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ANDROGEN RESISTANCE DUE TO DECREASED AMOUNTS OF ANDROGEN RECEPTOR: A REINVESTIGATION

PLACIDO B. GRINO, JAMES E. GRIFFIN and JEAN D. WILSON*
Department of Internal Medicine, The University of Texas Southwestern Medical Center, Dallas, TX 75235-8857, U.S.A.

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Summary—To provide insight into the pathogenesis of the androgen resistance in a previously described family with X-linked Reifenstein syndrome, we have systematically assessed a variety of parameters of androgen receptor function in fibroblasts cultured from scrotal skin biopsies. We assessed the amount of high affinity binding, the affinity of ligand binding to the receptor, the upregulation of androgen receptor levels by androgen, the stability of ligand binding in intact fibroblasts at high temperature, the dissociation of ligand from the receptor, the intranuclear localization and salt elution profiles of ligand–receptor complexes, and the ultracentrifugation characteristics of the ligand–receptor complexes. Since the only parameter found to be abnormal is a decreased amount of receptor, we conclude that the underlying mutation in this family influences the amount rather than the structure of the androgen receptor protein.

INTRODUCTION

Mutations of the androgen receptor cause resistance to the action of androgenic hormones and produce a spectrum of abnormalities in affected males, varying from 46,XY phenotypic women with the syndrome of complete testicular feminization to men with minor degrees of undervirilization [1]. The variability in expression is a function of differences in the severity of impairment of receptor function. A variety of methods have been utilized to characterize the androgen receptors in subjects with androgen resistance, and most of them exhibit either an absence of receptor binding or a qualitative abnormality in the receptor [1]. Indeed, analyses of the mutations at the DNA level have now been completed in a few patients, and the abnormalities identified to date include major structural rearrangements and/or defects in the gene that encodes the receptor [2] or single nucleotide substitutions that either cause premature termination of mRNA transcription [3] or single amino acid substitutions in the protein [4].

A third category of mutations of the androgen receptor has also been described in which the only abnormality detected is a decrease in the amount of what appears to be an otherwise normal receptor [5, 6]. Most patients in this category fall midway in the spectrum of phenotypic abnormalities associated with androgen receptor mutations and are usually categorized under the designation of the Reifenstein syndrome, namely a male with perineoscrotal hypospadias, azoospermia, and gynecomastia [1]. This phenotype also occurs in men with qualitative abnormalities of the androgen receptor [7], but it is of interest that three of the original families with the Reifenstein syndrome—namely the Australian family originally described by Ford [8] and subsequently by Walker *et al.* [9], the family described by Reifenstein [10, 11], and the Dallas family [11, 12] have been reported as having decreased amounts of what appears to be an otherwise normal receptor [5, 6, 13, 14]. Another distinctive feature of these three families is that there is considerable phenotypic variability in that most affected men have the Reifenstein phenotype but some have either less severe manifestations (isolated male infertility in three families) or more severe manifestations (pseudovagina formation in some men in the Dallas and Australian families).

Several implications can be deduced from this formulation. One, since these patients on average have about half normal levels of receptor, the amount of androgen receptor must be rate-limiting in virilization, e.g. the number of spare receptors is small or nonexistent. Two, since the mutation in the Dallas Reifenstein family appears to be allelic to those that cause absence of receptor binding [15], the mutation must influence either the rate of transcription or the stability of the messenger RNA that encodes the receptor rather than the structure of the receptor

*Correspondence should be addressed to: Dr Jean D. Wilson, Department of Internal Medicine, UT Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-8857, U.S.A. [*Tel.* (214) 688-3494].

molecule. In other words, the search for the molecular defect should focus on portions of the gene outside the coding sequence.

However, since the original category of decreased amount of normal binding was proposed, additional means have been described for identifying subtle mutations that cause qualitative abnormalities in receptor function, including receptor thermolability in monolayers [16], instability of cytosol receptor to density gradient centrifugation [17], defective upregulation of receptor levels [18], accelerated dissociation of the ligand from the receptor [19], lability of receptor under transforming conditions [20], and abnormal localization or anchorage of the androgen–receptor complex in the nuclei of cells [21]. We therefore decided to reinvestigate the question as to whether the residual receptor is qualitatively normal in the Dallas Reifenstein family by systematically examining all functional tests of receptor action.

EXPERIMENTAL

Materials

Nonradioactive dihydrotestosterone was from Steraloids (Wilton, N.H.). [1,2,4,5,6,7-^{3}H]dihydrotestosterone (180 Ci/mmol), [17α-methyl-^{3}H]mibolerone (85 Ci/mmol) and nonradioactive mibolerone were from Amersham (Arlington Heights, Ill.). Phenylmethylsulfonilfluoride (PMSF) and cycloheximide were from Sigma (St Louis, Mo.). The scintillation mixture was Pico-fluor 15 from Packard Instrument Co. Inc. (Downers Groves, Ill.). Thin-layer chromatography plates were from Whatman Ltd, Maidstone, Kent, England.

The following buffers were used: Tris–saline (50 mM Tris–HCl and 150 mM NaCl, pH 7.4), TMCM (10 mM Tris–HCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mM 2-mercaptoethanol, pH 7.4), Tris–sucrose (TMCM plus 0.25 M sucrose, pH 7.4), and TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.4). The pH of the buffers was adjusted at 23°C, and the buffers were stored at 4°C.

Methods

Cell culture

Fibroblasts were grown as described previously [5] from explants of genital skin obtained from punch biopsies of the scrotum under local anesthesia and were used before the 30th doubling. After monolayer cultures (15 cm dishes or 6 cm well plates) became confluent (usually day 7 after plating), the standard medium was changed to medium with no added serum, and the cells were used one day later unless otherwise indicated. The cell strains used in these experiments were derived from foreskin or scrotum. The cell strains included a control previously used in this laboratory (strain 704) and two strains derived from two previously unstudied patients, subjects 1 and 2 (strains 497 and 773 respectively) from the Dallas Reifenstein family (Fig. 1). Subject 1 was noted to have perineoscrotal hypospadias, an incompletely developed scrotum and bilaterally descended testes at the time of birth on 5/12/81. He underwent a two stage surgical repair of the hypospadias in 1984 and 1985. Subject 2 was born 5/28/85 with microphallus, perineoscrotal hypospadias, a chordee, and bilaterally descended testes, and the hypospadias was repaired in 1988 with a single operation.

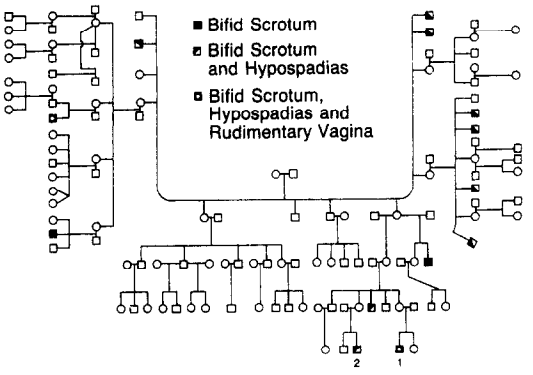

Fig. 1. Pedigree of the Dallas Reifenstein Family. Subjects 1 and 2 are the first affected members of the fourth generation of this family.

Monolayer binding assays

For saturation analysis, monolayers of fibroblasts (6 cm diameter wells) were incubated in duplicate at 37°C for 45 min with media containing various concentrations (0.05–1.2 nM) of [^{3}H]dihydrotestosterone. The cells were rinsed, harvested with trypsin–EDTA and homogenized by sonication after resuspension in water. Aliquots were taken for measurement of radioactivity and protein. Total dihydrotestosterone binding was plotted as a function of steroid concentration, and the maximal binding capacity (B_{max}), or the amount of high affinity binding, was calculated by extrapolating the plateau portion of the binding curve to the ordinate [6].

Upregulation of androgen receptor levels in fibroblast monolayers

Monolayers of genital skin fibroblasts were grown under standard conditions. On the day of the experiment the medium was removed, and 4 ml fresh medium containing 1 nM [^{3}H]dihydrotestosterone or [^{3}H]mibolerone were added to duplicate wells [22]. Parallel incubations in the presence of a 500-fold excess of nonradioactive ligands were used to estimate nonspecific binding. When mibolerone was the ligand, a 500-fold excess of nonradioactive triamcinolone acetonide was also added to all plates. Half of the plates were rinsed, the cells were harvested, and specific binding was determined at the end of 1 h at 37°C. The remaining plates were incubated for an additional 23 h at 37°C. Fresh medium containing 1 nM [^{3}H]dihydrotestosterone with or without nonradioactive dihydrotestosterone was

added to the corresponding plates at the end of the 23rd h after removing the old medium, and 1 h later the monolayers were rinsed, harvested, and assessed for specific binding as described above.

Effect of temperature on fibroblast monolayer binding

Two protocols were utilized. The first involved a standard 45 min incubation at 37 and 42°C as described [16, 17]. A more prolonged incubation was also utilized in which monolayers of genital skin fibroblasts were grown under standard conditions. On the day of the experiment, the medium was removed, 2 ml fresh medium containing 1 nM [^{3}H]-mibolerone were added, and the cells were incubated at 37°C for 1 h. One plate from each cell strain was harvested by trypsinization, and the remaining plates were transferred to an incubator at 42°C. At the indicated times, plates were harvested as above, the cells were sonicated in water, and aliquots were taken for protein and radioactivity. Parallel incubations in the presence of a 500-fold excess of nonradioactive mibolerone were carried out to measure nonspecific binding. A 500-fold excess of nonradioactive triamcinolone acetonide was added to all plates. Cycloheximide, 500 μM, was added to all plates to prevent the confounding effects of upregulation. Specific binding was determined by subtracting nonspecific binding from total binding. Results were plotted semilogarithmically as the percent of specific baseline binding remaining versus time at 42°C.

Dissociation of androgen–receptor complexes in monolayers

To determine the rate of dissociation of androgen–receptor complexes [22], 0.5 μM nonradioactive mibolerone was added to monolayers of cells that had been incubated with 1 nM [^{3}H]mibolerone for 1 h at 37°C. The percentage of baseline androgen receptor specific binding remaining was measured at various time intervals (up to 6 h), and the results were plotted semilogarithmically. The stability of the androgen–receptor complexes was monitored by parallel incubations without added nonradioactive mibolerone. To avoid the confusing effects of upregulation of androgen receptor levels, 500 μM cycloheximide was included in the incubation medium in all wells.

Nuclear localization and salt extraction of nuclear receptor

Nuclear localization of dihydrotestosterone was assessed by a modification of the method of Eil [21]. Cells were grown under standard conditions. On the day of the experiment, the cells were incubated with 1.2 nM [^{3}H]dihydrotestosterone for 1 h at 37°C in 15 cm dishes. The cells were rinsed, harvested and homogenized in TMCM buffer by repeated aspiration with a plastic syringe through a 25-gauge needle (an average of 10 times) until cells were disrupted, as assessed by microscopic examination. The homogenates were centrifuged at 800 *g* for 15 min at 4°C, and the supernatant was aspirated carefully and discarded. The nuclear pellet was further purified by sequential resuspension and centrifugation 3 times in Tris–sucrose buffer, with 0.5% Triton X-100 added to the second washing only. The nuclear pellets were resuspended in Tris–sucrose buffer, and 0.5 ml aliquots were mixed with increasing volumes of 4 M NaCl in 20 mM Tris, pH 7.4, to give the following final NaCl concentrations: 0, 25, 50, 75, 100, 200, and 300 mM. The largest volume of 4 M NaCl–Tris buffer added was 37.5 μl, corresponding to the 300 mM NaCl final concentration. The nuclear suspensions were incubated at 0°C for 30 min with frequent gentle vortexing and then centrifuged at 2000 *g* for 10 min at 4°C. Aliquots of the supernatants were transferred to counting vials and assayed for radioactivity. The results were plotted semilogarithmically as the percent of nuclear binding remaining versus the NaCl concentration, compared to the total binding present in 0.5 ml of the original nuclear suspension. DNA was measured by the Burton method [23].

Studies in cytosol preparations

For cytosol experiments fibroblast monolayers in 15 cm dishes were harvested by scraping in 4 ml ice-cold Tris–saline. The cells were pelleted by centrifugation at 800 *g* for 4 min at 4°C and washed twice in Tris–saline and once in TEGM-10. The supernatant was removed, and the cells were suspended in 1–2 equal volumes of TEGM-10. PMSF (1 mM) was added to the homogenization buffer from a stock solution (100 mM in 1-propanol). The cells were homogenized at 0°C by repeated aspiration with a plastic syringe through a 25-gauge needle (an average of 10 times) until they were disrupted, as assessed by microscopic examination. Homogenates were centrifuged at 250,000 *g* for 30 min at 4°C. The supernatant from this centrifugation contained 2–6 mg protein/ml and is termed the cytosol fraction. Protein was measured by the method of Lowry and coworkers [24] using bovine serum albumin as standard. Cytosol was incubated with 3 nM [^{3}H]dihydrotestosterone for 3 h at 0°C with or without 1.5 μM nonradioactive dihydrotestosterone to assess nonspecific binding. To remove unbound hormone, appropriate aliquots were treated with a suspension of dextran-coated charcoal (50 mg charcoal and 5 mg dextran T-70 per ml) equivalent to 25% (v/v) of the aliquot volume. The tubes were stirred, incubated for 5 min at 0°C, and centrifuged for 10 min at 2000 *g*. Aliquots of the supernatants were assayed for radioactivity or used for sucrose density gradient centrifugation.

For sucrose density gradient centrifugation 0.4 ml aliquots of labelled cytosol obtained as described above were layered on top of 5–20% sucrose density gradients in TEGM-10 buffer. Centrifugation was carried out at 4°C in a vertical rotor (Beckman VTi 65) at 400,000 *g* for 2 h. [^{14}C]BSA (15,000 dpm) was included as an internal standard (4.6 S) in all tubes. The gradients were fractionated into 0.2 ml aliquots

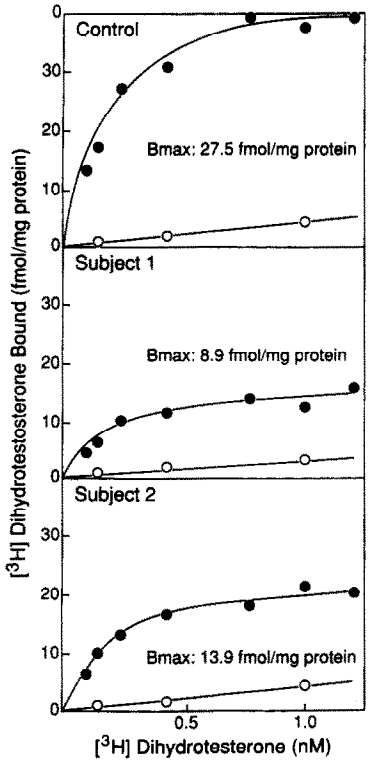

Fig. 2. Saturation analysis of androgen receptor binding in fibroblast monolayers from a normal strain (No. 704) and from the two subjects. Monolayers of genital skin fibroblasts were grown under standard conditions. On the day of the experiment the medium was replaced with fresh medium containing increasing concentrations of [^{3}H]dihydrotestosterone (0.05–1.2 nM) with or without a 500-fold excess of nonradioactive dihydrotestosterone. Cells were incubated at 37°C for 45 min, rinsed and harvested as described in Methods. Binding is plotted as a function of dihydrotestosterone concentration. The B_{max} was calculated by least squares linear regression from the points at higher concentrations of steroid after saturation. Half maximal saturation occurred at the following [^{3}H]dihydrotestosterone concentrations: control, 0.080 nM; subject 1, 0.080 nM; subject 2, 0.070 nM.

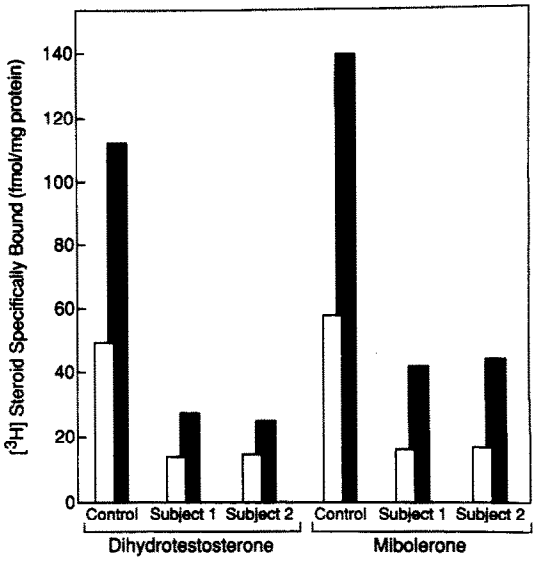

Fig. 3. Upregulation of androgen receptor levels in fibroblast monolayers from a control (No. 704) and from the two subjects. The cells were grown under standard conditions. On the day of the experiment the medium was removed, and 4 ml fresh medium containing 1 nM [^{3}H]dihydrotestosterone or [^{3}H]mibolerone were added. Parallel incubations in the presence of a 500-fold excess of nonradioactive ligands were used to estimate nonspecific binding. When mibolerone was the ligand a 500-fold excess of nonradioactive triamcinolone acetonide was also added to all plates. After 1 h at 37°C half of the plates were rinsed, and the cells were harvested and assayed. The rest of the plates were incubated for 23 h more at 37°C. Fresh medium containing 1 nM [^{3}H]dihydrotestosterone with or without nonradioactive dihydrotestosterone was added to the corresponding plates at the end of the 23rd h after removing the old medium. At the end of the 24th h the remaining plates were rinsed, harvested and assayed.

at the end of the centrifugation period on an ISCO gradient collector (Instrumentation Specialties Co., Lincoln, Neb.). 4 ml of scintillation counting fluid were added to each fraction, and the samples were assayed for ^{3}H and ^{14}C in a liquid scintillation spectrometer.

RESULTS

Characterization of the androgen receptor from the two subjects

Saturation analysis of [^{3}H]dihydrotestosterone binding was assessed in fibroblast monolayers from the two subjects and from a normal control (Fig. 2). Whereas the concentration of dihydrotestosterone that caused half-maximal saturation in the cells from the subjects was within the normal range and similar to the control (normal half-maximal saturation, <0.3 nM), cells from subjects 1 and 2 exhibited a decreased amount of androgen receptor binding under these conditions (8.9 and 13.9 fmol/mg protein, respectively) (B_{max} normal range, 15–30 fmol/mg protein).

Upregulation of androgen receptor levels

The effects of prolonged incubation of fibroblasts monolayers with dihydrotestosterone and mibolerone on the amount of high affinity androgen receptor binding are illustrated in Fig. 3. In the control strain both ligands produced a 2-fold or greater increase in androgen receptor levels at the end of 24 h of incubation at 37°C, as compared to the binding observed at the end of 1 h. In the cells from the two subjects 2.5- and 1.7-fold upregulation in androgen receptor levels were observed with mibolerone and dihydrotestosterone respectively, responses that are within the normal range reported by Evans and Hughes[25] and within the normal range for this laboratory as well [26].

Effect of elevation of temperature on the amount of high affinity binding in fibroblast monolayers

Raising the temperature of incubation from 37 to 42°C caused no difference in the rate of loss

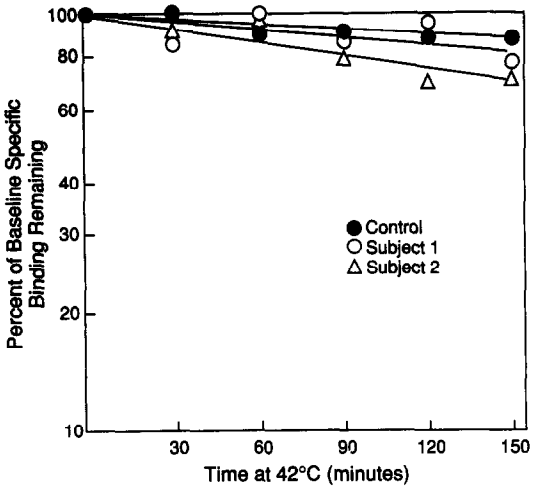

Fig. 4. Effect of temperature on androgen binding in fibroblast monolayers from a normal strain (No. 704) and from the two subjects. Monolayers of genital skin fibroblasts were grown under standard conditions. On the day of the experiment the medium was removed, 2 ml fresh medium containing 1 nM [^{3}H]mibolerone were added, and the cells were incubated at 37°C for 1 h. One plate from each cell strain was harvested by trypsinization and the remaining plates were transferred to an incubator at 42°C. At the indicated times, plates were harvested as above, the cells were homogenized in water and aliquots were taken for protein and radioactivity. A 500-fold excess of nonradioactive triamcinolone acetonide was added to all plates. Cycloheximide, 500 μM, was added to all plates to prevent the confounding effects of upregulation.

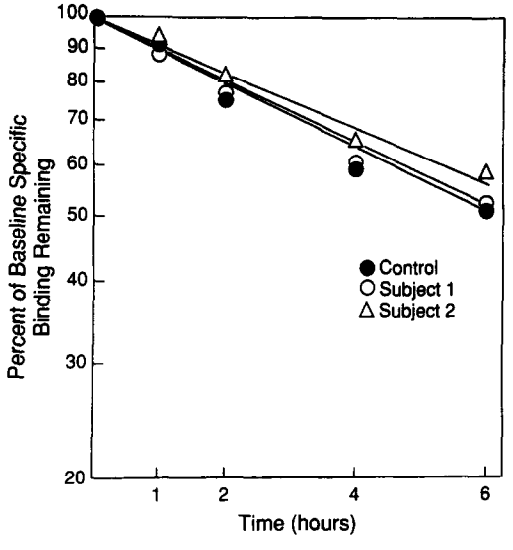

Fig. 5. Dissociation of androgen receptor complexes in fibroblast monolayers from a control strain (No. 704) and from the two subjects. Cells were grown under standard conditions. On the day of the experiment, cells were incubated at 37°C in the presence of 1 nM [^{3}H]mibolerone and 500 μM cycloheximide for 1 h, followed by a chase period of 6 h with 0.5 μM nonradioactive mibolerone. Triamcinolone acetonide, 0.5 μM, was also added to all plates. The percentage of baseline specific binding remaining was measured at the indicated times and the results were plotted semilogarithmically. The stability of the androgen–receptor complexes was monitored by parallel incubations without added nonradioactive mibolerone.

of specific androgen receptor binding in the two subjects as compared to the control either by our previous 45 min incubation protocol (results not shown) [16, 17] or when incubation was continued as long as 2.5 h at 42°C (Fig. 4).

Dissociation of androgen–receptor complexes in monolayers

Cells from the control and from the two subjects also displayed similar and linear rates of loss of androgen binding to the receptor following the addition of excess nonradioactive ligand, approximating 50% of baseline binding at 6 h after the onset of the chase period (Fig. 5).

Salt extraction of nuclear bound androgen receptor

Nuclei from cells that had been incubated with 1.2 nM [^{3}H]dihydrotestosterone exhibited lower absolute amounts of nuclear radioactivity in subjects 1 and 2 (162 and 284 fmol/mg DNA respectively) as compared to the control (675 fmol/mg DNA). However, the nuclear binding constituted 49% of whole cell binding in the control cell strain and 39 and 40% of whole cell binding in the two subjects, levels that are within the range of normal for this laboratory [27]. As shown in Fig. 6, when the radioactively

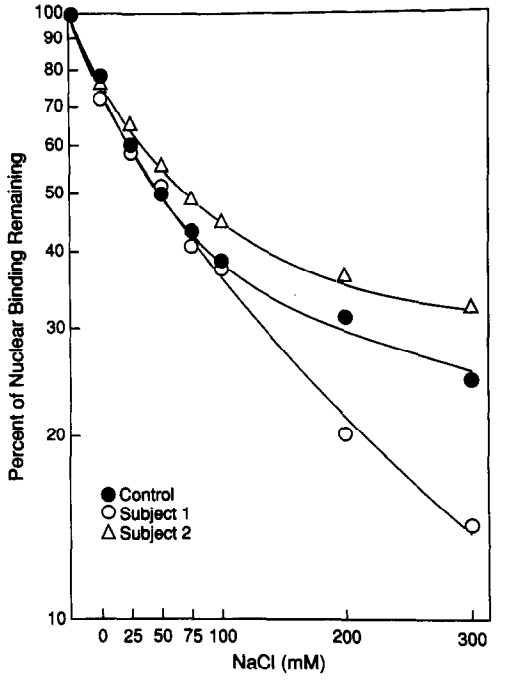

Fig. 6. Salt extraction of nuclear bound androgen receptor. Monolayers of genital skin fibroblasts were grown in 15 cm dishes under standard conditions. On the day of the experiment, cells were incubated with 1.2 nM [^{3}H]dihydrotestosterone for 1 h at 37°C, trypsinized, harvested and homogenized as described above. Purified nuclei were obtained as described in Methods and incubated at 0°C for 30 min with increasing concentrations of NaCl, followed by centrifugation at 2000 g for 10 min. The supernatants were assayed for radioactivity and the results were plotted semilogarithmically as the percent of baseline nuclear binding remaining at any given NaCl concentration.

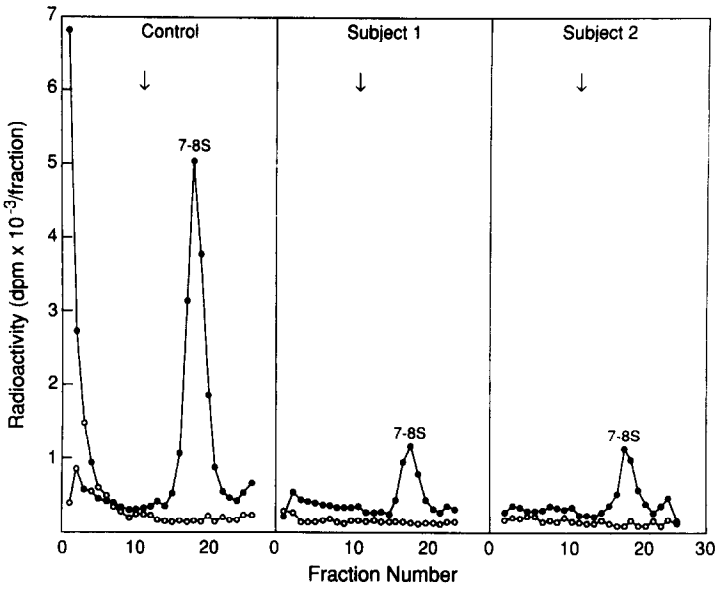

Fig. 7. Sucrose density gradient analysis of androgen receptor binding in fibroblast cytosol. Cells were grown under standard conditions in 15 cm dishes. On the day of the experiment, the cells were harvested by scraping in 4 ml of ice-cold Tris–saline and homogenized in TEGM-10 as described in Experimental. The cell homogenates were centrifuged at 250,000 *g* for 30 min. The supernatants of this centrifugation are termed the cytosol fraction. Aliquots of cytosol were incubated with 3 nM [^{3}H]dihydrotestosterone with or without a 500-fold excess of nonradioactive dihydrotestosterone for 3 h at 0°C. Free steroid was removed with dextran-coated charcoal and 0.4 ml aliquots were layered on to 5–20% sucrose gradients in TEGM-10 buffer. The tubes were then centrifuged in a VTi 65 vertical rotor for 2 h at 65,000 rpm (400,000 *g*). The gradients were fractionated in 0.2 ml aliquots and assayed for ^{3}H and ^{14}C radioactivity. The arrows indicate the sedimenting position of ^{14}C bovine serum albumin (4.6 S).

labelled nuclei were exposed to increasing concentrations of NaCl (0–300 mM), the nuclear bound androgen receptor was extracted at similar rates in both subjects and the control. The NaCl concentrations at which 50% of the nuclear bound radioactivity was released were 47 mM for subject 1, 75 mM for subject 2 and 52 mM for the control. We conclude that nuclear uptake and stability of the receptor within the nuclei are normal in these two subjects.

Analysis of androgen receptor in cytosol extracts by the charcoal assay and sucrose density gradient centrifugation

When cytosol extracts were prepared as described in Experimental and incubated with [^{3}H]dihydrotestosterone in TEGM-10 buffer, those from subjects 1 and 2 had less binding capacity for the ligand than that in the control strain (10.6, 15.8, and 32.8 fmol/mg protein, respectively) as measured by the charcoal assay. Subsequent centrifugation through sucrose density gradients in the vertical rotor yielded 7–8 S size peaks from all three cytosol extracts (Fig. 7). Recovery of high affinity binding loaded onto the gradients ranged from 57 to 78% in these peaks.

DISCUSSION

The two fibroblast strains chosen for these studies were derived from two prepubertal boys with the typical Reifenstein phenotype. Both subjects are members of a family in which it has been established by restriction fragment polymorphism studies that the mutant gene appears to be allelic to the gene that causes receptor negative androgen resistance [15], and hence the mutation almost certainly involves the X-linked gene that specifies the androgen receptor. It follows, therefore, that the fundamental mutation must influence either the structure or the amount of the receptor protein.

The present studies were designed to assay systematically the known functional capacities that have been utilized to identify qualitative abnormalities of receptor function in various laboratories, and we have been unable to identify any defect other than in the amount of androgen receptor in cultured skin fibroblasts. We, therefore, propose that the fundamental defect in this family is one that influences the amount of androgen receptor. This deduction will have to be confirmed by structural analysis of the androgen receptor cDNA in this family. If our deduction is correct, however, it implies that the mutation is outside the coding sequence of the androgen receptor gene and must influence the transcription and/or the stability of the androgen receptor mRNA. Whether this is a common type of mutation is not known at present, but in our laboratory approximately 14 of 80 families with androgen resistance are candidates for such mutations [1]. Characterization of the start site and the 5′ sequences that

regulate transcription of the gene may make it possible to define both the normal control mechanisms and the regulatory abnormalities that impair the amount of receptor.

Regardless of whether the androgen resistance in the present family is the result of a defect in the amount of normal receptor or due to a structural defect in the protein too subtle to be detected by the techniques used here, the variability in phenotypic manifestations in this family must be due to heterogeneity at genetic locus other than the coding sequence that specifies the androgen receptor itself. The nature of the factors that influence the variability of expression of androgen resistance in this and other families is unknown.

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