE I

Effect of Temperature and Ligand on Whole Cell and Nuclear Androgen Binding

Experiment	Cells	Incubation conditions		Androgen binding parameters			
				Whole cell uptake		Nuclear uptake	
		Temperature	Ligand	R_0	K_d	R_0	K_d
				sites/cell	$\times 10^{-9}$ *M*	*sites/cell*	$\times 10^{-9}$ *M*
1	D.W.	22°C	[^{3}H]DHT	6,250	0.82		
	W.W.	22°C	[^{3}H]DHT	6,860	1.20		
2	D.W.	37°C	[^{3}H]DHT			—*	—*
	W.W.	37°C	[^{3}H]DHT			—*	—*
	MCF-7	37°C	[^{3}H]DHT			4,280	0.36
3	Normal genital skin	22°C	[^{3}H]R1881	14,700	0.22		
	D.W.	22°C	[^{3}H]R1881	4,370	0.61		
	W.W.	22°C	[^{3}H]R1881	9,050	1.3		
4	D.W.	22°C	[^{3}H]DHT	4,800	0.78		
	MCF-7	22°C	[^{3}H]DHT	12,300	0.52	2,830	0.65
5	Normal genital skin	22°C	[^{3}H]DHT			1,700	1.7
	D.W.	22°C	[^{3}H]DHT			—*	—*
6	D.W.	37°C	[^{3}H]DHT	5,350	1.4		
	MCF-7	37°C	[^{3}H]DHT	9,800	0.27	2,890	0.39

* No detectable saturable binding. Fibroblasts or MCF-7 human breast cancer cells were grown to confluence, exposed to serum-free medium for 48 h, dispersed with trypsin, washed, and resuspended in medium. Cells were incubated in test tubes in 1 ml of medium with increasing concentrations of the ligand indicated for 60 min to determine whole cell uptake or for 45 min to determine nuclear uptake at the temperature indicated. Nonspecific binding was determined in the presence of 10^{-6} M R1881. After incubation the cells were collected by centrifugation, washed with PBS, resuspended and exposed to ethanol before scintillation counting. Nuclear uptake was determined following the incubation by further exposing the washed cells to an isotonic buffer containing 0.5% Triton-X, centrifugation to collect the crude nuclear pellets, resuspension in PBS, and ethanol extraction. The binding parameters were calculated by Scatchard analysis. The data are normalized to cell number determined in a hemocytometer.

minished DHT-receptor complex nuclear localization. They did not distinguish between a quantitative or qualitative defect in the cytosol as the cause for the diminished DHT-receptor binding.

TABLE II

Androgen Binding Parameters in the MCF-7 Human Breast Cancer Cells

Assay	n	Temperature	Binding capacity	K_d
			sites/cell	$\times 10^{-9}$ *M*
Whole cell	8	22°C	13,100±4,800	0.68±0.36*
	5	37°C	10,300±4,900	0.26±0.02
Nuclear uptake	4	22°C	2,360±550	0.87±0.22
	7	37°C	3,160±1,300	0.38±0.12

* Mean±SD.

Whole cell and nuclear uptake of [^{3}H]DHT in MCF-7 breast cancer cells were determined at the temperatures indicated as described in Table I.

The inclusion of certain patients with androgen insensitivity in the PRR category on the basis of fibroblast studies was until recently presumptive and by exclusion because at the time these observations were made there was no technique other than hormone binding with which to examine these patients' cells. Specifically, in patients with PRR, the demonstration of absence of an androgen-induced biological response until a short time ago had been lacking since androgen-dependent effects in cultured fibroblasts had not been reported. Fortunately, Ozasa et al. (34) have shown that DHT, albeit at high doses, induces synthesis of protein and collagen but not DNA in normal fibroblasts. This effect was not present in fibroblasts from a patient with CTF in which there were only 10% the normal number of androgen receptors. Moreover, Kaufman et al. (35) have recently reported that fibroblasts from patients with PRR fail to "up-regulate" their androgen receptors following exposure to androgens as do normally responsive cells. These observa-

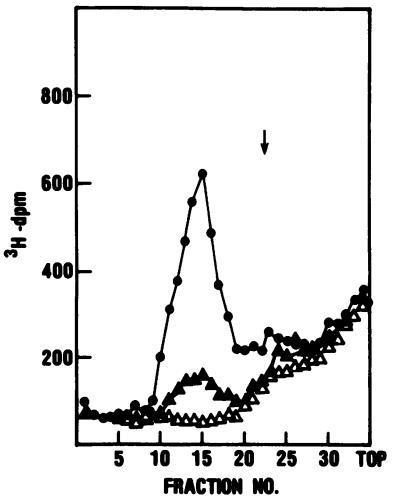

FIGURE 4 Sucrose gradient analysis of cytosol from normal male fibroblasts and from a patient with familial incomplete male pseudohermaphroditism, type I. Fibroblasts were grown to confluence, exposed to serum-free medium for 24 h, harvested, and homogenized in a low salt buffer containing 10 mM sodium molybdate (buffer B). The supernate obtained after 1 h centrifugation at 100,000 *g* was incubated with 5 nM [^{3}H]DHT for 3 h at 4°C in the presence or absence of 10^{-6} M R1881. After removal of unbound steroid with DCC, the cytosol was layered on a 5–20% sucrose gradient in buffer B and centrifuged for 18 h at 250,000 *g* at 0°C. Six drop fractions were assayed for radioactivity. The arrow indicates the position of [^{14}C]BSA that was added as an internal marker. The data have been normalized to reflect 1 mg of cytosol protein layered on the gradient. ●, normal fibroblast cytosol; ▲, W.W. fibroblast cytosol; △, W.W. fibroblast cytosol incubated in the presence of 10^{-6} M R1881.

tions may provide ways in vitro for verifying PRR in other patients with androgen insensitivity.

The results of the studies reported here indicate that familial disorders of androgen action, like some mutant cell lines with disorders of glucocorticoid action, appear to have defective nuclear transfer as a means of hormone insensitivity (23–25). The question remains, however, whether the inability of nuclear hormone uptake in these patients' fibroblasts is due to: (*a*) a qualitative abnormality in the cytosol receptor that has diminished affinity for chromatin or; (*b*) a defect in receptor activation analogous to the form of glucocorticoid resistance described by Schmidt et al. (25). A defect in the nuclear "acceptors" appears unlikely.

Another question remains as to why there is some evidence of androgen sensitivity in the patients; to wit, the presence of epididymes and vas deferentia and the partially suppressed breast development. One is forced to conclude either that the defective nuclear androgen uptake is specific for only certain tissues such as the skin and the external genitalia or is incomplete throughout the body with certain target tissues being more affected than others. In this regard, it is interesting to note that microscopic epididymides have been described in the *Tfm* mouse (36). The current techniques used here are relatively crude and would not preclude a small percentage of hormone-receptor complexes from binding to specific sites on the gene and triggering a partial virilizing response. In support of such a possibility Wieland and Fox have shown more than one kind of androgen receptor in *Tfm* mice using DNA-cellulose chromatography and isoelectric focusing (37, 38). In light of the unusual phenotype of these two patients (i.e., virilization of the Wolffian structures but no clitoromegaly or labioscrotal fusion), it is possible that these features constitute a unique phenotype associated with impaired nuclear androgen retention. Application of the methodology used in this study to evaluate fibroblasts from other androgen-resistant patients who have thermolabile receptors or those that fail molybdate stabilization will help resolve this issue.

It is worth noting again that fibroblasts from these same patients have been analyzed previously (8, 9),

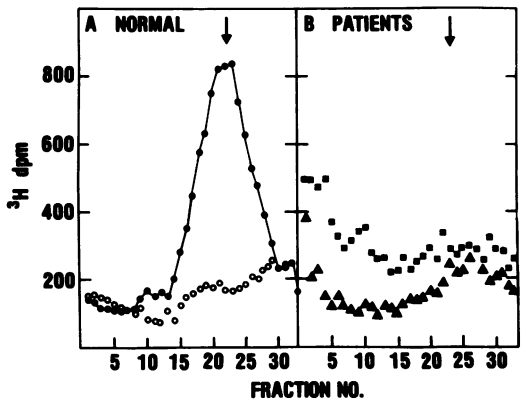

FIGURE 5 Sucrose gradient analysis of nuclear extracts from normal male and familial incomplete male pseudohermaphroditism, type I fibroblasts incubated with [^{3}H]DHT. Skin fibroblasts were grown and prepared for nuclear uptake of [^{3}H]DHT as described in Fig. 1. After incubation of the dispersed fibroblasts with 5 nM [^{3}H]DHT for 45 min at 37°C the cells were collected by centrifugation, washed twice with PBS, and homogenized in buffer B. The crude pellet, obtained by centrifugation at 2,000 *g*, was homogenized in buffer B supplemented with 0.3 M KCl. Following the addition of DCC and low-speed centrifugation, the supernate was layered on a 5–20% sucrose gradient in buffer B supplemented with 0.3 M KCl, centrifuged, and assayed for radioactivity as described in Fig. 4. The arrow indicates the position of [^{14}C]BSA which was added as an internal marker. The data have been normalized to reflect 1 mg of protein extracted from the fibroblast nuclei layered per gradient. A: ●, normal fibroblast nuclear extracts; O, normal fibroblast nuclear extracts from cells incubated with 10^{-6} M R1881. B: ■, D.W. fibroblast nuclear extracts; ▲, W.W. fibroblast nuclear extracts.

strain Nos. 93 and 94 in reference 8. Griffin has found that the androgen binding in these cells is labile at 42°C, but less so at 37°C, where the binding is diminished but detectable. Moreover, when the temperature was further lowered to 26°C, the whole cell binding appeared to be within the normal range as is reported here. Griffin and Durrant have further reported that fibroblasts from some patients with thermolabile receptors, including those reported here, also have cytosol androgen receptors that cannot be stabilized by sodium molybdate to form an 8S binding species on sucrose density gradient centrifugation (9). Although quantitatively much reduced compared with the normal control included in the same experiment (25%), Fig. 4 shows the presence of an 8S peak in the cytosol of the younger sibling's fibroblasts. Sucrose gradient analysis of cytosol from fibroblasts of the patient described by Medina et al. also showed a reduction in cytosol [^{3}H]DHT binding (i.e., 35–40%) vs. normal. The thermolability of the androgen receptor in the intact cell incubations at 42°C may be indicative of a receptor protein with altered stability so that after 3-h incubation and prolonged centrifugation at 0°C, the 8S binding species is markedly reduced and difficult to detect. Whether or not sucrose gradient analysis of cytosol from such patients' cells is helpful, failure of nuclear androgen uptake may be more physiologically pertinent and may explain the defective androgen action in these patients.

These data provide an example for the androgen resistance syndromes of defective nuclear hormone uptake analogous to several insensitivity states already described for glucocorticoids and calciferols (23–25, 27, 28). Currently, it is not clear whether the patient described by Medina et al. and the patients reported on here share the same molecular defect in androgen sensitivity. Although more rigorous studies will be required to ascertain the nature of the abnormality in the androgen receptor of patients with androgen insensitivity due to decreased nuclear androgen retention, assessment of fibroblast nuclear [^{3}H]DHT uptake may complement information obtained from studies of thermolability or sodium molybdate stabilization in identifying the nature of the androgen resistance and in assessing the functional integrity of the androgen receptor system.

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