NORMAL AND MUTANT REGULATION OF ANDROGEN RECEPTOR ACTIVITY IN HUMAN GENITAL SKIN FIBROBLASTS

by

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Short Titile--

Regulation of Androgen Receptor Activity

ABSTRACT

Confluent genital skin fibroblast monolayers were incubated with medium containing 3nM 5α-DHT at 37°C for 19 hours, then with fresh medium containing the same for 1 additional hour. They doubled their specific DHT-receptor activity. The acquisition of increased activity was time- and temperature-dependent, as well as suppressible by cycloheximide. This response is referred to as "up-regulation". Genital skin fibroblasts from four unrelated receptor-positive androgen-insensitive patients with partial or complete clinical phenotypes failed to up-regulate. The difference between controls and patients was emphasized when monolayers were assayed directly after a 20 hour incubation with only one feeding of medium containing DHT. Whereas controls increased their specific DHT-receptor activity to 150% of their basal (1 hour) values, patients lost all or almost all of their specific DHT-receptor activity.

RESUMÉ

Des fibroblastes provenant de tissue cutané genital, ont eté incubé dans un milieu contenant 3nM de 5α-DHT a 37°C. Après 19 heures d'incubation, ce milieu a été remplacé avec du milieu frais contenant la même concentration en DHT et a été incubé pendant 1 heure. L'activité spècifique du recepteur de la DHT est doublée. Cette augmentation est dependante du temps d'incubation et de la temperature, et est bloquée par la cycloheximide. On appelle cet effet "up-regulation". Les fibroblastes de quatre patients provenant de differentes familles, insensible a l'androgène, possèdent des recepteurs qui n'ont pas montré cette characteristique. La difference entre les temoins et les patients a été mis en valeur lorsque les fibroblastes furent testes directement apres 20 heures d'incubation sans changement de milieu. Cependant l'activité spècifique du recepteur DHT des temoins a augmenté jusqu'a 150% par rapport a leurs valeurs de base, mais les patients ont perdu presque toute leur activité.

To Davy, with love

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1. 5α-dihydrotestosterone, 5α-DHT, DHT 17β-hydroxy-5α-androstan-3-one
2. 5α-reductase . . . . . .NADPH:Δ^4-3-ketosteroid-5α-oxidoreductas
3. testosterone, T 17β-hydroxy-4-androstene-3-one
4. hCG . . . . . Human Chorionic Gonadotropin
5. LH . . . . . .Luteinizing Hormone
6. MRH . . . . . Mullerian Regression Hormone
7. AI . . . . . .Androgen Insensitivity
8. CAI . . . . . Complete Androgen Insensitivity
9. Tfm Mouse . . . . . Testicular Feminized Mouse
10. S.E.M. . . . . .Standard Error of the Mean

I. INTRODUCTION

One of the major discoveries in the field of medical endocrinology in the past decade has been the discovery of high-affinity proteins that selectively bind steroid hormones in target cells. The discovery of these "receptors", as they have come to be known, has opened up a new territory of important research into steroid-receptor interactions, a subject having widespread consequences in many areas--from reproductive disorders to cancer biology.

1. The Steroid-Receptor

The era of steroid-receptor research began with estrogen-localization studies (1,2). In these studies, organs that grow in response to estrogens, such as the uterus and vagina, retained physiologic doses of administered estrogens, whereas nongrowth-responsive tissues did not. Although the term 'receptor' was not used, these studies indicated that estrogens become bound with high-affinity to binding sites in target cells. These binding sites were shown to be stereospecific proteins in the cytoplasm (3).

A steroid-receptor was defined as a cytoplasmic molecule (or group of molecules), that interacts with a particular hormone through specific binding sites to form a hormone-receptor complex. This complex transfers the hormonal message to an executive site in the nucleus, which, in turn, mediates metabolic events in the

cell to produce physiologically recognizable responses to the hormone (4,5). Some general conditions established to identify receptors included: (i) high affinity of the target cells for active forms of the steroid; (ii) a higher concentration of receptors in target cells than in insensitive cells; (iii) inability of inactive steroids to bind to receptors at physiological doses; and (iv) interference with receptor binding by hormone antagonists. The pattern generally followed to identify receptor proteins included the identification of the hormone to which the cell responds, studies on the uptake and retention of that hormone in its target cells, and the detection and isolation of the protein that binds the active steroid (6).

Hormone-specific binding sites associated with protein molecules were subsequently shown to exist for mineralocorticoids, glucocorticoids, progestins, and androgens (4). These molecules clearly fulfilled the main criteria established for receptors: they were concentrated in target tissues, demonstrated stereospecificity, and bound to their hormones with high affinity.

2. The Androgen Receptor

Although studies on the uptake and retention of androgens were being carried out in the 1950's, the turning point in androgen-receptor research came in 1968 with the discovery that 5α-dihydrotestosterone (DHT), originating from testosterone, is the androgen preferentially retained by cell nuclei of the rat ventral prostate.

It binds to an acidic nuclear protein that is absent in tissues not sensitive to androgen.

Bruchovsky and Wilson (7) found that although DHT, 5∝-androstanediol, and testosterone were detectable in rat prostate cytoplasm within one minute of administration of ^{3}H-testosterone, only DHT and small amounts of testosterone were recovered from prostatic nuclei after two hours. By gel filtration studies, and the use of DNAase and proteolytic enzymes, they showed that steroid binding in the nucleus was associated with a nuclear protein.

Anderson and Liao (8) proved both in vivo and in vitro that prostatic nuclei selectively retain ^{3}H-DHT, whereas, liver, thymus, and other tissues insensitive to androgens do not retain DHT.

Autoradiographic studies showed too that androgen (but not estrogen) was selectively accumulated in the nuclei of rat prostate epithelium, and seminal vesicles, but not in the liver, diaphragm, or muscle (9, 10).

Fang and Liao (11,12) showed that cyproterone (an antiandrogen), as well as nonandrogenic steroids (such as estrogens), inhibited the formation of the DHT-protein complex in prostatic nuclei. This complex was shown to be extractable from cell nuclei by a 0.4 M KCl solution, and differed from a cytosolic DHT-binding protein with a slightly different sedimentation constant. Addition of a cytosol fraction to nuclei isolated from castrated rats, led to the retention of ^{3}H-DHT. This indicated that there was a two-step mechanism wherein DHT first combined

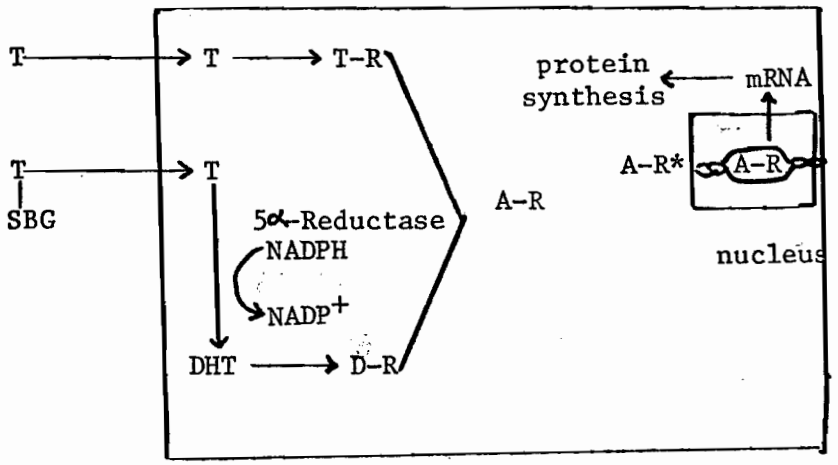
with the cytosolic protein, then entered the nucleus.

The transfer of the cytoplasmic receptor into nuclear chromatin was investigated and a nuclear acceptor for the DHT-receptor complex was hypothesized. Mainwaring and Peterkin (13) showed that the transfer of ^{3}H-DHT into chromatin was tissue-specific and maximal in androgen-sensitive tissues. This specificity was shown to be controlled by non-histone, chromatin-associated protein. The term 'acceptor' was used to describe that tissue-specific site on the chromatin which binds the receptor complexes (5).

Much of the initial research done to elucidate the mechanism of androgen binding was done in the rat ventral prostate, but receptors were also found and studied in many other mammalian androgen-sensitive tissues, including the testis, epididymis, uterus, kidneys, submaxillary glands, hair follicles, human skin fibroblasts, and various areas in the brain (6). More recently, androgen-receptors were reported to be found in human mammary tumors (14), human endometrial fibroblasts (15), human fetal fibroblasts (16), and human amniotic fluid cells (17). These receptors do not seem to differ from one another in their physicochemical properties, but rather in their affinities for various steroids (18, 19,20,21).

The mechanism for androgen action (according to current theory) is summarized in the following model of an androgen target cell, as described by Mainwaring (22) and Pinsky (24).

FIGURE A. ANDROGEN TARGET CELL



T. . .testosterone
T-R. . .testosterone-receptor complex
DHT,D. . .dihydrotestosterone
D-R. . .DHT-receptor complex
A-R. . .androgen-receptor complex
A-R*. . activated androgen-receptor complex
. .acceptor site on chromatin
SBG. . .Sex-Steroid Binding Globulin

1) Molecules of testosterone, that are transported in the blood by Sex-Steroid Binding Globulin, enter the cell by a currently unknown mechanism--probably by simple diffusion, and possibly by carrier-mediated transport (22).

2) Testosterone is reduced to DHT in some target cells.

3) Both testosterone and DHT bind specifically, with high affinity

and low capacity, to the cytoplasmic receptor protein, forming an androgen-receptor complex. (Testosterone, however, dissociates more rapidly than DHT (25).)

4) The androgen-receptor complex is "activated" at 25°C or higher in some incompletely understood way that is associated with a conformational change, and presumably is responsible for nuclear translocation and an increase in affinity for nuclear acceptor sites (22).

5) The activated complex translocates to the nucleus, and binds to specific acceptor sites on the chromatin, which are composed of non-histone proteins and presumably, underlying specific DNA sequences (24).

6) The acceptor-bound androgen-receptor complex initiates a series of biochemical events which result in the characteristic physiologic target cell response to the androgen. These events and the role the acceptor plays have not been completely elucidated, but they may include selective uncoiling of DNA (26), binding of RNA polymerase at specific sites (23), culminating in the production of mRNA which codes for the specific proteins that are to be synthesized by the cell.

3. The Androgen Receptor in Human Skin Fibroblasts

Until 1974 the only studies on androgen receptors in humans were those done on the prostate gland, both normal (27), and hypertrophied (28). In 1974, Keenan et al. (29) reported the identification of a highly specific, low-capacity DHT-binding protein in human skin fibroblasts. These receptors were not detectable in fibroblasts derived from the skin of patients with Complete Androgen Insensitivity (CAI) (29-33), a syndrome which had been attributed to insensitivity to masculinizing hormones.

The first experiments done to identify androgen insensitive patients used binding assays on skin fibroblasts derived from both nongenital and genital skin (31-34). Kaufman et al. (33,35) showed that the number of receptors in nongenital skin is extremely variable and approaches the limit of detectability. Binding in genital fibroblasts has a mean activity of three to four times that in nongenital skin. This finding was also confirmed by Griffin et al. (32) and Amrhein et al. (36). Herfert et al. (37) support this view and conclude that only genital skin fibroblasts should be used in DHT binding studies.

Monolayer cultures of human skin fibroblasts represent a controllable in vitro system which reflects the genetically determined properties and functions of the intact cells of their donor. Human genital skin fibroblasts: (i) convert testosterone to DHT by means of 5α-Reductase (NADPH:Δ^4-3-ketosteroid 5α-oxidoreductase) which reduces testosterone (38) (ii) synthesize receptor proteins which bind DHT specifically (29-35)

and (iii) translocate the DHT-receptor complex to the nucleus (29-33). Thus, monolayer cultures of human genital skin fibroblasts have become a useful system for the study of the actions of androgens, and the mechanism of sexual development, both normal and abnormal.

4. Normal Sexual Development (39,40,41)

The fetus is bipotential in relation to sexual differentiation, and male and female fetuses develop identically until approximately the seventh week of gestation.

The processes that are responsible for normal sexual differentiation are: (i) the development of gonadal sex and (ii) the development of phenotypic sex.

The development of gonadal sex is determined, firstly, by the genetic (chromosomal) sex of the zygote. This is established at the time of fertilization by either an X- or Y-bearing sperm. The Y chromosome contains a testis determining locus (loci) in its pericentromeric region, which "determines" the bipotential gonad to be a testis. This genetic locus seems to be closely linked to, or is synonymous with the H-Y antigen (histocompatibility Y antigen). In the absence of a testis, the phenotypic development of the fetus is female.

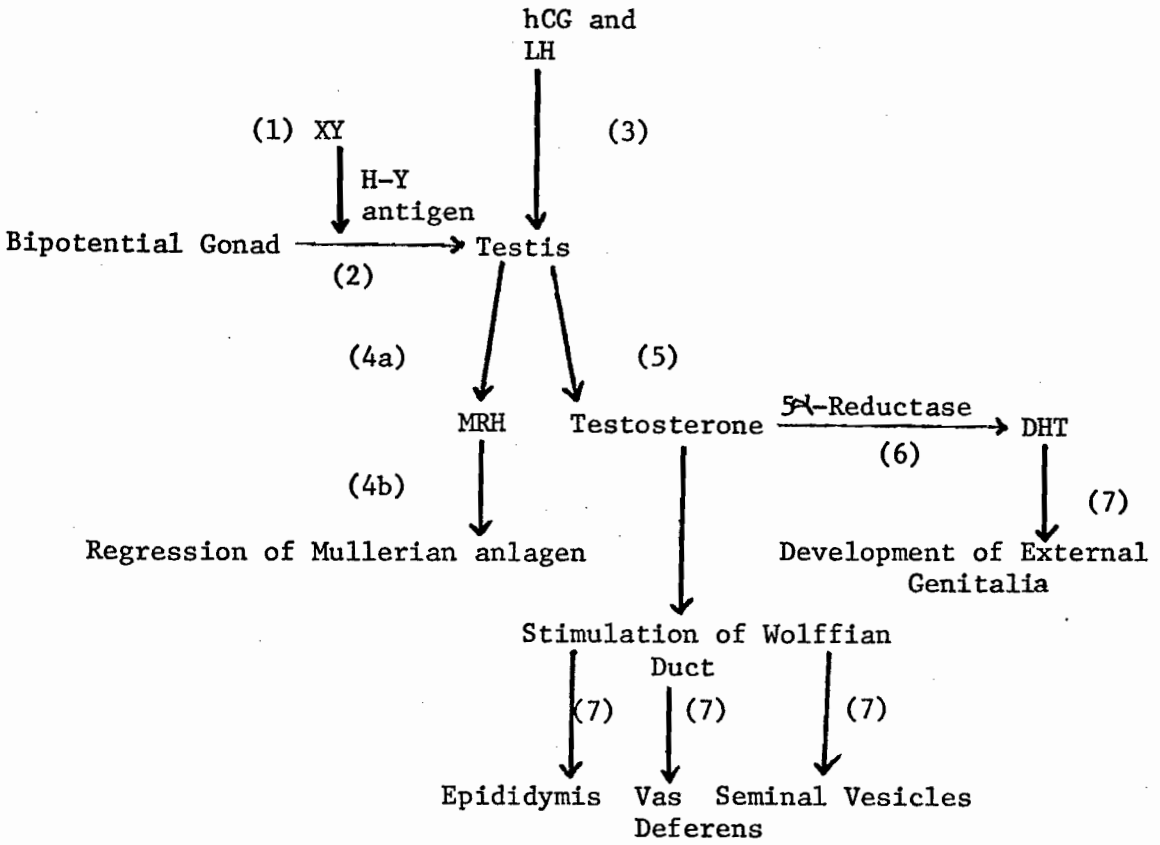
The internal accessory sex organs arise from either the Mullerian or Wolffian ducts. Both are present in the bipotential fetus. In the female, the Mullerian ducts differentiate to form the fallopian tubes, uterus, and upper vagina, while the Wolffian anlagen regress.

In the male, the Wolffian anlagen give rise to the epididymis, vas deferens, and seminal vesicle, and the Mullerian anlagen disappear. The testis secretes two hormones: a) Mullerian Regression Hormone (MRH), (by the Sertoli cells) which specifically inhibits the Mullerian anlagen; and b) Testosterone, (by the Leydig cells) which stimulates the differentiation of the Wolffian ducts.

The external genitalia of both male and female develop from common anlagen in the bipotential fetus: (i) the urogenital sinus; (ii) genital tubercle; and (iii) genital swellings and folds. The urogenital sinus gives rise to the urethra and lower vagina in the female, and to the prostate and prostatic urethra in the male. The genital tubercle develops into the clitoris in the female, and into the glans penis in the male. The genital swellings become the labia majora in the female, and the scrotum in the male, while the genital folds become the shaft of the penis in the male, and the labia minora in the female. The differentiation of the urogenital sinus and the development of the external genitalia is dependent on DHT, the 5α-reduced metabolite of testosterone, for which testosterone is the prohormone.

A defect in any of the steps in this mechanism will lead to abnormal male sexual differentiation.

FIGURE B. MECHANISM OF NORMAL SEXUAL DIFFERENTIATION AND POSSIBLE DEFECTS



Numbers 1-7: Possible Defects

1. Sex chromosome anomaly
2. Chromosome or gene deletions involving the Y chromosome
3. Gonadotropin unresponsiveness
4. Persistant Mullerian Duct Syndrome (PMDS) due to: 4a) MRH biosynthetic mutation or 4b) resistance to the hormone
5. Testosterone biosynthetic mutations--five enzymes (Kmax) involved
6. DHT biosynthetic mutation--5α-Reductase deficiency
7. Resistance to T and/or DHT

5. Abnormal Sexual Development (40)

Abnormalities of sexual differentiation can fall into four main categories:

A) Abnormal gonadal differentiation, which includes:

a. Klinefelter's Syndrome, and its variants (seminiferous tubule dysgenesis with 47,XXY complement and male sexual differentiation) Step 1

b. Turner's Syndrome and its variants (gonadal dysgenesis with 45,XO complement, or XX/XO mosaicism and female sexual differentiation) Step 1

c. XX and XY gonadal dysgenesis (46,XX or XY complement and female phenotype)

d. True hermaphroditism (XX, XY, or mosaics with both ovarian and testicular tissue present)

B) Female pseudohermaphroditism, where one with an XX sex complement, ovaries, and female ducts, displays a varying degree of virilization, usually due to androgens from tumors, luteomas, maternal drugs, etc.

C) Male pseudohermaphroditism, where one has an XY sex complement, testes, and varying degrees of female phenotypic characteristics.

D) Unclassified forms of abnormal sexual development, which may be associated with other congenital anomalies.

6. Male Pseudohermaphroditism (39, 40, 41)

Male pseudohermaphroditism can result from:

1) Target cell resistance to human chorionic gonadotropin (hCG) and/or luteinizing hormone (LH)--resulting in Leydig cell agenesis, or hyperplasia, and defective masculinization internally and externally--Step 3

2) Persistant Mullerian Duct Syndrome--Step 4

3) Testosterone biosynthetic defect--Step 5

4) DHT biosynthetic defect--Step 6

5) Androgen resistance syndromes--Step 7

7. 5α-Reductase Deficiency (24, 40, 41)

5α-reductase deficiency is due to homozygosity for an autosomal recessive gene, causing pseudovaginal perineoscrotal hypospadias. There is reduced conversion of testosterone to DHT, and therefore all DHT-dependent processes of sexual differentiation are defective.

Patients with this disorder have 46,XY karyotypes, and predominantly female external genital phenotypes at birth. The internal sex accessory organs (epididymis, vas deferens, seminal vesicle, and ejaculatory duct), which are Wolffian derived and dependent on testosterone alone, are present. At puberty, virilization occurs, and the patient undergoes male muscular development, growth of axillary and pubic hair, deepening of the voice, and enlargement of the external genitalia. There is little or no facial and body hair, no temporal hair recession, little

or no acne, and a very small prostate.

One way to determine whether a prepubertal patient is deficient in his 5α-reductase production is to measure his plasma levels of testosterone and DHT after hCG stimulation of his testes. Whereas the normal male will have a T:DHT ratio of 20:1 or less, the 5α-reductase patient will have a T:DHT ratio of over 30:1 (42). Other methods of diagnosis include measuring the ratio of urinary 5β-reduced to 5α-reduced steroids, which would be elevated in the patient; and measuring 5α-reductase activity in tissue slices, and in fibroblasts cultured from genital skin (41).

It has been confirmed that genital skin fibroblasts have more 5α-reductase activity than do nongenital skin fibroblasts (43,44,45). Even in genital skin fibroblast cultures, however, the amount of 5α-reductase activity appears to be extremely variable, ranging from 40-80 fold variation in foreskin strains, and approaching the limit of the assay sensitivity in labial strains (24, 46). Thus, the diagnosis of 5α-reductase deficiency in cultured fibroblasts seems to be less reliable than some of the other methods of diagnosis.

8. Androgen Resistance Syndromes

The spectrum of clinical androgen insensitivity (AI) is large, ranging from phenotypic females (as in Complete Androgen Insensitivity), to phenotypically normal males with infertility. These patients are insensitive to both testosterone and to DHT, and androgen-receptor

defects have been associated with all degrees of clinical androgen insensitivity.

a. Complete Androgen Insensitivity (CAI) (24, 39, 40, 41)

This clinical disorder, which has long been known as Complete Testicular Feminization, is an X-linked form of male pseudohermaphroditism, wherein the patient is phenotypically and psychosexually female. These patients undergo normal differentiation of the testes, and secrete normal amounts of MRH. However, all their androgen-dependent organs, including the brain, are insensitive to androgens. Thus, these people are characterized by a 46,XY karyotype, testes, absent uterus and fallopian tubes, blind vaginal pouch, and female external genitalia. At puberty, they exhibit some secondary sex characteristics, including breast development, and occasionally, scant pubic and axillary hair growth.

A diagnosis is usually made when the patient seeks medical attention for primary amenorrhea, but is occasionally made prepubertally, when inguinal "hernias" in a female child prove to be testes. Approximately one-third of the patients diagnosed have negative family histories and are thought to be the result of new mutations (39-41).

CAI was first shown to be due to a deficiency of androgen receptors (24,58)--a defect analogous to that of the testicular feminized mouse (Tfm mouse) (47,48). Cloning studies done on fibroblasts from heterozygotes showed that the gene for the androgen receptor and CAI is on the X-chromosome (49), as in the Tfm mouse. Some CAI patients were shown to have normal amounts of androgen-receptor acti-

vity (50,58), and eventually a receptor-positive form of CAI, in addition to the receptor-negative form, was found (51,52). Thus, there is heterogeneity of the genetic defect even within the uniform phenotype of Complete Androgen Insensitivity. The receptor-negative form, where little or no androgen-binding is measurable can be due either to an absence of the receptor protein, or to a structural alteration in the receptor preventing it from binding. The receptor-positive form, where a normal amount of binding is measured, can be due to a qualitative defect in the receptor, or to a post-receptor defect.

b. Incomplete Androgen Insensitivity (24,41,57)

The incomplete forms of androgen insensitivity include a wide range of undermasculinized male phenotypes. Reifenstein (53), Rosewater (54), Gilbert-Dreyfus (55), and Lubs (56), each described separate syndromes with defective virilization. These are thought by Griffin et al. (41), to be different manifestations of one syndrome, termed "Reifenstein Syndrome", caused by an X-linked mutation. Features of this syndrome range from gynecomastia and azoospermia at one end of the spectrum to hypospadias or pseudovagina at the other end, with varying degrees of masculinization in between.

The most common phenotype observed is the male with perineoscrotal hypospadias, who, at puberty, develops axillary and pubic hair, gynecomastia, and is azoospermic. He has little or no facial or chest hair. Many patients have cryptorchidism with small testes. Leydig

and Sertoli cells are present but the primary spermatocyte does not mature. Some patients also have defective ejaculatory systems, contributing to their infertility. Psychosexually, however, these patients are male and many do marry. Family studies of Reifenstein syndrome have recently found men who are phenotypically normal, and whose only manifestation of the syndrome is infertility (41).

As in CAI, there seem to be two categories of molecular defects responsible for the above phenotypes. Some patients have a partial deficiency of receptor binding activity, although the binding is of normal affinity, and receptor turnover is at a normal rate (32,57). Others have normal levels of receptor binding (31,51), and their defect is presumably qualitative or at the post-receptor stage.

Unlike pedigrees of patients with CAI, where the clinical manifestations are uniform, pedigrees of patients with Incomplete Androgen Insensitivity show more variability at the phenotypic level. Patients within one pedigree may range from almost normal males with micropenis, to patients with hypospadias, gynecomastia, and separate vaginal and urethral orifices. Griffin (32) reported variability within one pedigree at the molecular level as well. The binding activity in one member with Incomplete Androgen Insensitivity was 6 fm/mg of protein, and in two other affected members of the same pedigree--11, and 13 fm/mg protein. The normal values of binding activity for control strains ranged from 20 to 75 fmol/mg protein (32).

9. Qualitative Defects in Receptor-Positive Patients

Qualitative receptor defects in "receptor-positive" (Rpos) patients have been found in both completely and incompletely insensitive patients. Griffin (59) and Pinsky et al. (60-63) have independently shown reduction in androgen binding at 42°C in cells from AI receptor-positive patients, as compared to controls, which bind similarly at 37°C and at 42°C.

Griffin found thermal instability in female phenotypic CAI patients, but did not find the same in male phenotypic Reifenstein patients.

Pinsky et al. found thermal instability in the cells of one "incomplete" patient as well, although the defect was present to a lesser extent in these cells than in cells of patients with the "complete" phenotype. Pinsky et al. also found a three-fold increase in the dissociation rate of the DHT-receptor complex in cells of both patients compared to controls, when preincubated, assayed, and chased at 37°C with excess radioinert DHT. Both "complete" and "incomplete" patients displayed the same degree of dissociation (63).

Further evidence for a qualitative defect in the androgen-receptor was obtained from DHT affinity studies. Androgen receptors from the labial fibroblasts of a receptor-positive androgen insensitive patient were found to have a lower affinity for DHT and to be less stable at 23°C than receptors derived from normal genital fibroblasts (62).

* * * *

Through the study of the pathophysiology of the various forms of androgen insensitivity, we arrive at a deeper understanding of the normal mechanisms of androgen action and steroid-receptor interactions. Each mutation that we find in an androgen-insensitive cell elucidates one step in the normal pathway of androgen action.

In this thesis, I describe a normal form of regulation of the androgen receptor by its own hormone, and its mutant form of regulation.

II. OBJECTIVES AND RATIONALE

The present study had two aims: (i) to determine what factors regulate androgen-receptor binding activity in normal human genital skin fibroblasts; and (ii) to determine whether fibroblasts derived from receptor-positive androgen-resistant subjects behave differently with respect to any of these factors.

In the standard receptor-binding assay, we find very little intraexperimental variation; the results on replicates of (genital skin) fibroblasts within one assay will seldom deviate from the mean Bmax by more than 10%. However, results of assays of one strain on different occasions may vary as much as fourfold. This variation cannot be accounted for by donor or *in vitro* age (35).

Subtle changes in the microenvironment of a cell may influence its receptor-activity from day to day. These may include changes in the pH, temperature, inhibitory or activating factors, or the level of intracellular hormone. To determine what regulates receptor binding activity in normal genital skin fibroblasts, I chose to investigate whether (and how) the normal receptor responds to its ligand.

On the assumption that there would be a recognizable response in normal fibroblasts, my next step was to examine the response of fibroblasts of receptor-positive patients to the same conditions.

When one determines that complete AI is not due to a deficiency in receptor-activity, the next step is to seek a qualitative defect in the receptor, or a post-receptor abnormality. A qualitative abnormality in a receptor can be identified by a deficient or dissimilar response of the receptor to the same stimuli which elicit a specific, characterizable response in a qualitatively normal receptor. Thus, by observing the same or a different response, I could either rule out or define a new mutation in androgen insensitive patients.

III. MATERIALS AND METHODS

1. Subjects

All cell strains were derived from pieces of genital skin that were obtained with informed consent according to the local Ethics Commitee.

a. Controls

Control fibroblast strains were developed from pieces of preputial or labium majus skin of normal individuals with no clinical background or family history of androgen insensitivity. The foreskin donors varied from infancy to childhood. The labial skin donors varied from fetal life to adulthood. The results did not differ between strains from either site or with donor age.

b. Patients

Patient KIL was born with unambiguously female external genitalia. At 3.5 months, she was found to have inguinal testes and a normal XY karyotype. At 17 months, examination revealed a 1-2 cm deep vagina, but no cervix or uterus. A three-day course of hCG raised the plasma level of testosterone from 25 ng/100 ml to 942 ng/100 ml. The family history was negative.

Patient TCF was born with ambiguous external genitalia. At 12 years the hypospadiac phallus was 4 cm long, 1.5 cm wide, and was

bound by ventral chordae. The labioscrotal folds each contained a testis. A single urogenital opening, located at the base of the phallus, was enveloped by unfused labia minora. There was no uterus, and a prostatic utricle was observed on a voiding cystourethrogram. The karyotype was 46, XY. A course of testosterone enanthate resulted in progressive virilization and gynecomastia during the first 6 months. One year after the start of this treatment, a vas deferens and vestigial epididymis were identified on the right. At 13.5 years, a bilateral mastectomy and first-stage hypospadias repair were performed, and the testosterone treatment was stopped. The family history revealed a similarly affected older sister, as well as four other maternal relatives, who underwent feminine construction of external genitalia. They had spontaneous breast development, and were sterile.

Patient 14679 was born with ambiguous external genitalia. The penile phallus, bound by ventral chordae, measured 3.0 x 1.3 cm and was encircled by pigmented, well rugated labioscrotal folds. The urethra opened at the phallic mid-shaft. The testes were descended, the right completely. Laparotomy showed a "sac-like structure" communicating with the right vas deferens to be a dilated prostatic utricle, and revealed that the left vas deferens was extended to the left testis. The karyotype was 46, XY. A four-day course of hCG increased the plasma level of testosterone from 153 to 480 ng/dl. The family history was negative.

Patient 99900 was born with ambiguous external genitalia.

At 11 months the patient was referred for investigation of severe penoscrotal hypospadias. The penis was bound by chordae to the median raphe of a bifid scrotum. The width of the glans was 1.2 cm. Apart from the external genitalia, no significant abnormalities were noted. The karyotype was a normal male 46, XY, and the patient was reared as a male. Basal serum levels of testosterone, androstenedione, FSH, and LH were normal. The patient responded excellently to hCG stimulation. There was no family history for any intersex or related problems.

2. Cell Culture

Fibroblast strains were developed in the laboratory, as described previously by Pinsky et al. (64). The genital fibroblast monolayers were grown to confluence in Eagle's Minimal Essential Medium (MEM) made with Earle's salts and supplemented with 2mM glutamine, 1mM pyruvate, 1x non-essential medium, an equal mixture of newborn and fetal calf serum (10% v/v), penicillin G (60mg/l), and streptomycin sulphate (60mg/l). Approximately 50-100 thousand cells were plated in 30-60 plastic petri plates ($60mm^2$) and fed approximately twice a week with the above medium. After about 2-3 weeks in culture, when mitotic activity had stopped, the confluent monolayers were used for binding experiments.

3. Standard DHT-Binding Assay--General Principles

The standard assay to measure whole cell androgen-receptor activity involves:

1. preincubating confluent fibroblast monolayers in serum-free (s-f) medium overnight;
2. exposing them to ^{3}H-DHT in s-f medium at 37°C for about 60 minutes. At this time, the high-affinity androgen-receptor binding sites are saturated (31-33);
3. washing the monolayers several times with protein-containing and protein-free isotonic buffers to remove molecules of unbound radioactivity;
4. measuring cell radioactivity and protein content.

The principle of the assay is to use two sets of replicate monolayers to measure "total" and "nonspecific" binding respectively. The total binding is measured with ^{3}H-DHT alone, while the nonspecific binding (low-affinity binding due to various cellular macromolecules) is measured with the same concentration of ^{3}H-DHT, plus an excess of radioinert DHT. The excess unlabelled DHT binds to all the specific high-affinity receptor binding sites, thus, any radioactivity measured in the cell after the unbound radioactivity is washed off, is presumably attributable to the low-affinity (nonspecific) binding sites. The "total" minus the "nonspecific" radioactivity yields a measure of "specific" binding.

4. Standard DHT-Binding Assay--Specific Steps

1. Replicate 60mm^2 petri plates of confluent fibroblast monolayers were preincubated with s-f medium made with Hank's salts and buffered to pH 7.4 with 15mM HEPES (N-2-hydroxyethyl piperazine-N-ethane-sulfonic acid) overnight.

2. The s-f medium was removed and fresh s-f medium containing 3nM of (1,2,4,5,6,7-^{3}H) DHT (139 Ci/mmole; New England Nuclear Corp.) was added to one set of replicates, and 3nM ^{3}H-DHT plus 0.6μM unlabelled DHT was added to the other set. All plates were placed in a humidified 37°C incubator supplied with 5%CO_2:95% air.

3. After 60 minutes, the cells were removed from the incubator, and placed on a bed of ice.

4. The media containing DHT were removed, and the monolayers were washed five times with 3 ml of 20 mM Tris-HCl containing 0.9% NaCl and 0.2% Bovine Serum Albumin (BSA) at pH 7.4, and twice with the same buffer lacking BSA.

5. 3 ml of 0.1% trypsin were added to the monolayers which were removed from the ice and left at room temperature for 5 minutes.

6. The adherent cells were scraped with a rubber policeman and the cell suspensions were centrifuged at 800g for 5 minutes.

7. The cells were washed once more by resuspending the cell pellets in the Tris-NaCl buffer lacking BSA, and recentrifuging them.

8. The resulting cell pellets were resuspended in 1.5 ml distilled water, and sonicated by direct exposure to a 1 cm probe of a sonicator (Measuring & Scientific Equipment Co., London, England) at an amplitude of 2μ for 10 seconds.

9. One portion (125μl) of the sonicate was sampled for protein, according to the method described by Lowry et al. (65), and most of the remainder (1000μl-1 ml) was added to 10 ml of a toluene solution containing Omniflor (4 gm/l, NEN Corp.) for determination of radioactivity at an efficiency of 43% in an Intertechnique (SL-35) spectrometer

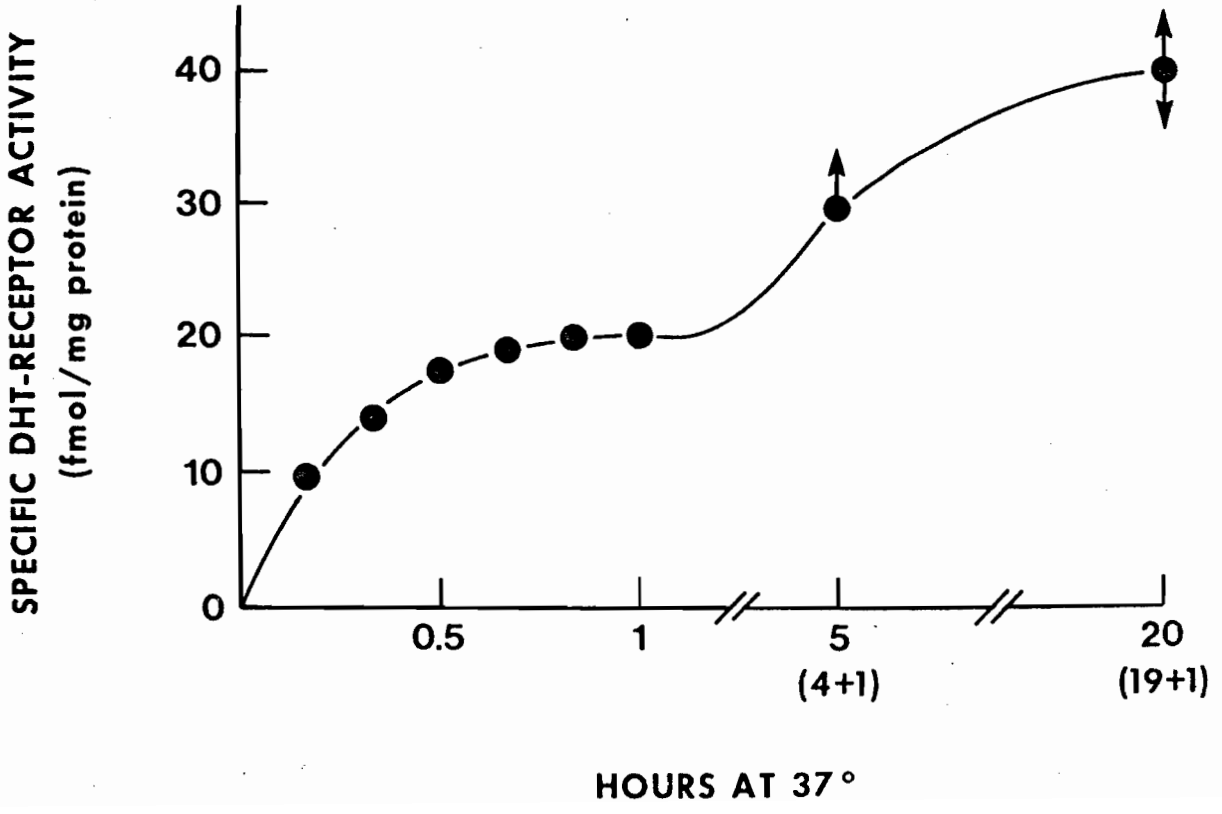
5. Extended Incubation with Ligand--General Principles

To determine the effect(s) of prolonged incubation with DHT, cells were incubated with ^{3}H-DHT for varying lengths of time. The experiment involved placing a number of sets of replicate monolayers with medium containing DHT in a 37°C incubator and removing one set at a time at different time intervals to complete the final processing.

The following figure shows the behavior of control strains with this procedure. Between 30 minutes to 1 hour, the specific receptor activity reaches a saturating level, after which it continues to rise. I refer to the procedure where a series of sets of fibroblasts undergo one continuous incubation with an initial concentration of 3nM DHT, as the "One-Pulse" or the "20 hour" protocol.

In order to estimate the extent to which the changes in receptor activity might be underestimated by possible ligand depletion due to cell catabolism of DHT, most experiments with both controls and patients were done by removing the medium from the plates one hour before the final processing and reincubating them for that remaining hour with fresh medium containing DHT. I refer to this procedure as the "Two-Pulse" or "19+1" protocol.

FIGURE C. DHT-RECEPTOR ACTIVITY CURVE



6. One-Pulse Assay

1. Replicate monolayers in petri plates were washed free of growth media, divided into three groups and fed either:
   a) s-f medium
   b) s-f medium with 3nM ^{3}H-DHT
   or c) s-f medium with 3nM ^{3}H-DHT plus 0.6μM unlabelled DHT

2. The first group was treated as the standard 1 hour control group, and processed according to the protocol above (p.25).

3. The second and third groups were incubated for 20 hours before processing (Steps 4-9, p.25).

Some experiments used intermediate and/or longer times of DHT incubation. These were done in essentially the same manner as those above.

1. Monolayer plates that were to be incubated for the longest time (20, 24, or 48 hours) were incubated with medium b or c, while all other plates were incubated with s-f medium alone.

2. At the desired time (usually 12 hours before the cells were processed), the plates were taken out of the incubator and incubated with Mediums b or c. They were reincubated for the appropriate amount of time, and both the 20 (or 24 or 48) and the 12 hour samples were processed at the same time.

3. The 3, 5, or 6 hour samples were incubated with s-f overnight and incubated with DHT in the morning, as in the standard 1 hour control sample. After the appropriate time of incubation, these samples were processed.

7. Two-Pulse Assay

Triplicate fibroblast monolayers, rather than being incubated for 20 hours "straight" with DHT, were first incubated for 19 hours with DHT, then drained of their medium, and refed fresh medium containing DHT.

Thus, if there were insufficient DHT to bind to every receptor due to catabolism, a replenishment of DHT would compensate.

In a period of 1 hour, an insignificant amount of metabolism occurs, approximately 20% (32,33,35). Therefore, the 1 hour control samples were treated in the standard way.

The experiments using intermediate times of DHT incubation were done where indicated, with an addition of fresh medium containing DHT, 1 hour before processing. (These are referred to by the amount of time they have been preincubated with DHT, followed by +1. Ex. 4+1, meaning 5 hours of incubation; 11+1, meaning 12 hours of total incubation.)

In certain experiments, the "One-Pulse" and "Two-Pulse" protocols were carried out simultaneously in a controlled manner, in order to assess if there was any difference between the two protocols.

8. Inhibitors

In order to test whether increases in receptor activity were due to the synthesis of new receptor protein, a protein inhibitor was added to half the 1 hour groups and half the 19+1 hour groups in some experiments. Solutions of 2μM of cycloheximide, an antibiotic causing a transient interruption of protein synthesis, due to an inhibition at the translation level, were added to the media of those experimental groups whose receptor synthesis was to be blocked.

9. Temperature Experiments

The effects of different temperatures on "up-regulation" were examined in control strains. 1 hour, 4+1 hour, and 19+1 hour incubations with DHT were done on fibroblasts under the "Two-Pulse" protocol in humidified incubators (supplied with 5% CO_2:95% air) set to either 37°C or 27°C.

10. Experiments Measuring Recovery from Cycloheximide Treatment

In order to measure the resumption of receptor activity after treatment with cycloheximide for 18 hours, I followed the following procedure:

1. Enough cycloheximide (in a propylene glycol solution) to yield a final concentration of 2.5μM was added to the medium of replicate sets of monolayer plates. One set of plates, receiving an equivalent amount of propylene glycol without cycloheximide, was set aside to serve as a control.

2. All plates were incubated for 18 hours in a humidified incubator (supplied with 5% CO_2:95% air) set to 42°C.

3. At the end of 18 hours, all plates were removed from the incubator, and the medium containing cycloheximide or propylene glycol was aspirated from the plates. Two sets of plates--one having been incubated with, and one without cycloheximide--were used for a standard DHT-binding assay, and their specific activities were compared. The cells treated with cycloheximide remained, on average, with 30-40% of the activity of the control plates.

4. Fresh medium containing the standard culture medium (p.23) was added to the remaining plates.

5. Every 3 hours, another set of plates was removed from the incubator, and assayed for specific receptor activity (according to the procedure outlined on p.25).

11. Statistical Analysis

Data was subjected to statistical analysis by Student's paired and unpaired t tests (78,79), or One Way Analysis of Variance (80).

IV. RESULTS

1. Controls:Two-Pulse Protocol

Twelve control cell strains were examined 1 to 3 times each within the "Two-Pulse" protocol described in Material and Methods, p. 27-28. The data summarized in Figure 1B. and in Table 1 show that a 19+1 hour incubation yields approximately twice as much receptor activity as a 1 hour incubation.

Although I observed variation in the basal receptor activity (the receptor activity after the standard 1 hour incubation) of a cell strain on different occasions, the 19+1 hour incubation almost always increased receptor activity by a factor of 2. This was a highly significant finding ($p < .001$, Student's paired t test). I refer to the increase as "up-regulation".

On three occasions an increase was not observed after 20 hours. This was not due to experimental error, but rather to temporal variation, i.e. the peak increase in receptor activity occured sooner than 20 hours (Fig. 3C). This is discussed in greater detail below.

One patient with 5α-reductase deficiency (Strain 61479) behaved as a control in these experiments, as would be expected from the nature of this defect. (See Introduction, p.12-13.)

2. Controls: One-Pulse Protocol

Similar experiments to the ones above were done by incubating control cell lines for either 20, 24, or 48 hours with 3nM DHT, after which the receptor activity was compared to that measured after a standard 1 hour DHT incubation. In these experiments, unlike those above, no additional DHT was added after 19 hours to overcome the possible problem of DHT catabolism. Nevertheless, receptor activity rose significantly ($p<.05$) after prolonged incubation with DHT (Table 2; Fig. 1A). Again, the only time a decrease in activity was noted after 20 hours, receptor activity had reached its peak by 5 hours (results not shown).

3. Patients: Two-Pulse Protocol

Patient strains, on the other hand, clearly did not exhibit an increase in receptor activity after prolonged incubation with DHT (Fig. 1). On those occasions where the cells of patient strains were given an additional boost of DHT for 1 hour (19+1 hour incubation), the receptor activity stayed the same or decreased slightly. These strains, in contrast to those controls which decreased their receptor activity on rare occasions, had never shown any prior increase in receptor activity (Table 3). The differences between strains was shown to be insignificant ($p=0.1$) by a One Way Analysis of Variance.

4. Patients: One-Pulse Protocol

When no additional boost of DHT was given and patient cells were exposed to one dose of DHT for 20 hours, receptor activity dropped dramatically

to 0 or almost 0 (Fig. 1; Table 4). I refer to this as "down-regulation".

5. Comparison of Both Protocols

There is a difference between the two protocols, and the difference is magnified tremendously in patient strains. Paired results from both patients and controls are summarized in Figure 2; unpaired results are summarized in Figure 1.

The increase in receptor activity in control strains after a one-pulse, 20 hour incubation with DHT is slightly less than that seen after a two-pulse, 19+1 hour incubation. Statistical tests were done on: (i) the pool of data for the 19+1 hour incubations versus the pool of data for the 20 hour incubations, using the Student's unpaired t test; and (ii) three sets of paired data of 19+1 and 20 hour incubations which were done simultaneously in controlled experiments, using the Student's paired t test. Both tests show a slight but significant difference between the two protocols ($p < .05$). With the unpaired data, the 19+1 and 20 hour incubations showed an average increase in receptor activity of 1.95 and 1.43 fold respectively. The paired data revealed almost identical results. (The 19+1 hour incubation showed a 1.99 fold increase versus a 1.43 fold increase for the 20 hour incubation.)

In the patient strains, pooled results of all strains showed 85% of the original activity after a 19+1 hour incubation versus 9% of the original activity after a 20 hour incubation. Results of eight paired experiments show 80% and 5% of the original activity for the 19+1 and 20 hour incubations respectively. Both differences are very highly significant ($p < .001$).

6. Time-Course Studies

Time-course studies were done on control as well as on some patient strains. Control strains, regardless of whether they were examined under the "One-Pulse" or "Two-Pulse" protocols, fell into three categories (Fig. 3):

a. Progressive Rise--in 68% of the experiments on controls, receptor activity increased progressively over time

b. Rise at the Beginning—in 18% of the experiments on controls, receptor activity increased rapidly within the first 5 hours, then remained the same over the next 15 hours

c. Rise and Fall--in 14% of the experiments on controls, receptor activity increased rapidly within the first 5 hours, then decreased to a higher level than the original activity, the same level, or a slightly lower level.

Patient strains always showed a rapid and progressive decline in receptor activity over 20 hours (100% of experiments), when given only one pulse of DHT. When given two pulses of DHT, patients' receptor activities either fell very gradually over time to a much lesser extent than in the "One-Pulse" protocol, or stayed the same within the first 5 hours, thereafter falling slowly, or decreased in the first 5 hours, then rose slightly to a lower level than its original activity.

7. Inhibitor Studies

Figure 5 shows typical results on a control and a patient strain, either with or without cycloheximide, upon prolonged incubation with DHT.

The increase in receptor activity due to up-regulation was blocked by cycloheximide. Receptor activity actually decreased slightly over time--probably the result of receptor degradation uncompensated for by synthesis.

A greater decrease was apparent in the cells of patients with cycloheximide. This seems to indicate that a certain amount of receptor synthesis does occur even in patient strains. This amount, however, seems too small to reflect a regulatory response to an external stimulus (such as incubation with DHT), and probably reflects a normal basal production of receptors which compensates for the basal degradation of receptors.

8. Temperature Studies

Receptor binding activity after 1 hour was similar at 37°C and 27°C. However, the increase in activity over time was smaller at 27°C than at the higher temperature. Regardless of the pattern of up-regulation in a 37°C incubation, the same cells incubated at 27°C showed a consistent pattern--the peak in receptor activity, however small, is reached at 5 hours, after which activity decreases to a lower level. These results are shown in Fig. 4.

9. Recovery of Receptor Synthesis after Cycloheximide Treatment

Table 5 summarizes the results on two control strains assayed twice each. Treatment with cycloheximide decreased the specific receptor activity to approximately 35% of the original receptor activity (i.e. percent of the specific activity of the same cells not treated with cycloheximide.) Within 6 hours, the specific receptor activity increased to approximately 56% of the original activity. This suggests that an increased amount of receptor synthesis (over the basal production of receptors) is taking place within the first 6 hours after the removal of cycloheximide from the cellular environment.

TABLE 1. EFFECT OF PROLONGED INCUBATION WITH DHT ON CONTROLS (TWO-PULSE PROTOCOL)

TYPE	STRAIN	ASSAY #	^{3}H-DHT-Receptor Activity (fm/mg protein) INCUBATION WITH DHT (hours) 1	19+1	Fraction of of Original Activity 19+1/1	Average Fraction of Original Activity 19+1/1
Control:	80055	1	27	53	1.96	1.96
	L32	1	15	31	2.00	2.14
		2	9	20	2.22	
	LCF	1	16	31	1.94	1.94
	XAF	1	40	65	1.63	1.64
		*2	40	32	0.80	
		3	20	50	2.50	
	SIS	1	30	59	1.97	2.35
		2	18	49	2.72	
	80044	1	21	49	2.33	2.58
		2	18	51	2.83	
	PUF	1	15	39	2.60	2.60
	70478	1	20	51	2.60	2.40
		2	30	63	2.10	
	L26	1	24	46	1.92	1.41
		*2	32	29	0.90	
	7777	1	12	17	1.42	1.44
		2	16	32	2.00	
		*3	22	21	0.91	
	L12	1	32	63	1.97	1.82
		2	29	51	1.76	

(cont'd next page)

TABLE 1. (CONTINUED)

TYPE	STRAIN	ASSAY #	^{3}H-DHT-Receptor Activity (fm/mg protein) INCUBATION WITH DHT (hours) 1	19+1	Fraction of Original Activity 19+1/1	Average Fracti of Original Ac 19+1/1
Control:	SGF	1	33	63	1.91	1.91
				** Mean ± S.E.M.:	1.95 ± 0.12	2.02 ± 0.12
5α-Reductase Patient:	61479	1	50	63	1.26	1.26

Table 1: Numbers representing receptor activity are means of triplicates of fibroblasts incubated with ^{3}H-DHT, minus the means of triplicates of fibroblasts incubated with ^{3}H-DHT and an excess of unlabelled DHT. Both sets of triplicates were either incubated for 1 hour, or 20 hours. Those incubated for 20 hours were fed an additional dose of DHT one hour before processing, and are therefore included in the column referred to as 19+1.

* Those strains which show a slight decrease in receptor activity at 20 hours, have actually shown a prior increase.

** Level of Significance of 19+1 vs. 1: $p<.001$
(Student's Paired t test)

Table 2: Numbers representing specific receptor activity are the means of triplicates of fibroblasts incubated with ^{3}H-DHT (representing total activity) minus the means of triplicates of fibroblasts incubated with ^{3}H-DHT and an excess of unlabelled DHT (representing non-specific activity) which were incubated for either 1 hour or 20 hours. The 20 hour samples were fed only once at the beginning of the incubation.

* This strain which shows a decrease in activity at 20 hours had shown a prior increase at 5 hours.

** Level of Significance of 20 vs. 1: $p < .05$
(Student's Paired t test)

TABLE 2. EFFECT OF PROLONGED INCUBATION WITH DHT ON CONTROLS (ONE-PULSE PROTOCOL)

TYPE	STRAIN	ASSAY #	^{3}H-DHT-Receptor Activity (fm/mg protein) INCUBATION WITH DHT (hours) 1	20	24	48	Fraction of Original Activity 20/1	24/1	48/1
Control:	L26	1	46	61	--	--	1.3	---	---
	80044	1	21	39	--	--	1.9	---	---
	LCF	1	15	16	--	--	1.1	---	---
	XAF	1	12	22	--	--	1.8	---	---
	1778	1	48	69	--	--	1.4	---	---
	L32	1	9	16	--	--	1.8	---	---
	7777	*1	12	8	--	--	0.7	---	---
	L26	1	22	--	60	--	---	2.7	---
	70478	1	30	--	68	--	---	2.3	---
	80033	1	19	--	--	68	---	---	3.6
						**Mean ± S.E.M.:	1.43 ± .17		

TABLE 3. EFFECT OF PROLONGED INCUBATION WITH DHT ON PATIENTS (TWO-PULSE PROTOCOL)

TYPE	STRAIN	ASSAY #	^{3}H-DHT-Receptor Activity (fm/mg protein) INCUBATION WITH DHT (hours) 1	19+1	Fraction of Original Activity 19+1/1	Mean ± S.E.M. of Fraction of Original Activity/ 19+1/1
Patient:	99900*	1	32	36	1.13	
		2	18	17	0.94	
		3	17	12	0.71	
		4	55	66	1.20	1.00 ± .11
	14679*	1	32	32	1.00	
		2	39	41	1.05	
		3	40	27	0.68	
		4	36	35	0.97	0.93 ± .08
	TCF**	1	24	19	0.79	
		2	28	17	0.61	
		3	37	37	1.00	
		4	24	21	0.88	
		5	13	9	0.69	0.79 ± .07
	KIL**	1	30	19	0.63	
		2	28	18	0.64	
		3	20	15	0.75	0.67 ± .04
				Mean ± S.E.M.:	0.85 ± .05	

Table 3: Level of Significance of Patient Strains vs. Control Strains in Two-Pulse Protocol: (Student's Paired t test)

* $p < .01$

** $p < .001$

TABLE 4. EFFECT OF PROLONGED INCUBATION WITH DHT ON PATIENTS
ONE-PULSE PROTOCOL

TYPE	STRAIN	ASSAY #	^{3}H-DHT-Receptor Activity (fm/mg protein) INCUBATION WITH DHT (hours) 1	20	Fraction of Original Activity 20/1	Mean ± S.E.M. of Fraction of Original Activity/S 20/1
* Patient:	99900	1	18	1	.06	
		2	32	6	.19	
		3	24	5	.21	
		4	12	1	.08	
		5	45	6	.13	
		6	17	0	.00	.11 ± .03
	14679	1	40	1	.03	
	TCF	1	29	3	.10	
		2	30	5	.17	
		3	24	3	.13	
		4	13	0	.00	.10 ± .04
	KIL	1	28	1	.04	
		2	20	0	.00	.02 ± .02
				Mean ± S.E.M.:	.09 ± .02	

Table 4: Level of Significance of Patient Strains vs. Control Strains in One-Pulse Protocol: (Student's Paired t test)

* $p < .001$

TABLE 5. RECOVERY OF RECEPTOR ACTIVITY AFTER CYCLOHEXIMIDE TREATMENT

TYPE	STRAIN	ASSAY #	TIME (hours)	Specific ^{3}H-DHT-Receptor Activity (fm/mg protein)	Percentage of Original Activi	Average/Strain
Control:	SIS	1	-18*	15	100	
			0**	6	40	
			6***	10	64	
		2	-18	38	100	100
			0	12	33	36
			6	20	54	59
	1778	1	-18	42	100	
			0	17	40	
			6	23	55	
		2	-18	46	100	100
			0	11	23	32
			6	24	52	54

* Specific Receptor Activity before treatment with cycloheximide
** Specific Receptor Activity after 18 hours of treatment with cycloheximide
*** Specific Receptor Activity 6 hours after removal of cycloheximide from the cellular microenvironment

Figure 1

A. In these experiments, fibroblasts were fed with only one pulse of DHT. Triplicates of fibroblast monolayers, incubated for 20 hours or 1 hour with DHT, were compared. The 20 hour results were taken as a percentage of the 1 hour results. The results in Figure 1.A. represent the mean $\pm$ the standard error of the mean (S.E.M.) of 7 experiments on control strains, and 13 experiments on patient strains.

B. In these experiments, fibroblasts were fed with two pulses of DHT--one pulse given 20 hours before processing, and the second given one hour before processing. The 20 hour incubation is referred to as "19+1" (i.e. 19 hours with the first pulse of DHT, and 1 hour with the second pulse.) The 19+1 hour results are recorded as a percentage of the 1 hour results. The results in Figure 1.B. represent the mean $\pm$ S.E.M. of 22 experiments on control strains and 16 experiments on patient strains.

FIGURE 1. EFFECT OF DHT-INCUBATION TIME ON RECEPTOR ACTIVITY

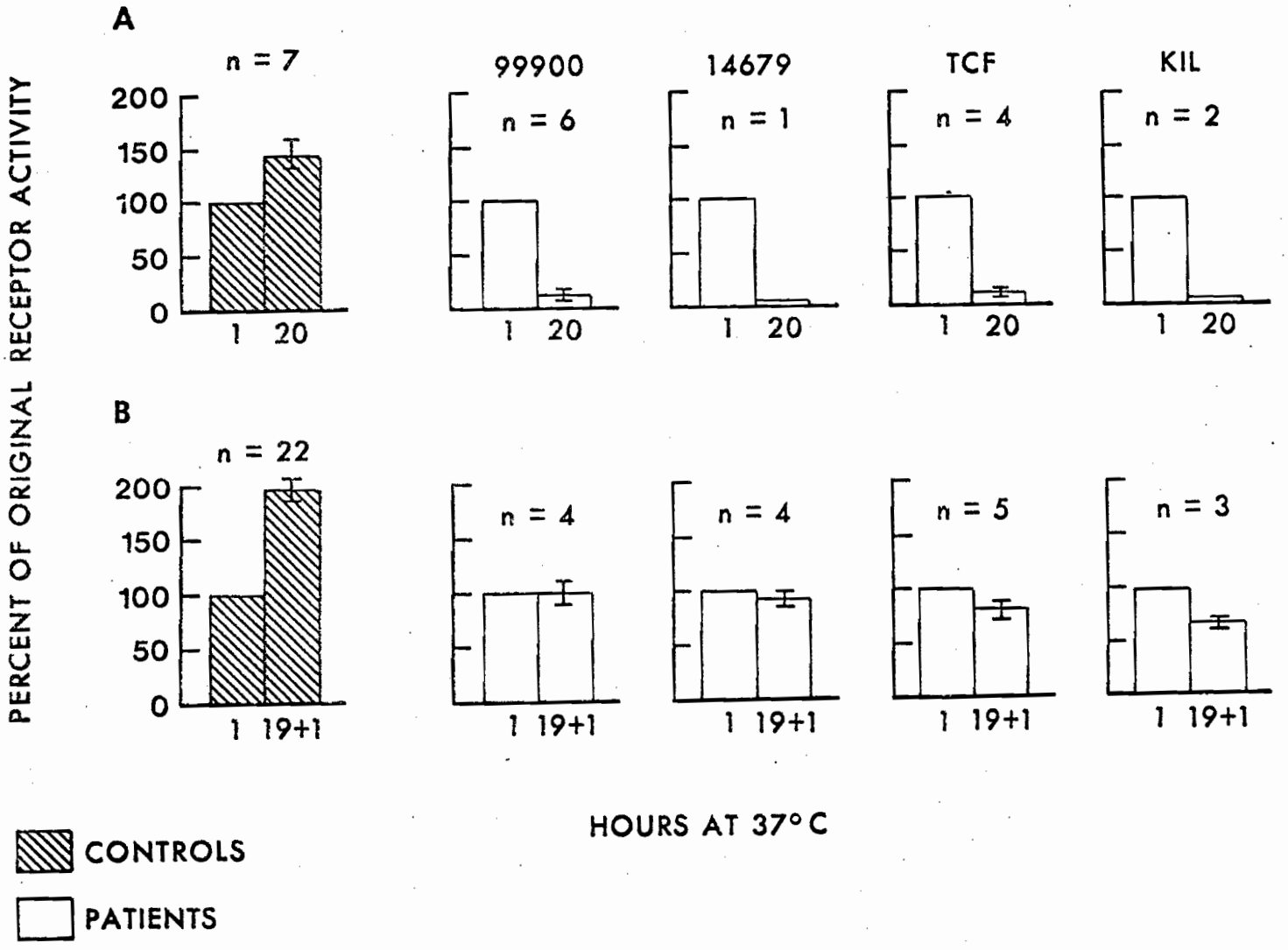


Figure 2 The effects of one or two pulses of DHT on receptor activity after 20 hours, were examined by treating one third of the monolayer plates with DHT for one hour, one third of the plates with DHT for 20 hours, and one third of the plates with DHT for 19 hours, then again for 1 hour (19+1).

A. Results on controls are the means $\pm$ S.E.M. of 3 experiments.

B. Results on patients are the means $\pm$ S.E.M. of 8 experiments done on 4 different patient strains.

FIGURE 2. EFFECT OF ONE PULSE VS. TWO PULSES OF DHT ON RECEPTOR ACTIVITY AFTER 20 HOURS (PAIRED DATA ONLY)

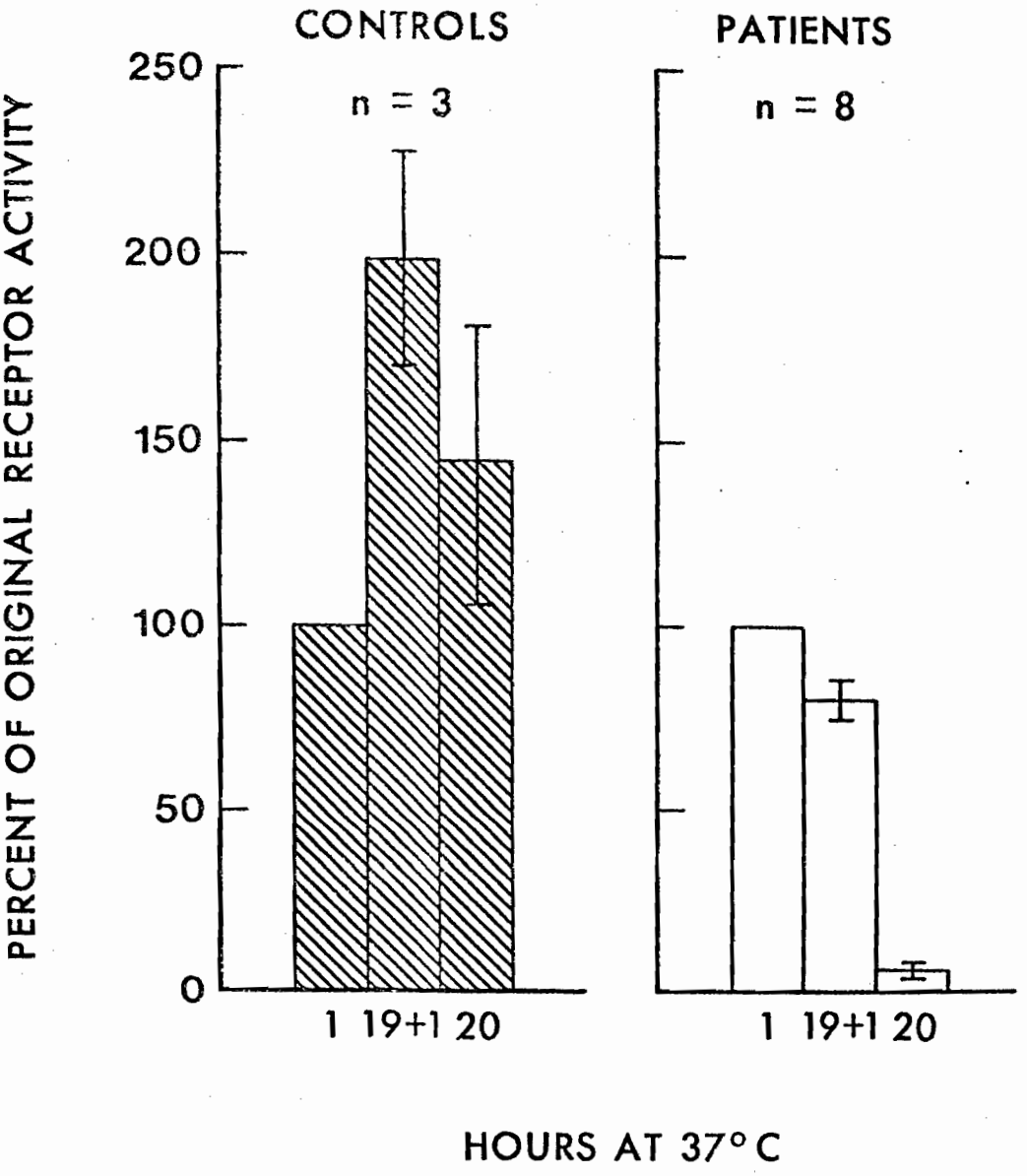


Figure 3 Controls were incubated for different time intervals with DHT--either with one or two pulses. The patterns of receptor activity increase did not differ with regard to the protocol. (The extent of increase did.) In both protocols, the predominant pattern was that depicted in 3.A. (These 22 experiments are not all the same ones as described in Table 1.)

Patients were incubated for 1, 5, or 20 hours with DHT. The pattern of receptor activity with one pulse of DHT was always that depicted in Figure 3.AA. Differing patterns occured only in the two-pulse experiments.

FIGURE 3. TYPICAL PATTERNS OF RECEPTOR ACTIVITY OVER A TIME COURSE

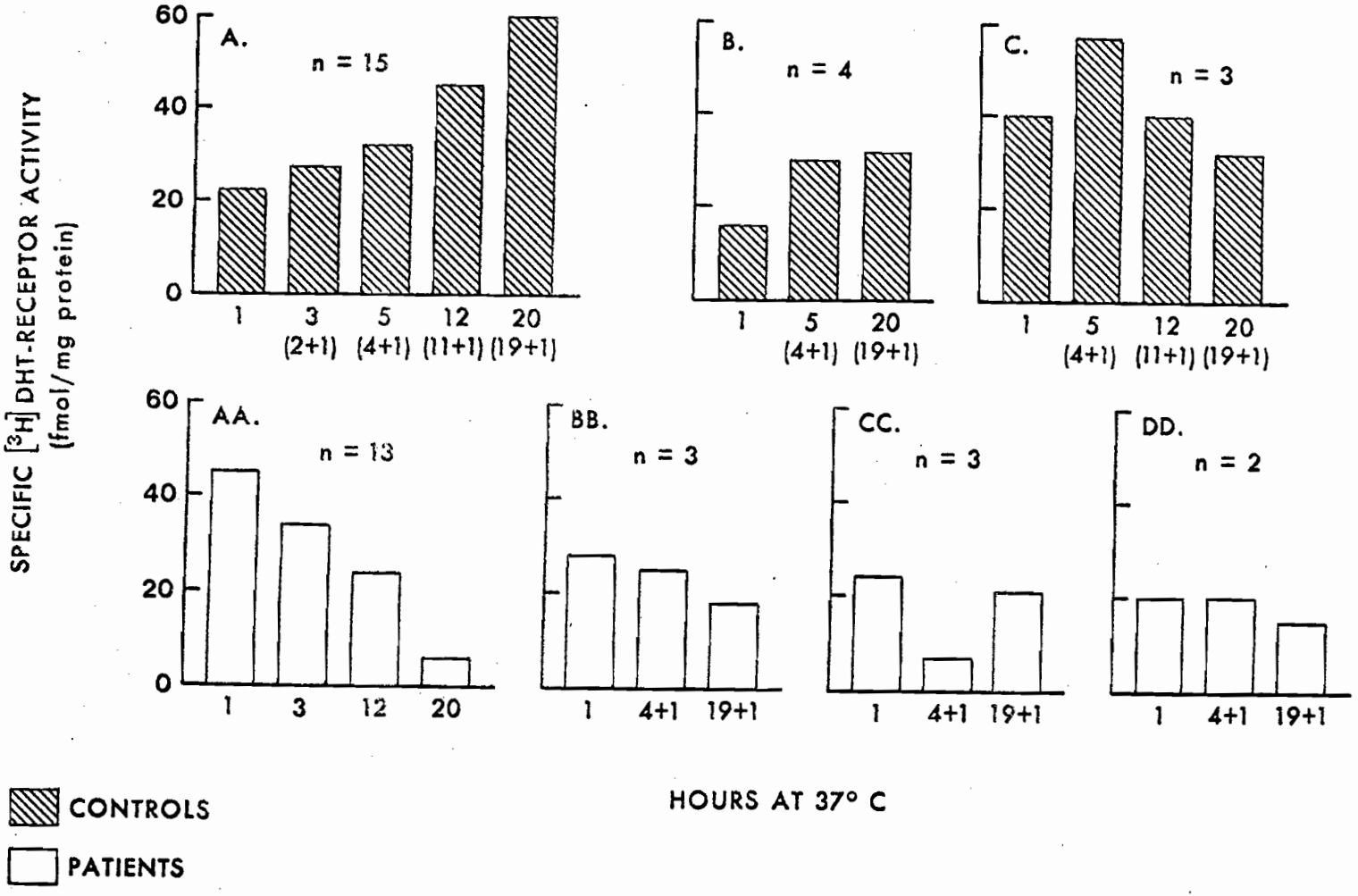


Figure 4 Comparisons between representative patterns of "up-regulation" in control strains at 37°C (Two-Pulse Protocol) and their corresponding behavior at 27°C is shown.

FIGURE 4. EFFECT OF TEMPERATURE ON "UP-REGULATION" IN CONTROL STRAINS

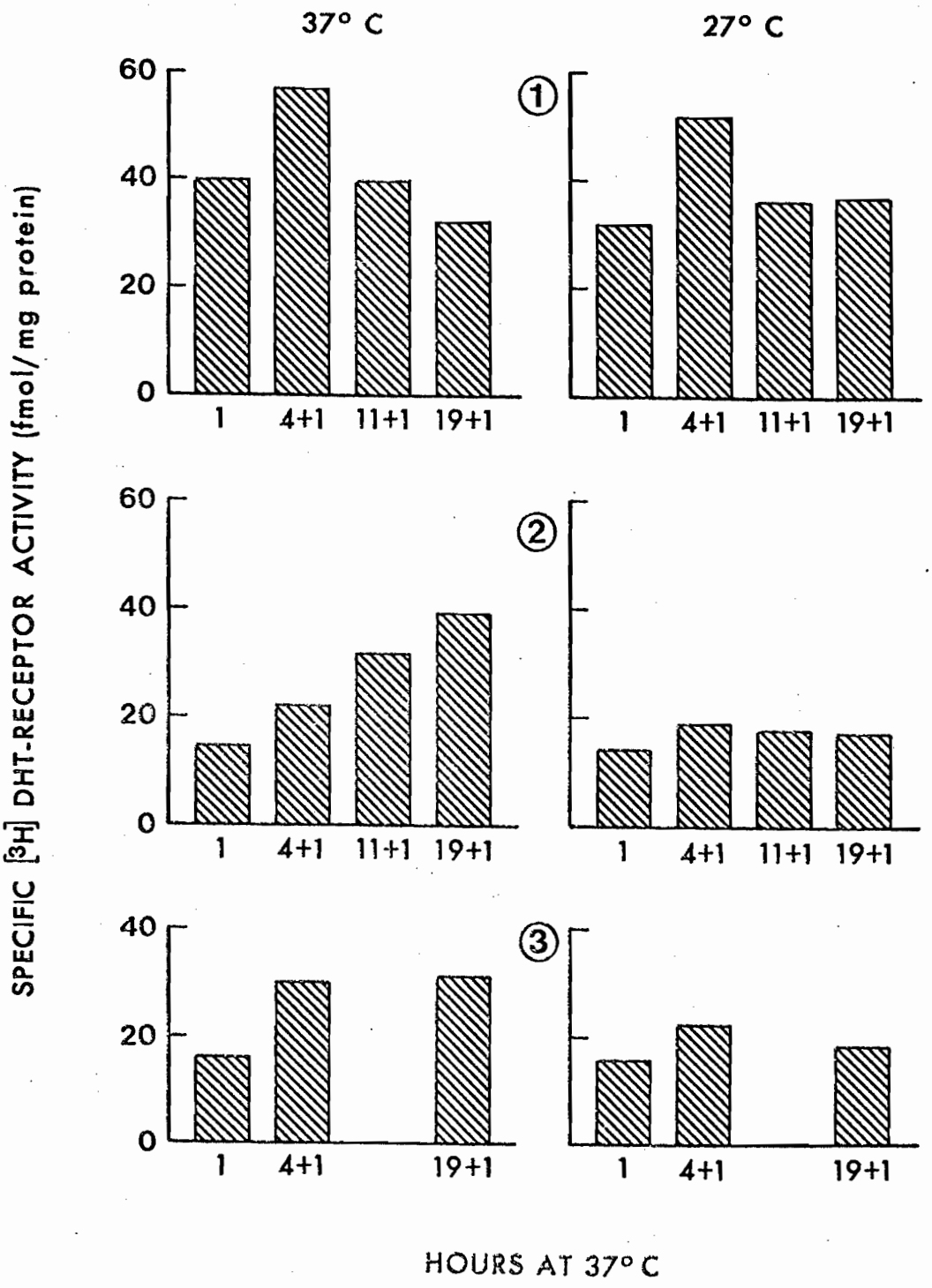
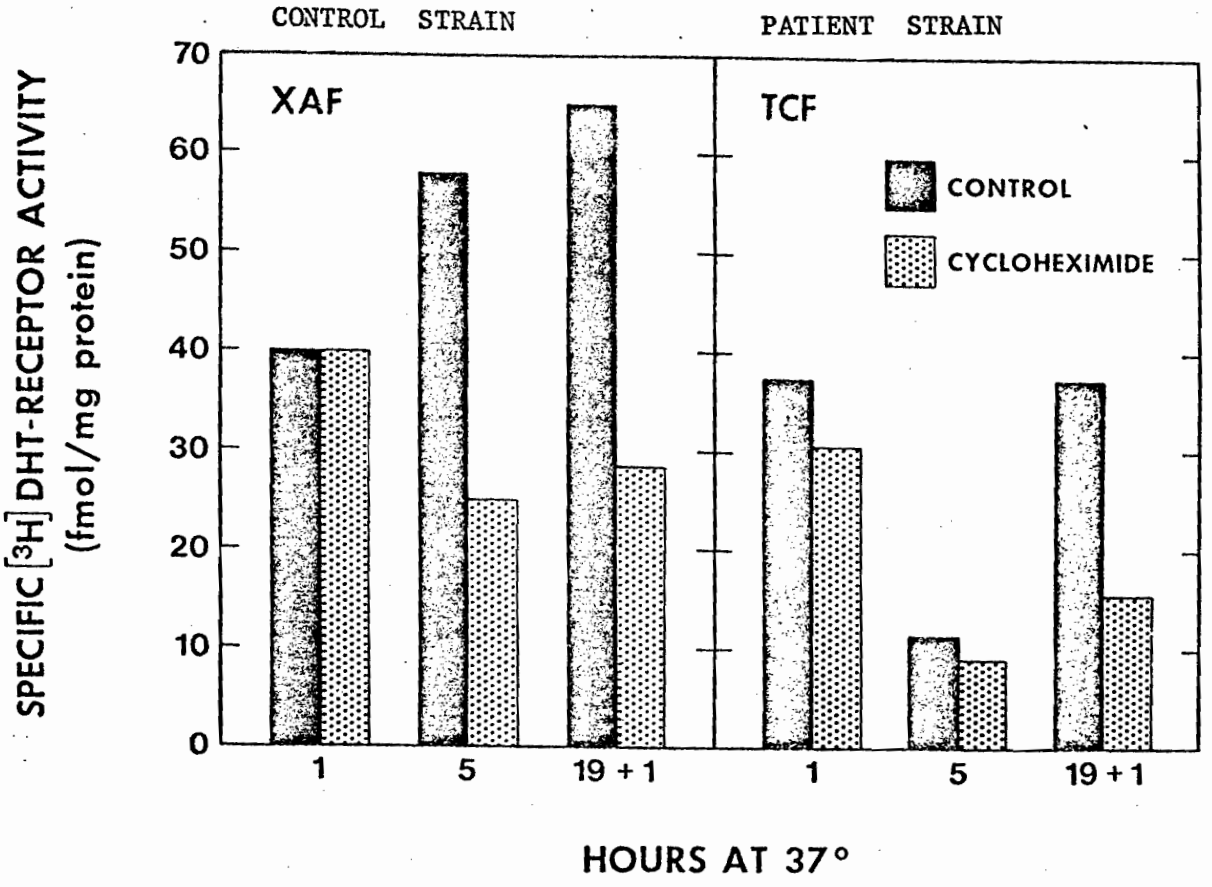


Figure 5 Fibroblasts were incubated with or without 2μM cycloheximide and with ^{3}H-DHT for prolonged periods of time. Figure 5 shows the representative behavior of a control strain which normally "up-regulates", and a patient strain, which does not normally "up-regulate", when incubated with cycloheximide.

FIGURE 5. EFFECT OF CYCLOHEXIMIDE ON "UP-REGULATION" IN CONTROL AND PATIENT STRAINS



V. DISCUSSION

These results demonstrate, to the best of my knowledge, the first instance of up-regulation of a normal hormone receptor by one of its natural ligands, *in vitro*; and the first case of *in vitro* ligand-induced regulation of an androgen receptor. Equally important is the demonstration of the mutant response of the fibroblast receptors of four unrelated patients to the stimulus of chronic exposure to androgen. This response serves as an *in vitro* marker for the *in vivo* androgen resistance of these patients.

1. Characteristics of Up-Regulation

Up-regulation of the androgen receptor in normal human skin fibroblasts is time- and temperature-dependent, as well as cycloheximide-suppressible. Although preliminary experiments done in this laboratory indicate that it is not concentration-dependent between 3-10 nM (L. Pinsky, and M. Kaufman, private communication), further work must be done to positively rule out concentration-dependency.

Androgen up-regulation requires a chronic exposure to its ligand. A significant increase in specific receptor activity is not obvious between 30 and 60 minutes after exposure to DHT, and does not become obvious until at least 2-3 hours after exposure, increasing thereafter with time.

Decreased temperature diminishes the regulatory effect. Androgen receptors are increased to a higher level at 37°C than at lower temperatures. An increase in receptor activity, however slight, occuring

at 27°C is observed after 5 hours of incubation with DHT, after which it stabilizes at a lower level. This pattern of increase at 27°C remains consistent from experiment to experiment, no matter what the pattern of increase at 37°C. Thus, decreased temperature limits the up-regulatory trend in normal human fibroblasts.

The fact that up-regulation is suppressible by a protein synthesis inhibitor indicates that it involves an enlarged pool of receptors. The fact that receptor activity, in the presence of cycloheximide, decreases over time instead of remaining constant, suggests that there is a regulated balance between receptor synthesis and degradation, or "processing". ("Processing", as defined by Horwitz et al. (66) for the estrogen-receptor system in the MCF-7 human breast cancer cell line, refers to the turnover of receptor protein after translocation to the nucleus.) It is possible that over an extended period of time, one or the other dominates, but in the prolonged presence of androgen, an increased amount of receptor synthesis is induced. If receptor turnover or "processing" is enzymatic in nature, cycloheximide might decrease receptor degradation as well as blocking synthesis, but I feel that it is probably receptor synthesis that is responsible for up-regulation. However, the actual extent to which up-regulation is due to de novo receptor synthesis, or decreased receptor degradation remains to be determined experimentally.

One interesting observation was that the nonspecific binding activity increased at each time interval by proportionately the same amount as did total receptor activity, resulting in a net increase

of specific receptor activity (results not shown). This could probably be explained by an enlarged pool of low-affinity as well as high-affinity proteins, either due to increased protein synthesis, or to decreased protein degradation. An interesting alternate possibility is that the initial steps in specific receptor synthesis is the synthesis of low-affinity, nonspecific binding proteins, which are subsequently modified stereochemically to become specific high-affinity receptors.

2. Daily Fluctuations in Basal Receptor Activity

In order to elucidate what specific factors were involved in the day-to-day variation of the basal receptor activity, I tried to change certain assay conditions. These included: (i) varying the pH of the system; (ii) incubating the fibroblasts with serum-free medium for various lengths of time before the assay, i.e. depleting the ligand from the medium, and (iii) measuring the recovery of receptor activity in the presence of serum (but no additional ligand) after treatment with cycloheximide for 18 hours, at 42°C.

Variations of pH within the buffering range of HEPES (6.8-8.3), had no effect on receptor activities (three experiments, results not shown). Preincubation with serum-free medium for various periods of time did not result in consistent changes in receptor activity (results not shown). Treatment with cycloheximide for 18 hours decreased the receptor activity to 30-40% of the original activity. Within 6 hours after the removal of cycloheximide, receptor activity increased to approximately 60% of the original activity (Table 5), after which receptor activity increased, decreased, or stayed the same.

The blockage of receptor synthesis by cycloheximide allowed me to estimate the amount of degradation, or processing, occurring in the cells. This may have been underestimated if cycloheximide inhibits cell degradation as well as protein synthesis. Assuming that synthesis and degradation are balanced to give a steady state level of basal receptor activity in the absence of cycloheximide, one would expect removal of the cycloheximide block to result in the resumption of receptor synthesis to the extent where it would balance degradation once more after 18 hours or more, depending on whether there is a time factor necessary for the cycloheximide to wear off after its removal from the cellular environment, and whether the amount of degradation is actually greater than estimated. It should thereafter maintain a constant level of activity at approximately 40% of its original activity. Instead, I found an even greater increase in receptor activity to about 60% of the original activity within 6 hours. Thus, an over-compensating increase in the rate of receptor synthesis occurs at 42°C.

The possibility that this may be an androgen-dependent mechanism, dependent on the amount of androgen present in the medium seems unlikely, as the amount of androgen in the serum has been measured, and found to be negligible (32). It has also been reported in the literature (35) that 4 days of incubation with serum-free medium results in no change in the binding activity of the fibroblasts, indicating that the presence of androgen in the serum is not sufficient to make a difference in the binding activity over a period of 4 days.

A more likely explanation of the overcompensating increase in receptor activity is that a separate androgen-independent mechanism, induced by the excessive depletion of receptor activity and the disruption of the synthesis-degradation balance at an increased temperature, is responsible for the increase in receptor activity. Thus, receptor synthesis appears to be subject to two types of synthesis mechanisms; one, androgen-independent, responsible for the basal receptor activity measured in a standard 1 hour assay, and responding to various stimuli, such as that described above; the other, androgen-dependent, responsible for up-regulation.

Although I did not identify all the factors responsible for the day-to-day fluctuation of androgen receptor activity in a single strain, the present results suggest that such fluctuation of receptor activity may be dependent on the balance between receptor synthesis and receptor degradation, which, in turn, is dependent on a complicated network of regulatory controls, one being ligand-induced receptor synthesis.

3. Mechanism of Up-Regulation

The mechanism of androgen-induced receptor synthesis is not completely known. Nor is it known what the effector is. It may be the hormone itself, or its derivatives; the hormone-receptor complex, or one of its derivatives; or simply a depletion of free receptor.

An increased amount of circulating ligand usually exerts a homeostatic negative-feedback control on the level of its own receptor

activity. Thus, a reduced biological response compensates for the high circulating levels of ligand. This has been documented for the insulin (67), human growth hormone (68), and low-density lipoprotein receptors (69). There is little precedent for positive-feedback control of receptor activity by high circulating levels of ligand, and it seems more likely that the effector in this system is either the hormone-receptor complexes or the depletion of free receptor.

The raising of a specific androgen-receptor antibody would give us a tool with which to determine what the effector in up-regulation actually is. Through receptor-antibody agglutination, one could compare the number of receptors present after chronic exposure to a saturating concentration of ligand with the number of receptors present before. The formation of a ligand-receptor complex could be prevented with the addition of an excess of antiandrogens, such as cyproterone acetate, or estradiol, which do not up-regulate. If the number of receptors after exposure are increased only without the addition of competing hormone, then one could conclude that the hormone-receptor complex is the effector. In the meantime, one can only speculate.

4. Ligand-Induced Regulation in the Literature

There is conflicting data in the literature on the ligand-induced regulation of androgen receptors *in vivo*. Some authors have reported that receptor activity in rat prostate or epididymis decreases to undetectable levels after orchiectomy. This decrease can be prevented by the administration of androgen immediately after orchiectomy, or

reversed by a later course of androgen treatment (70,71).

Others have denied androgen control of receptor activity and claimed that the decrease noted in receptor activity in the rat prostate after castration is followed by an androgen-independent increase after 4 days (72). Disappearance of receptor activity in rat epididymis after orchiectomy was later claimed to be an artifact (73), as the results of the first experiment could not be repeated.

One recent study on androgen-receptor activity in human skin cytosol concluded that androgens do not regulate their own receptor in that organ. This was based on the parity of values in receptor activity between men and women, old and young, despite differences in circulating androgen levels (74). However, the androgen-receptor concentration was shown to vary with the anatomical site, being highest in genital skin. This is likely to be a constitutive difference in the number of native androgen receptors at different anatomical sites. As these experiments were not repeated within a single individual over a specific course of time, however, there is not sufficient evidence to prove that ligand-induced regulation in this system does not occur within any single individual.

Further research must be done to clarify the issue of androgen regulation of its receptor in various target tissues in vivo. In light of our findings, in vitro, it does seem likely, however, that some form of in vivo androgenic regulation of the androgen-receptor does exist as does estrogenic regulation of the estrogen-receptor (75).

(In response to daily estradiol injections, normal adult ovariectomized mice show gradual increases in their uterine cytoplasmic receptor levels.)

Regulation of a receptor by its ligand, in vitro, has been demonstrated with polypeptide hormones (76,77,67,68), primarily with insulin. In these cases, chronic exposure to a high enough concentration of the ligand results in down-regulation, a reciprocal decrease in the receptor activity. The phenomenon is time-, temperature, and concentration-dependent.

It has been stated in the past that androgen-receptor activity in human genital skin fibroblasts is not regulated by its ligand (32). This statement was based on the fact that patients with 5α-reductase deficiency (who, thereby, have little or no circulating DHT) do show normal amounts of DHT-receptor binding in cell monolayer. In light of our results which show that a 5α-reductase patient up-regulated normally (Table 1), this reasoning must be incorrect. As has been discussed above--there may be more than one stimulus which induces receptor synthesis.

There does seem to be a retrospective hint in the literature that up-regulation of the androgen-receptor in cultured human fibroblasts does exist. Griffin, in a paper on the thermolability of the androgen-receptor of some androgen insensitive patients (59), compared receptor binding at 26°C for 16 hours to that at 37°C for 1 hour. The binding after 16 hours increased by approximately 50% of the 1 hour values. The increase in binding was attributed to stabilization of

receptor activity at the lower temperature. The reason given for the prolonged incubation at 26°C, was that this was the amount of time necessary for binding to reach equilibrium. In comparing time-course studies with ^{3}H-DHT at 37°C and 27°C (Fig. 4), I found that, contrary to Griffin's findings, receptor binding after 1 hour was similar at both temperatures, although binding after 20 hours was lower at 27°C. Thus, it is possible that the 50% increase in binding after 16 hours was due to DHT-induced up-regulation and not to the sparing effect of the lower temperature. Had binding after 16 hours been measured at 37°C, receptor activity might have been observed to up-regulate in normal cells.

5. Defects in Up-Regulation

In contrast to the control strains, receptor-positive patient strains did not exhibit any increase in receptor activity after a two-pulse, 20 hour exposure to DHT. In further contrast, they lost all or most of their activity after a one-pulse, 20 hour exposure. This was true of four different patient strains, three with a partial clinical phenotype, and one with the complete clinical phenotype. It was not possible to correlate the extent of the molecular defect with the extent of the clinical defect. Thus, the lack of up-regulation at 19+1 hours, or down-regulation at 20 hours, serves as a marker for all four patients.

Although the differences between the receptor activities of the various patients were not statistically significant, the trend seemed

to show KIL, the completely androgen insensitive patient strain, as having the least receptor activity after 19+1 hours, and 99900, a partially AI patient strain, as having the most (Table 3).

Two of the patients, KIL and TCF (partially AI) were shown to have defects in their thermolability when assayed at 42°C, and in their higher dissociation rates in monolayer (63), in addition to their up-regulatory defect. The receptors in cell free extracts of both patients were shown to dissociate with biphasic kinetics, as opposed to those of controls, which dissociated with monophasic kinetics, as in monolayer (M. Kaufman, and L. Pinsky, Cell. Molec. Endocrinol., in press). The two phases in the patient strains included a slowly dissociating component, presumed to represent the "activated" receptor, and a more rapidly dissociating component, presumed to represent the "pre-activated" receptor. In intact monolayers, patients dissociate with monophasic kinetics, representing only a rapidly dissociating phase, approximately 3-fold greater than that of controls in monolayer. The defect that was postulated to account for these data, was the inability of the patients' receptors to become activated prior to translocation in intact cells--a defect which could be rectified by removing the mutant androgen-receptor complexes from their cellular microenvironment.

The other two patient strains, 14679 and 99900, both partially insensitive, did not display the dissociative defect. Their failure to up-regulate is the only marker of their respective mutation(s) discovered so far.

The fact that TCF, 14679, and 99900 share only the up-regulatory defect, but not the dissociative defect, indicates that different mutations are responsible for similar clinical states of partial AI. The fact that TCF and KIL share their thermolability and dissociative defects but not their clinical defects indicates that this might be a general secondary expression of different primary mutations. Further support for a general secondary expression of different mutations, lies in the fact that both TCF and KIL share only their up-regulatory defect, but not their dissociative defect with 99900 and 14679. It is conceivable that all mutations affecting the androgen-receptor protein affect the up-regulatory site, resulting in a lack of up-regulation in patients. Other mutations might express themselves as thermolability and an inability to activate, as well as a failure to up-regulate. Depending on what structural part of the androgen-receptor protein is mutated, different manifestations of the mutation are identifiable. KIL, which shows a greater clinical defect than TCF, presumably has additional manifestations of its primary mutation which we have not yet identified, and which are not shared by TCF or any of the other patients.

Without the addition of DHT 1 hour before the final steps of the assay, patient strains lose almost all their receptor activity. Within 1 hour, control cells will metabolize 15-25% of the DHT in the medium (32,33). This is similar to the amount metabolized by patient strains over the same period of time (32). After 20 hours, it is reasonable to expect that most of the DHT in controls and patients is metabolized. Nevertheless, control cells increase their receptor activity after 20 hours

even without the addition of DHT, whereas patient cells lose almost all their activity. This may indicate that in controls, enough DHT remains in the medium to bind to the receptors, but in the patient strains DHT is hypermetabolized. The difference between control strains given one or two pulses of DHT is slight, and is probably due to DHT catabolism. Yet, this catabolism does not prevent controls from increasing receptor activity. The differences in receptor activity in patients given one or two pulses of DHT, cannot be accounted for by the same amount of metabolism. Thus, it appears as if there is excessive catabolism of DHT in patient strains. It remains to be determined, however, whether the correlation of excessive catabolism of DHT and "down-regulation" is causal or coeval.

Metabolism of DHT follows one of two pathways (77): (i) DHT to androstanedione, which is, in turn, converted to androsterone; and (ii) DHT to androstanediol, which is interconvertable with androsterone. There has been no evidence of any differences in the pathway or rates of metabolism between males and females, patients and controls. However, more definitive studies must be done on the qualitative and quantitative aspects of the metabolism of DHT in patients and controls.

VI. CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. Genital skin fibroblasts from individuals with no history or symptoms of androgen insensitivity or related problems, showed a progressive increase in specific DHT-receptor activity over time, during prolonged incubation with DHT.

2. Fibroblasts from four unrelated receptor-positive androgen insensitive patients showed no such increase.

3. When given two pulses of DHT over 20 hours i.e. 19 hours with one pulse, plus 1 hour with the second pulse, DHT-receptor activity in control strains rose to 200% of their basal (1 hour) activity, whereas patient strains showed 70-100% of their basal activity.

4. When given only one pulse of DHT for 20 hours, DHT-receptor activity of controls increased to 150% of their basal activity, whereas the activity of patient strains decreased to 0 or almost 0.

5. One patient with 5α-reductase deficiency, behaved as a control, and showed an increase in receptor activity after 20 hours.

6. The increase in receptor activity in controls was time-dependent, temperature-dependent, and suppressible with cycloheximide.

7. The basal receptor activity of controls measured after 1 hour with DHT, was similar at 37°C and at 27°C.

8. The receptor activity of controls measured after 20 hours with DHT, was higher at 37°C than at 27°C.

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