Chapter 5

Genetics of Steroid Receptors and Their Disorders

Leonard Pinsky and Morris Kaufman
The Lady Davis Institute for Medical Research
Sir Mortimer B. Davis–Jewish General Hospital
and Centre for Human Genetics
Departments of Pediatrics and Biology
McGill University
Montreal, Quebec, Canada H3T 1E2

INTRODUCTION

It is axiomatic that the extraordinary diversity in form and function of cells, tissues, and organs within a higher organism results not from differences in their genomic composition, but from the finely tuned selective expression of portions of their common genetic repertoires. The fact that steroids act as gene-regulating molecules, and that they do so, for the most part, by acting as allosteric modifiers of their respective receptor (R) proteins, has focused much attention on the manner in which steroid–receptor (S–R) complexes act to regulate gene expression, particularly at the level of gene transcription.

This chapter will deal with heritable germ-line and somatic alterations in the receptor-dependent portions of the molecular apparatus that underlies normal responsiveness in cells that are targets of steroid action. So defined, it will encompass variation in the structure–function attributes of steroid receptor (SR) proteins themselves, in "factors" (more or less well-defined) that modulate SR activity, and in cellular (specifically nuclear) components that cooperate with S–R complexes to effect steroid action.

Mutations that confer congenital androgen resistance (insensitivity)

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in man are by far the richest source of heritable germ-line variation in any SR system. We will discuss clinical, endocrine, and cultured skin fibroblast studies of such mutations in detail because they have contributed importantly and in several ways to knowledge of steroid-response systems in general (Pinsky, 1978; T. R. Brown and Migeon, 1981). First, they have revealed features that are common to the various androgen-target organs of the body. Second, they have exposed appreciable regional and temporal differences among such organs that can and need to be explored further. Third, they have enabled the recognition of some of the discrete, sequential steps that enable an androgenic steroid to evoke a specific response (or set of responses) from a target cell. Finally, they have pointed the way toward dissecting the remaining steps in the entire sequence, a task that will be greatly facilitated by the powerful techniques of modern molecular genetics. In addition, the aggregate body of knowledge that has accrued from the study of single, major-gene (Mendelian) mutations will inevitably help to understand androgen-response variation in the population that is multifactorial (partly genetic, partly environmental) in origin, whether such variation is deemed to be in or out of the normal range.

Mutations that cause various T-lymphoma cell lines to resist glucocorticoid-induced lysis have been another important genetic source of information about steroid-response systems. In addition, they have great potential relevance for improving the clinical management of lymphoproliferative disease. For these two reasons, we will also treat such mutations in some depth.

To provide a background, we begin with a general overview of steroids, SR proteins, S–R complexes, and steroid-hormone resistance.

BACKGROUND

Steroids

Evolutionarily Ancient Messengers

It is known that molecules structurally related to the steroid hormones are found in some prokaryotes and in most primitive eukaryotes. Thus, the mechanism for steroid biosynthesis was an early feature of evolution. The roles played by such "early" steroids are mostly unknown.

Sandor and Mehdi (1979) hold that some such early steroids had intracellular roles as "bioregulators," much in the fashion currently ascribed to autocrine substances, and in the case of *Pseudomonas testosteroni*, such a role has been documented (Watanabe and Watanabe, 1974). Karlson (1983) has suggested that such steroids initially had a structural role, and that a pre- or corequisite step in their ultimate adoption of a messenger (hormonal) role was their acquisition of autoregulatory control, that is, control of their own bioactivity, possibly by feedback inhibition of rate-limiting enzymes involved in their own biosynthesis. The ultimate question is: How did steroid molecules become recruited to the service of regulating transcription primarily and other steps in protein synthesis to lesser and variable degrees?

Karlson (1983) speculates that with the advent of oxygen in the atmosphere, cholesterol was " . . . invented and put to use for the control of the fluidity of lipid bilayers". With time a few hydroxylations would have made it sufficiently water-soluble for transport, yet not so lipid-(membrane-) insoluble that it could not be taken up by other cells. According to such a formulation, ecdysone has the structural attributes that might well have made it the first true steroid hormone, an hypothesis that fits with the fact that ecdysteroids are the steroid hormones of the protostomia. Figure 1 shows that the next step in the evolution of steroids, occurring either at the level of the archecelomates or the primitive chordates, might have been cleavage of the cholesterol side chain to yield a pregnane derivative, from which the progesterones might have quickly appeared. In the teleosts, 17-hydroxyprogesterone and one of its 20β-dihydroxy derivatives are used for oocyte maturation.

The most primitive of the corticosteroids, corticosterone, is found in the interrenal tissue of fish, and has been adapted for the control of sodium and potassium balance. Aldosterone and cortisol are differently specialized versions of corticosterone. Further cleavage of the side chain of 17-hydroxyprogesterone would give androstenedione, the immediate precursor of all natural androgens and estrogens.

Karlson (1983) believes that steroids, as a class, do not have structural properties that uniquely fit them for a protein-binding role. Rather, he suggests that other classes of small molecules, such as nucleotides and carbohydrates, had already been recruited for major regulatory roles early in the evolutionary biology of cells, and that steroids, once found accidentally to be able to serve as regulatory signals in partnership with proteins, were adapted by evolutionary divergence for the various discrete

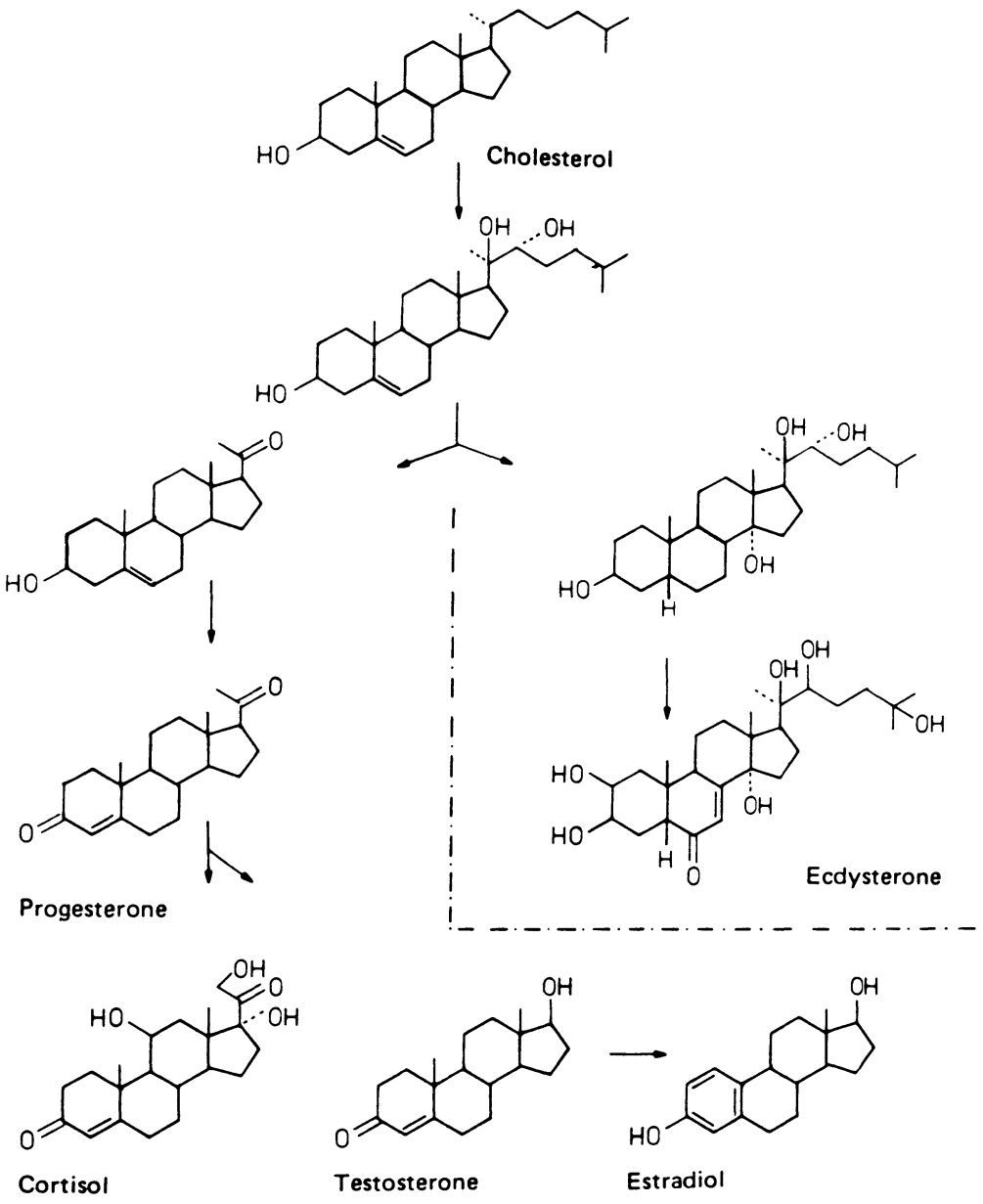


Fig. 1. An evolutionary scheme for the origin of steroid hormones according to Karlson (1983).

purposes that we now know them to serve in higher animals. It is important to point out that steroid hormones *per se* can bind to native DNA (Arya and Yang, 1975) and aminoacyl tRNAs (Chin and Kidson, 1971), and for prokaryotes, such an interaction might have sufficed to allow steroids to function as primitive bioregulators. On the other hand, SR proteins would have been a "logical" way for adaptive evolution to di-

versify the initially primitive regulatory roles of steroids into the manifold, highly specialized functions they now have in higher eukaryotes.

It is appropriate to point out here that we are currently ignorant about what precisely is the structure-function mechanism by which the partnership of a steroid and its receptor is able to endow the complex with the ability to act as an exquisitely specific signal for transcriptional regulation. In evolutionary terms one is entitled to ask the following "chicken-and-egg" question: Has the primitive regulatory information endowed in a steroid been modulated by its binding to a receptor? Or, conversely, has the primary regulatory content of a particular protein been modified by its assumption of a receptor-binding role toward a specific steroid? In either event, it is tempting to suggest that the earliest SR proteins were adapted from regulatory components of the earliest forms of RNA polymerase II, from proteins that transport steroids in the circulation or that facilitate their transfer across cell membranes, or even from enzymes involved in steroid metabolism. In the case of the last three suggestions, the acquisition by the protein of a DNA-binding domain by some form of genetic recombination would have been a mandatory step in its evolution as a SR. Thereafter duplication of the locus encoding the original SR, recombination, and divergence would have conspired to generate a family of related SR proteins, in much the same way as other families of genes and their products have been shown to have evolved.

Despite virtual certainty that this postulate is correct, it is notable that most of the antisera raised against highly purified preparations of the rat liver glucocorticoid receptor (GR) (Okret *et al.*, 1981; Eisen, 1982; Westphal *et al.*, 1982) do not cross react with the androgen receptor (AR) in rat prostate or the estrogen receptor (ER) or progesterone receptor (PR) of rat uterus. Moreover, most of these antisera recognize a 40,000–50,000 dalton, non-steroid-, non-DNA-binding fragment of the GR that has been assigned a modulatory, transducing role in GR action. This indicates clearly that evolutionary divergence of different SR proteins from one (or more) communal precursors has occurred not only in their discrete steroid- and DNA-binding domains, but also in relation to that still scarcely understood domain of a GR that is indispensable for it to accomplish its specific regulatory roles even after it has bound a glucocorticoid and the resulting G–R complex has found a particular portion of DNA with which to interact.

There is cogent evidence in favor of the idea that evolution of SR, as we know them in higher vertebrates, involved adaptation of preexisting

molecules. Androgen- and estrogen-binding proteins are found in the periplasm of the bacterium, *Pseudomonas testosteroni*, and displacement studies have suggested that it has two kinds of steroid receptors, one for C19 and C21 steroids, and another for 17β-estradiol (Watanabe and Watanabe, 1974). Feldman *et al.* (1982*a*) found that the unicellular eukaryote, *Saccharomyces cerevisiae*, has a protein that binds 17β-estradiol with high affinity, and an endogenous ligand that can bind to mammalian ER. GR exist in the intestine of eel (DiBattista *et al.*, 1983) and in the gill tissue of fish (Sandor *et al.*, 1984). And, not surprisingly, AR have been found in the thumb pad (a male secondary sex character) of the Green Frog, *Rana esculenta* (d'Istria *et al.*, 1979). Indeed, a low-molecular weight factor that inhibits the activity of mammalian G-R complexes has been found in such varied eukaryotes as yeast and lobster (Leach *et al.*, 1982).

Structure–Function Classes of Steroids

The structures of various steroid hormones from animals and plants are shown in Fig. 2. The sex hormones and the glucocorticoids (represented by cortisol) have well-known functions in morphogenetic and histogenetic differentiation and in postdifferentiative function of various organs. Aldosterone, the representative of mineralocorticoids, and calcitriol have equally well-known functions in sodium/potassium and calcium homeostasis, respectively. Judging from the structure of calcitriol (1α,25-dihydroxycholecalciferol), it is not surprising that it uses a typical SR to function as the regulator of transcription for a transport protein of calcium in the gut and of a calcium-binding protein in bone.

The structure of ecdysone, a regulator of moulting in insects (Berger and Morganelli, 1984), is closer to that of cholesterol than to any of the "classical" steroid hormones. Ecdysteroids are also found in plants, where, except for brassinolide (structure 14, Fig. 2), a growth-promoting hormone (Grove *et al.*, 1979), their functions are mostly unknown. Steroids (structures 12 and 13, Fig. 2) related to ecdysteroids are found in the ascomycete *Achylia ambisexualis*. where they act on differentiation of sexual organs (Horgen, 1981; Timberlake and Orr, 1984).

It is of considerable interest that the prothoracic gland of insect larvae produces principally ecdysone, and that its conversion to a more active derivative, 20-hydroxyecdysone, occurs in peripheral tissues (Koolman, 1982). This parallels the greater activity of 25-hydroxycholecalciferol once

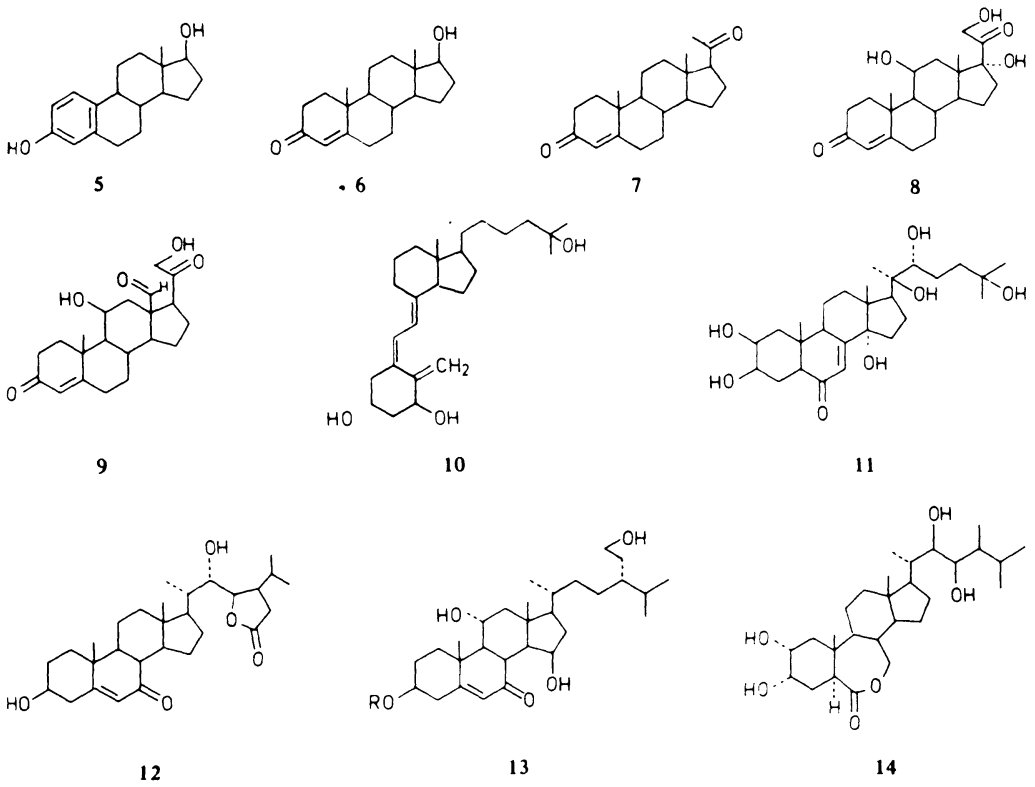


Fig. 2. Structural formulas of steroid hormones from animals and plants. (5) Estradiol; (6) testosterone; (7) progesterone; (8) cortisol; (9) aldosterone; (10) calcitriol; (11) 20-hydroxyecdysone (ecdysterone); (12) antheridiol; (13) oogoniol; (14) brassinolide. [From Karlson (1983) with permission.]

it is converted to its 1α,25-hydroxy derivative in kidney and the greater androgenic potency of testosterone when it is converted to 5α-dihydrotestosterone in skin and various peripheral androgen target organs.

Steroid Receptors

Basic Structure–Function Properties of Steroid Receptor Proteins (Fig. 3)

Steroid receptors are intracellular proteins synthesized preferentially by target cells—cells that respond to the action of particular steroid hormones. The molecular mechanism that enables various cells to differentiate as targets for the action of one or more specific steroid hormones is not understood. In some cells (tissues, organs) steroid hormones themselves are essential for terminal differentiation of the steroid-responsive

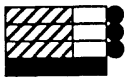
Form	Model	$S_{20,w}$ S	R_S nm	M_r x 10^{-3}	Axial ratio†
Tetramer		9-10	8-9	320-350	12-13
Monomer - RNA		6-8	~6	~130	—
Monomer		~4	5.3-6	90-110	12-14
Intermediate Fragment		3-4	2.5-4	40-70	3-7
Meroreceptor		2-3	1.9-2.4	20-25	1-3

Fig. 3. The structure–function attributes of a generic steroid receptor protein. Solid circle, steroid-binding domain, the meroreceptor; open box, DNA-binding domain; hatched rectangle, domain that modulates DNA binding (as defined in the GR); solid rectangle, non-steroid-binding monomer; stippled rectangle, RNA; axial ratio is calculated assuming a prolate ellipsoid. [Adapted from Sherman and Stevens (1984).]

state, as the latter is measured by steroid induction of specific products in mature target cells. For instance, morphogenesis of the mammalian male reproductive apparatus is under the essential control of androgenic steroids, just as postdifferentiative function of all parts of that apparatus is controlled by the same steroids.

Two properties of SR proteins have been major hindrances to the acquisition of a complete body of knowledge about them: they are sparse even within target cells (10,000–20,000 molecules per cell is a typical upper range); and unliganded (free) SR are very labile outside cells. These properties made it difficult to purify native SR according to modern standards of protein physical chemistry. Therefore, until recently, much that was known about SR proteins was inferred from the behavior of their respective S–R complexes within cells or tissues, or in partially pure subcellular preparations that were contaminated, more or less, by nucleic acids and nucleoproteins, and by enzymes of three types, nucleases, proteases and phosphatases.

Several technical advances have contributed to a major recent acceleration in understanding the structure-function relations of SR proteins. These include the synthesis of site-specific, covalent radioaffinity labels to overcome the easy dissociability of usual noncovalently-labelled

SR proteins, particularly under denaturing conditions, and of site-specific affinity matrices for "one-step" purification purposes, the development of monoclonal antibodies, and the application of recombinant nucleic acid technology for the direct analysis of SR genes or their respective cDNAs. In the past few years the use of these technical advances in various combinations has generated a clear picture of the structure-function relations of a typical SR protein (Fig. 3). When cytosolic* G–R complexes in low-ionic solutions containing 10 mM sodium molybdate are analyzed hydrodynamically, they have a Stokes radius (R_S) of 8–9 nm, a sedimentation coefficient ($S_{20,w}$) of 9–10 S, a molecular weight of 290,000–350,000, and an axial ratio of 12–13 (Sherman *et al.*, 1983; Sherman and Stevens, 1984). When the same preparations of G–R complexes are studied in the absence of molybdate, or in high-ionic media, and particularly in the presence of antiprotease "cocktails", an additional smaller form is seen that has an R_S of 5–6 nm, a molecular weight of 70,000–100,000, and other properties shown in Fig. 3. This must mean that molybdate stabilizes rather than generates the larger form (Holbrook *et al.*, 1984). It is known, in addition, that molybdate stabilizes free SR proteins, and keeps S–R complexes in their untransformed (low-DNA-affinity) state (Sherman *et al.*, 1983). How molybdate may do these two things, and the relation of the 5- to 6-nm form to the so-called "transformed" state of the S–R complex are discussed below.

The monomeric 4S steroid-binding protein has a linear structure–function arrangement that conforms with the "modular mode" of molecular evolution recently recognized repeatedly for various classes of proteins. Each has separate steroid- and DNA-binding domains (S. Green *et al.*, 1986; G. L. Greene *et al.*, 1986; Weinberger *et al.*, 1985), and a regulatory N-terminal portion has been recognized as well, but so far only in the GR protein. The N-terminal portion seems to be relatively immunogenic, and lacks steroid- or DNA-binding affinity; but, it plays an important role in modulating the affinity and productivity with which the GR binds to DNA, probably by exerting allosteric effects on the GR in virtue of its ability to interact with trans-acting transcriptional "factors". The existence and putative role of the N-terminal domain of the GR was

* Throughout this chapter, "cytosol" refers to the supernatant fraction following centrifugation of a cell, tissue or organ homogenate at 10^5 g for 1 hr, and not to the soluble portion of cytoplasm. Hence, the properties of a cytosolic SR or S–R complex are influenced by the conditions of homogenization and handling of the supernate before and during their analysis.

predicted by immunochemical studies on portions of the normal and the mutant forms of the GR produced by limit proteolysis (see p. 414). The recent evidence from recombinant DNA studies has been confirmatory. Interestingly, the ER protein, as inferred from its full-length cDNA, seems to have a much smaller domain corresponding to the N-terminal modulatory portion of the GR (S. Green *et al.*, 1986); the significance of this finding needs further evaluation. In the middle portions of the GR and ER, there is a ~60-residue long region that is rich in cysteine, lysine and arginine. This region is strongly homologous with a comparable portion of the erb A oncogene in the avian erythroblastosis virus (G. L. Greene *et al.*, 1986; Debuire *et al.*, 1984; Weinberger *et al.*, 1985) indicating first, that all three genes share an evolutionary origin, and second, that the erb A gene product is a DNA-binding protein, a property heretofore unknown. Interestingly, the erb A oncogene also lacks the N-terminal modulatory portion of the GR gene, and this lack has been implicated in its role as an agent of oncogenesis. The ER and GR share a proline-rich region, of unknown function, upstream of the cys-lys-arg segment, and more importantly, a C-terminal portion that is rich in hydrophobic aa residues and is responsible for steroid-binding. As Bishop (1986) has put it, "the means by which similar domains of a protein are nevertheless able to distinguish between glucocorticoids and estrogens pose marvelous puzzles for the crystallographer". Indeed, the identification of a steroidlike binding domain on the erb A oncogene product strongly implies that it has a specific steroid (or steroidlike) ligand, whereas none had previously been implicated in its action (Bishop, 1986).

Very recently, PR cDNA has been cloned (Loosfelt *et al.*, 1986), and used to detect an mRNA of ~5,900 nucleotides. Further studies of these clones will be very revealing, particularly in comprehending the origin of the two monomeric species of PR (A, 120,000 daltons; B, 94,000 daltons) that are found in various tissues of various species (Schrader *et al.*, 1981; Puri *et al.*, 1982; Suba *et al.*, 1986). Whatever the relation between the two PR proteins, their size in relation to the PR mRNA indicates that much of it is untranslated. This untranslated portion is probably at the 3′ end of the mRNA, since equally long 3′ untranslated regions have been found in ER and GR mRNA (G. L. Greene *et al.*, 1986).

In addition to these basic features of SR proteins, two new important facts have emerged whose significance remains enigmatic. First, the 9–10S moiety of S–R complexes is a heterooligomer, one component of which is equivalent to one of the well known heat shock proteins (hsp 90)

(Sanchez *et al.*, 1985; Housley *et al.*, 1985; Joab *et al.*, 1984). The stoichiometry of the oligomer is unclear, and it may not be a tetramer as originally modelled (Fig. 3) by Sherman and Stevens (1984). Second, there is an intermediate-size species of S-R complex (about 7 S) that contains RNA (Rowley *et al.*, 1986; Anderson and Tymoczko, 1985; Tymoczko and Lee, 1985; Tymoczko and Phillips, 1983). The existence of this species almost certainly explains the wide variation in $S_{20,w}$ values that has been reported for S–R complexes in low-ionic, molybdate-containing environments. The properties of this complex and the possible roles of RNA in S–R complex behavior and action are discussed further below.

Native Steroid Receptors May Exist in a Nonbinding (Inactive) State

N-Ethylmaleimide (NEM), a sulfhydryl (SH)-reactive reagent, eliminates steroid-binding capacity from cytosolic preparations of various steroid-target tissues. Glucocorticoid receptor activity is particularly liable to oxidation, and that in the lung is hardly measurable except with the addition of SH-reducing agents such as dithiothreitol (DTT). In contrast, liver cytosol has sufficient endogenous receptor-reducing activity that exogenous DTT is not enhancing. It has recently been proven that the endogenous, heat-stable, GR-activating factor in rat liver cytosol is thioredoxin, and that thioredoxin reductase together with NADPH is responsible for maintaining reduced thioredoxin levels (Grippo *et al.*, 1985). It is not yet known whether reduced thioredoxin must bind to the receptor in order for it to be activated to a steroid-binding conformation, or whether it simply reduces one or more disulfide bonds, the effect of which is to enable the receptor to adopt a steroid-binding state intrinsically. The following section discusses the relations among SH moieties, phosphate ions, and certain metal oxyanions in maintaining SR in their "active" or binding state (Dahmer *et al.*, 1984).

The GR in mouse L-929 cells (Housley and Pratt, 1983) and the PR in hen oviduct (Dougherty *et al.*, 1982) are proteins phosphorylated on one (or more) of their serine moieties. Phosphatases of various types inactivate the binding capacity of the GR in mouse L cells or rat liver cytosol (Housley *et al.*, 1982; C. J. Nielsen *et al.*, 1977), of the ER in rat uterine cytosol (Abou-Issa *et al.*, 1982), and of a low-affinity binding site on the chick oviduct PR (Maggi *et al.*, 1984). Several phosphatase inhibitors, such as fluoride, and some small phosphorylated substances, such

as glucose-1-phosphate, inhibit the endogenous, temperature-dependent form of inactivation that occurs for the GR and ER from various tissues; ATP can reactivate the binding capacity of GR in L-cell cytosol (Sando *et al.*, 1979) and of ER in rodent uterine cytosol (Abou-Issa *et al.*, 1982; Auricchio *et al.*, 1981). The AR of rat ventral prostate is rapidly deactivated by respiratory poisons such as 2,4-dinitrophenol, and reactivation is an energy-dependent, protein synthesis-independent process (Rossini and Liao, 1982). Together, these data indicate that phosphorylation promotes, and dephosphorylation inhibits, the binding activity of SR proteins. Further information is provided in the review by Carter-Su and Pratt (1984).

Another structural component of SR that is necessary for binding capacity is SH residues. This has been demonstrated particularly well for the GR (Rees and Bell, 1975), but it is true of all SR. Dithiothreitol can reactivate partially inactivated preparations of GR from various sources (Granberg and Ballard, 1977), and SH-oxidizing compounds, as pointed out above, are inactivating. Since GR can be labeled covalently with dexamethasone-21-mesylate, an affinity label that reacts with SH moieties, it is likely that the latter are positioned in or near the steroid-binding site (Eisen *et al.*, 1981).

Molybdate and other group IVA transition metal oxyanions, such as tungstate and vanadate, stabilize the binding activity of free receptors for all classes of steroids, including the sterol 1,25-dihydroxyvitamin D_3 (Leach *et al.*, 1979; Gaubert *et al.*, 1980; Krozowski and Murphy, 1981; Toft and Nishigori, 1979; Marver, 1980; Simpson and DeLuca, 1980), independently of their antiphosphatase activity. The most convincing evidence in favor of this conclusion is that molybdate must be present in order for phosphatase-inactivated preparations of SR to be subsequently reactivated by DTT (Housley *et al.*, 1982). The best interpretation of these data is that phosphorylation *per se* is not required for binding activity; rather, it helps to keep the SR in a state that is resistant to SH oxidation. Conversely, molybdate keeps the dephosphorylated receptor in a state that is susceptible to reactivation by DTT via S–S reduction. A model that visualizes this interpretation is presented in Fig. 4.

Intracellular Dynamics of Steroid Receptors

Just as the intracellular state of receptor proteins preceding their association to form S–R complexes is not well understood, so is their

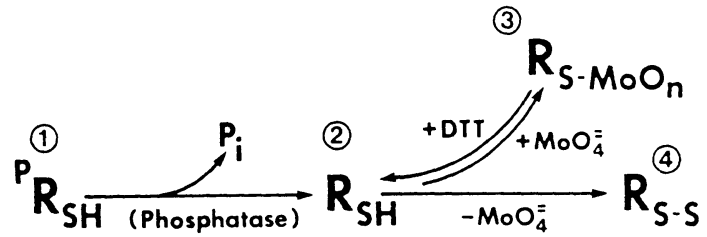


Fig. 4. A model of the various binding states of a steroid receptor in relation to its sulfhydryl (SH) and phosphate (P_i) groups and its interactions with dithiothreitol (DTT) and the molybdate ion (MoO_4^{2-}). States 1 and 2 are active, state 3 is inactive (nonbinding), but reversible by reduction. State 4 is irreversibly inactive. MoO_n indicates that its valence is unknown when it is bound to the receptor in state 3. [From Dahmer *et al.* (1983) with permission.]

intracellular fate once dissociation has occurred. For instance, it is not known whether a receptor is labile while still a component of a S–R complex, where S–R complexes dissociate, whether natural "small" or "large" molecular weight extra-receptor "factors" modulate their rate of dissociation, and whether the free receptor resulting from S–R complex dissociation (the "disliganded" receptor) is immediately, or ever, competent to associate with another steroid molecule. Such knowledge is crucial for understanding what terminates a SR-mediated response. In addition, steroids have been variously reported to increase or decrease the activity of their own cellular receptor or the receptor for other steroids, and it is already established that SR behave in different ways, in different cell types (Junker, 1983), with regard to each of these considerations. A brief survey of relevant data is given here so that the reader may appreciate their implications as potential markers of genetic variation.

When exposed to a given steroid a number of target cells respond by decreasing the homologous receptor activity. This is superficially similar to "down-regulation" of polypeptide and other cell-surface receptors, and has been shown to have diverse bases. In the case of estrogen target cells, where the response was originally called "processing" (Horwitz and McGuire, 1980), the decreased E–R activity results partly from a net loss of receptor protein (Eckert *et al.*, 1984) and partly from the appearance of chromatin-bound E–R complexes that are less extractable from nuclei and less dissociable than E–R complexes that newly appear in nuclei. The latter two properties account for an apparent loss of receptor activity when measured by an exchange assay. The same or similar mechanisms appear to explain the response of the 1,25-dihydroxyvitamin D_3 receptor in T47D human breast cancer cells upon incubation with the

vitamin (Sher *et al.*, 1985). By contrast, in the GH_1 rat pituitary (McIntyre and Samuels, 1985) and MCF-7 (Mullick and Katzenellenbogen, 1986) or T47D (Wei *et al.*, 1986) human breast cancer cell lines, exposure to glucocorticoid and progestin, respectively, causes a true decrease in the connate SR protein by virtue of the fact that it has a shorter half-life in the presence of steroid than in its absence. For the progestin – T47D system, true degradation of the PR protein in the nucleus has been demonstrated, and loss of hormone-binding capacity or recycling have been excluded (Wei *et al.*, 1986). Degradation may occur while the receptor protein is still complexed to a steroid, the view apparently favored by McIntyre and Samuels (1985). On the other hand, disliganded receptor might be more degradable, perhaps transiently, than a native receptor molecule that has never been liganded, or was so, but only remotely; recently reported data from our own laboratory (Kaufman *et al.*, 1986) support this contrary view. In any event, such disparate behavior could merely reflect the fact that a post-liganded receptor finds itself in a different (more proteolytic) cellular microenvironment than its never-liganded, or remotely liganded, counterpart. Nevertheless, it is perfectly reasonable to postulate that a post-liganded receptor may have a lower steroid-binding affinity than its never-liganded counterpart because of some endogenous, conformational imprint that it carries, perhaps transiently, as a consequence of its once having been part of a S–R complex. Such a receptor might be labelled "disactive" to distinguish it from an "inactive", biosynthetic precursor (proreceptor) that must be activated to attain the normal hormone-binding state. Indeed, we have recently recognized a class of mutant AR proteins (Kaufman *et al.*, 1986) that are more thermolabile, intracellularly, in their post- than in their pre-liganded state, indicating that the physical status or location of a once-liganded receptor can make an intrinsic contribution to its postliganded fate. Whether these observations and their provocative interpretations apply to the glucocorticoid-induced decrease of GR activity that is also observed in HeLa S_3 cells (Cidlowski and Cidlowski, 1981), the AtT-20 mouse pituitary tumor cell line (Svec and Rudis, 1981; Seigler and Svec, 1984), or human GSF (Berkowitz and Brown, 1986) is not known.

While glucocorticoid-induced loss of GR activity may be viewed as a means of terminating a glucocorticoid response, and therefore as being physiologically adaptive, it is noteworthy that glucocorticoid-induced depression of GR activity is the basis for resistance to glucocorticoid-induced lysis in a genetic variant of the W7 mouse T-lymphoma cell line

(Danielson and Stallcup, 1984). In other words, this variant has sufficient basal GR to initiate lysis, but the lytic response is aborted in the face of the ensuing down-regulation. Short-term exposure to mineralocorticoids (Claire *et al.*, 1981) and progesterone (Mockus and Horwitz, 1983) also induces decreased activity of their respective receptors, but this is not true for androgens. In fact, in MCF-7 human breast cancer cells, the ones originally used to demonstrate so-called "processing" of ER, androgens (acting through their own receptor) inhibit estrogen induction of PR activity. In so doing, the A–R complexes show neither of the features that characterize E–R processing (E. Shapiro and Lippman, 1985).

There is additional evidence that androgens behave differently from other steroids in the way they regulate their receptors. Thus, T rapidly increases AR activity in the ventral prostate of castrated rats by a process that is cycloheximide-suppressible (Blondeau *et al.*, 1982), and the synthetic androgen MT causes increased A–R activity in the ductus deferens smooth muscle tumor cell line derived from the hamster and in a cell line derived from the Dunning prostate adenocarcinoma (R. G. Smith *et al.*, 1984). In the former cell line, the augmentation has been shown to result from an increase both in the half-life and the rate of synthesis of the AR (Syms *et al.*, 1985).

We found that human genital skin fibroblasts (GSF) respond to prolonged incubation with physiological concentrations of 5α-dihydrotestosterone (DHT) and to a greater extent with equimolar concentrations of nonmetabolisable MT by augmenting their basal level of AR activity. This observation has since been confirmed in three other laboratories (Ring and Hodgins, 1984; Hughes and Evans, 1984; Gad *et al.*, 1986). For instance, incubation with 3nM MT will regularly induce a twofold to threefold up-regulation of AR activity at 37°C in 48–72 hr by a process that is temperature-dependent and cycloheximide-suppressible (Pinsky *et al.*, 1983). Indeed, the initial rate of up-regulation is often too fast to be explained simply by the stabilization of existing receptors with maintenance of *de novo* synthesis at a basal rate. For this reason, accelerated *de novo* synthesis and/or recruitment of mature receptor proteins from a precursor pool of nonhormone binding proreceptor proteins have been postulated to contribute to androgenic up-regulation of the AR in human GSF. Notably, evidence for a nonbinding biosynthetic precursor of the PR in MCF-7 human breast cancer cell line has recently been obtained by Mullick and Katzenellenbogen (1986). In any event, the up-regulation response of the AR in normal human GSF has become a very useful marker of

qualitative AR mutations that cause androgen resistance in man, as has the similar response of the Vitamin D receptor in SF exposed to 1,25-$(OH)_2D_3$ (Costa *et al.*, 1985). This use is discussed in detail below.

Anatomic Distribution, Ontogeny, Aging, and Possible Tissue-Specificity of a Particular Steroid Receptor

The earliest studies on differential organ retention of radiolabeled steroids administered systemically predicted that anatomic sites considered to be targets for the action of particular steroids would have higher concentrations of steroid-specific receptors than those not considered to be targets. This prediction has been upheld. Thus, sex SR are found at higher concentrations in sexual, and sexually dimorphic, tissues than those in other categories, GR are distributed widely, and mineralocorticoid receptors are concentrated in sodium-transporting epithelia of the kidney and the colon, although they are found in many other cells where their function, if any, has not yet been elucidated (Armanini *et al.*, 1985).

The intermediate lobe of the pituitary, at least in the rat, is one of the places where GR has not been found, even when sought immunologically, but GR appears in cultures of cells from this lobe, or *in vivo* after surgical interruption of the hypothalamic–pituitary axis. Antakly (1986; Antakly and Eisen, 1984) has recently shown that when the cultured cells are exposed to the dopamine agonist, bromocryptine, GR expression is inhibited. This indicates strongly that dopaminergic inhibition of GR synthesis occurs in the intermediate lobe of the pituitary *in vivo*, and this indication is supported by the existence of dopamine and its receptors in cells of the lobe.

The distribution of the two types of corticoid receptors in the rat kidney is interesting. Thus, both glucocorticoids and mineralocorticoids can stimulate Na/K–ATPase activity in the distal nephron, but aldosterone is the effector in the outer medullary thick ascending limb of the loop of Henle (Rayson and Lowther, 1984). The subtlety of this division of labor is matched by the cellular specificity of SR distribution within single tissues, a subject considered below.

The finding of an appreciable concentration of a particular SR activity in any structure is sufficient reason to look for an unknown action of that steroid class on the structure. In this regard, it is relevant that AR activity has been localized in human heart (McGill *et al.*, 1980) and gingiva (Southern *et al.*, 1978) and in fetal rat (Morishige and Vetake, 1978), rabbit

(Giannopoulos and Somers, 1982), and probably human lung (Pierce and Hocott, 1960). The action of androgen on the first two sites is unclear, but its inhibitory effect on fetal lung is intimately related to male inferiority in the rate of surfactant production during late fetal life (A. C. Nielsen and Torday, 1981).

In rabbit lung, there are no sex differences in the concentration of receptor activity toward androgen, glucocorticoid, or progesterone, but AR activity increases from late gestation to adult life (Giannopoulos and Somers, 1982). Similarly, genital skin (GS) cytosols of adult human males and females have comparable levels of AR activity. However pubic skin (PS) cytosol of adult females has more AR activity than that of adult males (Mowszowicz *et al.*, 1981), suggesting that circulating androgen levels have different effects on intracellular distribution of AR activiity in PS versus GS. This suggestion is supported by the fact that while total AR activity is independent of age in human foreskin, the nuclear/cytoplasmic distribution of it is greater in newborn, pubertal, and adult males, when circulating androgen levels are relatively high, than in prepubertal boys, when they are low (Fichman *et al.*, 1981).

Cell culture, particularly of skin fibroblasts, has helped to establish the anatomic distribution and ontogeny of SR. For instance, GSF from the external genital primordia of human male fetuses have newborn levels of AR activity as early as 8 weeks of gestation (Sultan *et al.*, 1980). This coincides with the appearance of Leydig cells and the start of masculine differentiation of the external genitalia. However, appearance of AR activity is not androgen-dependent, because female and male GSF have comparable levels of it, at least as early as 10 weeks of gestation. Furthermore, subjects with testosterone biosynthetic defects have normal AR activity in their GSF. The generally greater level of AR activity in GSF than PSF, and in both than in nongenital skin fibroblasts (NGSF) of both sexes (Mowszowicz *et al.*, 1983*b*; T. R. Brown and Migeon, 1981), is evident in early fetuses as well. It is an expression of the same hierarchy that is observed at all ages in cytosolic preparations of fresh male skin from these sites (Mowszowicz *et al.*, 1981).

Androgen–receptor activity is found in appreciable concentration in fibroblasts from human testis, kidney, and lung, but not intestine. In contrast to the variable distribution of AR activity in SF of various anatomic sites, GR activity is present equally in GSF and NGSF (Bruning *et al.*, 1979). Vitamin D receptors are present in the T47D line of human breast

cancer cells, in accord with the dose-dependent effect of the vitamin on growth of these cells (Sher *et al.*, 1985).

Most studies have not revealed qualitative differences in SR activities among various organs or tissues that are targets for a specific steroid, but the issue is controversial (Feldman *et al.*, 1978). The controversy stems from the fact that differences observed using impure preparations of SR activity are always subject to the criticism that they reflect the influence of extrareceptor, tissue-specific factors, rather than differences intrinisic to the SR protein itself (Menon *et al.*, 1978; Agarwal *et al.*, 1978). The question of tissue specificity has been a central concern with regard to the well-known effects of androgens on the liver. Only very recently has an androgen-binding macromolecule with the prototypic properties of an AR been identified in mammalian liver (Sheets *et al.*, 1985).

Skeletal muscle cytosol of genetically androgen-resistant mice (Schnochowski *et al.*, 1980; Dahlberg *et al.*, 1981) and rats (Max, 1981) has normal levels of AR activity. In the mice the AR activity is qualitatively defective in that it is unable to bind to DNA–cellulose. In all other tissues of these androgen-resistant animals, the level of AR activity is severely deficient. This tissue specificity in the expression of mutations at the presumably homologous X-linked loci of both species that encode the AR protein is an important observation. It indicates that differences in the nature of SR activity among different tissues may reflect the influence of extrareceptor factors rather than disparities at the level of the primary AR gene product.

In either situation and whatever their ultimate interpretation, the observations have substantial potential relevance for understanding the clinical expressivity of steroid-resistance mutations in man. As will be described below, there may be considerable variation in the anatomic distribution or chronologic expression of androgen insensitivity within families, and there is comparable variation among families that usually cannot be ascribed to known differences in the properties of the altered AR activity.

Similar considerations apply to changes in the quantity of SR and/or the quality of a SR apparatus that have been observed in various tissues of various species at different stages of the life cycle (Belisle *et al.*, 1985). A particularly interesting series of such studies has been conducted by Shyamala and her colleagues (1986). They have shown that ER are present at higher concentrations in estrogen-unresponsive lactating mammary glands of mice than in those of estrogen-responsive nulliparous partners.

This paradox is explained by the fact that E–R complexes in the former are less susceptible to transformation by 0.4 M KCl (Gaubert *et al.*, 1986), and by 15 mM ATP or elevated temperature (Carriero *et al.*, 1987), as judged by their relative ability to bind to DNA or chromatin (Shyamala *et al.*, 1986). A partially purified preparation of ER from lactating mice inhibits the chromatin binding of E–R complexes from other tissues (Shyamala *et al.*, 1986), thereby implicating an extrareceptor inhibitor. It has recently been reported that G–R complexes of lactating mammary glands share the same disability as their E–R counterparts (Ruh *et al.*, 1986), perhaps at the hands of the same inhibitory activity. In another study an extrareceptor factor has been clearly incriminated (Pellikka *et al.*, 1983). In still others they may be involved (Sakly and Koch, 1982), but differences indigenous to SR or the nuclear-binding sites for S–R complexes remain eminently possible (Sakly and Koch, 1982; Chuknyiska *et al.*, 1985; Giambiagi *et al.*, 1984). The latter explanation seems particularly likely in the case of the embryonic chick retina that is responsive to glucocorticoid on day 12, but not on day 7 (Sarkar and Lydigsen, 1978).

Steroid–Receptor Complexes

Steroid–Receptor Complexes Exist in Various Structure-Function States (Fig. 5)

The "untransformed" binding state of S–R complexes is characterized by a low affinity for nuclei, defined and undefined sequences of nucleotides, or anionic resins such as DNA–cellulose, and by high affinity for cationic resins such as DEAE–agarose. The "transformed"* state of the complexes is defined by a relatively higher binding affinity for nuclei, DNA, etc., and by their relatively lower binding affinity for polycationic substrates (Holbrook *et al.*, 1983). The transformed state is induced by warming intact cells, or by various treatments of steroid-incubated cytosols including: warming; dilution, gel-filtration or ammonium sulfate precipitation to remove transformation inhibitors; and exposure to high-ionic environments (0.4 M KCl) or ATP (5–15 mM). Dissociation of a

* Note that "activation" and "transformation" are often used synonymously in the steroid receptor literature. In conformity with growing practice, throughout this chapter "activation" refers to the process whereby a SR is converted from a nonbinding state to a binding state, and "transformation" to the change of a S–R complex from a state with low affinity for DNA to one with higher affinity for DNA.

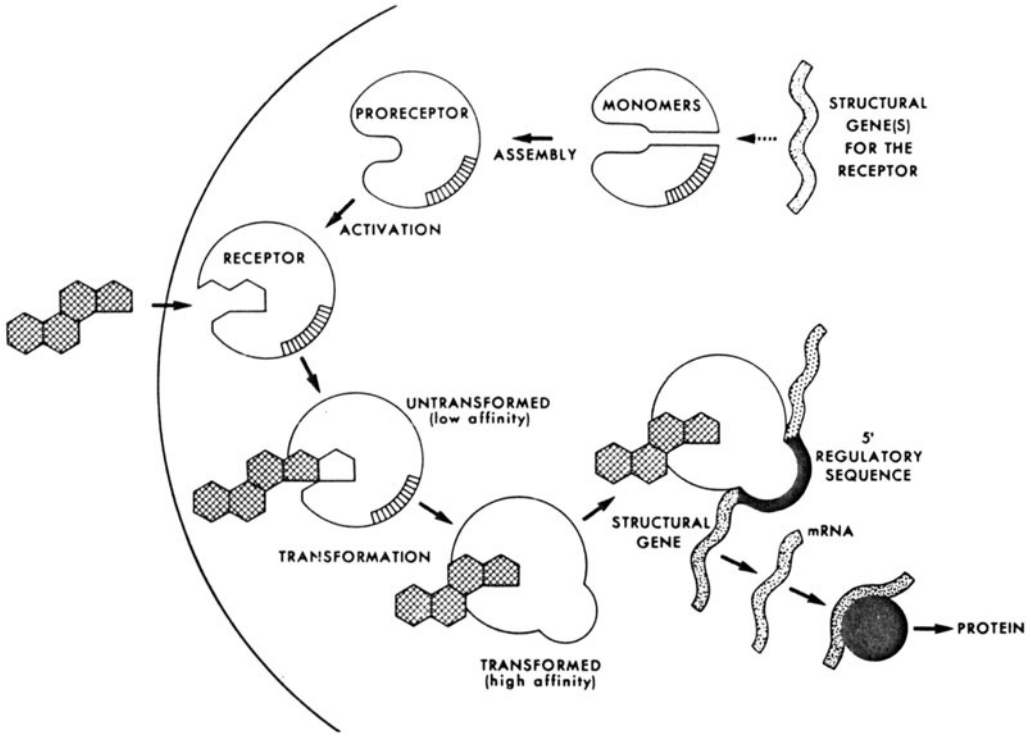


Fig. 5. A cycle of structure–function events applicable with various degrees of certainty to all steroid receptor proteins and steroid–receptor complexes.

steroid-binding protein monomer from one [or more (Suba *et al.*, 1986)] nonsteroid binding heteromers is a prerequisite rather than a concomitant of S–R complex transformation (Sakai and Gorski, 1984). Hence the increased surface positivity and hydrophobicity (Luttge *et al.*, 1984; Densmore *et al.*, 1986) of a transformed S–R complex may simply reflect its separation from an oligomeric partner that is relatively electronegative and hydrophilic. However, the changes may also partly reflect a conformational alteration in the steroid-binding subunit that exposes positively-charged residues, such as lysine and arginine (Muller *et al.*, 1983), and others that are hydrophobic, on the surface of the transformed moiety.

Relatively little attention has been given to the observation that transformation is also expressed by a state of increased affinity between a receptor and its steroid (Weichman and Notides, 1977; Traish *et al.*, 1984; McBlain *et al.*, 1981; Seeley and Costas, 1983; Murakami *et al.*, 1979; Mulder *et al.*, 1985; Sakai and Gorski, 1984; De Boer *et al.*, 1985). Indeed, there is now ample precedent for the belief that increased affinity between a receptor and its steroid can be a more reliable reflection of the physi-

ologically productive transformed state of a S–R complex than is its mere ability to bind to nuclei or the nuclear equivalents listed above. This is dramatized by a variety of receptor mutations that cause resistance to various steroid hormones despite the fact that the mutant S–R complexes have a normal (Pinsky *et al.*, 1981), or even an abnormally great (Gehring, 1983), ability to accomplish nuclear translocation or to bind to polyanionic substrates with increased affinity. It is also reflected in the behavior of certain steroid antagonists that bind to their respective receptors with affinities as high as (Rochefort and Borgna, 1981) or higher than (Moguilewsky and Philibert, 1984) agonists, whereas the antisteroid–receptor complexes cannot transform normally, judging by their rates of dissociation, even if they are able to achieve nuclear translocation (Rochefort and Borgna, 1981). Nor should the latter be surprising in view of the certainty that the process whereby S–R complexes regulate gene activity specifically and preferentially within various target cells must involve subtle interactions with multiple components of chromatin and of nuclear architecture.

Manipulations that induce transformation of cytosolic S–R complexes also promote their compartmentation within nuclei (and preferentially within homologous compared to heterologous, non-target-cell nuclei). Therefore, many assumed that transformation occurred within the cytoplasm of intact cells and was responsible for nuclear translocation of the complexes. But it had never been clear whether transformation enables a cytoplasmic S–R complex to traverse the nuclear barrier or whether it simply promotes nuclear retention of complexes that were initially formed in the nucleus. In other words, it had never been clear whether, in intact target cells, the site of S–R complex transformation is the cytoplasm or the nucleus. This is why "translocation", "transfer", "retention" and "compartmentation" have been used interchangeably in reference to various measures of the transformed state that have employed nuclei as a reagent. Some have distinguished between "transformation" and "translocation" using the former to refer to hydrodynamic alterations of transformed complexes, the latter to the ability of the altered (transformed) complexes to bind to nuclei or DNA. Indeed, this terminological confusion presaged the debate on whether cytoplasm or nucleus is the primary (or predominant) residence of unliganded SR within unstimulated target cells. In fact, it has recently become clear that different SR differ in this regard. Thus ER and PR are found predominantly if not exclusively in the nuclei (Greene, 1986), while GR appears to be primarily

in cytoplasmic residence (Antakly and Thompson, 1986; Gustafsson *et al.*, 1986).

Just as the intracellular distribution of SR and the site or chronology of S–R complex transformation may vary among different SR, so may they vary in the detailed mechanism of their respective transformation processes. Indeed, there is reason to believe that the "details" may vary for a given S–R complex in different cells or tissues. Thus, in addition to the basic feature—dissociation of a heterooligomer to produce a steroid-binding monomer that has the qualities of a transformed S–R complex—various adjunctive questions have occupied the attention of investigators: (1) whether the transformation process is reversible as a simple function of steroid occupancy of the receptor's binding site (McIntyre and Samuels, 1985); (2) whether steroid-induced transformation is irreversible in the absence of an energy-dependent regeneration of the untransformed S–R complex (Munck and Holbrook, 1984); (3) whether the steroid-binding monomer resulting from dissociation of the untransformed 9 S oligomer must or can undergo a monomolecular, first-order, reaction that involves a change in conformation but not in mass before it becomes fully transformed (Kaufman *et al.*, 1982*a*; Bailly *et al.*, 1980; Sakai and Gorski, 1984); (4) the homodimerization of penultimate 4 S monomers, in association with positive cooperativity of the estradiol-binding reaction, that has been incriminated in the ultimate transformation of uterine E–R complexes (Notides *et al.*, 1981); and (5) the extent to which transformation of various S–R complexes is dependent upon dissociation of macro- or micromolecular inhibitors (Sato *et al.*, 1980; Sekula *et al.*, 1981), dephosphorylation (Dahmer *et al.*, 1984), or proteolysis (Puca *et al.*, 1977).

Molybdate (as well as vanadate and tungstate) can reversibly block transformation of S–R complexes (just as they stabilize and promote the binding activity of unliganded receptors), but they will not interfere with the ability of transformed complexes to bind to DNA. How this effect is achieved is not understood, but it is likely to involve molybdate–sulfur interactions, because SH groups are necessary for S–R binding as well as for S–R complex transformation (Bodwell *et al.*, 1984).

A heat-stable factor within rat liver cytosol, having a molecular weight of <700, mimics the effect of molybdate by inhibiting GR inactivation and G–R complex transformation (Leach *et al.*, 1982; Goidl *et al.*, 1977). This factor is not pyridoxal phosphate: the latter acts on the transformed receptor complex to block its binding to DNA (Sekula *et al.*,

1981). A macromolecular factor in dialyzed rat liver cytosol that regulates GR functionality in a molybdate-like manner has also been discovered (Barnett and Speck, 1986).

Distelhorst and Benutto (1985) have recently identified an inhibitor of G–R complex transformation by Sephacryl S-300 filtration of rat liver cytosol. The inhibitor coeluted from the column with an albumin standard, and its activity was heat-stable, despite the fact that its size was reduced to <3500 daltons after heating. After separation of this inhibitor, G–R complexes transformed even in the presence of molybdate, and they did so by attaining the usual $S_{20,w}$ of ~4.

In addition to pyridoxal phosphate, at least one, and possibly several (Isohashi *et al.*, 1984; Milgrom and Atger, 1975; Liu and Webb, 1977), marcromolecular factors have been identified that inhibit the binding of transformed S–R complexes to DNA. For instance, the rat ventral prostate produces a glycoprotein, prostate α-protein, that inhibits the binding of transformed A–R complexes to nuclei and causes release of such complexes once bound (Shyr and Liao, 1978; Liao *et al.*, 1982). Colvard and Wilson (1984) isolated from rat serum a non-steroid-binding protein fraction, called "8 S A–R promoting factor," that inhibits binding of the transformed 4.5 S A–R complex to isolated nuclei in a concentration-dependent manner. They interpreted this behavior as supporting the hypothesis that the "8 S" A–R complex is an oligomer composed of a transformed 4.5 S complex and the "8 S A–R promoting factor," but they did not study the factor further.

It has been suspected, at least since 1973 (Liao *et al.*, 1973*a*; Liang and Liao, 1975) that SR action may involve types of RNA other than stimulation or inhibition of mRNA transcription (Liao *et al.*, 1980). Indeed, it has been established that SR are associated with the nuclear matrix—a structure composed in part of RNA—and that some positive effects of SR on expression of structural genes are mediated, in part, by stabilization of preformed mRNA, as discussed below on p. 336. But, a growing body of evidence, accumulated since 1981 (Lin and Ohno, 1981; Feldman *et al.*, 1981) indicates that certain species of RNA, yet undefined, may participate in the conversion of untransformed S–R complexes to their final transformed state. Tymoczko and Phillips (1983) reported that, in the absence of molybdate, RNase (A or T_1) treatment of cytosolic rat liver G–R complexes, previously transformed to a 7–8 S form by heating at 20°C for 30 min, increased their ability to bind to DNA–cellulose, and concurrently decreased their S values to 3–4. Tymoczko's group (Ty-

moczko and Lee, 1985; Anderson and Tymoczko, 1985) has proceeded to show that the RNase-sensitive 7–8 S form derives from the 9–10 S, and that a small (M_r <500) molecular weight factor that is neither protein or nucleotide can convert the 7–8 S form back to the 9–10 S parent. These data indicate that RNA is a component of both forms, and that loss of the small molecular weight factor allows the 9–10 S form to convert to its 7–8 S, RNase-sensitive, derivative. More recently (Tymoczko and Moses, 1986) it has been reported that RNA is associated with the 9–10 S GR receptor even when it is not bound to glucocorticoid, but that it is inaccessible to RNase until a G–R complex is formed and transformed to the 7–8 S form by heat.

Schmidt *et al.* (1986) have come to an important conclusion, and made provocative observations that bear on Tomyczko's results: They found that both RNase A and an enzymatically-inactive proteolytic fragment of it that contains the RNA-binding site can increase the extent to which heat-transformed G–R complexes combined to DNA–cellulose. Thus, they conclude that *in vitro* transformation of cytosolic rat liver G–R complexes is a 2-step process. The first step converts the 9–10 S form to a 7–8 S form that elutes from DEAE resins at relatively low-ionic strength, and that, in the second step, either form of RNase subsequently converts the 7–8 S form to the 3–4 S variety that has maximal DNA-binding ability. The fact that catalytically-inactive RNase works is surprising and implies that RNA hydrolysis is not essential for the second step.

Schmidt's results differ from those of Vedeckis *et al.* (1986) based on studies of the GR in the mouse AtT-20 pituitary cell line. The latter found that the intermediately transformed G–R complex (5.2–6.6 S, 6 nm, 132,000 daltons) was converted to the terminally-transformed variety of G–R complex (3.8 S, 6 nm, 96,000 daltons) by the loss of a small RNA (~36,000 daltons). But, they did not find RNA in the parental untransformed G–R complex (9.1 S, 8.3 nm, 319,000 daltons). Nor, indeed did they find that the latter had the expected hsp 90 non-steroid binding component. These disparate results may well reflect biological differences between rat liver and the tumor cell line. In either event, Ali and Vedeckis (1986) have pursued the nature of the RNA species involved in the second step of *in vitro* G–R complex transformation. They have so far found it in the fractions of cytosol that elute from DEAE–cellulose columns with 0.4–0.55 M KCl and have thereby reduced the search to about 5 percent of the total cytosol RNA.

Oharo-Nemoto *et al.* (1986) found that RNA is important in the trans-

formation of A–R complexes in the rat submandibular gland, and Rowley *et al.* (1986) reported confirmatory results for A–R complexes in the cytosol of the rat Dunning R3327H prostatic tumor. Similar results have been communicated from a study of the effect of RNase on the physicochemical properties of E–R complexes in rat and rabbit uterine cytosol (Thomas and Kiang, 1985).

The immediate profit of these important investigations on the role that RNA plays in different states of various S–R complexes is that they clarify why published data for the hydrodynamic properties of S–R complexes in various states have been so variable. Aside from purely subjective influences in the reporting of these data, it seems obvious that imperfect separation of untransformed from intermediately-transformed species of S–R complexes can yield weighted values that represent the proportions of each type in an apparently unimodal "peak" that appears on a density gradient or a gel exclusion chromatography column. And, the same issues apply to the distinction of the intermediately-transformed species from the terminally-transformed one.

An equally important by-product of these investigations is that they provide firm support for the idea that S–R complex transformation involves more than one step *within cells*. We (Kaufman *et al.*, 1982*a*) and others have made this suggestion previously, as discussed below (p. 400).

Speculation on the possible physiologic role of SR–RNA complexes is beyond the scope of this chapter, but, their mere existence and related observations in this section, illustrate how manifold is the potential substrate for genetic variation in the receptor-dependent portion of the molecular apparatus that underlies responsiveness to various steroid hormones. Indeed, the inhibitory activity of the macromolecular translocation inhibitor of G–R complexes described by Isohashi *et al.* (1984) is considerably greater in the cytosol of various glucocorticoid-resistant cell lines than in rat liver, a finding compatible with their involvement in the mechanism of glucocorticoid resistance. A similar observation has been made by Liu and Webb (1977) in Novikoff hepatoma cells.

The Two-Step Model of Steroid–Receptor Interaction: Old and New Versions

The old model originated from studies of naive and estrogen-stimulated target tissues (Jensen *et al.*, 1968; Gorski *et al.*, 1968). It held that

steroids, once they had traversed plasma membranes by diffusion, formed complexes with specific cytoplasmic receptor proteins, and that the S–R complexes were then translocated to nuclei, where they subsequently became bound to specific, so-called "acceptor" sites on chromatin. It was assumed that cytoplasmic S–R complexes had to be transformed from an initial state of low-nuclear-binding affinity to a subsequent one of higher nuclear-binding affinity in order for translocation to occur. However, it had never been clearly established that transformation was a cytoplasmic event: it could have been a nuclear event that caused increased nuclear retention of S–R complexes that had traversed the nuclear membrane in their pretransformed state.

Recent observations using immunocytochemistry (King and Greene, 1984) and nuclear enucleation (Welshons *et al.*, 1984) methods have generated a new model of S–R interaction (Jensen, 1984; Gorski *et al.*, 1984). It suggests that SR are primarily, or exclusively, in nuclear residence whether or not they are occupied by steroids, that transformation is a nuclear event, and that unoccupied SR (or pretransformed S–R complexes) are vulnerable to extraction from nuclei in low-salt solutions. The latter would explain the appearance of "cytosolic" SR during the conventional conditions used to homogenize steroid-target tissues. According to the new model, steroids would enter the nuclei either in their free state or bound, and, if bound, to a minority pool of specific receptors or to diverse proteins acting as nonspecific carriers. Considerable support for the new model is provided by the fact that in several situations SR are found in nuclear but not cytosolic fractions that have been prepared by conventional homogenization procedures. These include the 1,25-dihydroxyvitamin D_3 receptor in the intestinal mucosa (Walters *et al.*, 1980), the ecdysterone receptor in imaginal discs of *Drosophila* (Yund *et al.*, 1978), and the ER in the testis of the spiny dogfish (Callard and Mak, 1985). The latter is particularly interesting, since this species has a high body-fluid osmolarity (1000 mosM), and, appropriately, its nuclear E–R complexes require much higher than usual salt concentration to be extracted from nuclei or to be eluted from DNA–cellulose columns. Whatever the fate of the two models, each acknowledges the central role of S–R complex *transformation* in the mechanism of steroid action. This centrality is borne out repeatedly by evidence to be presented below.

The Reciprocal Structural Constraints for Specificity and High Affinity of Steroid–Receptor Binding and for Potency of the Resultant Steroid–Receptor Complexes

It is obvious that the correlative geometry of steroids and of SR proteins will determine whether any given steroid can combine noncovalently, with high affinity, but reversibly, with one or another receptor protein in order to form a S–R complex that is potent in terms of regulating gene expression. We must remark at the outset that inasmuch as nothing is yet known about the geometry of mature SR proteins, nothing can be said about the physicochemical properties that govern their contribution to the steroid-binding process. Much effort, however, has gone into trying to deduce what structural elements of steroid molecules determine their biopotency. These deductions have been based primarily on the apparent affinity with which they bind to a specific SR protein, as measured by displacement; that is, by the relative ability of a synthetic radioinert ligand (compared to a natural radioinert steroid) to compete with a radioactive version of the natural steroid for binding to a given receptor protein. In a few cases, a steroid analogue has been available in radioactive form to permit direct measurement of its equilibrium affinity constant, its nonequilibrium dissociation constant, and the affinity with which its S–R complexes bind to nuclei. The latter is a useful approximation of potential biopotency because high-affinity nuclear binding is often a valid measure of the extent to which initial S–R complexes transform to a subsequent conformational state that endows them with the capacity to effect transcriptional regulation. However, as will be documented below, nuclear affinity of S–R complexes is not always correlated with their biopotency. This indicates that subtle aspects of S–R complex geometry are critical for their effective collaboration with various substrates of chromatin in order to regulate gene transcription.

In addition to data on synthetic steroid agonists, important information about the structure–function relations of steroids has come from work with synthetic steroids and nonsteroidal compounds that antagonize steroid action by interacting with appropriate SR proteins. We briefly consider observations from both sources, and concentrate on androgenic and antiandrogenic compounds because the literature for all classes of steroids is immense, and the principal conclusions for this class ought to apply to the other classes as well. Indeed, it is appropriate to conclude

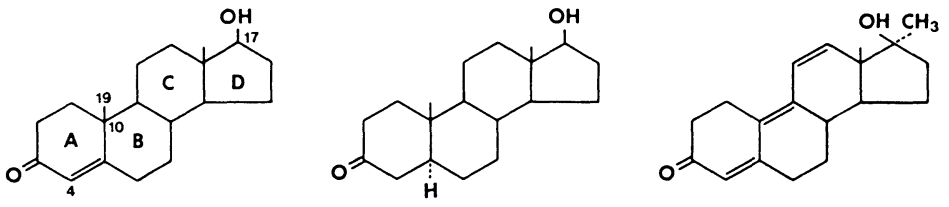


Fig. 6. The structures of testosterone (T), 5α-dihydrotestosterone (DHT), and methyltrienolone (MT). The steroid rings are lettered and particular carbon atoms are numbered to facilitate reference in the text.

these prefatory comments by declaring that it is impossible, based on present knowledge, to generate a coherent model of the structural attributes of a steroid that can predict whether its S–R complexes will be biologically agonistic or antagonistic.

Cunningham *et al.* (1983) used human foreskin fibroblasts to measure the affinities of various steroids for the AR relative to that of DHT [which was assigned a relative affinity (RA) of 100%]. They found that testosterone (T) had an RA of 66, reflecting its Δ^4 double bond in place of the 5α-reduced configuration in DHT, and that the C-3 ketone group of ring A, a planar (flat) configuration of the A/B-ring junction, and a 17β-OH group on ring D were critical for high-affinity binding (Fig. 6) Thus, the 17α-OH epimer of T had an RA of 0.4, 5β-DHT had an RA of 2 (because its configuration at the A/B-ring junction is bent rather than flat), and the androstanediols (3β- or α-reduced derivatives of DHT) had RA values of ~10. Interestingly, removal of the C-19 methyl group from DHT at its 10β position (19-NorDHT) increased its RA to 117, and addition of a 17α-methyl group enhanced it even more, to 140. In contrast, a 17α-ethinyl group decreased the RA to 43, indicating that the free 17β-OH group will tolerate only certain vicinal substituents. Despite the presence of a Δ^4 double bond, methyltrienolone (MT) had an RA of 89; this may reflect the benefit of its 17α-methyl group, but more importantly, it demonstrates that the Δ^4 double bond is not intrinsically inimical to AR binding.

Liao *et al.* (1973*b*) used rat ventral prostate as a source of AR and their principal observations coincided with those of Cunningham *et al.* However, some of the differences between the two studies presumably reflect disparate properties of the AR in human GSF versus rat ventral prostate, and the data on relative androgenicity (based on studies in castrated rats) provided by Liao *et al.* are particularly informative. For in-

stance, relative to DHT, 19-NorT had an appreciably greater competitive binding affinity than T in the study of Liao *et al.*, but not in the study of Cunningham *et al.* Yet 19-NorT is not more androgenic than T. Thus, the relative binding affinity of a steroid for the AR is a necessary but not a sufficient predictor of androgenic potency. Conversely, 19-NorDHT had a slightly higher RA than DHT in the study of Cunningham *et al.*, but an appreciably lower affinity than DHT in that of Liao *et al.* Appropriately, 19-NorDHT is quite an important androgen in the rat. Liao *et al.* found that addition of a 7α-methyl group to 19-NorT increased its relative binding affinity from 0.9 to 2.6, and the addition of a second methyl group in the 17α position increased it still more, to 3.5. Moreover, the increased relative binding affinities of the two methylated 19-NorT derivatives correlated well with their relative biopotency. These observations indicate again that a Δ^4 double bond is not an absolute hindrance to A–R binding or to bioeffective A–R complex formation if other parts of the steroid ligand are modified appropriately. At the same time, they reveal that the binding site of the AR is capable of interacting with spatially polar parts of the steroid molecule. Indeed, compounds with C=C bonds in rings A–C, such as MT, that also lack the C-19 methyl group at C-10 not only bind well to the AR, but do so in a manner that is androgenically potent. This is interpreted as reflecting the overall "flatness" of such multiunsaturated steroids that makes them more similar to 5α-reduced DHT than to T with its Δ^4 double bond.

Other groups have developed the concept that there is a division of labor among different regions of a steroid molecule in the attainment of a biopotent binding relation with its receptor protein (Lobl, 1980). Duax *et al.* (1981) suggested that the A-ring region is concerned primarily with binding, while the D ring is involved with activity, because when the structures of compounds that are agonists and antagonists of a specific steroid hormone are compared by X-ray crystallography, they generally exhibit similarities in their A-ring region and dissimilarities in their D-ring region (Fig. 7). Indeed, more recently, Duax *et al.* (1984; 1985) have postulated that the D ring confers activity on a S–R complex by interacting directly with DNA or chromatin. The idea that receptor-bound steroids act directly upon DNA, rather than only indirectly, by inducing changes in their respective receptor proteins has latterly been promulgated by others as discussed in a later section (p. 344).

Schmit *et al.* (1980) pointed out that the 17β-OH of natural androgenic steroids is not only essential for their binding to the AR, but that, in a

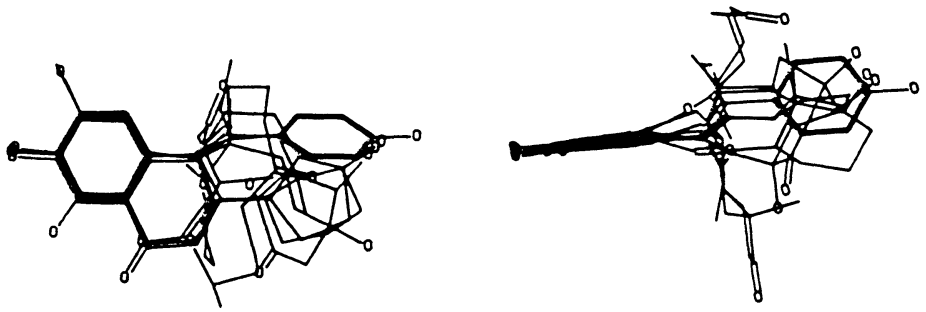


Fig. 7. Perpendicular views of the superimposition of the A rings of six different estrogens indicating the variability in orientation of the B, C, and D rings that is compatible with binding to the ER and some degree of estrogenic activity. [From Duax *et al.* (1981) with permission.]

different sense, it is responsible for allowing the AR to distinguish an androgen from a progestin or a mineralocorticoid. They proposed that the 17β-OH group acts as an acceptor for a proton donated by the receptor in the formation of a hydrogen bond and extended this proposal to explain the antiandrogen activity of some progestins.

Antiandrogens can be divided into two main groups: steroidal and nonsteroidal (Jänne and Bardin, 1984; Tindall *et al.*, 1984). This diversity immediately implies that it is difficult to construct a model that predicts the structural features that will endow a compound with the property of androgen antagonism. Cyproterone is a prototype of androgens that share a 17β-acetyl group with progesterone (Fig. 8a). It binds to the AR with about 20% of the affinity of T, and the complexes do not transform to the nuclear-binding state (Brinkmann *et al.*, 1983). Flutamide is the best-known nonsteroidal antiandrogen (Fig. 8b). Spironolactone (Fig. 8c), the well-known aldosterone antagonist, and cimetidine, the H_2 receptor antagonist, are also androgen antagonists by virtue of their binding to the AR. The structural diversity of these compounds (Fig. 8) indicates that the AR is strikingly indiscriminate in its choice of ligands. This conclusion is underlined by the observations of Liao (Liao *et al.*, 1985; Chang and Liao, 1986) on the interaction with steroid receptors of various nonsteroidal cyclic hydrocarbons. For example, 9,10-dihydrophenanthrene is about 20 times more active than its (1-4)-tetra- or its (1-8)-octahydrophenanthrene parents in competing with MT for the cytosol AR of rat prostate, and this compound acts as an antiandrogen by bioassay. This suggests that the two aromatic rings in 9,10-dihydrophenanthrene bind to

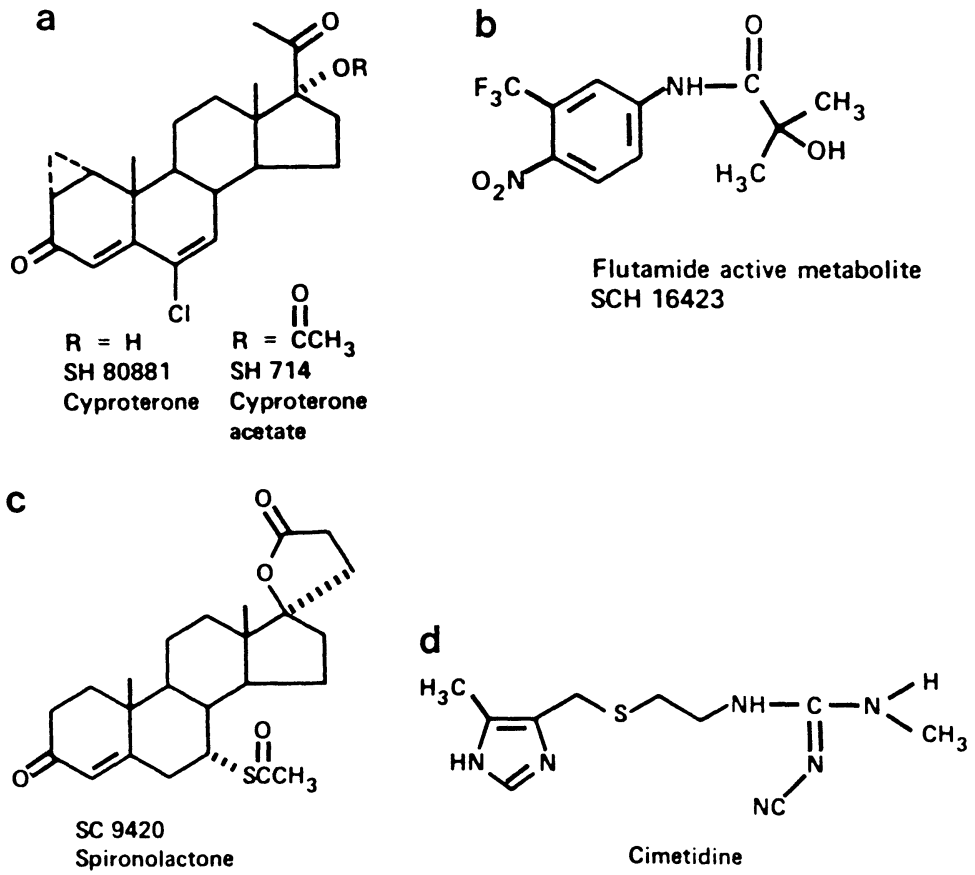


Fig. 8. The structural diversity of four compounds, two steroidal, two nonsteroidal, that act as antiandrogens by binding to the AR.

portions of the AR that normally interact with the B and D rings of androgens.

In contrast to the wide structural heterogeneity of antiandrogens, most estrogen antagonists have a phenol ring in common.

The first steroid capable of fully antagonizing the effects of glucocorticoids has been code-named RU 38486. It has a binding affinity for the GR about three times that of dexamethasone. Nevertheless, this steroid forms complexes with the GR that, upon heating, do not readily transform to the DNA- or nuclear-binding state, and this behavior is reflected in the fact that whereas G–R complexes become less dissociable upon transformation, the antiG–R complexes become more dissociable after exposure to the conditions of transformation (Moguilewsky and Philibert, 1984).

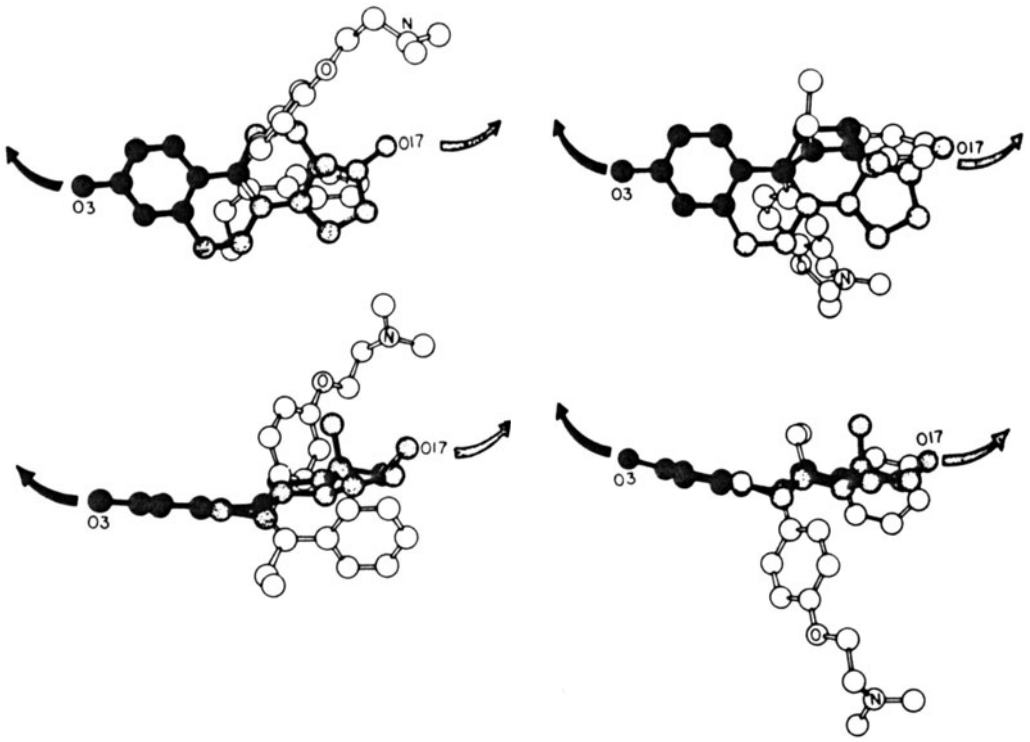


Fig. 9. Crystallographic models and hydrogen binding (curved arrow) potential of (shaded) 17β-estradiol and (open) tamoxifen. Common structures are black. The A ring of estradiol is superimposed on the phenyl ring of tamoxifen that is hydroxylated *in vivo*. The two possible superpositions of these rings are illustrated in perpendicular planes. [From Duax *et al.* (1981) with permission.]

Certain nonsteroidal estrogen antagonists (such as hydroxytamoxifen, Fig. 9) bind to the ER with as much (Rochefort and Borgna, 1981) or even greater (Keene *et al.*, 1984) affinity compared to E_2, and the antiE–R complexes even undergo a form of heat-induced conformational change that increases their ability to bind to nuclei or DNA. Under these otherwise normal circumstances, various observations appear to explain their lack of estrogenic activity. For example, Tate *et al.* (1984) have shown that a polyclonal antibody to the ER can reduce the affinity with which it binds E_2, but not hydroxytamoxifen. Furthermore, Rochefort and Borgna (1981) found that 10 mM molybdate blocked transformation of E–R and antiE–R complexes equally well as judged by acquisition of their ability to bind DNA. On the other hand, in the absence of molybdate, E–R complexes attain an intrinsically high-affinity state (as measured by a slow rate of complex dissociation) at the same time as they acquire the ability to bind DNA; in contrast, antiE–R complexes remain in a low-

affinity state (as measured by a high rate of complex dissociation) even when they have acquired the ability to bind DNA. Thus, the quality of transformation experienced by E–R and antiE–R complexes is different, and the difference is reflected in the different rates with which the two classes of complexes dissociate even when both have acquired the conformational state that endows them with an increased ability to bind DNA. Comparable results with the same implication have been obtained by Keene *et al.* (1984) using a different nonsteroidal, tamoxifenlike antiestrogen.

We have profitably used the difference in dissociation rates between untransformed and transformed states of S–R complexes to analyze a series of A–R mutations in man, as will be described in detail below.

The Sites and Modes of Action of Steroid–Receptor Complexes

The Nuclear Envelope. The nuclear envelope (NE) is a complex membranous structure that is composed of inner and outer nuclear membranes and so-called pore complexes, proteinaceous in nature, that are found in regions where the inner and outer membranes contact to form pores. A distinct, third layer—the nuclear lamina—is closely applied to the inner nuclear membrane.

Lefebvre and Novosad (1980) have found relatively high-affinity (k_D-8 nM), low-capacity binding sites for DHT on the NE of rat ventral prostate that represent about 10% of total nuclear DHT-binding activity. The NE sites are competed for well by androgens, moderately by estrogens, and not at all by glucocorticoids; castration 96 hr before assay reduces the binding completely (Lefebvre *et al.*, 1985*a*). Thus, these NE binding sites for DHT have some of the properties of typical AR. Differential preparations of NE have revealed that the outer nuclear membrane and the pore complexes do not contribute to the total NE DHT-binding activity (Lefebvre *et al.*, 1985*b*). Importantly, NE prepared from one androgen-responsive Shionogi mouse mammary cancer cell line had DHT-binding sites, while those from two androgen-unresponsive variant lines did not. Moreover, NE prepared from rat liver have androgen-binding sites with different qualities, a finding in accord with the fact that typical cytosolic AR have only recently been identified in this tissue (Bannister *et al.*, 1985). Whether NE DHT-binding sites are involved in translocation of DHT across the barrier of the NE, and, if so, whether they have another function, remains to be defined. It is of interest that Kaufmann and Shaper

(1984) have identified a glucocorticoid-binding site on the NE of rat liver labeled *in vitro* that differs from the one in intact nuclei that are labeled *in vivo*.

The Nuclear Matrix. The nuclear matrix (NM) is the architectural scaffolding of the nucleus, just as the cytoskeleton provides a structural basis for the functional organization of the components and organelles of the cytoplasm. NM is prepared by sequential extraction of the nuclei with the following: 1 percent Triton X-100, DNase I, low-ionic-strength buffer, and 2 M sodium chloride (NaCl). This treatment removes from a nucleus >95 percent of its DNA and phospholipid, >85 percent of its protein, and >60 percent of its RNA. The remainder is a spherical structure that consists of residual elements of the NE and lamina together with pore complexes, condensed residual nucleoli, and a granulofibrillar interchromatinic network which fills the interior of the sphere. It is composed predominantly of nonhistone nuclear proteins (Barrack and Coffey, 1982).

The NM seems to have a major role in DNA replication and in heterogeneous nuclear RNA synthesis and processing. Several observations hold specific relevance for the chapter. The first is that transcriptionally active genes, such as the one encoding ovalbumin in chicks, are associated with the NM of chick oviduct cells, but not with that of chick liver cells. Conversely, a transcriptionally inactive gene, such as the one for β-globin, is not associated with the NM of chick oviduct cells (Robinson *et al.*, 1982). The second observation is that receptors for various steroids, including all the sex steroids, are associated with the NM. Finally, phosphorylation of prostatic NM proteins is under androgenic control (Goueli and Ahmed, 1984). Thus, at a minimum, the NM seems to be the physical substratum that serves as the place where S–R complexes meet with the structural genes they are destined to regulate. A succinct review of the supporting evidence follows.

In several mammalian androgen- or estrogen-target tissues >50 percent of total nuclear S–R content resides in the NM (Rennie *et al.*, 1983). Furthermore, the native NM of rat ventral prostate can preferentially bind cytosolic preparations of transformed prostatic A–R complexes with high affinity and limited capacity, implying the existence of a saturable number of specific "acceptor" sites for the complexes (Barrack, 1983). The same is true for NM of rabbit uterus in relation to E–R complexes (Buttyan *et al.*, 1983). In contrast, nontarget (liver) NM have only 15–20 percent as many acceptors. Most of the NM-associated acceptor sites in the rat ventral prostate are associated with its internal network, rather than with its

peripheral lamina component, but specific, high-affinity binding sites for androgens have also been found in nuclear membrane preparations from rat ventral prostate (Lefebvre and Novosad, 1980).

Several groups have begun to study the composition and behavior of the acceptor sites in the NM. For example, one can show that exhaustive DNase I digestion that removes 99% of total nuclear DNA leaves 50 percent of the acceptor sites still associated with the NM. Thus, if DNA is a component of the NM-associated acceptor sites, its binding activity is enriched more than 50-fold in such preparations (Barrack, 1984). Buttyan *et al.* (1983) have reported that DNA extracted from prostate NM is a potent competitor of the binding of prostatic A–R complexes to DNA–cellulose. This strengthens the view that DNA is a crucial element in the binding activity of acceptor sites on NM.

It is important to note that the term "acceptor site" has been used in various operational ways: to refer to total saturable nuclear retention of transformed cytosolic S–R complexes, to the fractions extractable from nuclei by hypertonic salt solutions, and to the fraction that resists extraction by salt concentrations in excess of 0.6 M.

The Acceptor Fraction of Chromatin

Spelsberg has conducted a systematic investigation of the composition and behavior of the acceptor activity toward P–R complexes that is invested in chick and hen oviduct chromatin (Spelsberg *et al.*, 1983). His findings have been replicated substantially for chromatin of rat and bovine uterus with E–R (Ruh *et al.*, 1981), for that of rat prostate with A–R (Klyzsejko-Stefanowicz *et al.*, 1976), and for that of rat liver with G–R complexes (Hamana and Iwai, 1978). The essential observations are that native chromatin and a derivative freed of all histones plus 10 percent of the nonhistone proteins (NHP) have comparable levels of acceptor activity toward transformed S–R complexes. In contrast, a subsequent fraction, depleted of an additional 80 percent of its NHP, has markedly enhanced acceptor activity, thereby endowing the bulk of NHP with a function that "masks" much of the potential acceptor activity in native chromatin. Furthermore, removal of the remaining 10 percent of NHP leaves pure DNA that is nearly devoid of saturable acceptor activity. Thus, the "unmasked" fraction with enhanced acceptor activity (the nucleoacidic proteins, NAP) is composed of DNA and a minority population of acidic NHP (chromatin protein, fraction 3; CP-3). These observations

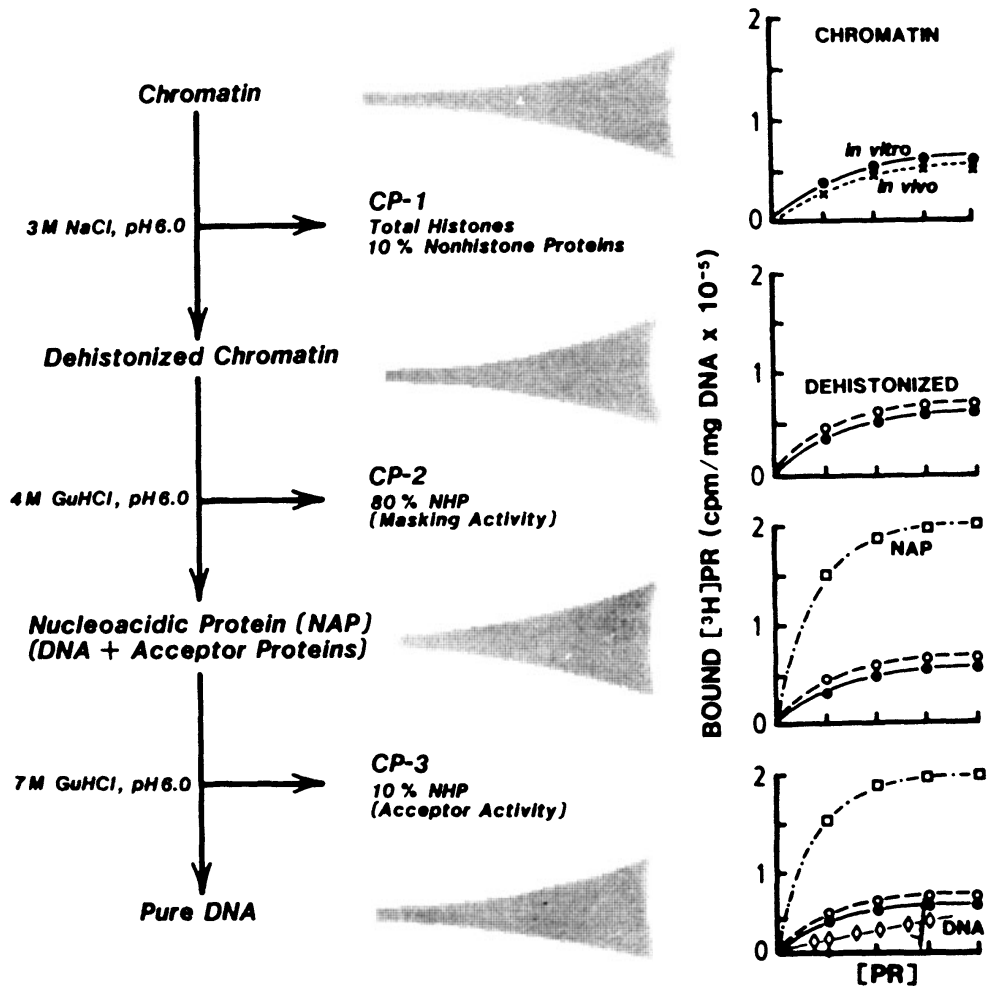


Fig. 10. A scheme for isolating the "acceptor" fraction of chromatin. [From Spelsberg *et al.* (1983) with permission.]

are summarized in Fig. 10. Indeed, reconstitution experiments have demonstrated that the binding of CP-3 to DNA is saturable, and that both components are essential. For example, preparations of plant or fish DNA in combination with oviduct NAP have much less acceptor activity than homologous combinations.

The physiological relevance of the "acceptor-enhanced" fraction is indicated by the fact that its activity varies with seasonal rhythms and developmental changes in total nuclear binding of hen and chick oviduct, respectively, and it is comparable in magnitude among target and nontarget cells, whereas their respective native chromatins differ markedly in this respect (Spelsberg *et al.*, 1983).

At this time there is every reason to believe that the "masking" activity invested in one or several classes of NHP is an important determinant of which genes are "available" for regulation by S–R complexes in different target tissues for a single steroid hormone. Moreover, the weight of the evidence is that the acceptor activity for S–R complexes that has been studied in the NM of steroid target cells at least overlaps, and may be substantially comparable, to that of the NAP fraction prepared from the chromatin of such cells. In the latter regard, it is noteworthy that the CP-3 fraction proteins protect the DNA of the NAP fraction against DNase I digestion (Spelsberg *et al.*, 1983), and that DNA has been solidly incriminated as an essential functional component in both types of systems (Buttyan *et al.*, 1983).

The relation between the DNA of the NAP and the S–R binding activity of the DNA in the "regulatory regions" to be discussed later remains to be defined, as does the relation between the protein component of the NAP and of the chromatin that carries the regulatory DNA sequences. In respect to this protein component, it is relevant that mild trypsin digestion can release the fraction of A–R complexes that resists extraction from prostatic chromatin by micrococcal nuclease digestion and solution in 0.6 M NaCl (Rennie *et al.*, 1983).

S–R complex binding to chromatin is correlatable with certain modifications in nonhistone proteins, particularly those in the "high mobility group" (HMG) of NHP identified as species 14 and 17. HMG proteins are subject to acetylation and ribosylation. For instance, exposure of mouse mammary tumor cells to glucocorticoid rapidly causes a 10-fold decrease in ADP ribosylation of these HMG species, but not of others in this class (Tanuma and Johnson, 1983). Acetylation and deacetylation of HMG proteins may also be involved in the extent of steroid responsiveness. Thus, butyrate inhibits histone deacetylase, and it selectively blocks estrogen induction of ovalbumin in avian oviduct (McKnight *et al.*, 1980), and glucocorticoid induction of tyrosine aminotransferase in HTC rat hepatoma cells (Plesko *et al.*, 1983).

Other Possible Sites and Modes of Action of Steroids and Steroid–Receptor Complexes. As will be discussed in some detail below, the tools of recombinant DNA technology have recently engendered a growing body of evidence that S–R complexes interact with specific sequences of nucleotides in regions adjacent to, or within, structural genes in order to regulate their transcription. Nonetheless, it is important to realize that there is evidence for, and proof of, various other foci of steroid

action. Indeed, it is not realistic to believe that direct, exclusive, interaction of S–R complexes with putative regulatory sequences of DNA is sufficient to explain all the monifold effects of steroids, ranging from organogenesis at one extreme to cell proliferation and postmorphogenetic function at the other. A brief survey of other such foci is in order, if only to indicate the breadth and depth of the intracellular substrate upon which genetic mutation can act to generate heritable variation in steroid responsiveness.

Aside from the action of S–R complexes on the synthetic rates of various RNAs, their possible roles (primary or not) in processing, transport, stabilization, degradation, or utilization of certain RNAs were given early attention (Liang *et al.*, 1977; Anderson, 1984). For instance, about 10 percent of the ribonucleoprotein particles extracted from prostatic nuclei can bind A–R complexes readily, while equivalent preparations from the liver do not (Liao *et al.*, 1973*a*). Indeed, androgen given to castrated animals has been shown to enhance the initiator tRNA-binding activity of prostate cytosol within 20 min of administration (Liang and Liao, 1975), and others have reported that it is needed to maintain elongation-factor activity during translation in rat ventral prostate (Ichii *et al.*, 1974). Furthermore, while androgen enhances the level of translationally active mRNA, as confirmed by experiments using mRNA from androgen-treated animals in *in vitro* translation systems (Liao, 1965), it may stimulate chromatin-bound RNA polymerase I activity (which catalyzes rRNA synthesis) even before it does that of the comparable RNA polymerase II activity (Mainwaring *et al.*, 1971). And, in both cases, there is reason to believe that androgen somehow enhances the "factors" that promote the activity of the polymerases, rather than the respective apoenzymes themselves (Liang *et al.*, 1977).

Similar information is available for other steroids. Glucocorticoids can regulate protein processing and compartmentalization (Firestone *et al.*, 1982). In addition, Gokal *et al.* (1986) have lately shown that Dex can decrease the level of an rDNA transcription factor in P1798 lymphoma cells. Gordon and Williams (1986) have recently reported that estrogen regulates the degradation of the mRNAs for apoliprotein II and vitellogenin II in chick liver, as Palmiter and Carey (1974) and Diamond and Goodman (1985) had previously shown in other steroid response systems. Similarly, posttranscriptional regulation by estrogen of albumin gene expression in *Xenopus* liver has been recognized latterly by Schoenberg *et al.* (1986).

Lastly, two aspects of steroid hormone action in somatic cells have

received relatively little attention largely, we suspect, on account of scientific faddism rather than sober assessment of their potential roles in the overall scheme of steroid hormone responsiveness.

The first is the relation of steroid hormones with the plasma membrane of target cells. It is commonly assumed that the lipophilicity of steroids enables them to pass through these membranes by simple diffusion. Yet, there is a body of evidence, albeit partly controversial (Sadler and Maller, 1984), indicating that steroids bind to SR-like molecules in the plasma membrane. Indeed, such molecules may not only influence the transfer of steroids across the membrane, but may also mediate, directly, certain effects of steroids on it (Pietras and Szego, 1979*a, b, c*). In this respect, it is notable that a mutant thymoma cell line has been described (Johnson *et al.*, 1984) that resists the lytic effect of Dex, but not of a closely related synthetic glucocorticoid (triamcinolone), even though the intracellular GR receptor of these cells binds Dex normally. Notwithstanding the immediate relations of steroids with the cell surface, there is good reason to believe that some steroid effects, that depend upon the classical intracellular SR apparatus, are nevertheless mediated by relatively rapid alterations in cell surface properties (Ballard and Tomkins, 1969; Fiskin and Melnykovych, 1971; Berliner and Gerschenson, 1975).

The second "low-profile" area of research on steroid hormone action is its relation with divalent metal cations. Colvard and Wilson (1984) have characterized well the ability of Zn^{2+} to potentiate A–R complex binding to rat prostatic nuclei, and there is reason to speculate that some of the well known noxious effects of Zn deficiency on male reproduction may be mediated in this fashion. Furthermore, the Zn^{2+} effect described above was not mimicked by even higher concentrations of six other divalent metal cations; hence, it seems to be specific. Interstingly, there is a hint of a Zn-binding domain in the GR (Weinberger *et al.*, 1985), and it has been profered that DNA-binding proteins, as a group, require Zn (Barton *et al.*, 1982). Rat uteri incubated with $2x10^{-4}$ M $CuCl_2$ for up to 1 hr have 30–50 percent less cytosolic and nuclear ER activity than controls (Smith and Kronenberg, 1984), and this may have some bearing on the way Cu-containing intrauterine contraceptive devices work. But, the physiological relevance of this observation is yet to be established. On the other hand, Ca^{2+} inhibits *in vitro* cytosolic GR activity from rat liver and kidney, or rat and human cell lines, in concentrations (0.1–1 μM) at which it effects its second messenger function in cells (Van Bohemen *et al.*, 1983). This

Ca^{2+} effect was also observed with the MR from rat kidney, but not with rat prostate AR (thus confirming the observation of Colvard and Wilson, 1984) or rat uterus ER and PR, indicating its relative selectivity. Various kinetic, thermodynamic, and hydrodynamic criteria are considered to suggest that Ca^{2+} alters receptor conformation (Rousseau *et al.*, 1984). Interestingly, GR activity and action can be modified in intact HTC rat hepatoma cells, or rat hepatocytes in primary suspension culture, by modulating their Ca environment directly (Rousseau *et al.*, 1982) or indirectly (Rousseau and van Bohemen, 1984). In aggregate, these data make it reasonable to consider that physiological variation in the level and distribution of cellular calcium can influence the properties of corticoid receptors sufficiently to modify cellular responses to them.

The Interaction of Steroid–Receptor Complexes with Regulatory Sequences of DNA. Two research efforts deserve to be identified as forerunners of the idea that S–R complexes regulate structural gene activity by controlling the levels of their respective mRNAs. Sekeris (1964) used *in vitro* translation to demonstrate that ecdysone stimulated dopa decarboxylase activity by increasing production of its mRNA. Peterkofsky and Tompkins (1968) exploited inhibitors of protein and RNA synthesis to deduce that glucocorticoids induce tyrosine aminotransferase (TAT) activity in HTC hepatoma cells by promoting the accumulation of TAT mRNA. The modern tools of recombinant DNA technology have enabled subsequent investigators to prove that S–R complexes promote the rate of gene transcription by interacting with particular regulatory sequences of DNA. In the following section, a sample of recent observations generated by these techniques will be described to indicate the extraordinary advances that have been made, and so identify the many important questions that have been raised at the same time.

Glucocorticoid-Inducible Genes. The mouse mammary tumor virus (MMTV) is a typical single-stranded RNA retrovirus that replicates via a double-stranded proviral DNA intermediate (Varmus, 1982). Glucocorticoids rapidly stimulate replication of native or cloned proviral transfected MMTV in the absence of protein synthesis within a variety of heterologous cell types that bear GR activity. During this process MMTV provirus is produced that contains direct repeats of sequences at the 3′ and 5′ ends of the viral mRNA. These long terminal repeats (LTR) consist of 130 base pairs from the 5′ end and 1200 pairs from the 3′ terminus of the retrovirus, and contain the major regulatory signals necessary for viral gene transcription. The latter include the core promoter

region, which is composed of the TATA and CAAT boxes centered about 25 and 65 nucleotides, respectively, upstream of the site of transcription initiation, and the glucocorticoid-regulatory region, which is located further upstream. In fact, there are four other regions, within the transcribed sequences of MMTV DNA, that bind purified G–R complexes selectively. Furthermore, each such region is composed of multiple, discrete G–R binding "sites" or sequence elements. For example, the upstream region contains five distinct sites that have been identified by nuclease protection (footprinting) experiments (DeFranco *et al.*, 1985). Linker scanning mutations (McKnight and Kingsbury, 1982) that abolish one footprint leave the others intact and, if the mutation disturbs a conserved octanucleotide sequence that is found within each footprint, glucocorticoid-responsiveness is reduced (DeFranco *et al.*, 1985).

The LTR of MMTV confers glucocorticoid-inducibility on various heterologous structural sequences that are fused with it (Ringold, 1985). A portion of the LTR primarily responsible for glucocorticoid-inducibility has been identified by assessing the glucocorticoid-responsiveness of chimeric gene constructions composed of deliberately deleted LTRs and heterologous coding sequences, the products of which are easily detectable. In this way, the site has been identified as having a 5′ border between −190 and −174, and a 3′ border between −151 and −138 nucleotides upstream of the transcription start site (Ringold, 1985). This site of the LTR is thus distinct from the "boxes" in its core–promoter region, and it will confer glucocorticoid-inducibility on heterologous promoters, such as the one from the herpesvirus thymidine kinase (Chandler *et al.*, 1983). Furthermore, the distance between the glucocorticoid-regulatory site and the "boxes" does not seem to be critical (Chandler *et al.*, 1983); thus, it has qualities reminiscent of so-called "enhancers" (Yamamoto, 1985). The latter are DNA sequences that can enhance the activity of promoters over relatively large distances, and regardless of their orientation relative to the promoters. It is probable that enhancers are sites of DNA–protein interaction; in this sense, also, their apparent commonality with the "sites" within glucocorticoid-regulatory region is tantalizing.

A variety of other genes are regulated by glucocorticoids, in some cases as shown by transfection into steroid-responsive heterologous or homologous cells; among them are the genes for human (Robins *et al.*, 1982) and rat (Karin *et al.*, 1984*a*) growth hormone, those for tyrosine aminotransferase (TAT) and tryptophane oxidase (TO) in rat liver (Shinomiya *et al.*, 1984), the one for human metallothionein-IIA (hMT-IIA)

(Karin *et al.*, 1984*b*), and the one for chick lysozyme (Renkawitz *et al.*, 1984). In the case of hMT-IIA, deletion analysis has placed the 5′ border of the glucocorticoid-regulatory region between −268 and −236 (Karin *et al.*, 1984*b*), and it is distinct from the region responsible for inducibility by heavy metals. For chick lysozyme, which can be induced by a variety of steroids, the 5′ border of the regulatory region for glucocorticoid and progesterone is between −208 and −164. It would be interesting to know whether a common sequence or two different ones serve the needs for both types of S–R complexes.

Not surprisingly, the glucocorticoid-regulatory region of the LTR is also the site of high-affinity binding with G–R complexes. Indeed, the ability of monoclonal antibodies against the GR to inhibit such binding has indicated that the receptor itself is directly involved in the binding process (Scheidereit *et al.*, 1983). There are other G–R binding domains in the LTR, but none has as great an affinity as the glucocorticoid-regulatory region itself.

The use of nuclease digestion ("footprinting") to identify DNA sequences protected by bound G–R complexes has identified the binding region of the hMT-IIA gene to be between nucleotides −266 and −241 (Karin *et al.*, 1984*c*). This is gratifyingly consistent with the placement of the 5′ border of this region at −268 on the basis of function studies (Karin *et al.*, 1984*b*). On the other hand, G–R complexes bind more tightly to the 5′ domain of the chick lysozyme gene between residues −74 and −39 than they do to the stretch between −208 and −161, yet the latter is responsible for functional inducibility of the gene by glucocorticoid or progesterone.

Within the domains that bind G–R complexes one always finds the sequence T-G-T-T-C-T or derivatives of the degenerate octonucleotide T/A-C-T-G-T/A-T-C-T, sometimes in multiple copies (Payvar *et al.*, 1983; Scheidereit *et al.*, 1983; Karin *et al.*, 1984*c*). Methylation at N-7 of guanine residues in the hexanucleotide abolishes binding (Scheidereit and Beato, 1984), and prebound G–R complexes protect these residues against methylation (Karin *et al.*, 1984*c*). Thus, these sequences are directly involved in the binding process. Furthermore, the spacing and topography of guanine residues are different in binding domains that are functional than in those that are not functional. Nevertheless, it is not known how this difference translates into function or nonfunction.

Dexamethasone 21-mesylate froms convalent complexes with the GR that transform normally to the DNA-binding form. Yet, this analogue is

a glucocorticoid antagonist. Recently, it has been found that these antiglucocorticoid–receptor complexes bind to defined sequences of the LTR in the MMTV in the same way as normal G–R complexes (P. A. Miller *et al.*, 1984). Clearly, the activity of this antiglucocorticoid must be due to other properties of its receptor complexes; for instance, those important for recognition of nonhistone chromosomal proteins and/or chromatin conformation, and those that are directly responsible for promoting gene transcription, once binding to regulatory DNA sequences has occurred.

Sex Steroid-Inducible Genes. The first application of recombinant DNA technology to the study of steroid hormone action concerned the effects of estrogen and progesterone on the level (Cox *et al.*, 1974) and transcriptional regulation (McKnight and Palmiter, 1979) of ovalbumin mRNA. Ovalbumin is a major egg white protein and is stimulated by estrogen in immature chick oviducts and by estrogen, as well as by other steroids, in mature (primed) chick oviducts.

Deletion analysis has been used to dissect the 5′ steroid-regulatory region of the ovalbumin gene after it has been fused to chick β-globin coding sequences and transfected into immature tubule gland cells of the chick oviduct. Progesterone-inducibility requires the presence of nucleotides between −222 and −95 (Dean *et al.*, 1983). It is significant that estrogen- and glucocorticoid-inducibility also depend upon this region, but it is not yet known whether the precise sequences required for each of these S–R complexes are identical and, if not, to what extent they differ or are overlapping (Dean *et al.*, 1984).

The secreted prostatic steroid-binding protein (''prostatein'') consists of three polypeptides. The gene for one, C3, retains its androgen inducibility when it is transfected into androgen-responsive Shionogi (S115) mouse mammary carcinoma cells along with 3.5 kb of its 5′ flanking sequence (Page and Parker, 1983). The same is true of the estrogen-inducible gene that encodes the pS2 protein in the human breast cancer cell line MCF-7 (A. M. C. Brown *et al.*, 1984). It is noteworthy that two regions of the C3 gene exhibit preferential binding of A–R complexes. One extends from −270 to +82 of the first exon, and possibly overlaps the 30-base sequence between positions −190 and −330 that is shared by the C3 component of prostatein and seminal vesicle secretory protein IV (Kandala *et al.*, 1985). The other contains 470 bases of the first intron, and shows even higher binding than the first (Perry *et al.*, 1985). The presence of one of these sites within the ''structural'' portion of the gene is reminiscent of the four G–R binding regions that have been located in

the MMTV within a stretch of 4–8 kb downstream of its 5′ end (Payvar *et al.*, 1983).

As expected, the progesterone-regulatory region of the ovalbumin gene binds P–R complexes with greater affinity than do nonspecific DNA sequences (Mulvihill *et al.*, 1982), but the relative affinity is only about tenfold. This is much less than the 1000-fold increment observed for the binding of G–R complexes to various glucocorticoid-regulatory regions, and even the latter increment cannot begin to explain the specificity with which a steroid induces the transcription of selected genes *in vivo*.

The E–R complexes bind to a 5′ flanking site of the chicken vitellogenin that is centered between nucleotides −660 and −550 (Jost *et al.*, 1984), and to a site within a 0.9-kb fragment of the rat prolactin gene whose 5′ border is 1.5 kb upstream of the transcription initiation site (Maurer, 1985). These sites are further upstream than physiologically relevant sites so far defined for other S–R complexes; and, in each case, the binding site is found within a region of DNase I hypersensitivity (Burch and Weintraub, 1983; Maurer, 1985). Most interestingly, the 5′ 0.9-kb fragment of the rat prolactin gene that binds E–R complexes selectively contains two regions with an alternating pattern of purines and pyrimidines. Such sequences can form DNA with a left-handed helix (Z-DNA), and conformational transitions from B- to Z-DNA have been incriminated in the regulation of gene transcription (Nordheim and Rich, 1983).

Mechanisms by Which Steroid–Receptor Complexes Regulate. Although it is clear that steroids can act at other levels, as pointed out above, positive (stimulatory) or negative (inhibitory) regulation of gene transcription is their primary mode of action. Therefore, it is reasonable to list the possible mechanisms by which they accomplish the latter (Anderson, 1984; Yamamoto, 1985; Ringold, 1985).

First, experimental disengagement of a core–promoter region from its steroid-regulatory region only rarely generates a higher than basal rate of transcription that is steroid-independent. Therefore, it does not seem as if S–R complexes counteract a constitutive form of negative regulation, as exemplified by bacterial repressors. Second, steroid-regulatory regions can be moved far from their normal core–promoter regions and still remain collaborative, and they can enhance more than one promoter region arranged in sequence. For these two reasons, it does not seem as if S–R complexes interact physically with RNA polymerases. One is left, therefore, with the view that S–R complexes can somehow alter the conformation of chromatin in such a way as to promote the efficiency with

which RNA polymerase itself can bind to DNA, or the efficiency with which various transcription "factors" can come into a fruitful union with the RNA polymerase apoenzyme. A relevant qualification of this view is that the physical "distance" between a steroid-regulatory region and its core–promoter region may not be equal to the "functional" distance between them, due partly to the possible topological contribution of the nuclear matrix, and partly to the solenoidal character of chromain. The latter means that two regulatory sites can be 1 kb apart in terms of DNA, and yet be physically, and therefore functionally, adjacent.

Strong general support for this view is provided by the fact that transcriptionally active eukaryotic genes exist in domains that have altered chromatin configuration as measured by their susceptibility to nucleases (Weintraub and Groudine, 1976). Within these domains, specific sites, hypersensitive sites (HS), are even more sensitive to DNase I. Indeed, it is known that steroids can alter the general DNase sensitivity of broad chromatin domains that contain structural genes susceptible to their influence, as well as the distribution of HS within these domains. In some cases, the alterations are steroid-reversible; in others, they are not. The former would more likely correspond to the epigenetic changes visualized to underlie cellular differentiation; the latter, to the short-term changes associated with transient steroid stimulation. While these conceptualizations are gratifying, they do not indicate the cause and effect relation between transcriptional activation and altered chromatin configuration. Indeed, even if altered chromatin configuration is a cause of transcriptional activation, it is not known whether S–R complexes *per se* are responsible, or whether they merely serve physically to focus the activity of other proteins (?enzymes) that carry out the task of altering chromatin configuration.

Finally, as impressive as the data are to this point that S–R complexes are intimately involved with activation of gene transcription, it must be understood that the primary products of steroid-regulated genes may themselves be gene-regulatory. Therefore, the overall effect of a steroid may depend on a cascade of primary, secondary, and higher order events that are intimately coordinated in chronological and spatial ways. Such coordination has been demonstrated for the "puffing" patterns that appear on polytene chromosomes of *D. melanogaster* after ecdysteroid exposure (Richards and Ashburner, 1984). And, it is clear that steroid-induced morphogenesis and histotypic differentiation in higher organisms must involve such cascades, as elegantly discussed by Yamamoto (1985).

It is worth concluding with a list of the important questions that remain about transcriptional regulation by S–R complexes and some pertinent comments about each of them:

1. To what extent is the steroid-dependent selectivity of this process embodied in the specificity of the binding sites for S–R complexes within the 5′ regulatory regions? Directed point mutagenesis of such regulatory regions and their transfection into receptor-bearing expressive cells will be useful approaches, but a steroid-dependent *in vitro* transcription system would be still better and, in the final analysis, correlation between molecular genotype and organismal phenotype will be indispensible.

2. Are there discrete, individual contributions of a steroid and its receptor to the overall signal content of a particular transformed S–R complex? For instance, does receptor-bound steroid interact directly with DNA? Indeed, indirect evidence for such interaction has existed for some time (Kidson *et al.*, 1970; Arya and Yang, 1975), and more of it has been generated recently. Thus, stereochemical considerations have led to the hypothesis that various classes of steroid hormones, and related agonists, are able to "fit" between base pairs in the partially unwound double helical sequence 5′-dTdG-3′ 5′-dCdA-3′ in a way that is commensurate with the size and shape ("perimeter") of the cavity defined by the bases, and concordant with the formation of hydrogen bonds between certain oxygen functions in the steroid and the phosphates in the DNA backbone (Hendry *et al.*, 1986). In the case of corticosteroids, additional hydrogen bonds are visualized to form with bases in the sequence (Bransome *et al.*, 1986*a*). Indeed, these considerations are able to predict agonist potency from the degree of "fit", and antagonist effect from the degree of ill-fit (Bransome *et al.*, 1986*b*). Lehner (1986) has observed, additionally, that the stereochemical complementarity of steroid–DNA interaction extends to the stacking of steroid A/B rings between dTdG and of C/D rings between dCdA, in the critical double-helical sequence. Recently, Lehner *et al.* (1986) have begun to use various physicochemical parameters to assess DNA interaction of a series of estrogens whose biopotency is predictable by stereochemical criteria: temperature of denaturation; UV/visible absorption spectroscopy; and fluorescence spectroscopy in the presence and absence of metal cations. Their initial study on coumesterol supports the idea that it inserts itself between base pairs of the critical sequence. Lehner (1986) has also pointed out that the critical sequence occurs consistently in reported steroid–receptor binding sites. Further

results of studies based on this novel approach to the molecular biology of steroid responsiveness are awaited with keen interest.

3. What are the structural properties of a given steroid, as distinct from a closely related one, that enable it not only to bind to a receptor with high affinity, but also to induce an allosteric (conformational) change in its receptor that endows the S–R complex with regulatory power?

4. Conversely, what are the properties of a receptor that, once liganded properly, enable it to function as a selective regulatory signal for transcription? For example, in the case of G–R complexes, what does the "modulating" domain of the receptor do in order to lower the binding affinity of the complexes for nonspecific DNA sequences and to raise their binding affinity towards specific DNA sequences? The ability to clone the genes that encode the structure of receptor proteins has begun to be a fruitful source of answers to the last question.

5. To what extent is S–R complex regulation of transcription, directly or indirectly, the effect of alterations in higher-order chromatin structure? Much will have to be learned about all forms of transcriptional regulation before this question can be answered in a worthwhile manner, but the way in which S–R complexes regulate transcription during acquisition of terminal cytodifferentiation will be invaluable in this regard.

6. How do S–R complexes act in relation to other positive or negative trans-acting "factors" to achieve vectorial regulation of gene expression at the level of transcription? For instance, it has become well-known that unknown cell-specific "factors" influence the extent to which transfected genes are expressed in target cells. Furthermore, even when a target cell is chosen to optimize these cell-specific factors, the transcriptional regulation of a gene may be demonstrably under the joint influence of a steroid hormone (or more than one) and one or more nonsteroid hormones or growth factors (Sanders and McKnight, 1985). For example, in regard to the rat prolactin gene (Somasekhar and Gorski, 1986), different sequences 5′ to the cap site are required for stimulation of transcription by EGF and its inhibition by Dex. Likewise, in the case of the rat growth hormone gene, the individual stimulatory effects of triiodothyronine and Dex are mediated by sequences within 236 base pairs of the cap site, whereas sequences which mediate their synergistic action either reside much further upstream, or possibly within the first intron of the structural gene itself (Samuels *et al.*, 1986). A particularly instructive example of steroid and nonsteroid interaction in the vectorial expression of a gene product has been partially elucidated by Gelehrter (1986). His group (Bar-

ouski-Miller and Gelehrter, 1984) has shown that Dex inhibits the plasminogen activator (PA) activity of HTC rat hepatoma cells by a receptor-mediated mechanism, although it stimulates the amount of PA synthesized by the cells, and it even enhances the stimulation of PA activity by cyclic nucleotides. The explanation for these apparently paradoxical observations is that Dex–R complexes also enhance the synthesis of a PA inhibitor, and the vectorial effect of Dex on PA activity is, therefore, negative.

7. What determines whether a particular S–R complex will enhance or inhibit gene transcription when it binds to a regulatory DNA sequence element? In the case of the pituitary pro-opiomelanocortin (POMC) gene, G–R complexes are inhibitory because they bind to the "CCAAT" box region, and presumably impede its function (Drouin *et al.*, 1986). It is tempting to suggest that this may be a general explanation for how S–R complexes inhibit transcription, since the core–promoter elements seem to be essential for transcription of most genes.

Finally, for all of the foregoing questions, the use of Mendelian mutations that singularly affect discrete steps in the process of transcriptional regulation by S–R complexes will be invaluable for dissecting the essential elements in the overall process.

Steroid Hormone Resistance

It is obvious that a target cell may resist the action of a steroid hormone for a variety of reasons unrelated to the receptor-dependent portion of the overall response apparatus. One such reason, failure of the steroid to enter a target cell, has been recognized in one type of glucocorticoid-resistant lymphoma cell (Johnson *et al.*, 1984). A second reason may be failure of prohormone to be converted to its active derivative, either within a target cell itself or in some other cell type. Human autosomal recessive 5α-reductase deficiency interferes with the conversion of T to DHT. Within certain androgen-target cells, T is an ineffective androgen; its inability to be converted to DHT within such cells endows them with a form of selective androgen resistance specific to T that coexists with a state of androgen sensitivity specific to DHT. Similarly, failure of 25-hydroxyvitamin D_3 to be converted to 1,25-dihydroxyvitamin D_3 in the kidney is the result of autosomal recessive 1α-hydroxylase deficiency in man. This results in a form of selective vitamin D resistance that is directed toward the prohormone, but not against its more active derivative. Finally, steroid hormone resistance may result from the failure of a per-

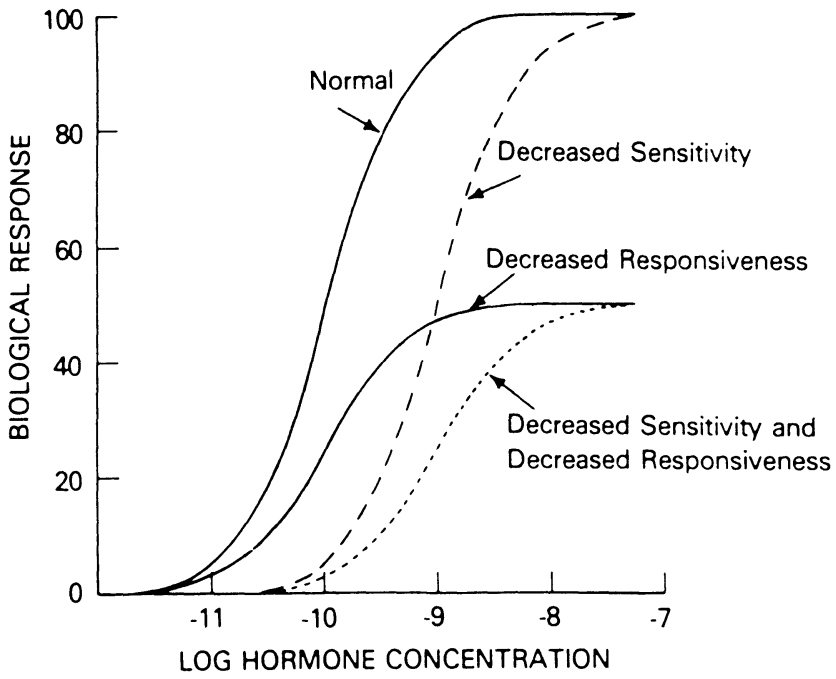


Fig. 11. Normal and various types of altered bioresponse to serial concentrations of a hormone. [From Kahn (1978) with permission.]

fectly normal receptor apparatus to effect the usual postreceptor events necessary to realize a steroid action. This is exemplified by allelic variation at the *Gur* locus in mice (Swank, 1978), which regulates the ability of androgens to induce β-glucuronidase activity in the proximal convoluted tubules of the kidney, but not other tissues (Swank, 1978), and by the epigenetic state of repression due to DNA methylation that is responsible for resistance to glucocorticoid-induced lysis in the SAK murine thymic lymphoma cell line (Gasson and Bourgeois, 1983).

The terms resistance, insensitivity, unresponsiveness, and refractoriness are often used synonymously in every-day parlance. In the case of inadequate reactivity to a hormone, some authors appear to favor "resistance" over "insensitivity," or the reverse apparently on stylistic grounds. It has been argued, however, that "resistance" ought to be reserved as a generic term, and that "insensitivity" and "unresponsiveness" should be used to distinguish specific types of resistance on biologic grounds, as depicted in Fig. 11. Thus, "decreased sensitivity" would apply to the situation in which a normal response to a hormone can be achieved by higher than normal levels of the hormone; while "decreased responsiveness" would describe the incapacity to achieve the maximal

response to a hormone, whatever its level. This scheme has the virtue of accommodating the dual situation of "decreased sensitivity" and "decreased responsiveness," but it suffers from the fact that "resistance" carries the connotation of an active process leading to lack of reactivity, while "insensitivity" connotes a passive process. For instance, accelerated conversion (gonadal or peripheral) of androgen to estrogen (hyperaromatization), as in the Seabright-bantam rooster (George and Wilson, 1982), would qualify as an active form of "resistance," while absence of a normal property of the A–R system would confer "insensitivity" passively. It is important to appreciate here, as will be explained below, that decreased sensitivity, as defined in terms of a biologic response, need not correspond to decreased sensitivity as defined by the kinetics with which an androgenic ligand binds to the AR. Notwithstanding these considerations, henceforth the term "steroid resistance" will be used in this chapter in its generic sense and with respect to receptor-related variation, unless otherwise specified.

THE ANDROGEN-RESPONSE SYSTEM IN DEVELOPMENT: IMPLICATIONS FOR GENETIC HEALTH AND DISEASE

The Fundamentals of Masculine Differentiation of the Mammalian Reproductive System

The basic prerequisites for such differentiation are depicted in Fig. 12. Testis-determining factors (structural or regulatory) are encoded by a gene (or genes) on the Y chromosome, usually on its short arm near the centromere. Together with other genetic factors, both on the X chromosome and the autosomes, the Y-linked determinants dictate that each bipotential gonad shall develop as a testis (Kidd, 1985; Kiel-Metzger *et al.*, 1985; Haseltine and Ohno, 1981). The Sertoli cells are responsible for elaborating a glycoprotein substance that, acting as a "short-range" (paracrine) hormone, is responsible for regression of the Müllerian duct on its own side (Josso *et al.*, 1977). It is important to appreciate that this hormone causes *regression* of a normal structure, and not inhibition of its development. Therefore, the appellation Müllerian Regression Factor is more appropriate than its more common name, Müllerian Inhibitory Factor. The Leydig cells secrete T, possibly autonomously, but probably

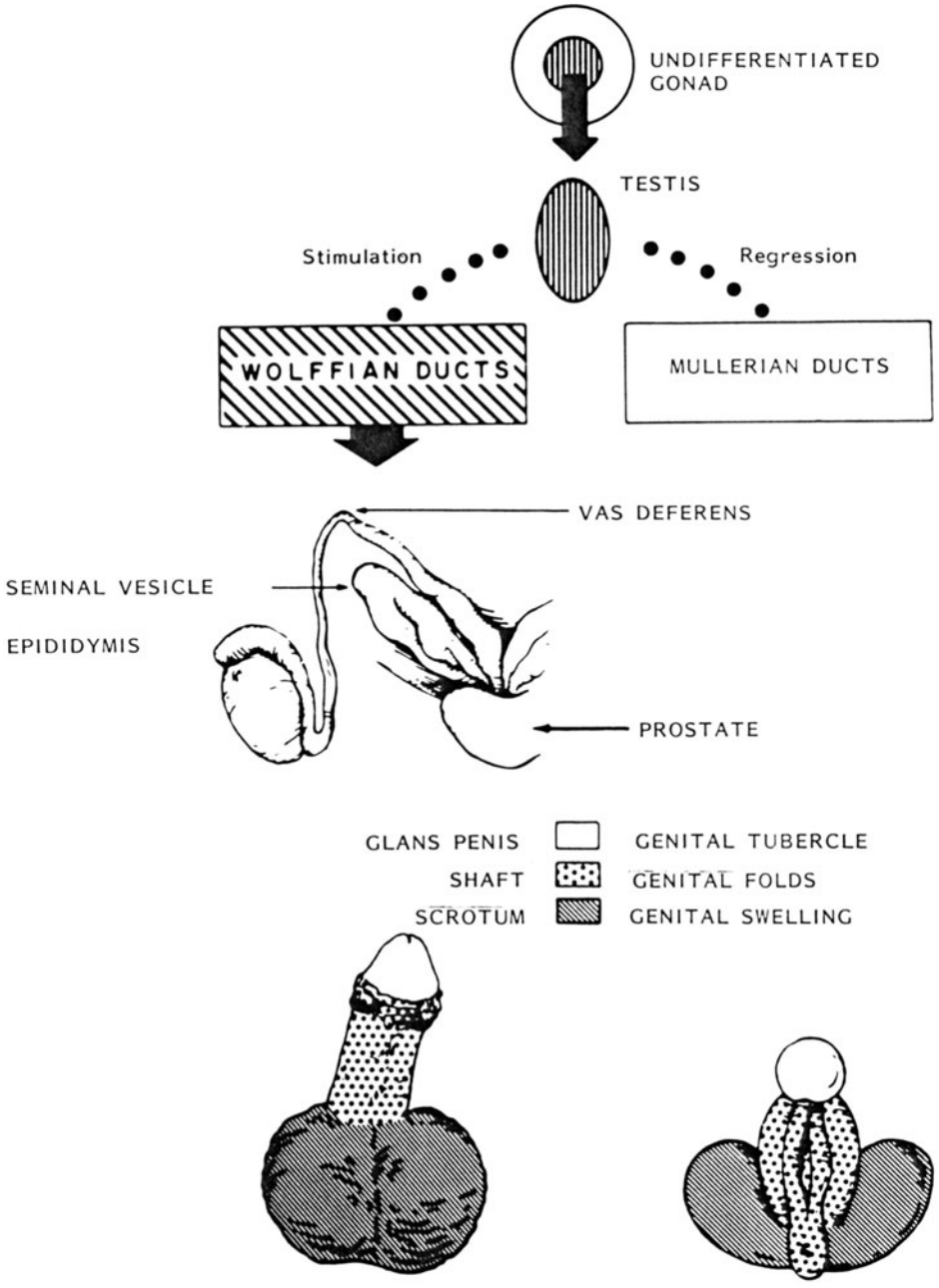


Fig. 12. The scheme of internal and external genital differentiation in males. [Adapted from Federman (1967) with permission.]

under the positive regulatory influence of chorionic gonadotropin, and T itself is responsible for differentiation of the Wolffian ducts into the vasa deferentia, the seminal vesicles, and the epididymes. By contrast, T is necessary but insufficient for imposing masculine differentiation on the urogenital sinus and the external genital primordia. For them it is a prohormone that must be converted to 5α-DHT, and the latter acts as the effector androgen (Siiteri and Wilson, 1974).

From studies of rodents mostly, and primates latterly, there is clear evidence that the hypothalamic–pituitary axis, various higher and lower regions of the central nervous system, and even organs not usually considered sexually dimorphic (such as the liver) are subject to the differentiating (organizing; imprinting) influence of androgen early in mammalian development. This imprinting process is essential for the evocation (activation) of masculine responses by androgen that appear at puberty. The degree to which humans are subject to such imprinting, to what extent conversion of androgen to estrogen (aromatization) is involved, and whether there is a division of labor between T and DHT at this level of sexual differentiation remain to be fully defined. The available evidence will be discussed below in a separate section.

Epithelial–Mesenchymal Interaction during Steroidogenic Histogenesis and Implications Thereof

The reproductive tracts of fetal/embryonic and neonatal rodents develop histotypic characteristics in response to their particular sex steroid environments. Thus, by autoradiographic analysis, it is found that nuclear E–R sites are restricted to the mesenchyme of the urogenital sinus and Müllerian ducts of embryonic mice (Stumpf *et al.*, 1980), and administration of estrogen to 3-day-old mice causes hyperplasia and cornification of the vaginal epithelium in the absence of epithelial nuclear E–R activity (Cunha *et al.*, 1982*b*). Indeed, the latter appears spontaneously only 10–20 days after birth (Cunha *et al.*, 1982*a*). These observations strongly suggest that epithelial differentiation in the female reproductive tract is a response to "signals" generated by adjacent mesenchyme after it is stimulated by estrogen.

This suggestion is proved by experiments in which epithelia and mesenchyme of various sources are recombined in organ culture systems and subjected to appropriate steroid stimulation. Thus, regression of mouse mammary epithelium will occur, whatever its genotype, only when it is

combined with homotypic, wild-type, mesenchyme that contains normal AR activity. If AR-deficient mesenchyme from Tfm (androgen-insensitive) mice is used, epithelial regression fails (Kratochwil and Schwartz, 1976). The same is true with regard to combination of epithelium and mesenchyme of the urogenital sinus when androgen-induced prostatic epithelial differentiation is the normal effect (Lasnitzki and Mizuno, 1979). Indeed, use of androgen-insensitive mesenchyme permits the development of vaginal in place of prostatic epithelium whatever the genotype of the epithelium (Cunha *et al.*, 1983). These recombination experiments are in complete accord with observations of normal male breast and prostatic development. In the prostate, epithelial growth and arborization of the epithelium occurs before it acquires any nuclear AR activity as demonstrable by autoradiography. The latter appears only with the initiation of secretory activity. In the male mouse breast, mesenchymal cells adjacent to epithelial breast buds acquire AR activity before epithelial regression occurs, and the autoradiographic concentration of the AR activity in these cells varies inversely with their distance from the bud (Heuberger *et al.*, 1982). This suggests that the epithelium plays an inductive role in the acquisition of AR activity by adjacent mesenchyme, and therefore that the epithelial–mesenchymal interaction that is responsible for suppression of mammary gland development in male mice is a reciprocal one.

In aggregate, these observations indicate that primary epithelial differentiation and/or morphogenesis of most reproductive tract organs is conducted under the influence of certain sex steroid hormones, but is mediated by mesenchymal messengers rather than by direct effects of the steroids on epithelium. Moreover, the mesenchymal signals are differentiatively specific and function across species. For example, uterine mesenchyme will induce vaginal epithelium to undergo columnar, instead of stratified squamous, differentiation, and vaginal mesenchyme evokes a stratified squamous epithelial response from uterine epithelium that is originally simple columnar (Cunha *et al.*, 1983). Similarly, mouse vaginal mesenchyme will support human vaginal epithelial growth and differentiation. In the male reproductive tract, the differentiative repertoire (competence) of epithelial differentiation is conditioned by its embryonic origin. Thus, urogenital sinus (UGS) epithelium (of endodermal origin) will develop as prostate, not as seminal vesicle, in response to seminal vesicle mesenchyme and seminal vesicle epithelium of mesenchymal origin will

develop as seminal vesicle in response to combination with UGS mesenchyme (Cunha *et al.*, 1983).

Haslam and Levely (1985) compared the estrogenic responsiveness of mouse mammary cells in mixed culture, and in cultures containing only epithelial cells or fibroblasts. 17β-estradiol increased PR concentration in mixed cultures, but not in pure epithelial or fibroblast cultures. Indeed, fibroblast cultures had no PR, under basal conditions or after estrogen exposure. Thus, estrogen induces PR in mammary epithelial cells by a primary ER-mediated effect in adjacent mesenchymal cells that is somehow communicated to their epithelial cell mates. This observation is very similar to the one made previously in relation to androgenic inhibition of "surfactant" production in mixed and purified cultures of lung cells (Smith, 1978).

The extent to which other steroid hormones conduct or affect differentiation and morphogenesis of other target organs (for example, intestinal epithelium; palatal shelf fusion) by a primary action on mesenchyme is unclear. However, in the case of fetal lung development, there is solid evidence that glucocorticoids have a primary effect on mesenchymal cells and that an oligopeptide signal released by them ("fibroblast-pneumonocyte factor") stimulates nearby alveolar type II cells to synthesize and release surfactant (B. T. Smith, 1978; Post and Smith, 1984). Indeed, male premature infants have less mature lung development (Torday *et al.*, 1981) and are more prone to hyaline membrane disease than females of equal gestational age, and benefit less from prophylactic glucocorticoids administered to mothers before delivery. There is good evidence that androgens interfere with this process at more than one step in the interaction between lung fibroblasts and adjacent type II alveolar cells (H. C. Nielsen *et al.*, 1982; Torday, 1984, 1985).

Of great potential importance is the fact that epithelial–mesenchymal interaction may not be limited to primary differentiation and morphogenesis of reproductive tract structures; it may also be involved in the histological changes associated with reproductive cycling, and to some extent with the genesis and metastases of sex steroid-responsive malignancies. These considerations are based in part on the fact that appropriate epithelium (breast, prostate) from adults can be induced to proliferate in combination with homotypic mesenchyme from embryos, and vice versa (Cunha *et al.*, 1983). It is simple to imagine that reactivation of the mesenchymal effect on otherwise quiescent adult epithelium may be involved in the initiation of benign prostatic hyperplasia (Wilkin *et al.*,

1980; Bolton *et al.*, 1981; McNeal, 1985), as well as of malignant changes in the prostate and the human breast. Furthermore, the steroid-responsiveness of cells that metastasize from primary foci of reproductive organ malignancy might well be conditioned by the foreign mesenchymal (stromal) environment in which they find themselves.

Sexual Differentiation of the Central Nervous and Immune Systems and the Liver

The Central Nervous System

5α-Reductase activity, the aromatizing enzymes responsible for converting androgen to estrogen, and receptors for androgen and estrogen are demonstrable in the mammalian brain before birth or during the first week of life (Martini, 1982; Tobet *et al.*, 1985; McEwan, 1982). Thus, the molecular apparatus for sex steroid hormone responsiveness is in place when experimental manipulation of these hormones can interfere with sexual differentiation of the rat brain, as normally reflected in sexual dimorphism of reproductive physiology and copulatory as well as nonsexual behavior. Adult male rats exhibit a tonic pattern of gonadotropin secretion and male copulatory behavior, while adult female rats exhibit a cyclical pattern of gonadotropin secretion and female copulatory behavior (the lordosis response). Male rats deprived of T during the critical (perinatal) period of brain differentiation do not exhibit normal male reproductive behavior and physiology at puberty, even if normal levels of T are provided at that time; female rats exposed to T during the critical period develop the male pattern of behavior at puberty. Thus, the adult male pattern of gonadotropin secretion and copulatory behavior depends upon two dissociable processes: suppression of the female pattern (defeminization) and development of the male pattern (masculinization). Indeed, there is a division of labor in the achievement of these effects. Estrogen, aromatized from T, suppresses the cyclical pattern of gonadotropin secretion and lordotic copulatory behavior, while androgen and estrogen promote the masculine counterparts of these processes, sometimes in a synergistic fashion (van der Schoot, 1980). Furthermore, there is cogent evidence for functional asymmetry between the left and right hypothalamus in their differentiative responses to estrogen (Nordeen and Yahr, 1982). Androgen-resistant (Tfm) rats experience normal defeminization, but inadequate masculinization (B. H. Shapiro *et al.*, 1980). Since

DHT is not aromatizable, one effect of normal 5α-reductase activity is to limit estrogen-dependent events. Conversely, one gratuitous consequence of deficient 5α-reductase activity is to promote estrogen-dependent events.

There are sex differences in the microscopic anatomy of various areas of the rodent, monkey, and human brain (Toran-Allerand, 1984; Swaab and Fliers, 1985). The hypophysiotropic area is concerned with sexually dimorphic reproductive and copulatory behavior, and implantation of T in those areas achieves the expected neuroanatomic and physiological effects (Christensen and Gorski, 1978). Furthermore, there are at least two nuclei in the lumbar spinal cord that concentrate androgen but not estrogen, control penile reflexes, are sexually dimorphic, and are absent in Tfm (androgen-resistant) rats (Breedlove and Arnold, 1981; Breedlove *et al.*, 1982; Jordan *et al.*, 1982). In fact, Nordeen *et al.* (1985) have shown that androgens achieve sexual dimorphism in the number of neurons within the spinal nucleus that innervates two penile muscles by preventing the cell death that occurs normally in females, or experimentally in males deprived of androgen during the early postnatal period. Toran-Allerand (1984) has described in detail the neurocytological effects of sex steroids as observed in organ culture of the developing CNS.

Beyond sex differences in reproductive physiology and behavior, there is sexual dimorphism in the social behavior of rats (Meaney and Stewart, 1981*a*) that is seen as well in primates (Mitchell, 1979), including man. For one of these behaviors, "play-fighting," it is a common observation that males are far more active than females. Indeed, perinatal T or DHT, but not estradiol, can induce male-level play-fighting in female rhesus monkeys or Norway rats (Meaney and Stewart, 1981*b*), and it is also known that human females exposed to increased levels of androgen prenatally, because of congenital adrenal hyperplasia or maternal therapy with androgenic progestins, exhibit more masculine play behavior (Ehrhardt and Meyer-Bahlburg, 1981). In addition, flutamide, an antiandrogen that competes for AR, blocks the masculinization of play-fighting, Tfm rats exhibit female patterns of social play (Meaney *et al.*, 1983), and girls exposed to diethylstilbesterol (DES) prenatally do not demonstrate male-like play (Hines, 1982). Thus, androgens masculinize play behavior by acting as androgen, not through aromatization, and they do so by acting organizationally (during development) rather than evocatively (at puberty). Recently, it has been found that lesions in the region of the amygdala reduce play-fighting in males to the female level (Meaney *et al.*, 1982),

and it is known that this is a region of androgen-dependent sex differences in microanatomy (McEwan, 1982). Indeed, in the neonatal period the level of occupancy of nuclear AR in this region is greater in males than in females, and T implants into the mediocortical region of the amygdala on days 2–6 of life masculinize the play-fighting of female rats at puberty. It is intriguing, furthermore, that glucocorticoids antagonize the effect of androgens on male play-fighting without affecting T concentration (Meaney *et al.*, 1982), and this effect is limited to the critical perinatal period of sexual differentiation of the rat brain. Thus, the observations on the male play behavior of human females with congenital adrenal hyperplasia may represent the combined defect of increased androgen and decreased glucocorticoid.

It is not possible to deduce from the foregoing what is the modal balance between sex-of-rearing and constitutional factors in the development of gender identity and orientation among human beings. For one thing, it is likely to be quite variable. However, it seems very unlikely that constitutional influences, as revealed by "experiments of nature" as well as systematic laboratory studies, play no role in the development of normal and abnormal sexual behavior in human beings. The dramatic shift in gender identity and orientation at puberty in human males with primary genetic 5α-reductase deficiency (Imperato-McGinley *et al.*, 1979; Savage *et al.*, 1980) and the evidence from human females with congenital adrenal hyperplasia exposed to high levels of androgen prenatally add credence to this view. Furthermore, behavioral studies on rhesus monkeys indicate that sex steroids influence their fetal brains, and Pomerantz *et al.* (1985) have identified receptors for these steroids in various regions of the developing rhesus brain, but so far only in late fetuses. They found that AR and ER concentrations were higher in hypothalamic–preoptic/amygdala areas than in cerebral cortex, but whereas AR and ER concentrations were equal in the former, that of AR predominated in the cortex. The latter is in accord with the hypothesis that sexual differentiation of rhesus brains differs from that of rodent brains in being more dependent on androgen than estrogen (Goy and Phoenix, 1972; Goy and Resko, 1972; Goy, 1981) and in various other ways (Resko and Ellinwood, 1984), and with a multitude of studies indicating that androgens influence sexual differentiation of cognitive (spatial, verbal) and higher behaviors in human beings (Hines, 1982; Waber, 1979; Maccoby and Jacklin, 1974, p. 634; Levy and Levy, 1978; Inglis and Lawson, 1981; Benbow and Stanley, 1980). For instance, Resnick *et al.* (1986) have found that females above

11 years of age with congenital adrenal hyperplasia that is well controlled by glucocorticoid therapy perform better in tests of spatial cognition than unaffected female relatives, a difference that is underlined by the similarity of the groups on objective tests of general intellect and psychologic status. In addition, the affected females showed a trend towards greater participation in spatial manipulation activity, and significantly lower participation in verbal activities. In accord with the foregoing, Hier and Crowley (1982) reported that males with congenital hypogonadotrophic hypogonadism have decreased spatial ability that is not improved by androgen therapy sufficient to normalize other aspects of virilization.

The Immune System

Males have "weaker" immune systems than females. This is reflected in the following combination of facts: males are more susceptible than females to most infections, but are less predisposed than females to most of the so-called autoimmune diseases, such as systemic lupus erythematosus, whether spontaneous or drug-induced (Batchelor *et al.*, 1980), Hashimoto thyroiditis, and rheumatoid arthritis. Indeed, sexual equality (for example, type I diabetes mellitus) or male predominance [for example, Goodpasture syndrome (Matthay *et al.*, 1980) and glomerulonephritis (Finn and Harmer, 1979)] is sufficient reason to consider an infectious origin for an autoimmune process (Southwest Pediatric Nephrology Study Group, 1985).

Aside from sexual inequality in the expression of genetic defects in the immune system that stems from X-chromosome hemizygosity in males (Powell *et al.*, 1982), there is a substantial body of evidence that androgens influence ontogeny, and that androgens and estrogens affect function, of both branches of the mature immune system. Androgens suppress development of the immune system in chick embryos by acting on the epithelial cells of the thymus and the bursa of Fabricius (Szenberg, 1970). The autoimmune disease of NZB mice and of NZB/W hybrids is more severe in females, can be ameliorated by giving them T, and can be exacerbated in males by castration (Roubinian *et al.*, 1978). Yet, orchiectomy increased, and T reduced, primary IgM responses of NZB mice to sheep erythrocytes only if accompanied by sublethal irradiation (Morton *et al.*, 1981). This suggested that androgens may preferentially affect the lymphoid-directed progeny of rapidly proliferating hematopoietic stem cells, and particularly those destined to be processed by the bone marrow

(Morton *et al.*, 1981). The same observation was made by Fujii *et al.* (1975) on C3H/He mice that are not prediposed to autoimmune disease.

Together, the above results fortify the thesis that androgens, at physiological concentration, have important effects on differentiation and postablative restoration of the lymphoid system, and there are additional data (Bullock *et al.*, 1980) indicating that the thymus-dependent limb of the differentiating immune system is also subject to androgenic influence. There are other clinical indications that androgens influence the immune system: Unlike adults, among children with lupus nephritis, males have more diffuse-proliferative disease, present more severly affected, and have worse prognosis than females (Arbus *et al.*, 1983); among 16 men with systemic lupus erythematosus, one had hypoandrogenemia, four had hyperestrogenemia, and one had elevated levels of serum FSH and LH (Inman *et al.*, 1982); danazol, an androgen with reduced virilizing potency, is an effective agent for treating idiopathic thrombocytopenic purpura (Ahn *et al.*, 1983).

Cohn (1979) provided another type of evidence in favor of an androgen effect on differentiation of the immune system, as distinct from postdifferentiative function, by showing that circulating antibody responses to a variety of antigenic challenges were inversely correlated with androgen sensitivity among male mice of a variety of inbred strains, whether or not they were castrated. Finally, data obtained from the NZB/W model of autoimmune disease are compatible with the suggestion that androgens affect different subsets of B lymphocytes (Morton *et al.*, 1981), or that they delay the switch in IgM to IgG antibody production (Roubinian *et al.*, 1978). Similarly, J. H. M. Cohen *et al.* (1983) have reported the absence of A–R activity in human peripheral T cells and restriction of E–R activity to OKT8-positive cells. Two groups have found that A–R activity is restricted to the reticuloepithelial cells of the thymus and is absent in thymocytes (Sasson and Mayer, 1981; Grossman *et al.*, 1979). In contrast, the AR has been identified in non-Hodgkin lymphoma B cells (Danel *et al.*, 1984). In summary, the immunomodulating effects of androgens are likely to be exerted on immature B or pre-B cells directly, while their action on the T-cell limb is mediated by effects on thymic epithelium rather than by direct effects on T cells or their precursors. Thus, under the influence of androgens, the reticuloepithelium of the thymus appears to collaborate in T-cell differentiation in a fashion that is homologous with that of mesenchyme during morphogenesis of the developing male reproductive tract and lung development, as discussed above.

The Liver

In the rat, large sex differences in hepatic metabolism are known for drugs such as ethylmorphine and hexabarbital and various classes of steroid hormone. Similar but smaller sex differences are present in humans. In the male rat, many of these adult sex differences in liver metabolism are imprinted by androgen early in development via a process that involves the hypothalamus and the pituitary and is presumably mediated by the AR (Gustafsson *et al.*, 1980). It is postulated that in the absence of such imprinting a "feminizing factor" elaborated by the hypothalamic–pituitary axis confers the female pattern of metabolizing enzymes on the adult liver. It is not known whether any sex-dependent differences in adult human liver function have such an "imprinting" basis.

Use of Cultured Skin Fibroblasts to Assess the Androgen-Response Apparatus

The properties that permit an androgen-target cell to respond to androgen are depicted in Fig. 13, according to the original two-step model of S–R interaction that considered target cells, in their basal state, to have most of their receptor molecules in cytoplasmic residence. T and DHT are thought to enter the cell by diffusion. In some target cells, there is

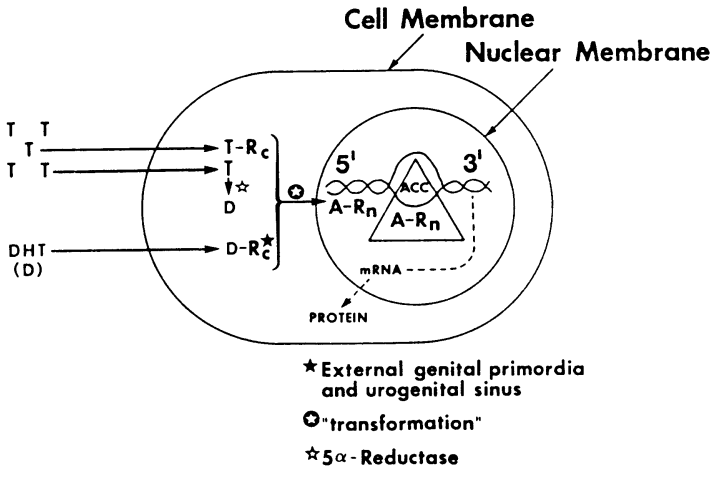


Fig. 13. A prototypic androgen-target cell depicted according to the original two-step model of steroid–receptor interaction. T, testosterone; DHT, 5α-dihydrotestosterone; R_c, receptor in the cytoplasm; $A–R_n$, androgen–receptor complex in the nucleus; ACC, "acceptor" site on the chromatin.

active conversion of T to DHT by the microsomal enzyme 5α-reductase. In some such cells, DHT rather than T is the functional effector of androgen action. Other target cells have little 5α-reductase activity ever, or at least at certain times of development, and in them T rather than DHT is the active effector hormone. For instance, the Wolffian ducts do not have 5α-reductase activity at the time they differentiate under the influence of T. In contrast, the urogenital sinus and the external genital primordia develop 5α-reductase activity before they differentiate so that they can autogenerate sufficient DHT for this purpose (Siiteri and Wilson, 1974). Furthermore, in some peripheral target tissues, 5α-reductase activity is inducible by androgen in a receptor-mediated way, and this can serve to amplify an androgen response or modulate the expression of androgen resistance, as will be elaborated upon below.

Both T and DHT bind to the same receptor protein, but they do so with appreciably different affinity (T < DHT), and the complexes produced have different properties. These differences (discussed in detail below) may be responsible, in part, for the division of labor between T and DHT during (1) morphogenesis of the male reproductive tract, (2) differentiation of other sexually dimorphic organ systems, and (3) acquisition of full virilization at puberty.

Cultured skin fibroblast (SF) lines provided the first vehicle for systematically measuring and characterizing the androgen-response system in human beings. These cells retain their differentiative ancestry as androgen-target cells; those derived from explants of pubic skin (PS) or genital skin (GS; scrotal, labial, preputial skin) have more 5α-reductase activity and AR activity than nongenital (NG) SF despite serial subcultivation to the point of senescence (T. R. Brown and Migeon, 1981; Kaufman *et al.*, 1977). A short generation time (24–48 hr) permits their economical growth in quantities sufficient for various types of assays. In addition, they can be cloned, cocultured, or hybridized with cells of other individuals or species, and their subcellular components can be studied separately, or, conversely, mixed with those of other cells, to yield additional information.

5α-Reductase Activity

The measurement of 5α-reductase activity in SF has yielded an arresting, occasionally conflicting, array of results. First, most laboratories report that PSF and GSF have more specific 5α-reductase activity than

NGSF, whether the comparison is made with cells from single individuals or groups. Second, two laboratories (Lambrigts *et al.*, 1979; Mowszowicz *et al.*, 1980) report an appreciable increase in 5α-reductase activity with increasing *in vitro* age of GSF lines, while another has noticed this to a minor extent (Moore *et al.*, 1975), and two others have not noticed this change at all (T. R. Brown and Migeon, 1981; Pinsky *et al.*, 1978). In fact, GSF metabolize T more actively than do NGSF (Pinsky *et al.*, 1972), whether the pattern of metabolism (Shanies *et al.*, 1972) is preferentially along the 17β-hydroxy pathway through DHT, the 17-ketonic pathway through androstenedione, or by a combination of the two (Mulay *et al.*, 1972). Furthermore, the pattern of metabolism is clone-specific (Kaufman *et al.*, 1975), starting with the earliest, visually isolated single fibroblasts around primary 1-mm^3 skin explants. Shanies *et al.* (1972) reported age- and sex-specific patterns of T metabolism in NGSF, but this was not confirmed (Pinsky *et al.*, 1974).

One remarkable finding, reported by several laboratories (Moore *et al.*, 1975; Pinsky *et al.*, 1978; Rodrigues-Pereina *et al.*, 1984), has been that the range of 5α-reductase activity among normal GSF lines is enormous: it extends from the lower limit of sensitivity of the assays over a range that can vary more than 200-fold. The apparent basis for this variability is that uncloned GSF lines are mosaics composed of cells that, constitutively, have low or high 5α-reductase activity, and that "high" clones can transform to the "low" phenotype on subcloning, while the reverse does not happen (Griffin *et al.*, 1981). Barring a trivial *in vitro* artifact, the simplest interpretation of these results is that cultured GSF bear markedly different (and/or "leaky") epigenetic imprints of their differentiative state *in situ*, and that this variation is somehow manifested *in vitro* as extreme variation in enzyme activity among uncloned GSF lines or by comparable variation of that activity in clones and subclones from a GSF line of one person. Considerable support for, and some extension of, this hypothesis is provided by the prior demonstration (mentioned above) of clonal specificity in the rate and pattern of T metabolism among the earliest SF that emanate from a single 1-mm^3 explant of genital skin (Kaufman *et al.*, 1975).

The practical consequence of this situation is that in most laboratories cultured GSF are used to exclude a diagnosis of primary 5α-reductase deficiency, but not to reliably verify it, and, except for one laboratory (Saenger *et al.*, 1978), NGSF are not used for either purpose.

It has been known since 1969 (J. D. Wilson and Walker, 1969) that

fresh slices of GS have more 5α-reductase activity than PS or NGS, and, since 1975 (Kuttenn and Mauvais-Jarvis, 1975), that the activity in slices of PS, but not GS, is subject to the androgen status of their donor. Thus, boys and women have lower PS 5α-reductase activity than men, and hypogonadal men have low PS 5α-reductase activity, which increases after T or human chorionic gonadotropin (hCG) administration. Such differences might be attributable to some or all of the epithelial components of intact PS. However, the recent report that serially subcultured PSF but not GSF have 5α-reductase activity that is inducible by androgen (Mowszowicz *et al.*, 1983*a*) indicates that dermal fibroblasts themselves are an important, if not the sole, source of the differences. Indeed, once confirmed, this observation will add further support, and another dimension, to the claim that cultured SF retain the ability to express their differentiative ancestry under conditions that must reflect properties indigenous to their epigenetic program, rather than any extracellular, adventitious property carried over from their previous *in situ* environment. Since androgen-inducibility of PSF 5α-reductase activity is AR-mediated, this behavior may provide a useful biologic marker of androgen responsiveness to add to the armamentarium of assays for characterizing androgen-resistance mutations in cell culture. Moreover, the finding that 5α-reductase activity is androgen-inducible in PSF, but not GSF, is in accord with the fact that 5α-reductase activity is normal in the GSF of subjects with complete androgen resistance (Pinsky *et al.*, 1981).

Androgen Receptor Binding Activity

The simplest assay for AR binding activity involves the incubation of whole cells (in monolayer or suspension) at 26–37°C with a tracer quantity of ^{3}H-labeled androgenic ligand alone ("total binding") or with a tracer quantity plus excess radioinert androgen ("nonspecific binding") to measure "specific binding" by subtraction (Fig. 14). Usually, the cells are preincubated in serum-free medium for 24 hr before assay, but this is not necessary. The assay is conducted for 30 min, by which time saturation of the specific binding sites is achieved, or for longer times, according to purpose. The separation of free from bound androgen is achieved in various ways; the simplest is by exhaustive washing of the cells. If replicate cultures are incubated with a series of concentrations of ^{3}H-androgen, a saturation curve is obtained that, when analyzed in various ways [usually the Scatchard (1949) plot] yields a measure of max-

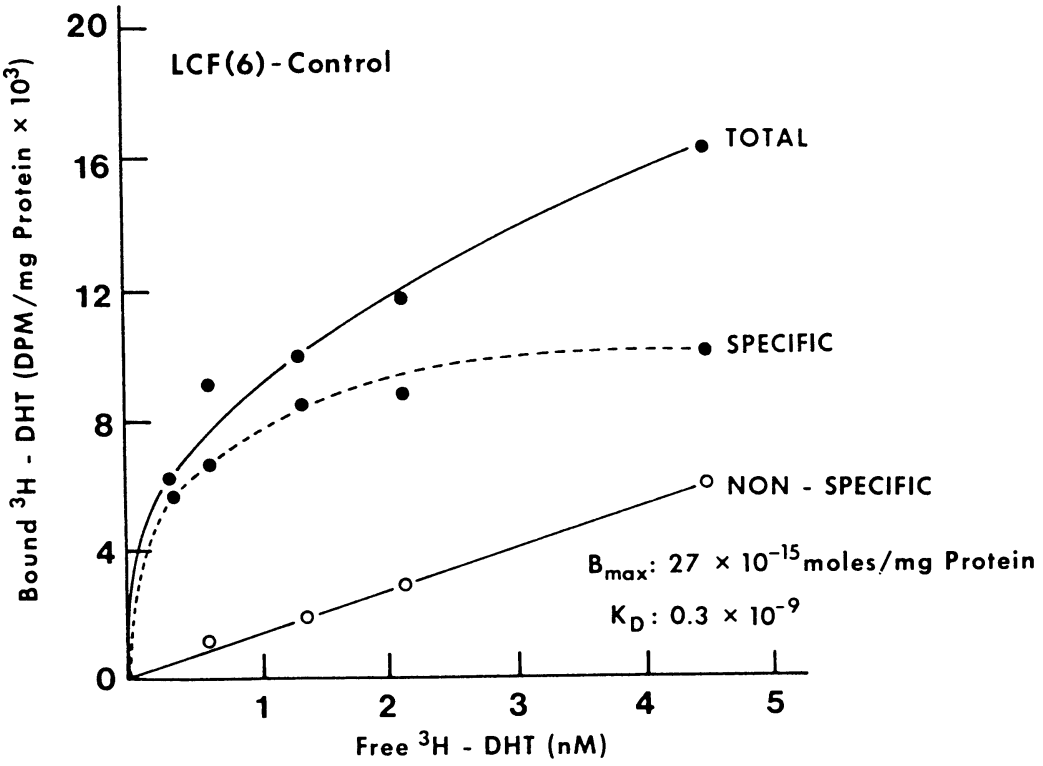


Fig. 14. A series of replicate monolayers of a control foreskin fibroblast strain are incubated with various concentrations of ^{3}H-DHT alone (total) or 200-fold excess radioinert DHT (nonspecific) and specific AR-binding activity is computed by subtraction.

imum binding capacity B_{max} and of the affinity with which the receptor binds the androgen. The latter is derived from the slope of the Scatchard plot K_A, and is usually expressed as its reciprocal, the equilibrium dissociation constant $K_D = 1/K_A$. The relation between a saturation curve and its Scatchard plot is shown in Fig. 15. Notice that a shallow saturation curve yields a Scatchard plot with a shallow slope and vice versa. The effect of temperature on specific binding can be assessed simply by bringing the "total" and "nonspecific" incubation media to a desired temperature and carrying out the assay in an incubator set at that temperature.

The cytoplasmic/nuclear ratio of A–R complexes is determined simply by lysing the cells in a medium that protects nuclear integrity and then measuring specific nuclear-bound radioactivity as a fraction of that bound by the whole cells.

The ability of heterologous androgens or other steroids to compete for binding with a given androgen can be measured by incubating a fixed concentration of radiotracer androgen with a series of concentrations of

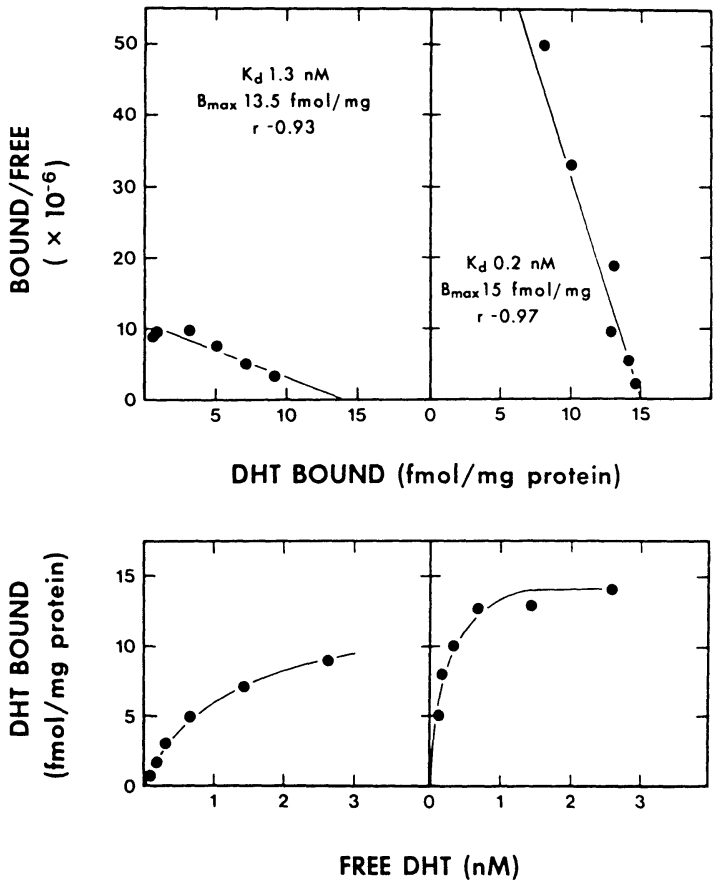


Fig. 15. Scatchard plots (top) and corresponding saturation curves (bottom).

radioinert competitor in a set of replicate cultures. The competition (displacement) curves so produced are a useful index of receptor quality.

The rate of dissociation k of radiolabeled A–R complexes formed within cells is measured by postincubating them with a great excess of radioinert ligand (a "chase") either *via* the culture medium or after the complexes have been extracted from the cells. The validity of this determination may depend on the concurrent demonstration that the level of unchased radiolabeled A–R complexes is stable in the presence of supersaturating concentrations of the radioligand during the interval of the chase.

The rate at which the receptor is lost from cells incubated with sufficient cycloheximide to nullify protein synthesis yields a measure of its half-life (or turnover) and, by inference, the rate at which the receptor is synthesized to achieve its usual steady-state level. Comparable information can be obtained by use of the heavy amino acids–density shift

technique to measure the rates of appearance or disappearance of the AR (Syms *et al.*, 1985). A suitable antibody for the AR is not yet available for these purposes.

Prolonged incubation with androgen beyond the time necessary to saturate the basal pool of AR binding sites causes the cells to acquire additional AR activity. This response is termed up-regulation (Kaufman *et al.*, 1983*a*), and is a useful index of receptor quality, particularly if a nonmetabolitable androgen is used.

The specific AR activity in GSF can be measured autoradiographically (Ozasa *et al.*, 1980), but this is inconvenient, since the small number of receptor molecules per cell demands exposure times of many months in order to see enough grains to distinguish receptor-positive from -negative cells.

Quantitative assays of receptor activity in SF cytosol have been used sparingly; they are more laborious than whole-cell assays and they yield an underestimate of total cell receptor activity. Nevertheless, qualitative characteristics of the A–R complex in cytosolic preparations have been determined. These include: (1) the mobility, according to molecular size (and shape), on sucrose-density gradients or gel filtration columns; (2) sensitivity to stabilization by molybdate during such procedures; (3) mobility according to net ionic charge on ion-exchange or DNA–cellulose columns; and (4) behavior in various media subjected to electrophoretic fields. These determinations are facilitated by using photoaffinity-labeled AR (Brinkmann *et al.*, 1985).

Two groups have used two-dimensional polyacrylamide gel electrophoresis of denatured GSF extracts to identify different sets of "spots" that are reputed to represent AR protein(s) (Risbridger *et al.*, 1982; Thompson *et al.*, 1983; Wrogemann *et al.*, 1986). These exciting reports await independent confirmation or interlaboratory cooperation before their significance can be assessed.

Various preparations of fresh human skin (from different anatomic regions) have been used to measure and characterize specific androgen-binding activity. These efforts are complicated by the presence of the sex steroid-binding globulin (SS-BG) in the interstitial space. This complication can be circumvented by using a synthetic androgenic ligand, such as methyltrienolone, that does not bind the SS-BG (Bonne *et al.*, 1977) and by separating the AR from the SS-BG by prior purification (Wilbert *et al.*, 1983). It is noteworthy that cultured GSF lack progesterone or estrogen receptors, and that the SS-BG is not a factor during measurement

of their AR activity, since the cells can be washed to remove it or preincubated in serum-free medium before assay.

Needed: A Marker of Androgen Response in Cultured Skin Fibroblasts

In routine practice androgen resistance is diagnosed presumptively by excluding other causes of male pseudohermaphroditism or hypogonadism using clinical and endocrine criteria. There is no simple and sensitive *in vivo* test that can expose a state of androgen resistance due to a defect within target cells, and protocols to assess total body nitrogen and phosphorus balance during prolonged periods of androgen supplementation are cumbersome and costly. Furthermore, the discovery of at least one state of constitutional androgen resistance for which clinical expression is restricted to one process within one organ (spermatogenesis in the testes) (Aiman *et al.*, 1979) makes it unlikely that any one systemic *in vivo* test will be capable of detecting all types and degrees of androgen resistance.

For these reasons, an early goal of research on the androgen-response apparatus in cultured SF was to identify a biochemical, perhaps anabolic, marker of androgen response in such cells. Since skin is obviously a sexually dimorphic organ *in vivo* and, to some extent, the dimorphism must reflect differences in the response of the dermal component of skin to androgen, it seemed possible that the proliferation rate of cultured SF and, very likely, the level of various metabolic activities in them would be regulatable by manipulating the concentration of androgen in the culture medium. Among the latter, the metabolism of collagens and glycosaminoglycans appeared to be suitable candidates. There has been only one report of a receptor-dependent androgenic response of GSF (Ozasa *et al.*, 1981), and it was based on a comparison between the SF of one subject with complete androgen resistance and those of one control, in terms of the incorporation of proline into noncollagen protein and of derived hydroxyproline into the "collagen" portion of the TCA-precipitable cell fraction. Interestingly, the effect on noncollagen protein was seen at a DHT concentration of 10 ng/ml (approximately 0.3 nM) and that on collagen protein required 100 ng/ml, but there was no effect on DNA synthesis as measured by thymidine incorporation, even at a level of 1000 ng/ml. The latter observation is not surprising, since SF of patients with receptor-negative complete androgen resistance proliferate as well as con-

trol fibroblasts in regular cell culture medium containing 10% untreated fetal calf serum, with or without added T or DHT. The relation of the added effective DHT concentrations to the circulating level of DHT in normal males may or may not be relevant, taking into consideration the fact that binding to the SS-BG leaves only a small fraction of the total circulating DHT in its free form. What is relevant, however, is that DHT was added to medium made with serum that had been stripped of steroids by preexposure to dextran-coated charcoal. This raises the possibility that supraphysiological levels of DHT were required to induce either of the observed effects because "factors" (other hormones?) that normally potentiate androgen action were removed inadvertently from the charcoal-treated serum. Another important consideration was the protocol used by Ozasa *et al*: The effects they observed were measured 6 days after confluent cultures were first exposed to medium with added DHT, and since DHT is metabolizable, fresh experimental medium was exchanged for DHT-depleted medium every 48 hr in order to replenish the concentration of DHT.

In a recent preliminary communication (Loire *et al.*, 1984), DHT, in unspecified concentrations between 10^{-10} and 10^{-5} M, was reported to double the concentration of "collagen" secreted into the medium of control GSF, but not into that of GSF from an unspecified number of patients with complete androgen resistance. Furthermore, the mutant GSF had about a fivefold lower rate of basal collagen synthesis than control cells, and the added DHT appeared to have little effect on this parameter. It will be necessary to confirm these observations when the details of these experiments become available.

Skin glycosaminoglycans (GAG) are under androgenic control *in vivo* (Kofoed *et al.*, 1970), but we have not found any effect of T or DHT on their metabolism in SF (Pinsky and Weksberg, 1969). With regard to GAG, it is noteworthy that T increased the hyaluronic acid concentration of rat gingiva twofold (Kofoed, 1971), and human gingiva contains relatively high concentrations of AR activity (Southern *et al.*, 1978). Perhaps human gingival fibroblast cultures would display a hyaluronic acid response to medium supplemented with androgen.

Two other strategies that should be exploited in order to generate a reliable marker of androgen response in SF deserve to be mentioned. One is based on experience with a line of chick comb fibroblasts (Froble *et al.*, 1979), which suggested that androgens may have certain effects on cells in exponential growth, rather than on confluent monolayer cultures.

The other strategy is based on the observation that hydrocortisone has a variety of well-known effects on human SF in culture (Russell *et al.*, 1978; Rowe *et al.*, 1977), and it has been suggested (Harvey *et al.*, 1976*a*,*b*) that androgens, acting through their own receptor, may be able to antagonize some effects of glucocorticoid that are mediated by the GR.

Finally, it should be noted that a search for a reliable marker of androgen action in cultured SF deserves to be pursued with determination for at least two practical reasons. First, failure to demonstrate an androgen response using such a marker would rationalize the investment in long-term studies of receptor-positive, androgen-resistant SF lines that fail to reveal a known qualitative receptor defect that interferes with androgen action. It would also motivate systematic pursuit of postreceptor defects in androgen action. None has yet been identified. Second, the availability of such a marker would permit the conduct of whole-cell and recombinant DNA experiments designed to dissect the elements of the androgen-response apparatus. The latter would, of course, be especially true if the marker could be adopted as the basis of media that selected for or against the androgen-resistant phenotype, just as the use of lymphocytolysis has been particularly valuable as a selectable marker of glucocorticoid resistance in various mutant lymphoid cell lines (see below).

The Division of Labor between T and DHT

Primary 5α-reductase deficiency is an autosomal recessive, sex-limited disorder that causes XY males to be born with external genitalia that are predominantly feminine (Imperato-McGinley *et al.*, 1980). Their internal genital organs are normal except for prostatic hypoplasia. This means that inability to convert T to DHT interferes with masculinization of the neutral external genital primordia and the urogenital sinus, but not with androgenic differentiation of the Wolffian ducts into the vasa deferentia, the seminal vesicles, and the epididymes. This coincides precisely with the fact that 5α-reductase activity appears in the Wolffian ducts after their differentiation is completed, but in the external genital primordia and the urogenital sinus before their differentiation begins. Furthermore, at puberty there is deepening of the voice, a spurt in statural, muscular, and penile growth, increased rugosity and pigmentation of the scrotum, and testicular descent. However, the prostate remains impalpable, facial and body hair is sparse, and pubic hair growth, although appreciable, has a female contour. Acne is rare, and temporal hairline recession does not

develop. Spermatogenesis can be normal in descended testes, and the subjects have erections and ejaculations. The mean blood level of LH is elevated despite normal or elevated levels of T, indicating that DHT has a role in the negative feedback control exerted by androgens on the hypothalamic–pituitary gonadostat. Conversely, DHT in pharmacological doses causes a drop in blood T with a consequent loss of libido and development of impotence (Peterson *et al.*, 1977; Jukier *et al.*, 1984; Imperato-McGinley, 1983), indicating that T is the important effector of these behaviors. From this aggregate of clinical and endocrine information, it follows that some aspects of pubertal virilization are dependent on T, while others are DHT-mediated.

Of very great interest with regard to the division of labor between T and DHT is the fact that at puberty most affected males adopt a male sexual identity and orientation, despite having been reared as females. This change occurs not only in the special social situation of rural inbred isolates in the Dominican Republic (Imperato-McGinley *et al.*, 1979), but also in more usual environments (Savage *et al.*, 1980). It is obvious that failure to alter surgically the congenitally feminine external genitalia to a masculine appearance facilitates the psychological change, but it cannot explain the unusual propensity for these subjects to desire, indeed tolerate, the change instead of wishing to preserve their female sex-of-rearing. Rather, it seems very likely that exposure of affected males to T during critical periods of development *in utero* and postnatally—and perhaps to relatively increased levels of it because of reduced T to DHT conversion in the brain—somehow imprints their brains with a masculine program of differentiation that is evoked by the rise of circulating androgens at the time of puberty.

The fact that spermatogenesis can be normal in affected individuals with descended testes indicates that T itself is sufficient for this process. On the other hand, if DHT is responsible for imparting certain aspects of masculine differentiation to the CNS, these subjects should be lacking one or more of such DHT-dependent behaviors. However, we are not aware of results from studies directed toward this issue.

The division of labor between T and DHT, as revealed by the Mendelian form of 5α-reductase deficiency, has implications in three other areas. First, XX females homozygous or heterozygous for 5α-reductase deficiency have full or partial biochemical expression of the disorder, respectively, but neither has clinical signs of it. Specifically, their pubic (and presumably axillary) hair is normal in amount and distribution, and

they do not have delayed menarche. These two signs, singly or in combination, are not unusual (indeed, they are diagnostic) in XX females who are presumptive heterozygotes for mutations causing receptor-dependent androgen resistance of various degrees. Second, the absence of gynecomastia in affected males reveals that DHT is not needed to suppress mammary gland development at puberty. And third, imposition of 5α-reductase deficiency by pharmacological inhibitors of 5α-reductase activity might be expected to be efficacious in the treatment of prostatic hypertrophy and premature temporal hairline recession in men, acne in both sexes, and hirsutism in women.

Finally, the selectivity of T and DHT action that is exposed by primary 5α-reductase deficiency as an "experiment of nature" is an excellent model for analyzing the ligand-selective form of receptor-dependent androgen resistance that is discussed below. Indeed, primary 5α-reductase deficiency can be appropriately labeled as a form of androgen-selective androgen resistance in which sensitivity to T is retained by some targets and resistance to T is expressed by other targets that can respond to DHT exclusively or preferentially.

The molecular and pathophysiologic bases for the dramatic division of labor between T and DHT, both prenatally and during virilization at puberty, remain unresolved. Various explanations have been proffered, including: (1) proximity of the fetal testes to the Wolffian ducts as the basis for a paracrine effect of fetal testosterone secretion on differentiation of the ducts; (2) higher levels of T at puberty than during organogenesis, with or without the consequent attainment of a threshold-effective plasma DHT level at puberty despite an unchanged level of residual 5α-reductase activity; (3) the possibility that T itself, i.e., T–R complexes, may be sufficient for pubertal growth, but not morphogenesis of the external genitalia; (4) a chronologic increase at puberty in the sensitivity of the external genitalia to T–R complexes; and (5) a difference of T–R complexes from DHT–R complexes in subtle but important physicochemical properties that influence or determine their disparate capacities as effectors of androgen action.

Several studies on GSF have generated data that bear on the latter suggestion. For instance, T–R complexes dissociate faster than DHT–R complexes in or out of GSF (Maes *et al.*, 1979; Hodgins, 1982), and, once out, they dissociate not only more rapidly than DHT-R complexes, but with a different (biphasic) character (Kaufman and Pinsky, 1983; Kaufman *et al.*, 1983*b*). Indeed, it is possible to induce DHT–R complexes ex-

tracted from GSF to display dissociative behavior much like that of T–R complexes by treating them with agents like pyridoxal 5′-phosphate (the aldehyde group of which is capable of forming a Schiff base with lysine and arginine residues of AR protein) or sodium thiocyanate, a chaotropic salt (Kaufman *et al.*, 1982*b*). Prostatic T–R complexes also dissociate faster than DHT–R complexes (Wilbert *et al.*, 1983).

Since 5α-reductase deficiency also causes reduced conversion of progesterone to its 5α-reduced derivatives, and since progesterone is a better competitor for the AR than its derivatives, Hodgins (1982) has suggested that the failure of T to accomplish normal urogenital sinus and external genital virilization in 5α-reductase deficiency is due in part to interference with its action by increased concentrations of progesterone.

Further evidence that T–R complexes differ qualitatively from DHT–R complexes is provided by the description of a family with partial androgen resistance in which the mutation causes DHT–R complexes to acquire a type of lability that normal T–R complexes express under conditions designed to promote transformation of A–R complexes to their DNA-binding state (Kovacs *et al.*, 1984).

Androgen Sensitivity As a Continuous Variable in the Normal Population

There are several lines of evidence suggesting that androgen sensitivity is a multifactorial, quasicontinuous variable among normal people. They include the data discussed above on variation in 5α-reductase and AR activities among samples of fresh skin from different anatomic sites of donors of different ages and androgen status, and among cloned or uncloned SF maintained in different androgen environments. In aggregate these data indicate that intrinsic (differentiative) variation in basal genital skin 5α-reductase activity, and the potential for variation in the degree of androgen inducibility of PS 5α-reductase activity, may be important sources of overall variation in the androgen sensitivity of the pubogenital and other areas of skin in normal human beings.

The levels of several enzymes are androgen-inducible in kidney but not in other organs of the mouse. These include β-glucuronidase, alcohol dehydrogenase, arginase (Swank *et al.*, 1977), and ornithine decarboxylase (Kontula *et al.*, 1984, 1985; Berger *et al.*, 1984). Hypophysectomy does not alter basal kidney β-glucuronidase activity, but it reduces its androgen inducibility to about 5% of normal. In contrast, hypophysec-

tomy causes a large reduction in kidney arginase activity that is largely restored by androgen therapy. Similarly, in the rat, hypophysectomy abolishes the androgen inducibility of α_{2u} globulin, a protein synthesized in liver and excreted in urine. In fact, maximum inducibility of α_{2u}-globulin by androgen requires the synergistic influence of glucocorticoid, growth hormone, thyroxine, and insulin (Roy *et al.*, 1983). Thus some effects of androgens require pituitary and other hormones, or products of their actions, in order to be realized.

Furthermore, it is clear that multiple factors affecting the postreceptor steps of androgen action will contribute to overall variability in net sensitivity to androgen. To illustrate this, Judge *et al.* (1984) formed cell hybrids from rat ventral prostate epithelial cells and a line of mouse renal tumor cells selected for resistance to 8-azaguanine. Selection of hybrid cells was facilitated by the greater sensitivity of the rat than the mouse parental cells to ouabain. A variety of independent hybrid clones was isolated that differed in modal chromosome number, mean doubling times, cellular morphology, and in proliferative response to 10 nM DHT. In the latter regard, some clones exhibited >40% reduction in doubling time, others had little reduction, and still others had an intermediate response. However, AR activity (total or nuclear) could not explain the variation in proliferative response to DHT among the different classes of clones. This indicates that nonreceptor factors were important modulators of DHT responsiveness. Indeed, some clones with relatively low AR activity were among the most responsive to DHT. Comparable observations have been made regarding glucocorticoid inducibility of tyrosine aminotransferase (TAT) activity in various rat hepatoma cell lines and their hybrids (Thompson *et al.*, 1977*a*; Croce *et al.*, 1973).

From all these observations it seems intuitively likely that variation in the extracellular background, involving multiple hormones, or in intracellular factors not necessarily related to the AR, may be important sources of variability in the androgen sensitivity of human beings. Indeed, there are cogent clinical indications in support of this statement. For instance, women with sustained hyperandrogenemia usually present with hirsutism and/or acne in association with amenorrhea. But some hyperandrogenemic women may be amenorrheic without hirsutism, acne, or any other clinical features of their androgen excess (McKenna *et al.*, 1983), and others may express more advanced signs of virilization, such as deepening of the voice and clitoromegaly (Scully *et al.*, 1982) in the absence of hirsutism.

In a large study of 400 women examined because of hirsutism, 24 were found to have the late-onset form of adrenal hyperplasia due to partial 21-hydroxylase deficiency (Kuttenn *et al.*, 1985). Interestingly, nine siblings who had the same enzyme defect, as defined endocrinologically and by their HLA-identical genotypes, did not have hirsutism. The 5α-reductase activity in the skin of these nine individuals was not specified, but among 12 patients studied, the activity varied at least 30-fold. In a study conducted by Reingold and Rosenfield (1984), plasma free T was related to an arbitrary score for hirsutism among 52 Caucasian female volunteers 18–21 years of age and seven Caucasian females of the same age range referred for hirsutism. The hirsutism score correlated with the free T level, but among those with elevated free T (>13 pg/ml), eight had hirsutism, four had acne without hirsutism, and one had neither. In contrast, four individuals had hirsutism with normal plasma levels of free T. Cryptic hyperandrogenemia thus represents a state of relative target organ resistance to androgen, while idiopathic hirsutism represents the opposite end of the spectrum, a state of androgen hypersensitivity. Indeed, Lorenzo (1970) arrived at the same conclusion on the strength of a family study that indicated a polygenic, multifactorial genetic basis for hirsutism.

In women with idiopathic hirsutism, androgen hypersensitivity is consistently reflected by increased activity of 5α-reductase but not the AR in fresh preparations of pubic skin (Kuttenn *et al.*, 1977) and frequently in cultured PS fibroblasts (Mowszowicz *et al.*, 1983*b*).

Another indication of androgen sensitivity as a continuous constitutional variable in the human population is the common condition acne vulgaris. This is well known to have an androgenic component in pathogenesis, yet circulating levels of androgens do not appear to be at fault. In a study by Sansone and Reisner (1971) acne-bearing skin produced 2–20 times more DHT from T than did normal skin. It is likely that genetic variation in the distribution of acne-related 5α-reductase activity is one basis for the well-known familiality of acne. An extreme expression of such variation has been suggested by Solomon and Fretzin (1970), who reported moderately severe acne with a typical distribution, as well as in the atypical site of the forearms, in nine postpubertal patients with Apert syndrome.

In theory, the degree of peripheral aromatase activity should also contribute to variation in androgen sensitivity, and therefore to the clinical expressivity of particular defects in androgen responsiveness. Aromatase activity has been measured in human hair roots (Schweikert *et al.*, 1975)

and characterized in NGSF and GSF, but except for one inconclusive report (Schweikert *et al.*, 1976), it has not been invoked as a cause of variable expressivity in androgen-resistant or hyperandrogenemic states. Estrogen produced by peripheral aromatase activity would, of course, depend on ER for its effect, and the latter has been identified in normal facial skin (Hasselquist *et al.*, 1980) at higher concentrations than in skin from the breast or thigh.

The foregoing observations indicate clearly that otherwise normal women vary in the degree and/or focality with which they will express the somatic consequences of hyperandrogenemia. Since men are just as likely to vary in the same way, it follows that such constitutional sources of variation may modulate the clinical expressivity of androgen resistance among affected members of one family who carry an identical AR defect. Indeed, such variation is not uncommon, and will be discussed below.

HEREDITARY ANDROGEN RESISTANCE IN MAN

History

Resistance to androgen was the second form of hormone resistance to be described in man; the first was resistance to parathyroid hormone.

Modern clinical and genetic descriptions of complete androgen resistance (CAR) appeared in the first quarter of this century, but Pettersson and Bonnier (1937) are credited with providing the first insight into its pathogenesis. They concluded that genetic males developed into externally unambiguous females because of a failure to diverge from the basic female design of sexual morphogenesis, and they suggested that a gene mutation, X-linked or male-limited autosomal dominant, was at fault. Morris (1953) defined the clinical–endocrine criteria for the disorder and gave it the name "testicular feminization," thereby solidifying the idea that feminization occurred in the presence of normally functioning testes. It was Wilkins (1965), however, who prescribed pharmacological doses of methyltestosterone for a castrated subject and showed that she was resistant to its usual androgenic effects. It remained to determine the primary basis for such resistance, and Migeon (Keenan *et al.*, 1974) was the first to show that several members of Wilkins' original families lacked specific AR-binding activity in their serially subcultured NGSF. The year 1974 was pivotal in the evolution of knowledge about androgen resistance,

since in that year publications defining two important types of partial androgen resistance (PAR) also appeared. One, labeled the Reifenstein syndrome (J. D. Wilson *et al.*, 1974), is sometimes associated with deficient levels of A–R activity; the other is due to 5α-reductase deficiency (Imperato-McGinley *et al.*, 1974; Walsh *et al.*, 1974). These reports heralded the recent era of knowledge about androgen resistance. Before embarking on a review of the remarkable progress made during these 12 years on defects of the androgen-response system in human target cells, we will introduce a classificatory system for them.

A Classificatory System for Hereditary Androgen Resistance

A system incorporating five criteria is listed in Table I. The known patterns of inheritance associated with androgen resistance in man are X-linked recessive and autosomal recessive with male-limited expression. What needs formal study is whether more than one X-linked locus is involved, and whether autosomal dominant mutation(s) with male-limited expression is the mode of transmission in some families assumed to be segregating for an X-linked recessive gene.

"Severity" relates to the degree of androgen resistance as reflected in masculinization of the external genital phenotype. Thus, "complete"

TABLE I. Classification Criteria for Constitutional Androgen Resistance

Feature	Type
1. Inheritance	X-linked recessive (? number of loci)
	Autosomal recessive, male-limited
	? Autosomal dominant, male-limited
2. Severity	Complete: female external genitalia
	Partial: ambiguous external genitalia (of any degree)
	Minimal: male external genitaliaa
3. Distribution	Universal: all targets affected
	Multifocal: more than one target affected
	Isolated: only one target affected
4. Chronology	Congenital onset; unchanging with time
	Congenital onset; increasing/decreasing with time
	Adult (pubertal) onset
5. Selectivity	Resistance to T alone; T and DHT
	Resistance to natural and synthetic androgen
	Resistance to natural but not synthetic androgen

refers to unambiguously female genitalia, "partial" to genitalia that are ambiguous to some degree, and "minimal" to external genitalia that are male. Despite their heuristic value as separate indices, in practice there is considerable overlap among the criteria of "severity," "distribution," and "chronology." Thus, the more masculine the external genital phenotype, the more likely it is that the distribution of affected target organs will be restricted. Similarly, there is a positive correlation between the less severe, more restricted phenotypes and a temporal change in their expression.

Selective resistance to one or another androgen, whether natural or synthetic, is a particularly informative criterion for classifying the various androgen-resistance disorders. It exposes the fact that the functional competence of an A–R complex is exquisitely interdependent on the combinatorial properties conferred upon it by the individuality of its androgenic ligand and the quality of the receptor protein to which it is bound. Selective androgen resistance is an "experiment of nature" which mimics the fact that many steroid antagonists (Rochefort and Borgna, 1981) can bind to their respective receptors and translocate to the nucleus normally, yet are either completely incompetent or weakly agonistic.

Hereditary Androgen Resistance: Homeotic Mutation in Man

Homeotic mutations of Diptera are almost unique in that they cause a change from one normal morphogenetic capacity to another normal one, rather than a loss or a qualitatively abnormal expression of the original capacity. Mutant homeotic genes in the bithorax and antennapedia complexes of *Drosophila melanogaster* specify the morphogenetic fate of segments of the fly so that, for example, a leg may appear on the head in the place of an antenna (Ouweneel, 1976). Morphogenetic maldevelopment, in the homeotic sense, is rare in man, except in the domain of sexual dysmorphogenesis: differentiation of ovarian along with testicular tissue within a biopotential gonad (true hermaphroditism) represents one example, and pseudohermaphroditism represents another. Strictly defined, pseudohermaphroditism refers to the development of genitalia that are opposite to the sex of gonads that are histologically normal. In a female with ovaries, this is usually the result of an unusual source of androgen. In a male with testes, a defect in androgen biosynthesis or responsiveness is the cause. Either situation, if genetic, reflects the expression of a hom-

eotic mutation, albeit one that has a molecular basis very different from that incriminated for homeotic mutations in *Drosophila* (McGinnis *et al.*, 1984).

Differential Diagnosis of Androgen Resistance

The clinical–endocrine suspicion of androgen resistance must be pursued by the most parsimonious route possible. The two basic possibilities to be ruled out are defects in testis determination or differentiation due to chromosomal or genetic causes, and defects in androgen biosynthesis (Saenger, 1984; Imperato-McGinley, 1983; Simpson, 1982).

Family History

A positive family history, taken with sophistication, can be exceedingly useful if a suspect has been shown to have a normal 46,XY karyotype. Thus, evidence for affected individuals linked through their maternal ancestry points to many forms of androgen resistance, as well as those types of "gonadal dysgenesis" in which presumptively X-linked mutations have interfered with testicular differentiation (that is, with the translation of "chromosomal sex" into "gonadal sex"). Conversely, parental consanguinity and other features of autosomal recessive inheritance point toward inborn enzymatic errors of T biosynthesis or primary 5α-reductase deficiency.

In the familial setting, the evolution of the clinical phenotype with age in a 46,XY individual with unambiguous female external genitalia at birth can supply important evidence for differential diagnosis of a younger relative suspected of being affected. Thus, XY females with so-called "pure gonadal dysgenesis" have primary amenorrhea, but little evidence of pubertal sexual development in either the masculine or feminine direction. In contrast, those with female external genitalia due to severe, non-salt-losing forms of defective T biosynthesis may have appreciable bidirectional changes at puberty (for example, breast development and beard growth), while those with complete androgen resistance (CAR), if they are not exposed by the appearance of inguinal testes in childhood, will have primary amenorrhea and sparse axillary and/or pubic hair as components of a puberty that is otherwise normally feminine.

Of equal importance with regard to family history are questions re-

garding age of menarche and amount, distribution, or symmetry of sexual hair (pubic, axillary) in the 46,XX females of the maternal ancestry. It is not widely known that such females often express their carrier state for CAR by delayed menarche and/or sparse/asymmetric sexual hair. This is presumably because random inactivation of one X chromosome in each somatic cell of females permits the development of tissue mosaicism composed of some target cells with the normal phenotype and others that are androgen-resistant. When such mosaicism occurs in the hypothalamic–pituitary axis, it presumably interferes with the "gonadostat" that fixes the timing of menarche; and when it occurs in the hair follicle-bearing regions of the pubis or axillae, it may result in sparse, nonuniform, or asymmetric hair growth. In contrast, carrier females of the presumptive X-linked gene responsible for familial, XY, "pure gonadal dysgenesis" display no evidence of their carrier state, and neither do female heterozygotes for the various autosomal recessive mutations causing various defects of T biosynthesis.

The family history is likewise informative in the case of a propositus with ambiguous external genitalia. Here, the differential diagnosis involves testicular maldifferentiation ("testicular dysgenesis"), *partial* defects of T biosynthesis, and *partial* forms of androgen resistance (PAR). Intrafamilial variable expressivity is not uncommon in the first and third situations, but it is distinctly uncommon in the second, where the phenotype typically "breeds true" (Imperato-McGinley, 1983). There is, however, one form of T biosynthetic defect, that due to 3β-hydroxydehydrogenase deficiency, in which affected 46,XX females may be born with clitoromegaly because the precursor that builds up behind the enzymatic block, dehydroepiandrosterone, is sufficiently androgenic to masculinize external genital development in females, although it is too weakly androgenic to cause normal masculine external genitalia in affected 46,XY males (New *et al.*, 1983). In addition, it is not widely appreciated that 46,XX carrier females of X-linked PAR mutations may express their tissue mosaicism by delayed menarche or sparse/asymmetric sexual hair (Pinsky, 1978), just as do many females who are heterozygotes for the X-linked genes that cause CAR. By contrast, neither females heterozygous for autosomal recessive partial T-biosynthetic defects nor those for presumptively X-linked testicular dysgenesis have any clinical expression of their genotypes.

Another cause of PAR is that due to primary, autosomal recessive, 5α-reductase deficiency. The family history can be very informative, since

affected males, who have usually been reared as females, experience a prominent, distinctive pattern of virilization at puberty. Despite the fact that affected males and females have comparably severe endocrine consequences of the enzyme deficiency, only the former express these consequences clinically; heterozygotes of either sex do not, even if they demonstrate intermediate endocrine defects (Imperato-McGinley *et al.*, 1985).

Minimal androgen resistance (MAR) in 46,XY individuals born with male external genitalia may for a time be clinically indistinguishable from so-called familial delayed puberty. In addition, the differential diagnosis may involve consideration of gynecomastia of diverse origin (J. D. Wilson *et al.*, 1980), primary familial disorders of the hypothalamic–pituitary axis governing normal gonadotropin secretion, and the familial causes of oligo- or azospermia (J. D. Wilson *et al.*, 1980; Chaganti *et al.*, 1980; Chaganti and German, 1979; Hargreave, 1983; de Kretser, 1979; Aiman *et al.*, 1979).

Clinical–Endocrine Criteria

The prototypic clinical–endocrine criteria for androgen resistance in men are a hypoandrogenic phenotype, of whatever degree, in the presence of elevated levels of luteinizing hormone (LH), T, and 17β-estradiol (E_2), the latter being partly of direct testicular origin and partly the result of peripheral aromatization of androgen to estrogen (J. D. Wilson *et al.*, 1983). The concurrently elevated levels of T and LH constitute in themselves an inferential endocrine diagnosis of androgen resistance: they reflect increased secretion of T by testes that are being stimulated by an increased level of LH, which, in turn, reflects hypothalamic–pituitary resistance to the feedback effects of T. However, elevated levels of T and/or LH are frequently *not* demonstrable in random specimens of plasma and, in the case of T, even estimates of daily plasma production rates may not be elevated in subjects with proven androgen resistance (Boyar *et al.*, 1978). Repeated sampling of plasma LH during a day will reveal a greater than normal amplitude and number of cycles of LH release per day, but this practice is not routine. Therefore, when other circumstantial evidence, such as the family history, does not point strongly toward androgen resistance, normal random levels of T and LH in the plasma of a suspect with androgen resistance may have to be distinguished from a partial T biosynthetic defect in which normal steady-state levels of plasma T are achieved by the compensatory increase in mean plasma

LH levels as a result of feedback disinhibition of the hypothalamic–pituitary axis.

In the latter situation, normal levels of plasma T are achieved at the expense of various precursors that "build up" behind the various specific blocks of T biosynthesis. The elevated levels of these precursors can be measured in plasma or urine, either in the basal state or in response to stimulation–suppression tests of the hypothalamic–pituitary–adrenal–testis axis by sequential or combined administration of LH, ACTH, and dexamethasone.

Failure of exogenous T to suppress elevated basal levels of LH, and exaggerated release of LH in response to luteinizing hormone-releasing hormone (LH-RH), are inconstant expressions of hypothalamic–pituitary resistance to androgen that are still sought relatively often (Price *et al.*, 1984). However, failure of exogenous T to cause increased sebum production, decreased binding capacity of the thyroxine-binding globulin, and positive total body nitrogen and phosphorus balance are seldomly used (Vagenakis *et al.*, 1972). In fact, cultured GSF and PSF have become the favored vehicles for making specific, definitive diagnoses of androgen resistance in various families and for basic dissection of the normal and mutant androgen-response system in man.

TYPES OF HUMAN ANDROGEN RESISTANCE DUE TO MAJOR-GENE MUTATIONS AFFECTING THE ANDROGEN–RECEPTOR APPARATUS

Complete Androgen Resistance (CAR)

Clinical Features

This disorder, formerly "testicular feminization," is characterized by congenital, universal, severe, and persistent failure of target cell-responsiveness to androgen at all stages of life. Vasa deferentia and seminal vesicles (of Wolffian duct origin) fail to develop because they are resistant to the differentiating influence of T, while urogenital sinus and external genital primordia development are allowed to pursue the female route because of resistance to DHT. Internal female genitalia fail to develop because regression of the Müllerian ducts is androgen-independent. Epididymes and rudimentary derivatives of both duct systems may be found

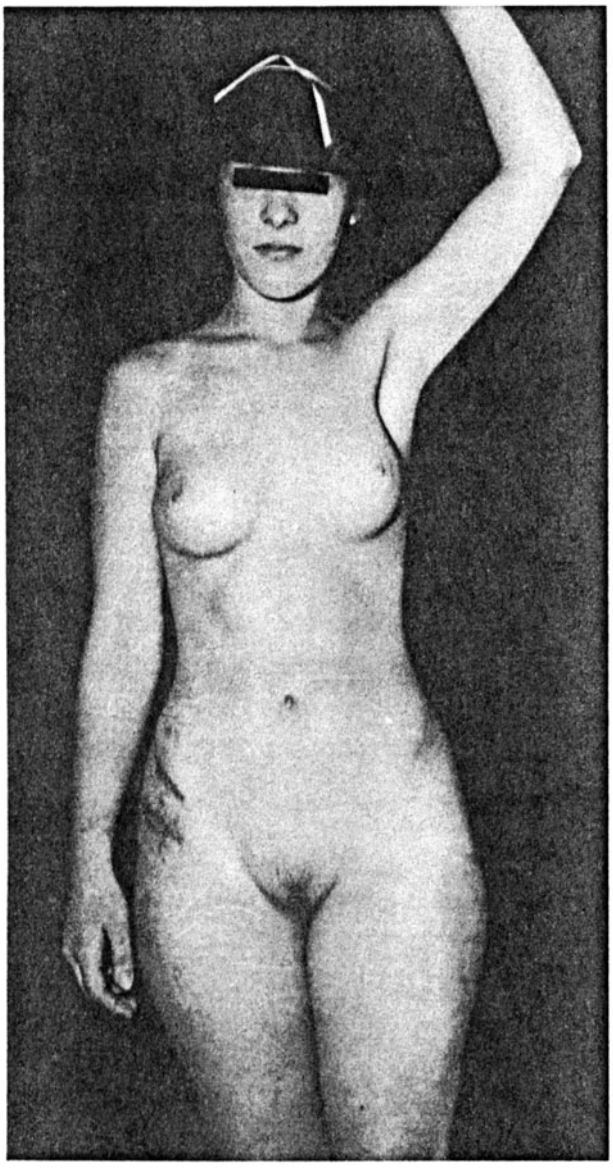


Fig. 16. A subject with complete androgen resistance. Note female body contours, absent axillary and sparse pubic hair, and full breast size.

at surgery. In the case of the Müllerian rudiments, it is not clear whether this finding is more common than in normal males (Naftolin and Judd, 1973). The gonads are labial, inguinal, or abdominal. In the intermediate situation, the subject is usually discovered surgically to have hernial sacs containing testes; in the latter, she is considered a normal girl until the cause of her primary amenorrhea is determined in late adolescence (Fig. 16). The labia minora are often described as hypoplastic, and the vagina is usually short because it lacks its upper, Müllerian duct-derived com-

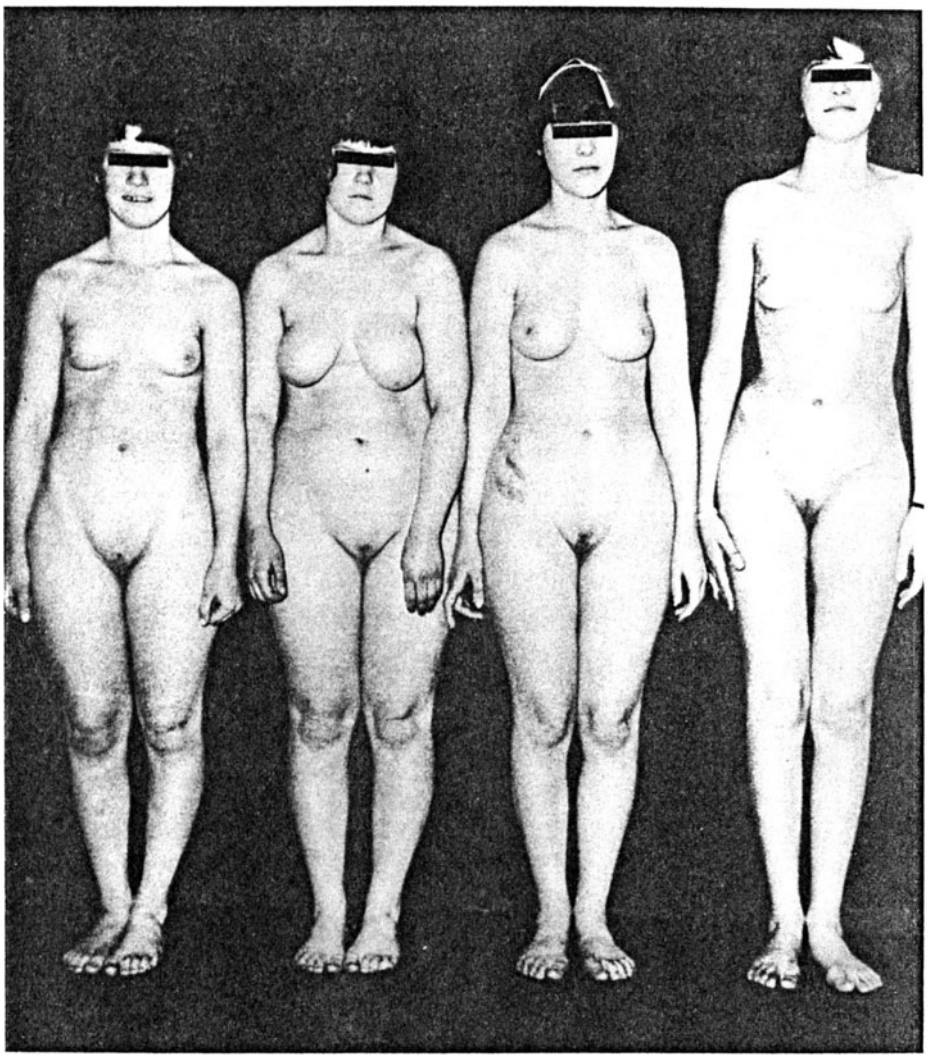


Fig. 17. A sibship with complete androgen resistance. Note variable breast size.

ponent. The testes are histologically normal before 5–7 years of age. Later, they have poorly developed seminiferous tubules, aberrant spermatogenesis (J. Müller, 1984), the usual changes due to cryptorchidism for any reason, and Leydig cell hyperplasia that reflects stimulation by increased levels of LH at puberty. Breast size is often greater than average, but it can be quite variable even within families (Fig. 17). Mean adult height (D. W. Smith *et al.*, 1985) and permanent tooth size (Alvesalo and Varrela, 1980) are greater than those of normal females, but general body contours are typically feminine. The former reflect the operation of Y-linked genes that are androgen-independent. There is no or scanty pubic

hair, and axillary hair is usually absent. Gender orientation is normal female and, except for the emotional consequences of primary amenorrhea, gender identity is entirely feminine as well.

Hormonal and Pathophysiologic Features

Subjects with CAR, as a group, have elevated levels of LH, T, and E_2 in their plasma, but, as pointed out previously, there is great variation even among affected siblings within families. Indeed, none of the three may be elevated in random specimens, and it may be necessary to measure multiple samples of plasma in order to recognize an elevated mean level during all or part of a day (Boyar *et al.*, 1978). In fact, Δ^4-androstenedione, the immediate biosynthetic precursor of T, is more often elevated in random or aggregate samples of plasma than is T itself (Imperato-McGinley *et al.*, 1982), a fact that suggests secondary, relative deficiency of the enzyme 17-ketosteroid reductase, and is reminiscent of the same finding in the androgen-resistant rat (Schneider and Bardin, 1970).

The prototypic combination of elevated basal LH and T is considered to reflect hypothalamic–pituitary resistance to the normal negative feedback effect of T, for two reasons: first, the concurrently elevated level of SS-BG is insufficient to explain decreased feedback on the basis of a lower than normal fraction of free to bound T in the blood; and second, because large doses of exogenous T only weakly suppress basally elevated levels of LH. The LH response to LH-RH is inconstantly elevated, whether assessed by the absolute increment or the factor of increase. Plasma FSH levels vary from normal to elevated, even among affected members of one family (Imperato-McGinley *et al.*, 1982).

Plasma levels of DHT are repeatedly reported as normal, yet relatively low levels of it compared to T may yield a plasma T/DHT ratio that is significantly and appreciably higher than normal, as documented in a large family with CAR by Imperato-McGinley *et al.* (1982). This finding is in accord with previous data that peripheral (as distinct from hepatic) 5α-reductase activity is androgen-inducible via an AR-mediated process. The absence of those primary and accessory sexual structures that are normally responsible for much of the body's peripheral 5α-reductase activity may be the basis for the secondary 5α-reductase deficiency in subjects with CAR.

Feminization at puberty is simply the consequence of normal (or elevated) levels of estrogen in the absence of countereffective androgen.

The absence or sparsity of sexual hair means that androgen is responsible for its development in both sexes. There is no clinical–endocrine parameter of CAR that correlates with any of the relatively slight variation (breast size; more or less sexual hair) that is recognizable even among the phenotypes of affected subjects in one family.

Studies on Cultured Skin Fibroblasts

The level of specific AR binding activity in GSF is classifiable as (1) very low (R-negative, R^-; at or near the lower limit of sensitivity of the assay; <5 fmole/mg protein), (2) easily measurable, but less than normal (R-deficient, R^{def}; 5–10 fmole/mg protein); or (3) normal (R-positive, R^+; 10–40 fmole/mg protein). In normal NGSF lines the lower limit of binding activity is so low that recognition of the R^- state is unreliable and that of the R^{def} state is impossible (Fig. 18). Migeon and his collaborators

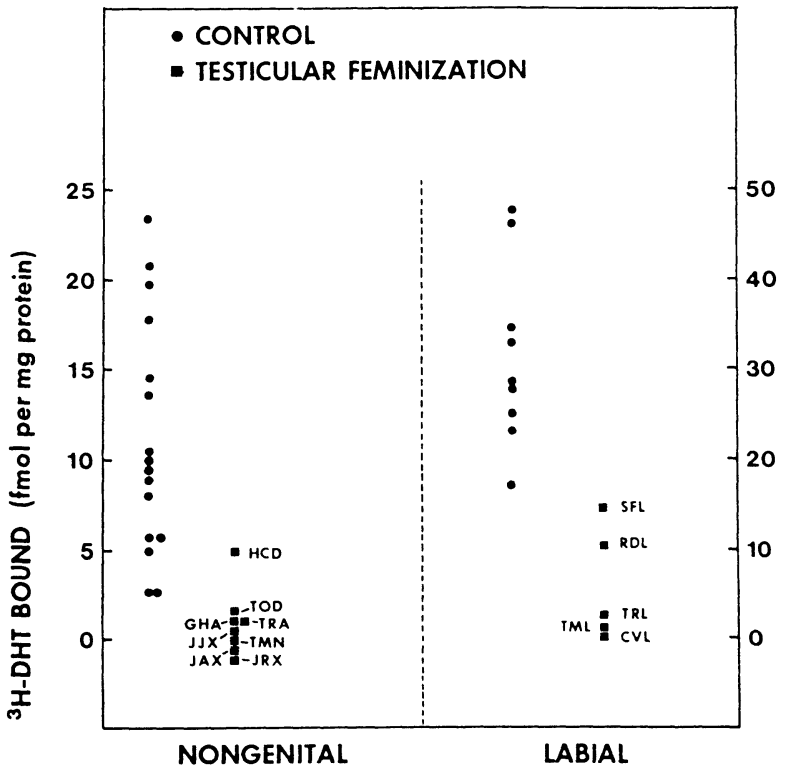


Fig. 18. Specific AR activity with DHT as ligand in NGSF and labium majus SF of controls and subjects with complete androgen resistance (testicular feminization). Note that the scales of the two panels differ by a factor of two.

(Amrhein *et al.*, 1977) deserve credit for being the first to recognize the R^- and R^+ varieties of CAR, while R^{def} CAR was identified in our laboratory (Kaufman *et al.*, 1976) (see RDL in Fig. 18).

The existence of R^{def} CAR implied that the easily measurable residual AR activity was qualitatively defective, since, in the absence of such defectiveness, one would expect a phenotype of PAR rather than CAR. In fact, qualitative defects of the binding activity were soon delineated in some patients with R^{def} CAR by demonstrating that this activity was unusually thermolabile to assay at 42 versus 37°C (Griffin, 1979).

The existence of R^+ CAR implied either a postbinding defect of A–R complex function or a postreceptor defect at one of the steps in the target cell-response apparatus, beginning with interaction of the A–R complex at selected (acceptor) sites in the chromatin. Postbinding defects were soon recognized in the form of assay thermolability at 42°C (Griffin, 1979; Pinsky *et al.*, 1981), failure of molybdate ion to stabilize A–R complexes during sucrose density gradient centrifugation (Griffin and Durrant, 1982), an increased rate of dissociation of A–R complexes formed within GSF and then allowed to dissociate within GSF or after their extraction from the cells (Pinsky *et al.*, 1981; Kaufman *et al.*, 1982*a*; T. R. Brown *et al.*, 1982), and by thermolability of synthetic A–R complexes within intact GSF (Pinsky *et al.*, 1985*a*).* The provocative findings of increased AR activity in two siblings with CAR points to a different type of postbinding defect (Evans *et al.*, 1984). Postreceptor defects of the androgen-response apparatus in man have not been identified in CAR (T. R. Brown *et al.*, 1983). More refined assays, using cloned sequences of specific oligonucleotides, promise to be more successful.

Analysis of single-cell clones derived from obligate heterozygotes for the gene causing R^- CAR has been valuable in confirming the X-linked location of the locus that encodes the AR in man. Meyer *et al.* (1975) found the expected mosaicism of such clones among NGSF of one family, and Elawady *et al.* (1983) found such mosaicism among GSF clones of a heterozygote for a mutation causing CAR of the R^{def} type, using the marker of assay thermolability. To identify heterozygotes among presumptive or possible carriers of R^- mutations, a low level of AR activity in uncloned GSF may be sufficient (Hodgins *et al.*, 1984).

* The title of the Pinsky *et al.* (1985*a*) paper is incorrect; the subject has complete, not partial, androgen resistance.

Partial Androgen Resistance (PAR)

Clinical Features

Individuals in this category constitute a wide clinical spectrum (Fig. 19). One extreme is represented by those, labeled as having "incomplete testicular feminization" (Madden *et al.*, 1975), in whom the external genitalia are predominantly feminine, their only external masculinizing features being clitoromegaly, mild posterior fusion of the labioscrotal folds, and, on the average, a greater amount of sexual hair than that seen in subjects with CAR. The other extreme is represented by those with predominantly masculine external genitalia at birth (i.e., some degree of penile hypospadias) who develop gynecomastia and a more or less male habitus at puberty with normal or near-normal sexual hair, but diminished chest and facial hair. They remain infertile because of spermatogenetic failure. The polar phenotypes that appear to be typical of certain families coexist in other families in which variable expressivity covers the entire spectrum between the two extremes (Griffin and Wilson, 1980). The latter families make it self-evident that background factors, whether genetic or environmental, can modulate appreciably the overall severity and the regional disparity of expression of PAR due to a single mutant genotype. Whether certain families that fall within or at either extreme of the spectrum represent a single-gene mutation or several distinct ones remains to be determined. If the same mutation is at fault, then sampling bias due to the small size of human families may be at work. On the other hand, there is much precedent for the idea that some of the interfamilial phenotypic variation represents the actions of allelic mutations at the X-linked locus that encodes the AR protein, and recent studies on cultured GSF (see below) offer evidence for this alternative.

Hormonal Features and Pathophysiology

As in subjects with CAR, those with PAR typically have elevated plasma levels of LH and normal (or elevated) levels of T and E_2. In fact, in some subjects with PAR, E_2 secretion and production rates are even higher than in CAR, yet feminization at puberty is less than in CAR, reflecting presumably a more virilizing androgen/estrogen balance.

A provocative recent finding in our laboratory has been that secondary 5α-reductase deficiency may occur in subjects with R^+ PAR due

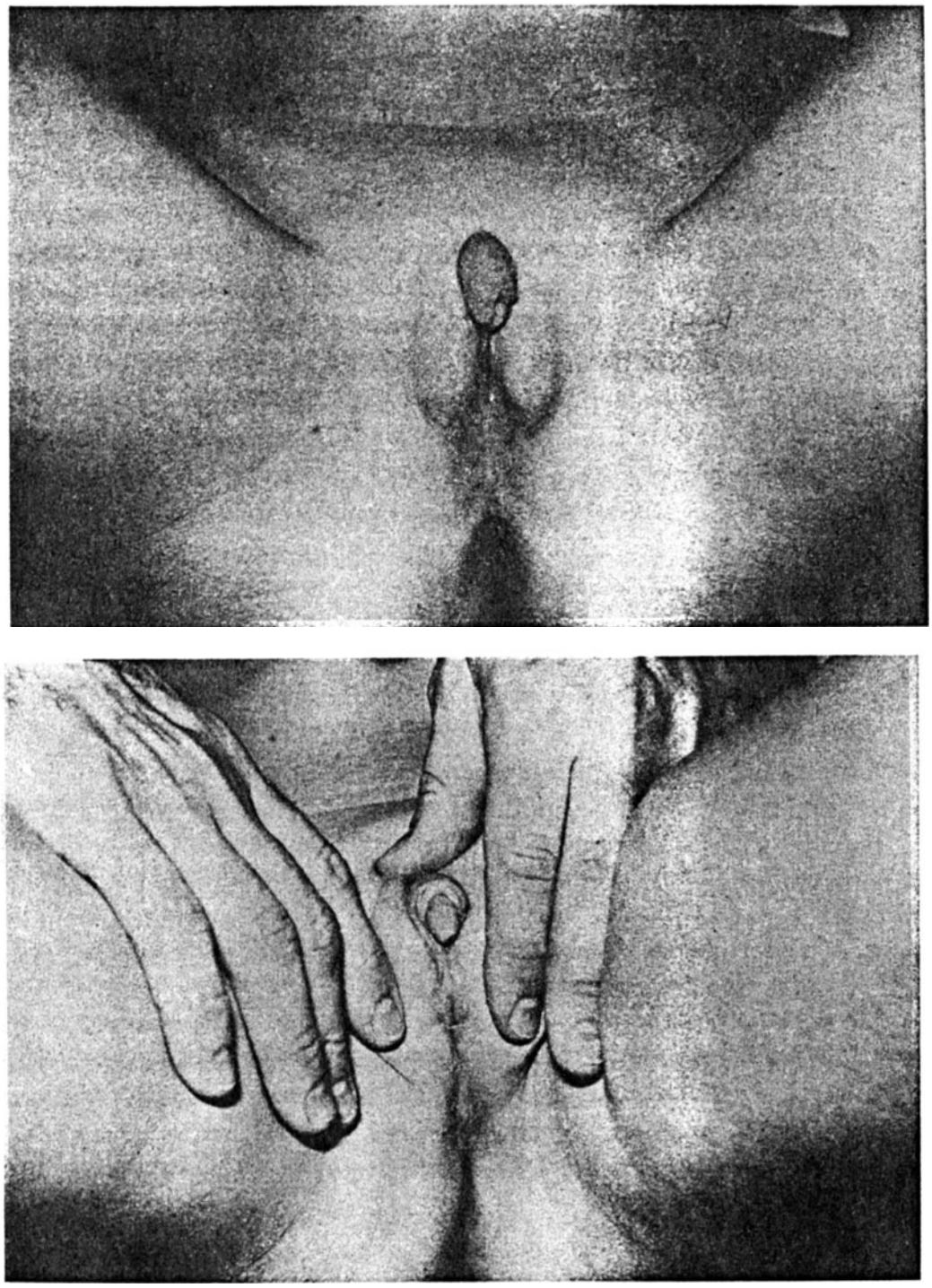


Fig. 19. An individual with partial androgen resistance. He is coded patient 1 in Figs. 20–23.

to a qualitative defect of the AR activity. In one such family, the severity of the clinical phenotype was in accord with the presence or absence of the secondary 5α-reductase deficiency (Jukier *et al.*, 1984). This suggests that secondary 5α-reductase deficiency is one of the factors modulating the clinical expression of PAR due to a partial receptor or postreceptor defect, and therefore the variable expressivity observed within, and perhaps among, certain families.

Studies on Genital Skin Fibroblasts

These studies have been disappointing in the narrow sense that they have not revealed a simple correlation between the level of AR activity and the clinical severity of the PAR, either among or within families. On the other hand, they have been very helpful in (1) identifying the genetic heterogeneity of PAR, (2) inferring discrete structure–*dys*function relations of various mutant receptors, and (3) beginning the process of assigning different structure–function domains to the normal AR.

First, some families (or individuals) have no specific AR activity, others have decreased levels of it (J. D. Wilson *et al.*, 1983), while in still others it is normal (Pinsky *et al*, 1981, 1985*b*; Kaufman *et al.*, 1986; Jukier *et al.*, 1984; Evans *et al.*, 1984). Various explanations have been proferred for those with no activity (Sultan *et al.*, 1983): (1) chance or systematic selection *in vitro* of a clonal cell line with no activity; (2) marked instability of the A–R complexes *in vitro*; and, most intriguing, (3) that AR activity is present during the critical period of morphogenesis in sufficient concentration to sustain partial masculinization of the external genitalia, but then disappears according to some mutational aberration in temporal regulation of the A–R system.

Among those with decreased levels, qualitative defects in the form of assay thermolability (Griffin and Durrant, 1982), failure of molybdate stabilization (Griffin and Durrant, 1982), and decreased nuclear retention (Eil *et al.*, 1983) have been absent, present together, present separately, or were not sought (Rodrigues Pereira *et al.*, 1984).

Among mutations that cause PAR in these different families despite a normal level of binding activity, a variety of qualitative defects have been described in various informative combinations. In one family, assay thermolability and failure of up-regulation are combined with increased apparent equilibrium (K_D) and nonequilibrium (k) dissociation constants, whether DHT or MT (R1881, the synthetic, nonmetabolizable androgen)

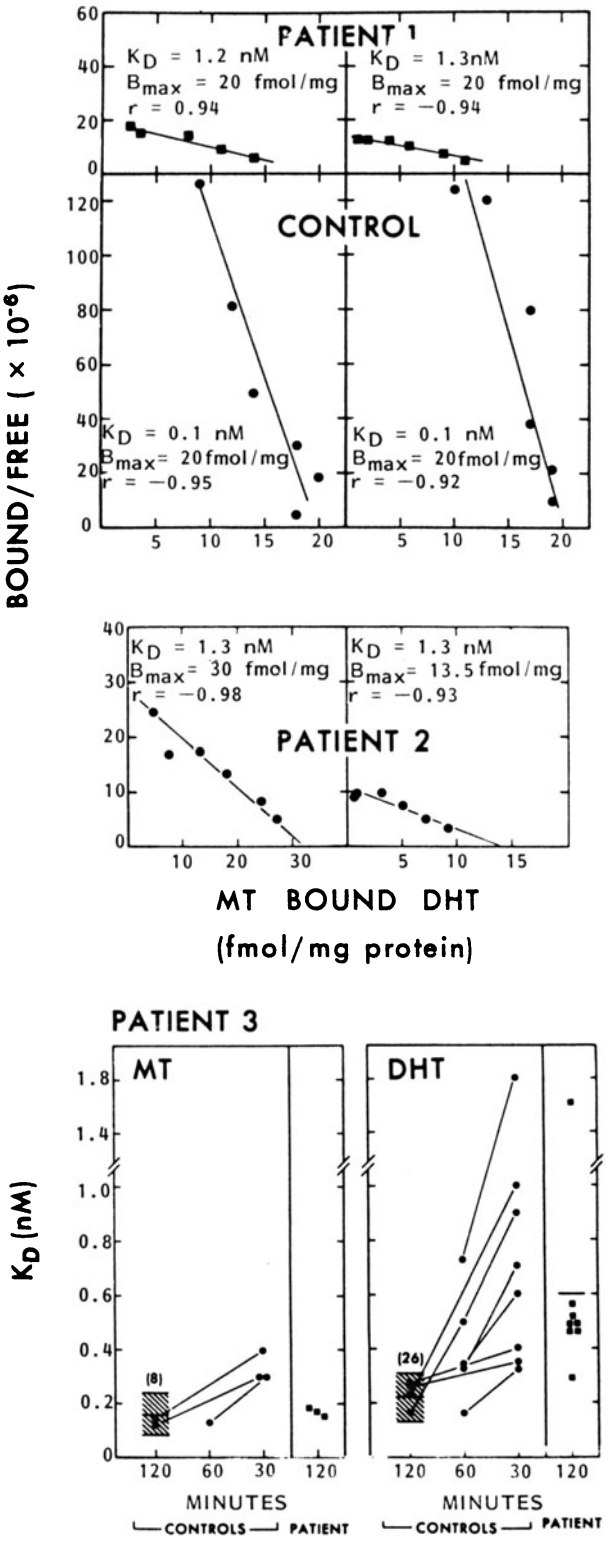


Fig. 20. (Top, middle) Representative Scatchard plots from 2-hr assays and (bottom) individual K_D values from assays of various duration on GSF of controls and typical patients 1–3 representing families 1–3 respectively, with partial androgen resistance discussed in the text.

is the ligand [patient 1, Figs. 20–22 (Kaufman *et al.*, 1984*a*)]. In a second family, failure of up-regulation and an increased K_D with both ligands are accompanied by a normal rate of dissociation of the complexes formed with either ligand [patient 2, Figs. 20–22 (Jukier *et al.*, 1984)]. In a third family, the AR activity has normal binding parameters and the ability to

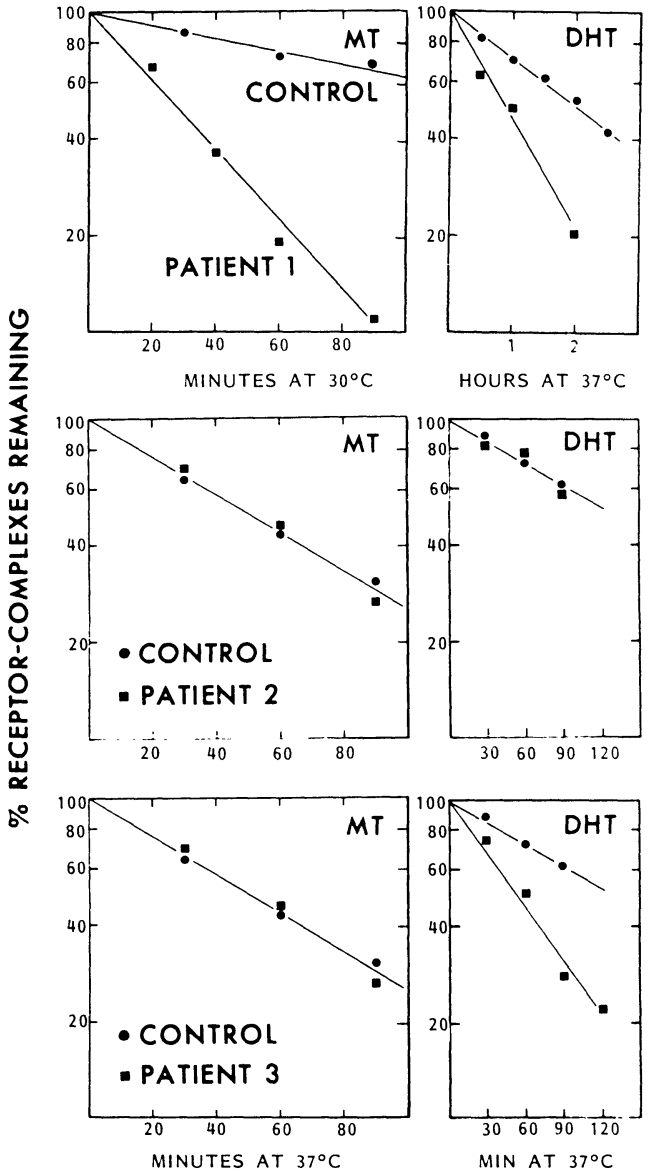


Fig. 21. The rates of dissociation of MT–R and DHT–R complexes in GSF of controls and patients 1–3 representing families 1–3 with partial androgen resistance discussed in the text. Note that receptor from patient 2 can form complexes with either ligand that dissociate normally, even though its apparent equilibrium affinity constant with either ligand is abnormally high, as shown in Fig. 20.

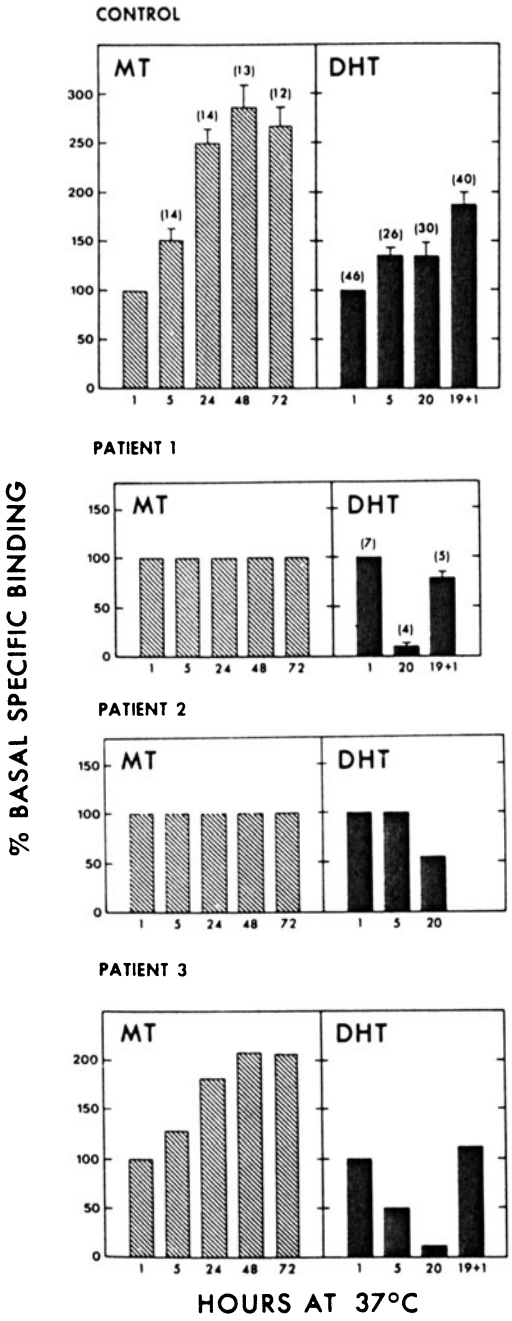


Fig. 22. The response of basal AR activity (1 hr) to prolonged incubation with MT or DHT in GSF of normal controls (sample size in parentheses) and typical patients representing families 1–3 with partial androgen resistance discussed in the text. On the abscissa, 19 + 1 means that a saturating concentration of DHT was added to the medium at the 19th hr of incubation to replenish the DHT consumed by metabolism during the interval. This allows the true level of up-regulated activity to be measured in control cells, and reveals that the 20-hr values in the mutant cells are artificially low because of insufficient ligand. Note that the cells of patient 2 do not up-regulate their AR activity with either ligand even though their receptor can form complexes with either ligand that dissociate at the normal rate, as shown in Fig. 21.

up-regulate with MT, but is abnormal in both respects with DHT as ligand [patient 3, Figs. 20–22 (Pinsky *et al.*, 1985*b*)]. In the last family, the rate of DHT catabolism is normal; this rules out the possibility of hypercatabolism of DHT (to derivatives that are much less androgenic) as its primary abnormality. In the same family, the K_D defect with DHT as ligand is incomplete and variable. In the first family (Fig. 23) and the

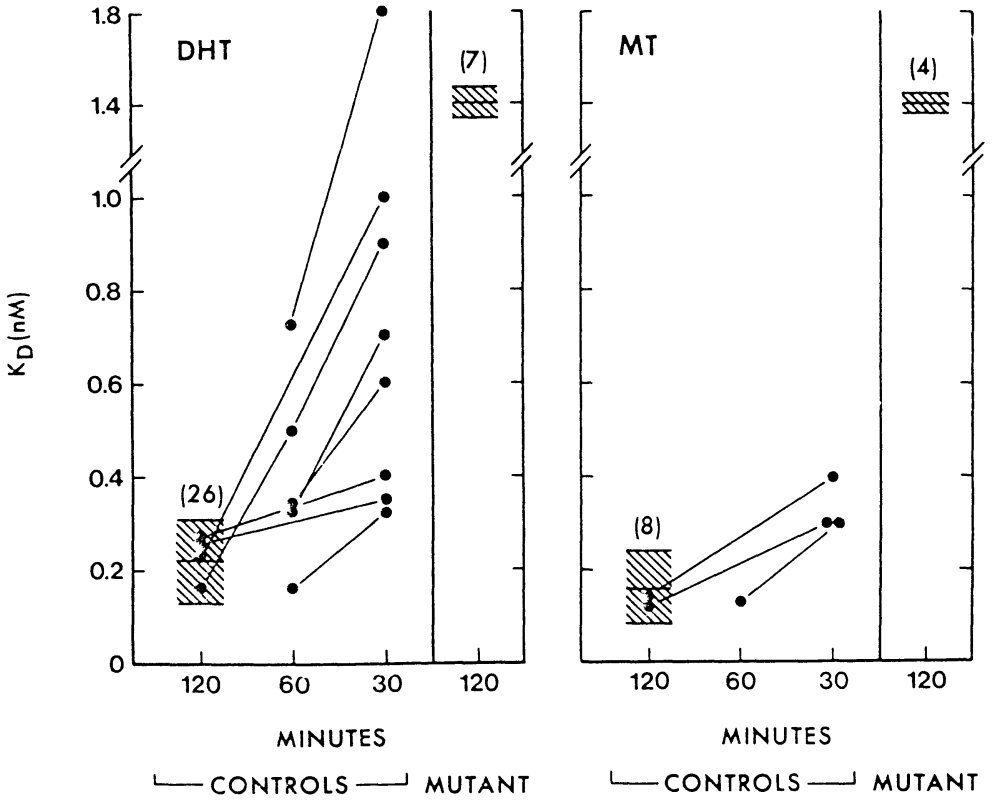


Fig. 23. K_D values with DHT and MT as ligands of the A–R activities in GSF of controls in assays for various times and of the patient from family 1 with partial androgen resistance discussed in the text. Note that, in contrast to the patient from family 3 (Fig. 20), there is very little scatter of the K_D values with DHT.

second it is complete and invariant. In all three families the decrease in apparent K_D (increase in affinity) seen normally with increasing time of incubation (0.5–2 hr), and despite progressive catabolic consumption of DHT, does not occur (Fig. 23). These and other data (summarized in Table II) have led us to propose a model for GSF in which low-affinity A–R complexes formed initially are transformed, in one or more time-, temperature-, and ligand concentration-dependent steps, to a high-affinity state that makes them competent to effect androgen action at the level of chromatin (Table III). Indeed, in two of these families the "low-affinity" A–R complexes exhibit normal nuclear concentration within intact cells, thereby indicating that the high-affinity state, but not nuclear residence, is a valid marker of receptor-mediated androgen action. Further evidence for invalidity of nuclear residence itself as a marker of competent S–R complexes is derived from two sources. In the family with PAR

TABLE II. The Properties of Various R^+ Androgen-Receptor Mutations in Three Families with Partial Androgen Resistance[a]

	Family 1	Family 2	Family 3
K_D (~0.2 nM)			
DHT	×6	×6	×3
MT	×6	×6	+
$k_{37°}^{DHT}$ (0.006 min^{-1})	×3	+	×2
$k_{37°}^{MT}$ (0.012 min^{-1})	×6	+	+
Up-regulation			
DHT	—	—	—
MT	—	—	+

[a] —, mutant; +, normal, ×, factor of elevation; parentheses enclose normal values.

described by Kovacs *et al.* (1984), nuclear–cytoplasmic distribution of DHT–R complexes in intact GSF is normal, but cytosolic preparations of the complexes are labile under conditions (25°C; 5 mM molybdate) that normally permit their transformation to the DNA-binding state. This behavior is reminiscent of E–R complexes that acquire nuclear residence normally in the estrogen-resistant mammary cancer of C3H mice (Baskevitch *et al.*, 1983) and of antiE–R complexes that differ from their normal counterparts in their ability to attain the high-affinity state, but

TABLE III. A Model of Normal and Mutant Androgen–Receptor Complex Transformation in Three Families with R^+ Partial Androgen Resistance[a]

Normal:	A—R	⟶	A=R	⟶	[A≡R]	⟶	up-reg$^+$
Mutant:							
Family I	DHT, MT > R	⟿					up-reg$^-$
Family II	DHT—R	⟿	DHT=R	⟿	DHT≡R		up-reg$^-$
	MT—R	⟿	MT=R	⟿	MR≡R		up-reg$^-$
Family III	DHT—R	⟶	DHT=R	⟿			up-reg$^-$
	MT—R	⟶	MT=R	⟶	[MT≡R]	⟶	up-reg$^+$

[a] A—R, A=R, A≡R: Low-, intermediate- (inferred), high-affinity states of the complexes. [A≡R]: High-affinity state of the complex that is competent to effect up-regulation. ⟿: Complete block in transformation with either ligand. ⟿: Impeded transformation. Sufficient concentrations of either ligand can generate a high-affinity, but up-regulation-incompetent, state of the complex. ⟿: Variable incomplete block in transformation with DHT. With MT as ligand, transformation to the high-affinity, up-regulation-competent state is normal.

not in their ability to achieve nuclear retention or accumulation (Rochefort and Borgna, 1981).

In the study of PAR by Gyorki *et al.* (1983), total GSF AR activity was normal, but percent nuclear accumulation was lower (<20) than normal (>40), and remained low when foreign normal cytosolic A–R complexes were combined with native mutant nuclei. This suggests a primary defect in traverse of the nuclear membrane or in retention of the complexes by the nuclei, and, if the latter, presumably because of defective binding at mutant "acceptor" sites on chromatin.

Therapeutic Considerations

Long-term, high-dose, parental administration of testosterone esters that raise plasma T levels above the normal range have been shown to lower plasma LH levels, promote nitrogen retention, and improve virilization in one subject with PAR (Reifenstein syndrome) due to a qualitatively abnormal R^+ mutation (Price *et al.*, 1984). In a second, with PAR due to a severe R^{def} mutation, comparable elevation of plasma T stimulated well-being and muscle strength, but did not cause significant virilization or depress the plasma LH level, despite an appreciable increment in nitrogen balance in response to acute T administration. Regrettably, the latter subject illustrates that virilizing responsiveness to long-term androgen supplementation cannot be predicted by assessment of nitrogen balance as a measure of sensitivity to acute T stimulation. Price *et al.* (1984) suggested that depression of LH, and perhaps of FSH, may be a useful correlate, if not an indicator, of virilization by subjects with PAR in response to long-term androgen therapy.

Minimal Androgen Resistance (MAR)

Relation to Other Classes of Androgen Resistance

There is a degree of familial, systemic androgen resistance that does not interfere at all with masculine differentiation of the external genitalia, but may interfere with growth of the penis or may only begin to express itself by gynecomastia and a variable pattern of undervirilization at puberty. We call this minimal androgen resistance (MAR), realizing that it may be artificial to segregate such families from those, labeled as having

PAR, in whom similar degrees and patterns of undervirilization at puberty are accompanied by minimal penile maldifferentiation [for example, by first-degree (coronal) hypospadias]. The heuristic value of such a classificatory segregation will become apparent when we come to discuss those clinical situations in which hypospadias, oligoazospermia, or gynecomastia occur as apparently isolated expressions of apparently constitutional androgen-resistance states.

Clinical Features

In the family described by Larrea *et al.* (1978), two sets of maternal first cousins with a normal adult male habitus experienced a puberty that included normal external genital growth, normal pubic but absent axillary hair, and progressive bilateral macromastia. Their ejaculates had low volumes, but, surprisingly, a normal concentration of sperm that exhibited normal motility. Their prostate glands were extremely small. Their maternal grandfather had a history of bilateral postpubertal gynecomastia and if, as seems likely, he was also affected, the gene mutation responsible for the androgen resistance in this family does not confer infertility.

In the family we have studied (Pinsky *et al.*, 1984), two sets of maternal first cousins had a communal phenotype that overlaps the one described by Larrea *et al.* They shared normally differentiated male external genitalia, postpubertal gynecomastia, low to normal ejaculate volume with a low sperm count, and normal pubic and leg hair, but sparse axillary, facial, and chest hair (Fig. 24). They differed appreciably, however, in penile size (probably congenitally, but certainly after puberty), the chronology of their spontaneous virilization, and their responsiveness to long-term pharmacological doses of T.

Hormonal and Pathophysiologic Features

In the family studied by Larrea *et al.* (1978), variable but moderately elevated levels of serum LH coexisted with elevated levels of T and E_2. In addition, LH-RH stimulated an exaggerated LH response, and chronic administration of T failed to suppress the elevated levels of LH. Thus, the basic endocrine criteria for diagnosing androgen resistance were fulfilled. Remarkably, however, testicular biopsies revealed normal spermatogenesis. This is the only form of androgen resistance, other than that

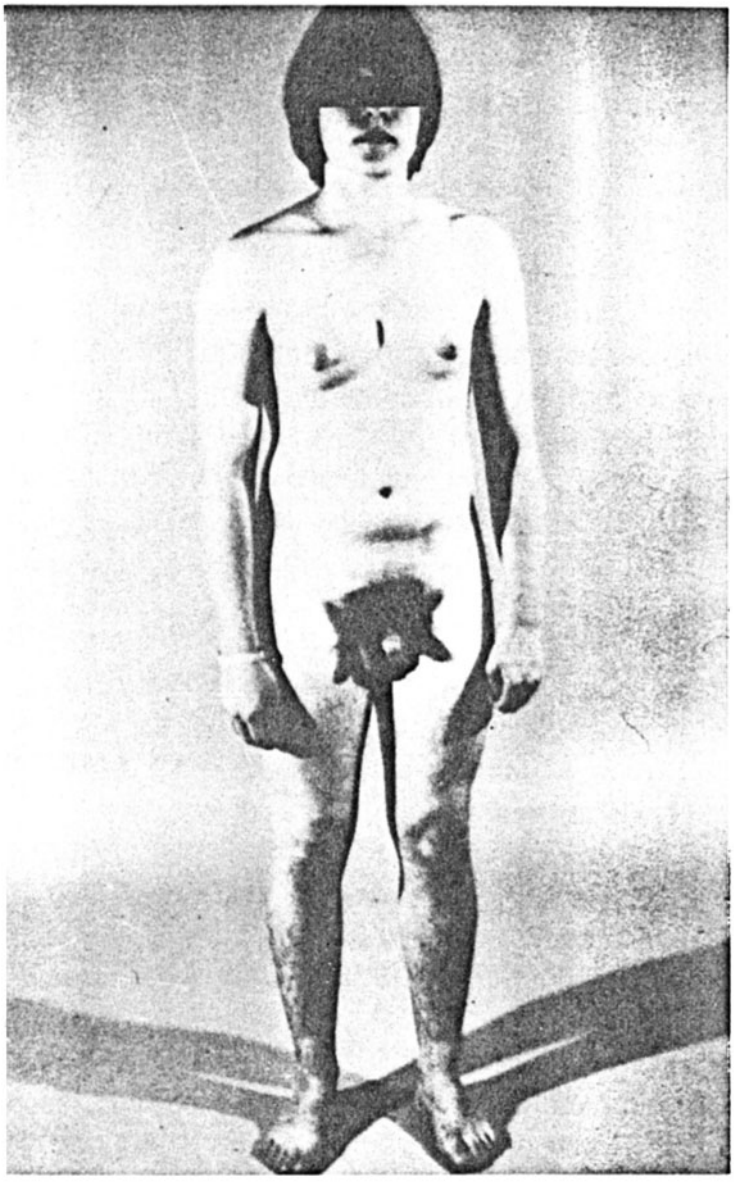


Fig. 24. An affected maternal first cousin (at age 14) of the brothers with minimal androgen resistance referred to in Fig. 25. He is individual III-2 in Fig. 1 of Pinsky *et al.* (1984). Note that he had prominent gynecomastia, which required bilateral mastectomy, despite a male habitus, normal pubic hair, normal penis and scrotum, and normal lower leg hair.

due to 5α-reductase deficiency, in which spermatogenesis is spared. Furthermore, it supports the inference that the gene mutation (presumably X-linked) responsible for their androgen resistance was transmitted to the two sets of maternal first cousins from their maternal grandfather through their respective mothers.

In the family we have studied, T and E_2 levels in the plasma were normal, but LH levels were not high, and LH-RH did not produce an exaggerated response of LH. This illustrates once again that hypothalamic–pituitary criteria for androgen resistance may not be fulfilled even in situations where it is strongly suspected on clinical grounds and, as shown below, is eventually proven by definitive studies on the AR activity in GSF.

Larrea *et al.* studied the uncultured breast tissue of one of their patients, and found that it had specific receptor activity for T and DHT at a normal level and with a normal binding affinity. Despite these results, it is legitimate to ask, as will be discussed below, whether mammary stromal fibroblasts of their patients might not have an AR activity that is abnormal, particularly when assessed in other ways.

Cultured GSF Studies

In the family we studied, the AR binding activity was shown to have normal binding parameters (K_D, k) with MT, but not with DHT (Figs. 25 and 26), and prolonged incubation with MT, but not with DHT, caused a normal up-regulation response of the basal receptor activity (Fig. 27).

Recently, we have studied the ligand-selective, mutant AR activity in three additional ways: by measuring the turnover of the native receptor and its affinity parameters with a second, synthetic, nonmetabolizable androgenic ligand, mibolerone (MB), and by characterizing the thermostability of its synthetic A–R complexes (preformed at 37°C) within cells that are incubated at 41–43°C in the presence of 10 μM cycloheximide and saturating concentrations of either synthetic ligand MB or MT (Kaufman *et al.*, 1984*b*). To our surprise, the ligand-selective mutation causes the mutant receptor to have an increased k with MB, but not with MT, both at 37 and 42°C, despite the fact that its K_D with MB at 37°C is normal, as it is with MT. Thus, it forms complexes with MB that are more dissociable than normal, while its counterparts with MT are not. Furthermore, in the thermostability assay, both its MT–receptor and MB–receptor complexes are less stable than normal and this behavior is worse with MT (from which the receptor dissociates normally) than with MB (from which it dissociates abnormally quickly). Thus, the additional data provide two new, temperature-dependent, ligand-sensitive markers of the ligand-selective mutation. Indeed, the thermolability marker is particularly intriguing because it is expressed by complexes formed between a

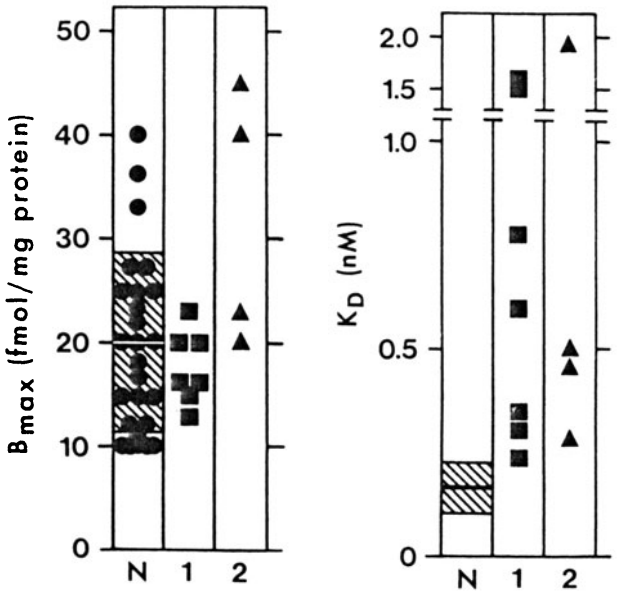


Fig. 25. B_{max} and K_D values for the AR activity in GSF with DHT as ligand in (N) 26 normal controls, and (1, 2) two brothers with ligand-selective minimal androgen resistance. Brothers 1 and 2 are coded D3295 and B1016 and correspond to individuals III-6 and III-5, respectively, in Fig. 1 of Pinsky *et al.* (1984). Note that the scatter of K_D values is comparable to that observed for patient 3 with the ligand-selective form of partial androgen resistance, as shown in Fig. 20. With MT as ligand, the B_{max} and K_D values are normal (results not shown).

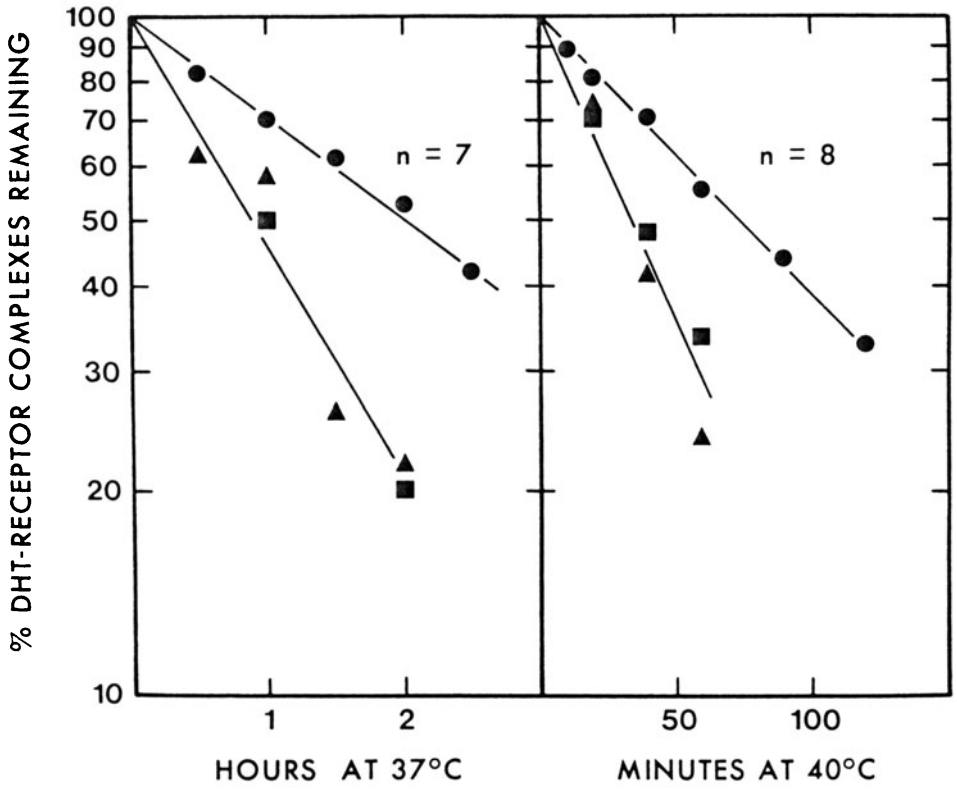


Fig. 26. The rates of dissociation of DHT–R complexes within GSF of (●) controls and (■, ▲) two brothers with ligand-selective minimal androgen resistance.

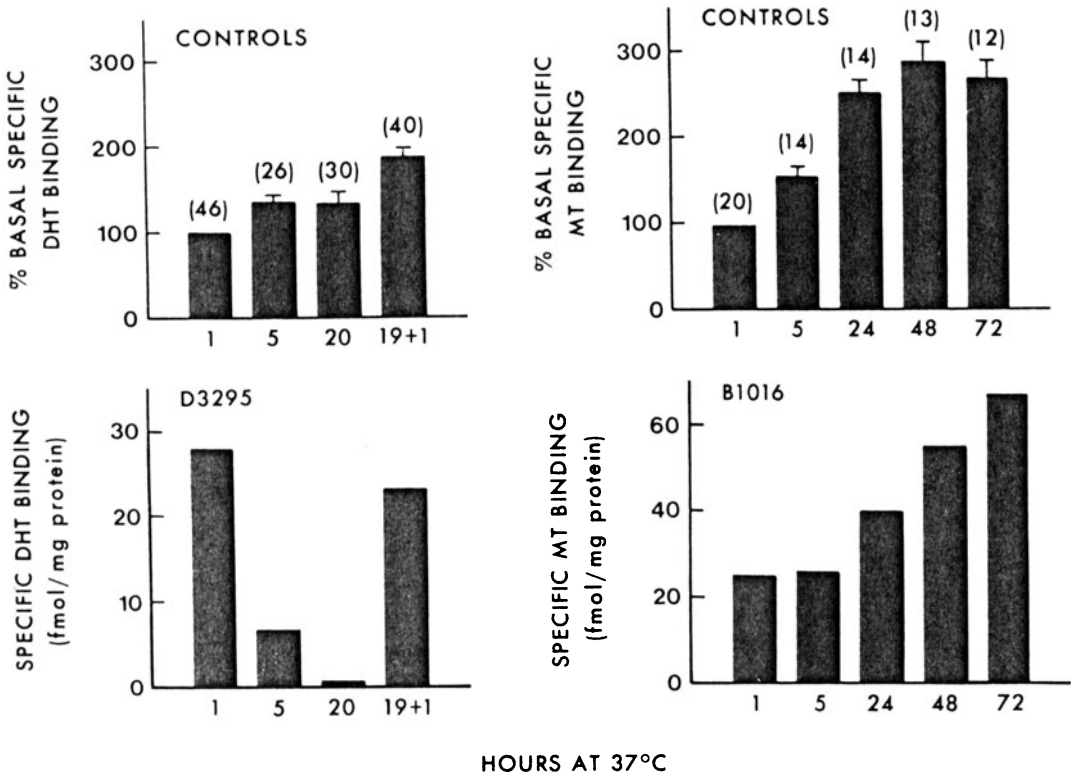


Fig. 27. The effect of prolonged incubation with DHT or MT on specific AR activity in GSF of normal controls (size of sample in parentheses) and the brothers B1016 and D3295 with ligand-selective minimal androgen resistance, for whom B_{max} and K_D values are presented in columns 1 and 2, respectively, of Fig. 25. With MT, the patients up-regulate their basal activity normally; with DHT they do not. In fact, in the absence of a normal uup-regulation response to DHT, concurrent metabolic consumption of DHT (at the normal rate) yields a measure of AR activity that is artificially low. Provision of a saturating concentration of DHT to the DHT-depleted medium at the 19th hr (column 19 + 1) allows the true, unchanged level of AR activity to be expressed. Similarly, the up-regulation response in control cells incubated with DHT appears to be the same at 5 and 20 hr, but supplementation of the culture medium with a saturating concentration of DHT at the 19th hr (column 19 + 1) allows the true level of up-regulated AR activity to be expressed.

synthetic androgen and the mutant receptor at the same elevated temperatures that do not cause the native mutant receptor to exhibit an increased rate of turnover (Kaufman *et al.*, 1986).

Theoretical and Therapeutic Implications of the Ligand-Selective Androgen–Receptor Mutation

Our observations on the ligand-selective mutation hold great potential significance for elucidating the combinatorial properties of A–R com-

plexes that endow them with the ability to act as differential regulators of gene transcription. The ability of MT, a synthetic androgen, to unite with the mutant receptor protein in such a way as to generate MT–R complexes that behave normally by all except one (thermostability) of the criteria we have used to assess normality argues that the structure–function attributes of MT are somehow able to complement most of the structure–dysfunction attributes of the mutant receptor protein. Although we do not yet know anything about the structural mutation that alters the gene encoding the AR or about the resultant alteration(s) in the primary or higher order structure of its product, it is obvious that this information, when it becomes available, will certainly help us to understand more about the structure–function domains of a normal A–R complex that endow it with the property of regulating gene transcription selectively in various androgen-target cells.

The ligand-selective AR mutation is functionally homologous with the one described by M. A. Miller *et al.* (1984) in two clonal variants of the MCF-7 human breast cancer cell line. These two variants are sensitive to natural estrogen, both in terms of growth promotion and PR responsiveness, but, in contrast to the parental cells, they are insensitive to the growth-resistant effect of antiestrogens. No differences have been observed in the properties of antiE–R complexes formed by the variants and the parental cells, suggesting that the mutational alteration in antiestrogen-responsiveness of the variant cells must occur at steps beyond the initial interaction of ligand with cellular estrogen receptors.

Another aspect of the thermal behavior of the ligand-selective mutation that is very provocative from a theoretical point of view is the fact that the stability of the native mutant receptor is normal at the same elevated temperatures that cause its synthetic A–R complexes to be labile. Since thermostability is measured in the presence of saturating concentrations of ligand (supersaturating concentrations of ligand protect the thermostability of the complexes), and since the mutant MT–R complexes are more thermolabile than normal mutant MB–R complexes even though they are not more dissociable than normal, it is clear that this assay does not measure the turnover of intact complexes, but rather that of free mutant receptors ("disliganded") that have recently been dissociated from the synthetic androgens. In view of the normal thermolability of the native mutant receptor, this observation can have only two explanations: Either the disliganded mutant receptors find themselves in a new cellular

microenvironment that is inimical to them but not to their native or normal counterparts, or, their environment is constant but they misbehave because of some conformational imprint left on them by their steroid ligands. The fact that others have reported increased rates of SR turnover in the presence of their ligands (McIntyre and Samuels, 1985; Mullick and Katzenellenbogen, 1986) suggests that the ligand-sensitive AR mutation is simply expressing a normal process to an abnormally great extent. In any event, either explanation offered above bears serious implications for intracellular "cycling" of SR, an aspect of their biology about which little is known. Furthermore, the idea that SR may carry a physiochemical imprint, perhaps transient, of their once-liganded state is compatible with the suggestion that some SR-mediated effects may be carried out by steroid-imprinted, but currently unliganded, SR proteins (Kaufman *et al.*, 1986).

The fact that MT is able to normalize most of the abnormal properties of the ligand-selective mutant receptor suggests that it could be prophylactic or remedial for patients who carry it—for instance, in preventing gynecomastia and the need for surgical mastectomy, and in promoting a more normal virilization, including, perhaps, normal spermatogenesis. However, MT is very hepatotoxic, at least when taken orally (Kruskemper and Noell, 1966), and therefore other synthetic androgens with comparable corrective effects *in vitro* will have to be assessed, first *in vitro* and then carefully *in vivo*, before attempting to use them therapeutically.

Several young adult subjects in the familiy we studied with ligand-selective MAR have received long-term, high-dose testosterone ester therapy, to which they have responded with relatively minor virilization (Pinsky *et al.*, 1984). In relation to the recent report of Price *et al.* (1984) on a subject with R^+ PAR, our observations indicate, contrary to expectation, that therapeutic androgen responsiveness after puberty may not vary inversely with the degree of androgen resistance as it is expressed by the extent of masculinization of the external genitalia during prenatal life.

A SINGLE-BINDING-SITE, ALLOSTERIC MODEL OF ANDROGEN–RECEPTOR INTERACTION IN HUMAN GENITAL SKIN FIBROBLASTS

Various analyses of the specific binding of T, DHT, MT, and MB within GSF from controls and subjects with different mutational defects

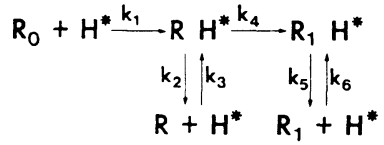


Fig. 28. Allosteric model of sequential steroid–receptor interaction.

of the AR have indicated that A–R complexes can exist in multiple affinity states. A single-site, allosteric model that integrates these states is shown in Fig. 28. The essential features of the model are that: (1) the native receptor (R_0) binds hormone (H) at a rate k_1 to form a low-affinity complex (RH); (2) as a consequence of the binding event, the receptor is altered conformationally such that upon RH dissociation, the free, once-liganded receptor R is no longer equivalent to the native version R_0; (3) the low-affinity type of complex undergoes a second conformational change, at a rate k_4, which endows it with higher affinity status. According to this model, the time- and concentration-dependent effects on specific hormone binding activity (and the reflection of these effects on the character of the resultant Scatchard plots) merely represent the redistribution of A–R complexes between their low- and high-affinity states as a function of time. We have tested these hypotheses by solving the set of linear dif-

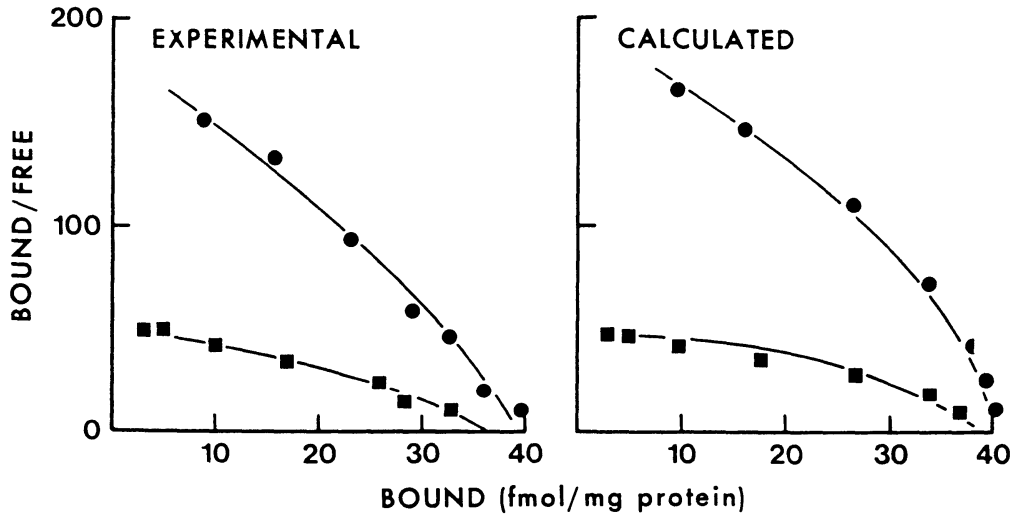


Fig. 29. (Left) Replicate confluent monolayers were incubated (37°C) with ^{3}H-MB (0.05–3 nM) alone (total binding) or in the presence (nonspecific binding) of a 200-fold excess of nonradioactive hormone for (■) 30 min or (●) 120 min. The amount of specific receptor activity was plotted by the method of Scatchard. (Right) Simulation of experimental Scatchard plots according to the allosteric model.

ferential equations developed from this model to simulate the binding of MB to the AR within GSF (Fig. 29).

THE SEARCH FOR ANDROGEN–RECEPTOR ABNORMALITIES IN PRESUMPTIVE ANDROGEN RESISTANCE THAT IS TRANSIENT OR ANATOMICALLY RESTRICTED TO ONE OR FEW SITES IN THE BODY

Background

One of the families with MAR described in the preceding section illustrates that a mutation that affects the quality of the AR activity can, in particular individuals, only take its toll on late (pubertal) events in male sexual development, while sparing completely those androgen-dependent events that occur earlier. To try to interpret this situation, it is important to appreciate that the overall degree and the topographic/chronologic character of the impaired pubertal virilization in such families may not differ at all from that in other families in which AR defects do interfere with genital morphogenesis. In this light, the families that appear to express a late onset of their androgen resistance may indeed represent mutations that in some way alter the normal temporal regulation of the AR apparatus in various parts of the body. On the other hand, the delayed onset of the phenotypes in these MAR families may represent nothing more than a greater sensitivity or vulnerability of certain pubertal than of certain prenatal events to the specific quality of the gene mutations that they carry. It is self-evident that the same set of alternative explanations could apply to individuals with types of androgen resistance apparently restricted to one or very few functions or sites in the body.

There is a firm foundation in mammalian genetics for either of the alternative mechanisms noted above (Paigen, 1979). In the mouse, the structural locus (*Gus*) for the androgen-inducible kidney enzyme β-glucuronidase is on chromosome 5. It is very closely linked to two kinds of "controller" genes. One, *Gut*, controls the temporal appearance of β-glucuronidase during the life of the animal. The other, *Gur*, is responsible for controlling the degree to which androgen, such as DHT, acting through the AR, can induce β-glucuronidase activity in proximal convoluted tubule cells of the kidney. Allelic variation among inbred strains of mice

results in "high" or "low" inducibility varying from 5- to 30-fold. The *Gur* locus acts, only in the *cis* conformation, to increase the concentration of β-glucuronidase mRNA in response to androgen stimulation, primarily by regulating transcription of the *Gus* gene. It is easy to imagine that resistance restricted to androgen-inducibility of β-glucuronidase activity in the mouse kidney might occur as the result of: (1) an AR defect that specifically prevents the usual interaction of A–R complexes at a normal *Gur* locus, (2) an "acceptor" defect at or near the *Gur* locus that interferes with its ability to interact with normal A–R complexes, or (3) a mutation at the *Gut* locus that alters primarily the temporal appearance of β-glucuronidase and, secondarily, the extent of its inducibility by androgen.

On this background it is appropriate to proceed with the following discussion.

Oligo/Azospermia

Several reports have described men, including a pair of identical twins (Smallridge *et al.*, 1984), who presented to infertility clinics with oligo- or azospermia, but usually no other clinical–endocrine expressions of androgen resistance, and who have quantitative (Aiman and Griffin, 1982) or qualitative (Griffin and Durrant, 1982; Warne *et al.*, 1983) abnormalities of the AR activity in their GSF. In one study (Aiman and Griffin, 1982), 9 of 22 men with idiopathic oligospermia fell into this category, but in 6 of them, serum levels of T and LH were individually normal, as was their product, indicating that the androgen resistance, presumably constitutional, was not expressed by defective feedback inhibition of their hypothalamic–pituitary–testis axes. Schulster *et al.* (1983) used the T × LH product as an index of androgen insensitivity and found it to be above 200 in 10 of 86 men with idiopathic azo- or oligospermia. It would now be valuable to know how many of these ten men have AR defects that are detectable in their GSF. Oligospermic subjects with other minor clinical signs of undervirilization [for example, decreased facial or axillary hair (Aiman *et al.*, 1979)] would qualify for the label of MAR, according to the classification used in this chapter, if they had positive family histories. But, except for the identical twins mentioned above, all the subjects (to date, a total of ten) have been sporadic. If these individuals, who are "genetic lethals," carry mutations at the X-linked locus that encodes the AR protein, then, under certain assumptions, one-third of them should represent new mutations. The re-

mainder might have a positive family history. It will be interesting to learn whether the negative family history data so far collected represent a chance phenomenon or not. The fact that various qualitative (Aiman and Griffin, 1982; Warne *et al.*, 1983), not just quantitative, abnormalities of the AR have been found in at least some of these men with oligo/azospermia suggests that mutation at the AR locus is involved. If this is verified, then such infertile males will constitute a convincing example of one (or more) gene mutation(s) that confers androgen resistance on a specific part of the body (the seminiferous tubules) and on a specific process (spermatogenesis). This may have one of the following bases: spermatogenesis may be the ultimate androgen-dependent process in the body and, therefore, the most sensitive of all to any aberration of the AR system (Chowdhury and Steinberger, 1975); spermatogenesis is singularly vulnerable to certain types of AR defects; mutations that occur at sites responsible in part for normal temporal regulation of the AR locus may cause the appearance of abnormal AR activity only after differentiation and growth of the male reproductive system has occurred. The latter possibility is supported by the fact that decreased and, in some cases, qualitatively abnormal AR activity is demonstrable in skin fibroblasts cultured from skin of the penis, scrotum, or prepuce of such subjects, all of which have differentiated normally. This implies, at least, that there was a morphogenetically effective, if not an entirely normal, complement of AR activity in these structures at the time they were undergoing morphogenesis and growth.

To our knowledge, meiotic studies (Chandley, 1979; Hembree *et al.*, 1977) have not yet been done on any subject considered to have isolated oligo/azospermia as the result of an AR disorder. It is important, therefore, to point out that desynaptic meiosis (normal pairing at pachytene and failure of chiasma formation at diakinesis) with spermatogenic arrest at the level of primary spermatocytes has been described in one human family with the pattern of transmission expected of X-linked inheritance (Chaganti and German, 1979). It has also been described in a number of infertile men with negative family histories (Chaganti *et al.*, 1980). Clearly, such subjects, particularly those with possible X-linked inheritance, merit investigation for AR defects.

The fact (McKenna *et al.*, 1983) that women with hypertestosteronemia can have secondary amenorrhea without other signs of androgen excess (hirsutism, acne, etc.) is additional evidence for differences in androgen sensitivity among different regions or processes in the body.

Finally, Sertoli cells of the rat have typical AR activity (Sanborn *et al.*, 1977); an impairment of their androgen-response system might thus interfere with their sustaining-nutritive function in spermatogenesis (Lyon *et al.*, 1975). Similarly, the epididymis is essential for normal sperm maturation (Cameo *et al.*, 1971). A localized impairment of its AR apparatus could cause male infertility in the absence of oligo/azospermia.

Despite the foregoing considerations, the finding of a beardless male with or without oligospermia should not lead to the automatic presumption of focal androgen resistance: subjects with this phenotype have been identified as members of a family in which females of three generations had hirsutism with polycystic ovarian disease (P. N. Cohen *et al.*, 1975).

Simple Hypospadias (SH); Micropenis

Simple hypospadias (SH) is defined as incomplete fusion of the penile urethra without a urogenital sinus. The severity of the defect is classified according to the position of the urethral orifice: coronal (on or within 1 cm of the corona); phallic (>1 cm from the corona, <0.5 cm from the perineum); perineal–scrotal. SH is one of the more common birth defects of man. In all its degrees of severity, incidence has been estimated to be as high as 0.8%; in its second and third degrees, as high as 0.2% (Carter, 1973; Sweet *et al.*, 1974). The high familial incidence of SH [20–30% in various series (Bauer *et al.*, 1979)] and the success in finding AR abnormalities among subjects and families with "complicated" forms of hypospadias have stimulated efforts to search for similar abnormalities in SH.

There have been at least five reports of such studies, using uncultured foreskin preparations or cultured GSF. The problem with four of them is that they have been performed on children with apparently negative family histories and without full investigation for intersexuality; thus, the "simple" status of their hypospadias is suspect (Rajfer and Walsh, 1976). In one report (Evain *et al.*, 1977) four subjects (three coronal; one penoscrotal) had normal levels of specific DHT-binding activity. In a second (Svenson and Snochowski, 1979), 2 of 11 subjects (one distal–penile; one scrotal) had specific MT-binding activity not significantly below the normal range. In a third report (which defines neither the number of subjects studied nor their degrees of hypospadias) differences from normal were observed in the molecular sieve chromatographic behavior of MT–R com-

plexes from hypospadiac tissue, but not in its level of specific MT-binding activity (Coulam *et al.*, 1983).

In the first (Warne *et al.*, 1983) of three studies that used cultured GSF, a defect in nuclear accumulation of MT–R complexes not attributable to the quality or quantity of the cytosolic-receptor activity was discovered in one of 30 SH children by reciprocal reconstitution experiments using cytosolic and nuclear preparations from the subjects and controls. In the second (Keenan *et al.*, 1984), sonicated preparations of GSF were used to measure AR activity in $20{,}000 \times g$ supernatants. The results revealed that activity was lower in 26 subjects than in 18 controls (6 versus 10 fmole/mg protein), but there was considerable overlap between the two groups, and there was no correlation between residual activity and severity of the defects among the subjects. Thus, the evidence that "simple" hypospadias (hypospadias not part of frankly ambiguous genitalia) may result from various anatomically restricted defects of the AR apparatus is vague at best. Systematic studies (including formal family histories and long-term follow-up) of this problem are needed, not only to answer the basic biologic question of interest, but also to determine how often focal expression of defects of the androgen-response apparatus can be identified as one class of genetic factors that contributes to the genetic heterogeneity (Page, 1979) and multifactorial inheritance (Bauer *et al.*, 1979) of "simple" hypospadias. In the third study (Eil *et al.*, 1984), there was no difference between PSF or GSF of 20 subjects with hypospadias compared to 26 controls either in whole-cell AR binding activity or in the representation of this activity within nuclei prepared from whole cells by detergent treatment.

Micropenis is defined as a penile phallus (without hypospadias) that is below the third percentile of the normal distribution for age. In one study on 13 subjects witih micropenis, only one was documented to have a normal T response to hCG stimulation, and he had a normal level of DHT receptor activity in his GSF (Amrhein *et al.*, 1977). In another study (Warne *et al.*, 1983) two of seven subjects had AR activity in their GSF below the normal range.

Postpubertal Macromastia

This condition is defined as pubertal gynecomastia that persists, and may progress, beyond male adolescence without other evidence of sexual maldevelopment. No hormonal abnormalities have been found. Areolar

and PSF studies have revealed a normal level and affinity of the AR activity in such subjects (Eil *et al.*, 1983). The question remains whether the implied mammary gland-restricted androgen resistance in these subjects would be reflected in other qualitative defects of their androgen-response apparatus. Furthermore, it is not obvious that areolar SF are valid representatives of the cells (and tissues) that are responsible for macromastia; it may be necessary to study fibroblasts cultured from mesenchyme within the glands, or even epithelial–mesenchymal interaction by coculture of the two cell types.

ANIMAL MODELS OF HEREDITARY ANDROGEN RESISTANCE

Hereditary androgen resistance (testicular feminization; Tfm) has been described in several mammals, including the chimpanzee (Eil *et al.*, 1980), but has been studied intensively only in the rat and mouse (Bullock, 1982). In these species the trait is transmitted as if it were X-linked, and in the mouse this has been proven by linkage analysis with *Tabby* and *Blotchy*, marker genes known to occupy the distal half of the mouse X chromosome (Lyon and Hawkes, 1970). Indeed, severe reduction of AR activity has been demonstrated in numerous tissues of affected animals in both species, indicating that the responsible mutations have occurred at the X-linked locus that encodes the A–R protein in the mouse and, due to evolutionary conservation of the X chromosome, very probably in the rat as well. Further support for homology of these loci comes from the lack of complementation in hybrid cells formed from SV40-transformed Tfm mouse kidney cells and labial skin fibroblasts from a human subject with R^- CAR (Migeon *et al.*, 1981).

When examined by various criteria, including isoelectric focusing and DNA–cellulose chromatography, the residual AR activity in Tfm rats is indistinguishable from normal (Naess *et al.*, 1976; Wieland and Fox, 1981). In contrast, that in mice carrying the original Tfm mutation (Lyon and Hawkes, 1970) may have a lower affinity for DHT (Attardi and Ohno, 1974) and, according to some investigators, behaves like a normal minor species of the A–R complex on DNA–cellulose chromatography (Wieland and Fox, 1979; Wieland *et al.*, 1978) and isoelectric focusing (Fox and Wieland, 1981). In preliminary experiments, the residual AR activity associated with an independent mouse Tfm mutation is lower than that

of the original one, and elutes from DNA–cellulose columns at salt concentrations corresponding to the major wild-type species of the AR (Fox *et al.*, 1983).

In addition to the anatomic consequences of androgen resistance that stem from interference with androgen-dependent morphogenesis of the male reproductive tract, other expressions of the androgen-resistant phenotype in these animal models have been studied profitably. For instance, T. R. Brown *et al.* (1978) exploited the mouse Tfm mutation to show that some androgenic effects on the liver are mediated by the AR (e.g., ethylmorphine *N*-demethylase activity), while others are independent of it (liver weight and microsomal protein content). Indeed, this evidence indicated that mammalian liver had a classical AR despite the failure of initial efforts to demonstrate it.

The Tfm rat has partial rather than complete resistance to androgen, which is reflected in reduced, but not absent, responses of preputial, adrenal, and pituitary gland weights and pituitary gonadotropin secretion to elevated doses of exogenous androgen (Bullock, 1982). Of greater interest is the fact that Tfm rats display feminine patterns of drug and steroid metabolism in their livers (Goldman *et al.*, 1973; Goldman and Klingele, 1974) and a mixture of masculine and feminine sexual and nonsexual behaviors. The feminine pattern of liver metabolism appears to result from interference with an androgen-initiated (imprinting) effect on the liver that is mediated by the anterior pituitary (Gustafsson *et al.*, 1980), but there does not seem to be a clear counterpart for this in man. On the other hand, certain aspects of the division of labor between androgens and estrogens in accomplishing masculinization and/or defeminization of the male rodent brain appear to have been retained during primate evolution, and thus are relevant for man. For example, the presence of a short, blind vagina in some Tfm rats permitted the observation that many of these animals are in constant estrus, indicating a tonic (masculine) rather than a cyclic (feminine) pattern of gonadotropin secretion (B. H. Shapiro, 1985). This observation and the fact that Tfm rats have levels of FSH equal to normal males but about twice as high as normal females were two of the first indications that masculinization of the hypothalamic–pituitary axis could occur in AR-deficient animals, thereby implicating estrogen mediation (by local aromatase activity) of a process initially thought to be carried out strictly by androgens. Conversely, the early observation that Tfm rats have a feminine pattern of saccharin preference and four- to fivefold (Shapiro and Goldman, 1973) higher levels of LH

than normal males or females indicated that some sexually dimorphic patterns under the control of the central nervous system do arise by the AR-mediated action of androgens *per se*. This division of labor between androgens and estrogens in the attainment of the overall masculinization of the central nervous system has since been verified in other ways, as discussed above.

The availability of the mouse Tfm mutation has spawned a number of interesting experiments designed to elucidate various aspects of androgen action. For instance, Lyon *et al.* (1975) made male mice chimeric for the Tfm/Y and +/Y genotype by the technique of blastomere aggregation. They found, by fertility analysis, that normal spermatozoa could be produced from the Tfm germ cells of these chimeras. This indicated that the role of androgen in normal spermatogenesis is not carried out within the germ cells themselves, but perhaps through neighboring Sertoli or other cells of the seminiferous tubules, which, by virtue of their +/Y genotype, can sustain the formation of spermatozoa. A similar partnership, involving cellular metabolic cooperation, would apply to maturation of Tfm-bearing spermatozoa that occurs in the epididymes of fertile chimeric mice. Indeed, Sxr, the sex-reversal mutation, can be used to produce XX male mice that are heterozygous for the Tfm mutation (Drews and Alonso-Lozano, 1974). The epididymes of such mice are mosaic by virtue of random X-chromosome inactivation, and the two cell types can be distinguished histologically as flat Tfm cells and columnar wild-type cells. After stimulation by T, the ^{3}H-thymidine labeling index is similar in both cell types, indicating that metabolic cooperation between the + and Tfm cell types is taking place (Drews and Drews, 1975).

Sex-reversed XX, Tfm/+ mice have also been used to study the nucleocytoplasmic behavior of multinucleated muscle fibers in the striated musculature of the urethra. Thiedemann and Drews (1980) found, as expected, that the diameter of such fibers was intermediate between those of normal males and females. However, the nuclei within such intermediate-size fibers were clearly bimodal (mosaic) in size, indicating that nuclei derived from Tfm myoblasts were somehow determined to be unresponsive to the AR activity contributed to multinucleated fibers by the wild-type myoblasts.

Lyon and Glenister (1980) used males chimeric for the mouse Tfm gene to breed female mice homozygous for the Tfm gene. Such animals were fertile, indicating that AR activity is not necessary for reproduction in female mice, but they underwent reproductive aging prematurely com-

pared to heterozygous Tfm/+ females, as judged by long-term reproductive performance and ovarian histology. The same conclusion was reached from a separate study that demonstrated the fertility of XO mice hemizygous for the Tfm mutation (Lyon and Glenister, 1974).

The submaxillary (submandibular) salivary gland (Dunn and Wilson, 1976) and kidney (Kontula *et al.*, 1985) of mice are sexually dimorphic and androgen-dependent. The inability of androgen to induce various products of these organs in Tfm mice has yielded useful indices of androgen action (Barthe *et al.*, 1974; Lyon *et al.*, 1973; Toole *et al.*, 1979; Hastie *et al.*, 1979). In particular, β-glucuronidase activity in proximal convoluted tubules of mouse kidney has been exploited profitably to study various aspects of its androgenic regulation, as discussed previously.

We have previously noted that skeletal muscle of Tfm rats (Max, 1981) and mice (Snochowski *et al.*, 1980) has a normal concentration of AR activity. Since other tissues are severely deficient in it, tissue-specific expression of the gene mutation is apparent. It is of great interest that Tfm rats have a defect in testicular T synthesis, apparently at the level of 17-ketoreductase enzyme that is responsible for the conversion of androstenedione to T (Schneider and Bardin, 1970). This is highly reminiscent of the fact that, on the whole, plasma androstenedione levels are relatively higher than those of T in 24-hr samples from human beings with androgen resistance (Boyar *et al.*, 1978), and it is compatible with the fact that Leydig cells, at least in the rat testis (Sar *et al.*, 1975), have AR activity. Furthermore, Tfm rats develop testicular tumors with a cumulative incidence of 25% (Vanha-Perttula *et al.*, 1970), but the relation of these neoplasms to the ones that develop in human beings with androgen resistance has not been defined (Welch and Robboy, 1981).

GENETIC VARIATION IN RECEPTOR-MEDIATED SENSITIVITY TO OTHER CLASSICAL STEROID HORMONES

Glucocorticoid Resistance

Hereditary Cortisol Resistance in Man

This condition has been documented in two families. In one (Chrousos *et al.*, 1983), the propositus had elevated plasma levels of cortisol,

ACTH, deoxycorticosterone, and corticosterone, and was hypertensive, hypokalemic, and alkalotic (Vingerhoeds *et al.*, 1976), but had no signs or symptoms of Cushing syndrome. His son and several other relatives distributed through three generations had biochemical evidence of hypercortisolism, but were clinically normal. High-dose dexamethasone therapy corrected the hypertension and hypokalemic alkalosis in the propositus without complications of glucocorticoid excess. The GR was shown to have a reduced affinity for dexamethasone in circulating mononuclear leukocytes and cultured SF and in cytosolic preparations from the cells (Chrousos *et al.*, 1982*c*). In the latter, a reduced receptor concentration was found as well. Reduced affinity was not observed in two asymptomatically affected relatives, but, as in the propositus, their G–R complexes were unstable during thermal induction of the high-affinity state, and Epstein–Barr-virus transformation of their peripheral lymphocytes induced GR activity to a lesser extent than normal (Chrousos *et al.*, 1984*a*).

In the family described by Iida *et al.* (1985), a mother and her son had hypercortisolemia, partial resistance of the adrenal to dexamethasone suppression, and mild hypertension, but neither had clinical expression of Cushing syndrome, hypokalemia, or an increased plasma level of ACTH. Both subjects had about half-normal GR binding capacity in their peripheral blood mononuclear cells and cultured SF (Gomi *et al.*, 1986), but its apparent equilibrium dissociation constant (K_D) was normal. The absence of hypertension or hypokalemia in this family compared to the one of Chrousos *et al.* (1983) is explained by their mild elevation of corticosterone and deoxycorticosterone. Kontula *et al.* (1980) reported one patient with hypercortisolemia and decreased GR activity in the peripheral lymphocytes.

The genetic basis for this condition remains to be defined, but the disparate biochemical phenotypes of the mutant GR activity in the two families indicates heterogeneity, and male-to-male transmission and susceptibility of both sexes points to autosomal inheritance. The variable expressivity of the biochemical and clinical phenotypes in the family of Chrousos *et al.* (1982*c*; 1984*a*) could not be explained by cell-free mixing experiments designed to identify a non-receptor modulator of receptor activity. Indeed, the normal father in the family of Iida *et al.* (1985) incriminates a variably-expressed autosomal dominant mutation.

Glucocorticoid Resistance in Lymphoma and Other Cell Lines

Glucocorticoids are well-known agents in the management of human T-cell lymphomas and leukemias. They cause lysis of the tumor cells just as they do of normal T lymphocytes during early stages of their differentiation. Recently, Compton and Cidlowski (1986) have recognized a family of proteins (12,500–19,000 daltons in size)—putative nucleases—whose concentration increases concordantly with Dex-stimulated degradation of rat thymocyte DNA at internucleosomal sites.

A large variety of spontaneous and mutagenic dexamethasone-resistant (Dex^r) derivatives of glucocorticoid-sensitive parental mouse and human lymphoid cell lines has been isolated in order to study the mechanism of glucocorticoid-induced lysis. Among the minority without some incriminating defect in G–R function, decreased permeability of the cell membrane for steroid (Johnson *et al.*, 1984) and methylation of putative steroid-response loci (Gasson *et al.*, 1983) have been identified. However, the great majority of such mutant lines have been shown to have quantitative (R^-) or qualitative (R^+) aberrations of their GR apparatus. Somatic cell hybridization has never revealed complementation among any of the latter mutants (Harmon *et al.*, 1985). This is not surprising, since the monomeric GR protein has discrete domains for binding glucocorticoid and DNA that are encoded by a single locus.

Among the qualitative (R^+) mutants, deficient nuclear transfer (translocation; nt^-) and increased nuclear transfer (nt^i) of G–R complexes have been particularly interesting. In the case of the 143 nt^i variant of the murine S49 T-cell lymphoma line, Gruol *et al.* (1984) have recently found that the greater capacity to organize G–R complexes into compartments within the nuclei of intact cells is, paradoxically, accompanied by a greater sensitivity to release of these complexes by DNase I digestion of nuclei prepared by NP-40 treatment. Indeed, high-salt treatment of these nuclei also extracted a greater fraction of the nt^i complexes than it did of complexes within nuclei of wild-type cells. This implies that a greater than normal fraction of nt^i complexes are bound to nuclear components in an unproductive manner. Conversely, the fraction of G–R complexes that are bound to wild-type nuclei in a manner that resists DNase I digestion and high-salt extraction can be inferred to be physiologically important.

An important glucocorticoid-resistant mutant (4R4) of the CEM-C7 human lymphoblastic leukemia line has been characterized by Schmidt *et al.* (1980). In contrast to the typical R^+ nt^- variant of the S49 mouse

lymphoma line (in which nuclear translocation of G–R complexes is 40% of normal), the 4R4 variant accomplishes no nuclear transfer. This is due to lability of the 4R4 G–R complexes under conditions that promote conversion of normal untransformed complexes to their transformed state, the state characteristic of normal G–R complexes in nuclear residence. When sodium molybdate was added to 4R4 complexes, they remained stably in the untransformed state. This suggested either that it is the transformed state of the 4R4 G–R complexes that is labile or that molybdate is somehow able to stabilize the otherwise unstable untransformed type of 4R4 G–R complex. In all respects, this GR mutant is similar to the human AR mutant described by Kovacs *et al.* (1984), which is as unstable with DHT as ligand as normal cells are with T as ligand, under conditions that normally promote conversion of wild-type A–R complexes from their untransformed to their transformed state. Activation-labile, molybdate-resistant mutants of the CEM-C7 have been isolated as well. They do not complement molybdate-sensitive mutants that are activation-labile, indicating that defects at the receptor locus are primary in both types of "activation-labile" mutants (Harmon *et al.*, 1984).

Recently, Gasson and Bourgeois (1983) have conducted important studies on the SAK cell line, which was derived from a spontaneous thymic lymphoma in an AKR mouse and is resistant to glucocorticoid lysis, despite the presence of an intact GR apparatus. The latter was ascertained by hormone-binding studies and by the ability of glucocorticoid to promote the accumulation of murine leukemia virus and metallothionein mRNAs within the lysis-resistant cells. When SAK cells were mated with a receptor-defective variant of the W7 murine lymphoma line, the resulting hybrids were sensitive to glucocorticoid lysis, indicating complementation. Conversely, when SAK cells were fused with parental glucocorticoid-sensitive W7 cells, the hybrids were sensitive to glucocorticoid lysis, indicating that lysis resistance of SAK cells is recessive. Backselection of the hybrids yielded resistant derivatives that contained an intact GR apparatus, but had lost two chromosomes, demonstrating loss of gene(s) necessary for the lysis function.

It will be interesting to see what the relation is between the genetic determinants of the lysis function in SAK cells and those responsible for the loss of this function in the Dex^r clone (CEM-C1) recently isolated from a Dex-sensitive human lymphoblastoid cell line by Yuh and Thompson (1986). In any event, treatment of SAK cells with 5-azacytidine (an inhibitor of DNA methylation) yielded glucocorticoid-sensitive clones at

high (nonmutational) frequency. Thus, the gene(s) responsible for the lysis function appears to be intact, but in a state of methylated repression as a result of a process akin to differentiation. Clearly these observations suggest that normal immature T cells lose their sensitivity to glucocorticoid lysis merely by undergoing a process akin to methylation-dependent maturation. Such a behavior would have import for strategies directed to the treatment of human T-cell malignancies.

Equally informative observations have been made by Gehring and Hotz (1983) not only on the 143r nt^i clone of S49 cells, but also on one other nt^i clone and on a pair of nt^- clonal variants. First, they showed that wild-type G–R complexes were eluted from DNA–cellulose with about 180 mM, while nt^- and nt^i complexes required lower (~80 mM) or higher (~210 mM) KCl for elution, respectively. Second, they photoactivated receptor bound to the glucocorticoid triamcinolone and used SDS–PAGE of these covalently labeled G–R complexes after subjecting them to limited proteolysis with chymotrypsin and trypsin, in order to relate the molecular size of the complexes with their affinity for DNA. They found that wild-type complexes had a molecular weight around 94,000 and that chymotrypsin decreased it to a molecular weight of 40,000 with a concomitant increase in affinity for DNA. Both nt^- clones had a native and postchymotryptic molecular weight equal to that of the wild type, but one of them acquired a greater (but still less than normal) affinity for DNA. In contrast, both nt^i clones had a native molecular weight around 40,000 and chymotrypsin changed neither their molecular size nor their affinity for DNA. In contrast to chymotrypsin, trypsin (and a separate endoproteinase with extreme specificity for cleaving polypeptides at lysine residues) abolished the DNA affinity of wild-type complexes and of those in both types of mutant clones, indicating either that trypsin caused dissociation of the steroid- and DNA-binding domains of their respective receptors or that it destroyed the activity of the latter domain. Judging from the fragments produced by trypsin (about 39,000, 29,000, and 27,000 daltons), the former is more likely than the latter. In fact, Eisen *et al.* (1985) showed directly that a nonsteroid-binding, but DNA-binding domain lies in a M_r ~16,000 fragment that is tryptically separated from various fragments of M_r ~30,000 which retain the steroid-binding site, but not the one for DNA binding.

From these results in aggregate, Gehring and Hotz (1983) inferred that GR with molecular weights around 40,000 have abnormally high affinity for DNA, because the 45,000-dalton fragment (which is missing from

native nt^i receptors or from chymotryptic fragments of wild-type receptors) has the function of modulating the affinity, and more importantly, the productivity with which an intact G–R complex binds to DNA. This inference is strongly in accord with a prediction of Wrange and Gustafsson (1978) and data published by Carlstedt-Duke *et al.* (1982) on the basis of comparable proteolytic studies, and with two independent observations: (1) that antibodies raised against wild-type rat liver GR did not react with the nt^i variant of glucocorticoid-resistant P1798 lymphoma cells (Stevens *et al.*, 1981); and (2) that similar antibodies did react with a chymotryptic fragment of the same size generated from rat liver GR, which is responsible for neither steroid- nor DNA-binding.

Monoclonal antibodies against the modulating domain have also been useful in analysing the process of transformation. One such antibody reacts better with wild-type and nt^- GR after heat-induced transformation of G–R complexes, while another does not (Westphal *et al.*, 1984). Thus, the conformational change attending transformation exposes an epitope within the modulating domain that is recognized by the first antibody but not by the second. Furthermore, this change is not essential for transformation itself, since nt^i receptors can be transformed even though they lack the modulating domain.

There are several possible explanations for the origin of the native ~40,000-dalton GR in glucocorticoid-resistant lymphoma cells of the nt^i type: (1) independently synthesized (or intracellularly sequestered) binding and modulating domains fail to form a single functional receptor; (2) a mutation alters gene transcription or posttranscriptional modification to yield a truncated receptor or a receptor precursor with increased protease sensitivity; and (3) a mutation engenders increased endogenous proteolytic activity. Mixing experiments have essentially ruled out the last explanation (Stevens *et al.*, 1983), and an early observation with a cDNA probe for the GR revealed that the 40,000-dalton nt^i receptor corelates with an mRNA transcript that is 1 kb shorter than normal (Miesfeld *et al.*, 1984). To our knowledge, this result reflected the first application of recombinant DNA technology for characterization of SR gene mutations.

Further use of this technology has corroborated and extended our understanding of R^-, nt^- and nt^i mutations at the GR locus. Thus, the R^- forms lack the N-terminal steroid-binding site of the GR protein or have a critical alteration in it (Hollenberg *et al.*, 1985), the nt^- types have changes in the "middle" domain of the protein that contains the DNA-binding site (Ringold, 1986, in Bishop, 1986), and most importantly, the

nt^i variants lack the C-terminal portion, the hyperimmunogenic domain that modulates DNA-binding affinity and the transcription-regulating activity of that binding (Yamamoto, 1985).

Hawkins *et al.* (1982) studied the GR system in solid tumors derived from Dex-sensitive and Dex-resistant cells cloned from a melanoma in the Syrian hamster. They found higher levels of cytosolic GR activity in the Dex-resistant tumors than their Dex-sensitive relatives, but the transformed complexes of the Dex-resistant tumors appeared to bind more tightly to DNA than those of their counterparts. This suggests that the GR in the Dex-resistant melanoma clone is defective in its N-terminal modulatory domain, like the nt^i lymphoma mutants discussed above.

The glucocorticoid-resistant variants of the W7 mouse thymoma cell line studied by Danielson and Stallcup (1984) have been particularly interesting. These variants have 30–60% of the GR activity of control cells, a level that should not in itself preclude glucocorticoid lysis. These variants resist lysis because exposure to glucocorticoid causes them to down-regulate their GR activities. Thus, it is a combination of basally reduced GR activities plus the down-regulation response that conspires to make these variants glucocorticoid-resistant. Such combinations are likely to be generated in patients with lymphoproliferative disease who are treated with glucocorticoids. Similar medically significant considerations apply to the types of variants isolated by Johnson *et al.* (1984), which have a defect in the membrane permeability of a particular glucocorticoid (dexamethasone, but not triamicinolone) as well as other, nonsteroidal compounds, such as puromycin, colchicine, and vincristine.

Thompson *et al.* (1977*b*) studied variants of the HTC rat hepatoma cell line that expressed their glucocorticoid resistance by low tyrosine aminotransferase inducibility. By all the criteria they used, these resistant cells had a normal GR apparatus, suggesting that they were defective in a postreceptor step necessary for glucocorticoid responsiveness.

Garroway *et al.* (1976) analyzed two cell lines that secrete ACTH. One was R^+ and glucocorticoid-responsive (its ACTH production was inhibited) and the other was R^- and glucocorticoid-resistant. The nuclei of each line had equivalent ability to bind normal G–R complexes. Such lines will be useful for studying, at the chromatin level, how negative regulation of gene expression by glucocorticoids differs from positive regulatory systems.

Venetianer and Bösze (1983) compared the expression of various liver-specific functions among different dexamethasone-resistant variants

derived from the well-differentiated dexamethasone-sensitive Reuber H35 rat hepatoma cell line. They found that the dexamethasone-resistant cells lost most liver-specific functions during long-term culture, whereas only selected functions were lost in the sensitive cells. Similar observations were made by Chou (1983) on a glucocorticoid-responsive rat adult hepatocyte cell line that is temperature-sensitive for growth and differentiation. Such cell lines will be useful for probing the differential susceptibility of various genes to regulation by G–R complexes.

Dexamethasone causes growth inhibition, rather than lysis, of L cells, and R5020, a synthetic progestin, has the same effect despite the fact that it has little affinity for the GR. Studies by Gal and Venetianer (1984) on R^+ Dex^r variants in mouse L cells have also revealed complementation among hybrids formed from certain pairs of variants. It is interesting that some Dex^r clones were also found to be resistant to R5020, and this was true in the presence of normal PR activity. This coordination is somewhat reminiscent of the generalized steroid insensitivity in New World primates (see below), and it suggests that in these studies the complementation involves two or more nonreceptor factors that modulate the behavior or effects of distinct S–R complexes.

Glucocorticoid–Receptor Activity and Susceptibility to Experimental Glucocorticoid-Induced Cleft Palate

It has been known for many years that pharmacological doses of cortisone (F. C. Fraser and Fainstat, 1951) and other glucocorticoids (Pinsky and DiGeorge, 1965) produce cleft palate in the offspring of mice treated during gestational periods that correspond to the stage of palate closure. Presumably, this results from interference with cell proliferation and extracellular matrix formation in the palatine shelves that first appear in a vertical position on both sides of the tongue, before they rotate to a horizontal position above the tongue, finally to meet and fuse in the midline (Shah, 1984). Physiological concentrations of glucocorticoids, together with other hormones, are important regulators of mesenchymal proliferation and function during normal palate formation *in vivo* (Salomon and Pratt, 1979), and similar agonist effects have been demonstrated in palatal mesenchyme cells in culture (Sasaki and Kurisu, 1983). The mechanism by which agonism is converted into antagonism as a function of glucocorticoid concentration is not understood (Pratt *et al.*, 1984). A related issue is that the teratogenic potency of a series of glucocorticoids

does not accord with their relative antiinflammatory potency as conventionally defined in clinical terms (Pinsky and DiGeorge, 1965; Walker, 1965).

There are large differences in susceptibility to glucocorticoid-induced cleft palate among various inbred, congenic and recombinant inbred strains in mice. The genetic basis of these differences is not well defined, but several major loci have been identified by classical forms of genetic analysis (Vekemans and Biddle, 1984), and their identity varies among different sets of strains that are studied. These analyses are made both more complicated and more interesting because of maternal interaction effects that are expressed by differences among the offspring of reciprocal F1 crosses.

The morphogenetic and biochemical substrates for such multigenic variability and susceptibility to glucocorticoid-induced cleft palate must be manifold. In the former category the stage of gestation during which palate closure is initiated and the speed with which the process of closure occurs have been documented as important variables (Walker and Fraser, 1957). At the biochemical level, variation in GR activity has received much attention as one factor that might cause strain differences in susceptibility to glucocorticoid-induced cleft palate. Interestingly, there are data, albeit contradictory (Hackney, 1980), suggesting that mesenchyme cells from the oral–facial region and the palates themselves of strains sensitive to glucocorticoid-induced cleft palate have more GR activity than comparable cells from resistant strains (Pratt *et al.*, 1984). The former are, appropriately, more sensitive than the latter to the inhibitory effects of pharmacological doses of glucocorticoid (Salomon and Pratt, 1978). Indeed, synthetic glucocorticoids such as triamcinolone and dexamethasone have somewhat higher affinities for the GR than natural glucocorticoids, and this may partly explain their relatively greater teratogenic potency (Pinsky and DiGeorge, 1965; Walker, 1965).

There is evidence suggesting that allelic variation within the D and K regions of the H-2 (major histocompatibility) locus on chromosome 17 in the mouse contributes to sensitivity to glucocorticoid-induced cleft palate (Pratt *et al.*, 1984). Indeed, it has been suggested that this H-2-dependent variation is mediated by differences in levels of G–R activity (Katsumata *et al.*, 1981). The evidence, however, is conflicting at both levels (Butley *et al.*, 1978) and resists explanation by a simple correlation with particular H-2-linked alleles. It is noteworthy, nevertheless, that the locus encoding the GR in the mouse has been assigned to mouse chro-

mosome 18 (Francke and Gehring, 1980), so that whatever H-2-related genetic factors may be involved are most likely to be of a nonreceptor nature.

The relation and significance of the foregoing observations to the occurrence of "sporadic" cleft palate in human infants are not known. In man, cleft palate behaves as a multifactorial character with appropriately increased risks of recurrence among first- and second-degree relatives of affected individuals (F. C. Fraser, 1980). A teratogenic association with natural glucocorticoid excess has been suggested (Strean and Peer, 1956), but questioned (F. C. Fraser and Warburton, 1964). It is of some interest, nevertheless, that the frequency of spontaneous cleft palate among genetically different strains of mice studied by Butley *et al.* (1978) correlated with their relative levels of hepatic GR activity, but not with their respective H-2 haplotypes.

Yoneda and Pratt (1982) found that vitamin B_6 given to pregnant mice on a B_6-containing diet reduced the frequency of glucocorticoid-induced cleft palate in their offspring, and that vitamin B_6-deficient diets had the opposite effect. Vitamin B_6 also reduced the GR activity of cultured palatal mesenchyme cells, suggesting that this is how it protected against the glucocorticoid induction of cleft palate.

To our knowledge, the possibility that vitamin B_6 may be a protective agent against spontaneous cleft palate in man has not been considered.

A preliminary report has indicated that there is reduced GR activity in dermal fibroblasts of human beings with facial clefting, that is, cleft lip with or without cleft palate (Yoneda *et al.*, 1981). No confirmatory data have appeared. Nevertheless, one is provoked to consider the possibility of some systemic disturbance in the regulation of GR activity that might be expressed by an interference with the action of glucocorticoids on differentiation of the secondary palate in man (Yoneda and Pratt, 1981).

Glucocorticoid Resistance in New World Monkeys as One Expression of Generalized Steroid Insensitivity

In an interesting series of studies Chrousos *et al.* (1982*a*, 1984*a*) have found that New World monkeys have lower sensitivity to glucocorticoid than do Old World primates and prosimians. This is reflected in markedly elevated urinary free-cortisol excretion that is not attended by pathophysiologic effects, is resistant to dexamethasone suppression, and is presumably caused by a GR activity (as sampled in mononuclear leukocytes

and cultured skin fibroblasts) that has a normal concentration but a reduced affinity for dexamethasone.

In evolutionary terms the responsible mutation must have occurred after divergence of the Old and New World primates (about 60×10^6 years ago), and before speciation among the latter (about 15×10^6 years ago). A very provocative feature of this form of glucocorticoid resistance is that it is only one component of generalized insensitivity to various steroids as well as the vitamin D (sterol) hormone (Shinki *et al.*, 1983). In regard to vitamin D, Takahashi *et al.* (1985) reported that the common marmoset, a New World monkey, has much higher circulating levels of 1α-25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) than the rhesus monkey or man, without being hypercalcemic. The marmoset has one-sixth as much vitamin D–receptor activity in its intestinal cytosol as the rhesus without a difference in affinity of their receptors for the vitamin. In addition, the marmoset's transformed vitamin D–receptor complexes elute from DEAE–cellulose columns like those of the rhesus (with about 0.1 M KCl), yet their DNA-binding ability is much less than those of the rhesus. The latter defect is similar to one found in a human family with vitamin D resistance (Hirst *et al.*, 1985), as discussed on p. 426, and the disparate behavior of the complexes on DEAE and DNA alludes to the nature of the molecular aberration in the marmoset's vitamin D receptor protein. Among the steroid insensitivities, those directed toward estrogen and progesterone are associated with reduced concentration, but not affinity, of their respective uterine receptors, and circulating levels of plasma estradiol and progesterone are increased to compensate for the respective target-organ resistances (Chrousos *et al.*, 1984*a*). Elevated plasma concentrations of androgen (M. I. Wilson *et al.*, 1978; Pugeat *et al.*, 1984*b*) and aldosterone (Chrousos *et al.*, 1984*a*) in the same animals predicted that aberrations in their respective receptors will be found as well. Indeed, a twofold lower affinity of the AR for MT has recently been reported in New World compared to Old World primates (Siiteri, 1985), and aldosterone-receptor concentrations are two- to threefold lower in the New World squirrel monkey than in the Old World cynomolgus macaque (Chrousos *et al.*, 1984*a*). In addition, more cortisol is needed to displace aldosterone from the mineralocorticoid receptor of the squirrel monkey than from the cynomolgus macaque.

It is difficult to imagine what property of all steroid/sterol response systems may be at the root of this phenomenon of generalized steroid/sterol insensitivity, particularly in view of the fact that the New World

receptors do not seem to share a communal aberration. Although it has become clear that the genetically discrete steroid-binding moieties of steroid receptors share intimate structural relations that derive from an evolutionary ancient precursor, it does not seem reasonable, from the viewpoint of molecular evolution, that these relations underlie the generalized steroid/sterol insensitivity of New World primates. It is more likely that New World primates inherit a variant "factor" that alters the activity or behavior, or both, of the receptors for all steroid hormones and the vitamin D sterol. One candidate for such a factor, albeit unlikely, is the hsp 90 phosphoprotein that appears to be a common subunit of all socalled untransformed S–R complexes (Catelli *et al.*, 1986; Sanchez *et al.*, 1985; Housley *et al.*, 1985).

Another aspect of this phenomenon is that a variety of changes have occurred apparently as secondary expressions of adaptive evolution. Thus, the cortisol-binding globulin in New World primates has a lower affinity and is present in lower concentrations than in Old World primates (Pugeat *et al.*, 1984*a*; Klosterman *et al.*, 1986). This would permit a greater percentage of total blood corticosterone to be "exposed" for action on target cells, and contribute to the compensated nature of the corticosterone-resistance state. Similarly, although the level of SS-BG in the New World male squirrel monkey is 10- and 3-fold higher than in humans and rhesus monkeys, respectively, the former has a lower affinity for T than either of the latter. The vectorial result of these changes is that the androgen target tissues of the squirrel monkey are exposed to a relatively high level of unbound T (Murai *et al.*, 1986). Interestingly, Siiteri (1985) has found that 5α-reductase activity is very low in the New World squirrel monkey compared to the Old World rhesus monkey, but it is difficult to understand how this can be adaptive. On the contrary, it suggests some still unknown link between the AR protein and the 5α-reductase enzyme protein.

Equally provocative observations have been made with regard to the steroids that interact with the mineralocorticoid receptor of the New World squirrel monkey to regulate overall sodium-retaining activity. Thus, as noted above, its mineralocorticoid receptor has relatively low binding affinity for cortisol, which is a mineralocorticoid agonist, and its plasma concentrations of aldosterone do not rise during the reproductive cycle or pregnancy, times when progesterone levels are 10- to 20-fold higher than in reproductively inactive females. This suggests that pro-

gesterone is a poorer mineralocorticoid antagonist in the squirrel monkey than in the cynomolgus macaque (Chrousos *et al.*, 1984*a*).

Mineralocorticoid Resistance: Pseudohypoaldosteronism

Pseudohypoaldosteronism is a serious sodium-wasting, potassium-conserving disorder of infancy that may be episodic clinically, and that typically improves with age (Cheek and Perry, 1958; Armanini *et al.*, 1985), despite persistence of the basic biochemical abnormality. In the majority, sodium loss occurs from all mineralocorticoid-responsive brush-border epithelia, including those of the kidney, colon, salivary and sweat glands, despite elevated plasma aldosterone concentration and renin activity (Savage *et al.*, 1982; Oberfield *et al.*, 1979). The disorder is familial with a high rate of parental consanguinity (Armanini *et al.*, 1985; Bosson *et al.*, 1985; Savage *et al.*, 1982), indicating autosomal recessive inheritance. An autosomal dominant form has also been proposed (Limal *et al.*, 1978), although variable expressivity among heterozygotes might explain the appearance of genetic heterogeneity (Kuhnle *et al.*, 1986). In one patient sodium-wasting was demonstrated in sweat and salivary glands but not in the kidneys, suggesting an oligofocal form of aldosterone resistance (Anand *et al.*, 1976); in another (Satayaviboon *et al.*, 1982) only the kidney was involved, indicating a monofocal form. In some families clinical improvement with age has been accompanied by normalization of plasma aldosterone concentration and renin activity (Savage *et al.*, 1982). Nonetheless, the peripheral mononuclear leukocytes of the one healthy affected adult tested so far had no specific aldosterone receptor-binding activity (Armanini *et al.*, 1985). If this result reflects the status of this activity in his kidneys, then an aldosterone-independent compensatory mechanism that improves with age must be invoked; a likely candidate is progressive maturation of sodium-retaining systems in the proximal renal tubules. Salt-wasting crises in infancy, such as those precipitated by relatively mild infection, are treatable with parenteral sodium supplementation. Adequate dietary sodium is sufficient to maintain normal health and development after infancy.

Stewart (1975) has related the specific binding of aldosterone to kidney with its physiological effects on the kidney in two strains of mice. In the CBA strain, aldosterone reduced sodium and increased potassium excretion in the urine. In the Peru strain, aldosterone binding was re-

duced, and while its effect on sodium was negligible, that on potassium was increased. This indicated that aldosterone's effect on potassium excretion is dissociable from that on sodium excretion. F1 hybrids behaved like their Peru parents. Interestingly, the progeny of F1 mice backcrossed to their recessive parents (CBA) responded to aldosterone in a qualitative pattern that correlated with the proportion of high-salt-extractable aldosterone–receptor complexes in their nuclei. These results imply that the quality of aldosterone action is dependent on the type and fate of aldosterone–receptor complexes formed in its target cells. They also illustrate the potential power of genetic analysis for elucidating physiological–molecular correlation in steroid action.

Estrogen Resistance in Human Breast Cancer

Antiestrogen therapy has become an effective treatment for breast cancer in women, yet 40% of patients with ER-positive tumors fail to respond to this form of treatment. To try to understand the basis for such treatment failure, a number of experimental models have been used. Of these the estrogen-responsive MCF-7 human breast cancer cell line is most notable. M. A. Miller *et al.* (1984) have studied two mutant clones of the MCF-7 line that are resistant to the antiestrogen hydroxytamoxifen (HT), but not to the natural estrogen 17β-estradiol. This observation is analogous, and may be genetically homologous, with the type of AR mutation discussed above that causes ligand-selective androgen resistance in man (Pinsky *et al.*, 1984, 1985*b*). It fortifies the belief that thorough investigation of spontaneous germ-line mutations that cause androgen resistance in man should help us to understand why the success of steroid manipulation of human breast cancer is so unpredictable. By all the physicochemical criteria used, the HT–R complexes produced by the two MCF-7 variant clones were equal to those of the parental cells. In addition, no differences could be discerned between the parental cells and the variant clones with regard to the class of HT-binding site that does not compete for estrogen. In aggregate, these findings indicate that very subtle differences in the quality of the HT-binding properties of the variant clones must be at the root of their resistance to the growth-inhibitory effects of HT.

In this context, it is noteworthy that Tate *et al.* (1984) have detected differences between E–R and HT–R complexes of MCF-7 cells and human breast tumors in their reaction to a polyclonal antibody against

the ER. These differences should help to define the type of ER mutation that could confer resistance to HT but not to 17β-estradiol.

An equally subtle difference has been incriminated in the estrogen-resistant form of mammary cancer that occurs spontaneously in C3H mice. 17β-Estradiol does not induce PR activity, and ovariectomy does not decrease the growth rate of this tumor. Baskevitch *et al.* (1983) found that E–R complexes formed by the estrogen-resistant tumor had an increased affinity for DNA compared to that of E–R complexes in uterus and in the type of mammary cancer induced in rats by dimethylbenz(*a*)anthracene. Such a defect is reminiscent of the nt^i type of glucocorticoid resistance that occurs in certain mouse lymphoma cell lines, as discussed above.

RESISTANCE TO VITAMIN D, A STEROL

Vitamin D_3 (cholecalciferol) is synthesized photochemically in the skin, and its hormonal, effector form results from successive hydroxylation steps: at carbon 25 in the liver to yield the prohormone 25-hydroxyvitamin D_3, and subsequently at carbon 1 by the renal 1α-hydroxylase enzyme (Fig. 30). Decreased responsiveness to vitamin D occurs in two forms. Vitamin D-dependent rickets is an autosomal recessive disorder due to deficiency of the renal hydroxylase (D. Fraser *et al.*, 1973). It is treatable with 1,25-dihydroxyvitamin D_3, and is homologous in genetic and physiological terms to the state of androgen resistance (DHT dependence) that results from testosterone 5α-reductase deficiency and that is the cause of one form of male pseudohermaphroditism.

A second form, vitamin D resistance, is also an autosomal recessive disorder. It was recognized first by Root and Harrison (1976), and has received much attention in the past 10 years. It is characterized by rickets, often by alopecia, and is accompanied by hypocalcemia, secondary hyperparathyroidism, and elevated circulating levels of 1,25-dihydroxyvitamin D_3. The basic defect is target-organ resistance to the vitamin D_3 hormone. It is demonstrable in the intestine (Rosen *et al.*, 1979), in bone, as reflected by normocalcemia despite elevated serum levels of parathormone, and in cultured SF, which fail to induce their 24-hydroxylase activity (Feldman *et al.*, 1982) or inhibit their proliferation (Clemens *et al.*, 1983) in response to incubation with 1,25-dihydroxyvitamin D_3. The

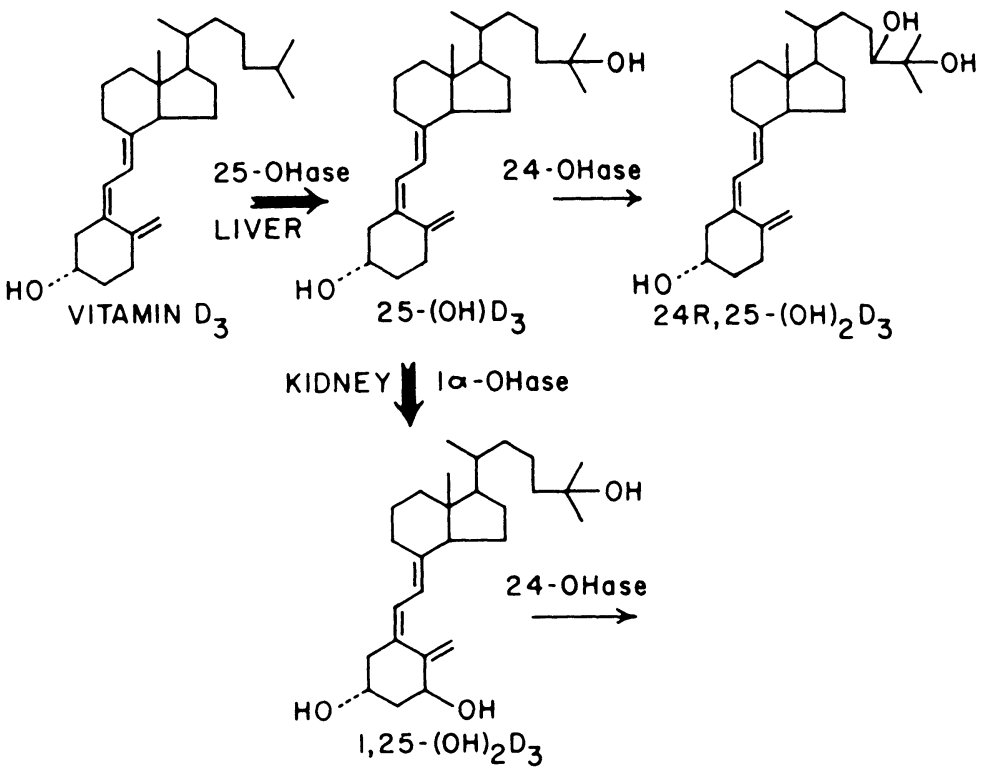


Fig. 30. Metabolism of vitamin D_3

mutant phenotype is also expressed by cells cultured from bone that have osteoblastic properties (Liberman *et al.*, 1983*a*).

The role of 1,25-dihydroxyvitamin D_3 in skin has not been defined completely. The fact that it cannot stimulate 7-dehydrocholesterol production in receptor-deficient keratinocytes (Clemens *et al.*, 1983) suggests that it regulates the synthesis of vitamin D_3. The relation of this defect as expressed in the keratinocytes to the development of total body alopecia is unclear, nor is it understood why alopecia is a variable component of the phenotype. Another unexplained clinical facet is that the severity of the rickets has been intermittent or has improved with age in some patients and, if so, in a manner not attributable to concurrent therapy with pharmacological doses of 1,25-dihydroxyvitamin D_3 or to any demonstrable changes in the severity of their basic target-cell defect (Hirst *et al.*, 1985). These observations, once elucidated, could suggest potential therapeutic strategies that might prevent the development of any clinical abnormality in affected children diagnosed preclinically.

The basic defect in target-organ resistance to vitamin D is etiologically heterogeneous in molecular terms (Liberman *et al.*, 1983*b*), as studied in cultured SF, keratinocytes, and osteoblasts. Some patients have negligible receptor activity (R^-) or a deficient amount of it (R^{def}), others have normal levels of receptor activity but cannot organize vitamin D–R complexes into compartments within their nuclei to a normal extent, and still others with near-normal nuclear compartmentation form mutant D–R complexes that have reduced affinity for DNA (Hirst *et al.*, 1985). By exclusion, some patients appear to have postreceptor defects.

Molecular analyses of the target-cell defects responsible for vitamin D resistance have been instructive in ways that apply to steroid resistance in general. First, several of the patients with negligible, deficient, or normal DR activity have been shown to have normal amounts of immunoassayable receptor protein concentration by a monoclonal antibody raised against the chick vitamin D receptor (Pike *et al.*, 1984). Second, the mutation that is expressed by near normal nuclear compartmentation of D–R complexes (Hirst *et al.*, 1985) has been informative in two ways: the fact that its complexes have clearly reduced affinity for DNA–cellulose (Fig. 31) and are too easily extractable from their nuclei by buffers containing 0.3 M KCl (Fig. 32) is another indication that simple measurement of nuclear compartmentation within intact target cells is not a reliable measure of the quality of sterol– or steroid–receptor complexes; and, judged by sucrose gradient centrifugation, the mutant complexes do not aggregate (to the 6 S form) in low-salt solutions to the extent that normal complexes do. This suggests that the DNA-binding domain of SR plays a role in a property that characterizes all S–R complexes; namely, that they sediment as larger molecules in low-salt than in high-salt environments.

DR activity has been identified in many normal cells and tissues (Colston *et al.*, 1980; Pike *et al.*, 1979; Provvedini *et al.*, 1983) and malignant cell lines (Colston *et al.*, 1981; Reitsma *et al.*, 1983; Eisman *et al.*, 1979). In some of these normal cells and tissues, the presence of DR activity implies a role for vitamin D that remains to be discovered. On the other hand, the ability of vitamin D to impair the proliferation of some malignant cell lines (Colston *et al.*, 1980) has immense clinical implications. In the case of the HL-60 cell line established from a patient with promyelocytic leukemia, near-physiological concentrations of 1,25-dihydroxyvitamin D_3 inhibit its proliferation and induces it to acquire features of monocyte maturation. Furthermore, these changes are preceded by a marked de-

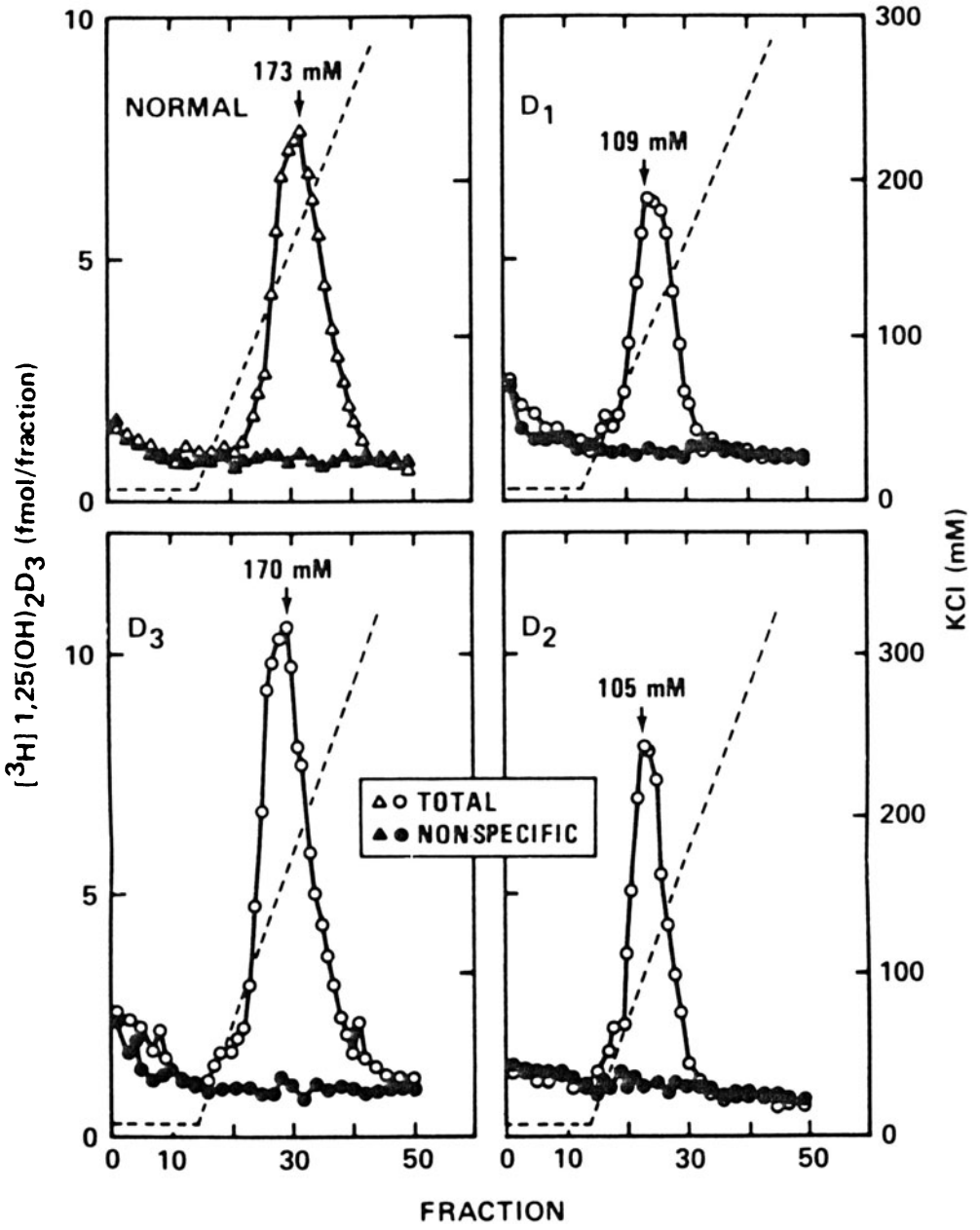


Fig. 31. Continuous KCl-gradient elution of DNA–cellulose columns loaded with 1,25-dihydroxyvitamin D–receptor complexes formed within SF of two siblings (D_1, D_2) with vitamin D resistance, one normal sibling (D_3), and one unrelated control (normal). [From Hirst *et al.* (1985) with permission.]

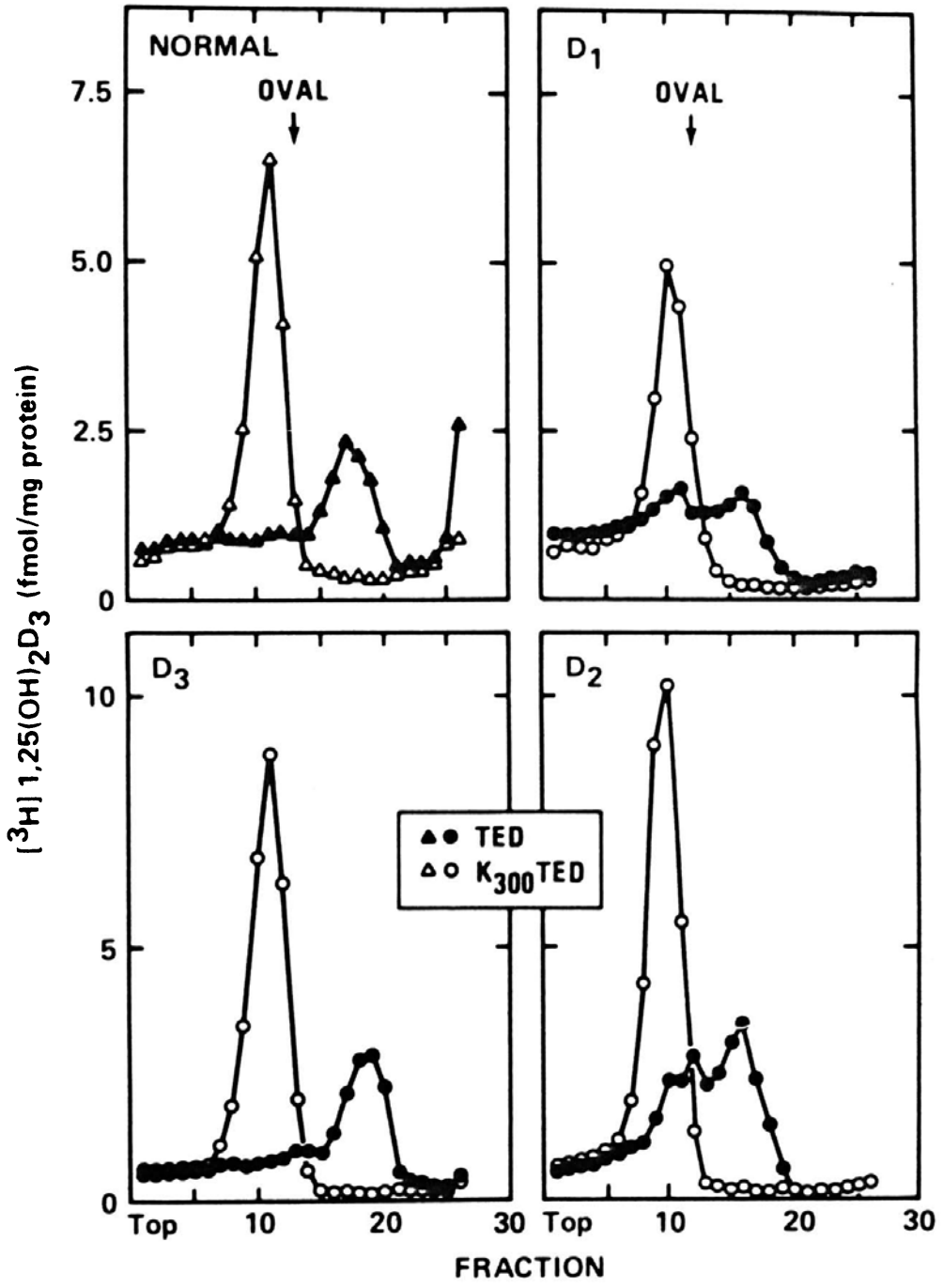


Fig. 32. Stepwise KCl-extraction of 1,25-dihydroxyvitamin D_3–receptor complexes from nuclei of normal or (●) vitamin D_3-resistant SF. [From Hirst *et al.* (1985) with permission.]

crease in the level of mRNA for the c-myc oncogene (Reitsma *et al.*, 1983). Similar observations have been made on a human promonocyte cell line (Amento *et al.*, 1984). One is entitled to wonder whether some form of vitamin D resistance that is expressed *in vivo* may be a factor in the pathogenesis of promyelocytic leukemia or of any malignancy that is related to the malignant cell lines whose growth is suppressed by vitamin D *in vitro*. The therapeutic strategies that can derived from this possibility are evident.

MISCELLANEOUS CONDITIONS THAT MAY INVOLVE ALTERED STATES OF STEROID SENSITIVITY

Altered Steroid Sensitivity in Skin Dysplasia

Unilateral Nevoid Telangiectatic Syndrome (UNTS)

Unilateral nevoid telangiectatic syndrome (UNTS) is a dermal vascular disorder that is restricted to the upper half of the body and upper extremities. It is usually acquired in relation to states of estrogen excess: pregnancy, male or female puberty, liver disease, and during the menstrual cycle or estrogen therapy. Occasionally, it occurs congenitally or prepubertally, or in adults for unknown reason (Duong and Raymond, 1983). Uhlin and McCarty (1983) found that ER and PR activity were increased manyfold in affected chest skin compared to the unaffected contralateral skin of a pregnant patient who first developed the lesions coincident with the onset of oral contraceptive usage. The concurrent elevation of ER and PR is provocative because induction of PR activity is a well-known effect of estrogen. A genetic component of UNTS has not been identified, but it is relevant that hereditary hemorrhagic telangiectasia, an autosomal dominant disorder, is ameliorated by high levels of circulating estrogen (Koch *et al.*, 1952; Heyde, 1954; Harrison, 1970; Flessa and Glueck, 1977) and is exacerbated by low levels of it (Koch *et al.*, 1952; Heyde, 1954). Hasselquist *et al.* (1980) have identified relatively low levels of ER activity in normal facial skin, and even lower levels in normal breast or thigh skin. This accords witih the typical propensity for UNTS to affect the upper half of the body.

Becker's Nevus

Becker's nevus is a unilateral, hyperpigmented, hairy, cutaneous hamartoma, often accompanied by acne, that usually appears on the upper portion of the trunk in adolescents. Its occurrence mainly in males suggests that androgens are involved pathogenetically. The AR activity level in the cytosol of a pectoral lesion from one patient (measured with MT as ligand) was 630 fmole/mg protein, while the unaffected contralateral pectoral skin had undetectable AR activity (Person and Longcope, 1984). The AR activity in the lesion was at least ten times greater than in normal genital skin (Evain *et al.*, 1977). Thus, receptor-mediated hypersensitivity to androgen may be at the root of Becker's nevus.

Keloids

Keloids are benign, predominantly fibrous, dermal tumors that arise in or near a site of wound healing in genetically predisposed individuals (Murray *et al.*, 1981). Fibroblasts cultured from keloids exhibit an abnormal set of responses to glucocorticoids. Compared to fibroblasts from skin or normal scars, they hyporespond with regard to proliferation and collagen production, but hyperrespond in respond to induction of "system A" amino acid transport (Gadson *et al.*, 1984). These differences in response to glucocorticoid occur despite the fact that GR in keloid and normal SF are indistinguishable by a variety of criteria. In both normal and keloid fibroblasts glucocorticoid induction of amino acid transport involves an increase in its V_{max} and appears to require RNA and protein synthesis. Furthermore, progesterone counteracts this effect of glucocorticoids strongly, indicating that it is mediated by the GR. Together, these observations suggest that keloid fibroblasts differ from normal ones at one or more postreceptor steps in the overall response system to glucocorticoid. Interestingly, insulin itself stimulates "system A" amino acid transport in SF, and it can replace serum in the medium required to sustain glucocorticoid induction of the system. It has been suggested that insulin is required for the expression of a protein, induced by glucocorticoid, that is necessary to promote "system A" amino acid transport (Gadson *et al.*, 1984). Perhaps keloid and nonkeloid SF differ in their ability to generate or respond to this protein.

Altered Progesterone Sensitivity

Pulmonary Lymphangiomyomatosis

Pulmonary lymphangiomyomatosis occurs almost always in women of reproductive age. It is characterized by proliferation of smooth muscle in perilymphatic regions of the lung and often in adjacent extrapulmonary areas (Corrin *et al.*, 1975). Very similar pulmonary lesions develop rarely, but principally in women, in the autosomal dominant disorder tuberous sclerosis (Liberman *et al.*, 1984), and as a metastatic expression of "benign" leiomyoma of the uterus (Banner *et al.*, 1981). McCarty *et al.* (1980) found substantial levels of PR activity in the pulmonary lesions of a patient with relatively early disease who did not have tuberous sclerosis or leiomyoma of the uterus. The patient improved with pharmacological doses of medroxyprogesterone acetate and relapsed with temporary discontinuation of therapy. Several anecdotal experiences (McCarty *et al.*, 1981*a*,*b*) have affirmed the original report, particularly in patients with early disease. Others have suggested that oophorectomy is valuable in management of pulmonary lymphangiomyomatosis, whether or not it is associated with leiomyoma of the uterus (Banner *et al.*, 1981; Kitzsteiner and Mallen, 1980).

Desmoid Tumors (Aggressive Fibromatosis)

Lanari (1983) has recently reported improvement in 8 of 11 patients with desmoid tumors treated with 100 mg of progesterone daily for 3 months followed by long-term maintenance therapy. The role of the receptor in the pathogenesis and therapy of this disorder is undefined. The therapy is based on the success with which progesterone was able to cause disappearance of fibrous tumors that appeared in the retroperitoneum, mesentery, and omentum of guinea pigs treated with estrogen for prolonged periods.

Infertility Due to Uncompensated Progesterone Resistance?

An infertile woman, initially considered to have corpus luteum deficiency, was shown to have normal circulating levels of estrogen and progesterone, but inadequate development of luteal phase endometrium

(Keller *et al.*, 1979). Exogenous progesterone did not correct the abnormality, and a cytosol preparation of her endometrium contained half the PR activity as two normal subjects.

Altered Steroid Sensitivity in Cystic Fibrosis?

Lobeck and McSherry (1963) noted decreased sensitivity of sweat electrolyte concentration to 9α-fluorohydrocortisone in patients with cystic fibrosis (CF). Their results were confirmed and expanded by Grand *et al.* (1967*a*,*b*). In response to aldosterone, sweat sodium concentration decreased to a lesser extent in patients with cystic fibrosis than in normal individuals, and the impaired responsiveness was more severe in adults than in children. Conversely, aldosterone provoked a greater increase in sweat potassium concentration among adults with cystic fibrosis compared to normal adults, but not in children with cystic fibrosis compared to normal children. The positive effects were blocked by spironolactone, indicating that they are mediated by the mineralocorticoid receptor. However, since the basic defect in the cystic fibrosis gene or its product is still unknown, the pathogenetic meaning of these observations, if any, remains obscure. In any event, Knowles *et al.* (1985) recently analyzed the relation between aldosterone and the raised transepithelial potential difference (PD) that exists across various respiratory (including nasal) epithelia in patients with cystic fibrosis. This marker reflects active sodium absorption across a membrane that is relatively chloride impermeable and could, in theory, result from focal hypersensitivity to aldosterone. However, systemically effective doses of spironolactone did not change the nasal PD in CF patients or normal subjects, even though both groups experienced the decrease in rectal PD that is expected in response to aldosterone antagonism. These observations indicate that CF patients have normal rectal responsiveness to aldosterone, that their nasal epithelium is not hypersensitive to aldosterone and that, in any event, the normal nasal PD is not under the influence of aldosterone.

A series of observations from one laboratory have indicated that SF of patients with CF have a membrane defect that is expressed by resistance to the cytotoxic effects of dexamethasone, all three sex steroids, ouabain (a sterolic compound), and cyclic AMP (Epstein *et al.*, 1977, 1978; Breslow *et al.*, 1978). These effects were unrelated to the activity of the classical intracellular receptors for the steroids, and their validity was challenged strenously on methodological grounds (Kurz *et al.*, 1979). De-

spite a rebuttal to this challenge (Breslow and Epstein, 1980), there has not been independent confirmation of the original observations. Furthermore, results indicating a SF sodium-transport defect in CF after ouabain exposure (Breslow *et al.*, 1981) could not be repeated (Breslow and McPherson, 1981).

In spite of these discouraging developments with regard to the quest for basic understanding of CF and for useful markers of it in SF, it is worth recalling here that Johnson *et al.* (1984) have recently isolated a genetic variant of the W7 murine lymphoma cell line that resists dexamethasone-induced lysis by a mechanism unrelated to the classical intracellular GR. The cross-resistance of this variant to other membrane-active substances (for example, colchicine) and the ability of procaine, a membrane-active anesthetic, to promote dexamethasone uptake by the variant cells suggest that dexamethasone resistance originates in its plasma membrane. Others have reported synergistic responses to steroids (particularly androgens) and cyclic AMP, using membrane and overall cell morphology as the end point (Puck *et al.*, 1972; Porter *et al.*, 1974). Indeed, the web of circumstantiality surrounding the foregoing observations is thickened by the report of Forrest (1981) that SF of cystic fibrosis patients have decreased colchicine-binding activity.

Altered Glucocorticoid Sensitivity in Depression and Cushing Disease

In addition to the fact that patients with Cushing Disease often have signs of clinical depression (Starkman *et al.*, 1981), there is evidence for a disturbance among corticotropin-releasing hormone (CRH), ACTH, and cortisol in people with the depressive type of primary affective disorder (Lowy *et al.*, 1985). The cardinal expression of this disturbance in both types of subjects is hypercortisolism. Furthermore, in some patients with depression, dexamethasone fails to suppress serum cortisol or prolactin normally (Meltzer *et al.*, 1982), or the competence of their lymphocytes to respond to mitogens (Lowy *et al.*, 1984). It is noteworthy that the latter study did not consider the association of HLA-B7 with increased phytohemagglutinin (PHA) stimulation, or of HLA-A10 with decreased sensitivity to glucocorticoid inhibition of PHA stimulation (Erickson *et al.*, 1985). Nonetheless, such findings have generated the hypothesis that GR dysfunction is somehow involved in the pathogenesis or expression of primary depression. Recently, Gold *et al.* (1986) have used CRH stimu-

lation to determine that the pituitary corticotroph cells have attenuated ACTH responses in patients with Cushing disease, but not in those with depression. This indicates that the pituitary adenomas found in at least 75% of patients with Cushing disease (Daughaday, 1985) have an impaired sensitivity to the normal feedback effect of basal hypercortisolism. It will be interesting to see whether the feedback defect in these adenomas occurs at the level of the regulatory DNA sequences adjacent to the 5′ portion of the structural pro-opiomelanocortin (POMC) gene that interact with G–R complexes to decrease its rate of transcription (Drouin *et al.*, 1986), or at another, posttranscriptional site that regulates the final expression of this gene product. The same question applies to the minority of patients witih Cushing disease who do not have a pituitary adenoma. Indeed, in view of the evidence, cited by Gold *et al.* (1986) that the proximal cause of hypercortisolism in primary depression is at, or above, the hypothalamic level, as reflected by increased concentrations of CRH, one is entitled to ask whether defective GR-mediated feedback is also involved in its pathogenesis (Mayo, 1986). An affirmative answer to this question would provide a useful marker for investigating the genetic basis of susceptibility to the depressive form of primary affective disorder.

CONCLUSION

Genetic variation has been indispensable for dissecting the discrete components of steroid-response systems, both in their receptor and post-receptor limbs. It will continue to be so. In the near future this advantage will apply only to the androgen- and glucocorticoid-response systems, because they are the two for which rich sources of mutation are currently available. However, it is certain that modern techniques of molecular genetics will hasten the acquisition of detailed knowledge about other steroid-response systems. For instance, directed mutagenesis has not yet been applied to the structural genes encoding SR proteins. In fact, at the time of this writing (August, 1986) cDNA clones exist only for the ER, GR and PR loci, and nothing is known about their genomic organization. The structural similarities and differences among various natural steroid hormones or their synthetic analogues, particularly in relation to those portions of their respective receptor proteins that are necessary for achieving the high-affinity binding state, ought to facilitate the delineation of properties common to all or many S–R complexes. Conversely, it will

be exciting to learn what critical structure-function features impart unique regulatory information to S–R complexes of well-defined relatedness. For instance, although some clues have begun to emerge, it is not understood why an antisteroid–receptor complex fails to achieve the effects of its related agonist–receptor complex, even when it can apparently bind as well to appropriate regulatory sequences of DNA.

The various components of chromatin with which S–R complexes collaborate, the organization of chromatin that permits S–R complexes to bind to selected DNA sequences, and the reorganization of chromatin that is likely to accompany and reflect their action are becoming subjects of closer study. S–R complexes are the best signals currently available for designing experiments to define the general rules that govern transcriptional regulation of gene expression in vertebrates.

In aggregate, the broadly diverse aspects of steroid action covered in this chapter are indicative of the important biomedical implications attached to further research in this field. We conclude by listing some of them:

1. Sexual differentiation of the brain as a source of enormous variation in sexual identity and orientation among individuals in one sex, and as a contributor to sexual inequality of the various cognitive behaviors.
2. The roles of sex steroid responsiveness in the pathogenesis, metastasis, and therapy of certain benign and malignant neoplasms that are common (for example, of the breast and the prostate).
3. The influence of sex steroids on normal differentiation of the immune system and on the pathogenesis and management of autoimmune disease.
4. The impact of testis-restricted androgen resistance as a cause of male infertility.
5. The contribution of sex steroids to sexual inequality in the incidence of premature atherosclerosis or of hyaline membrane disease in the premature infant.
6. The role of corticosteroids in the pathogenesis of essential hypertension.

We confidently predict that 5 years from now a review of the subject of this chapter will have a very different character from the one just presented.

ABBREVIATIONS

ACTH	adrenocorticotropic hormone
AR	androgen receptor(s)
B_{max}	maximum binding capacity
CRF	corticotropin-releasing factor
Dex	dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione
DHT	5α-dihydrotestosterone
DTT	dithiothreitol
E_2	17β-estradiol
ER	estrogen receptor(s)
E–R	estrogen–receptor complex
FSH	follicle-stimulating hormone
GR	glucocorticoid receptor(s)
G–R	glucocorticoid–receptor complex
GS	genital skin
GSF	genital skin fibroblasts
hCG	human chorionic gonadotropin
HT	hydroxytaxomifen
k	rate constant of dissociation
k_D	equilibrium dissociation constant
LH	luteinizing hormone
LH-RH	luteinizing hormone-releasing hormone
MB	mibolerone, 7α,17α-dimethyl,19-nortestosterone
MR	mineralocorticoid receptor(s)
MT	methyltrienolone, 17β-hydroxy,17α-methyl-4,9,11-estratriene-3-one
NGSF	nongenital skin fibroblast(s)
NHP	nonhistone proteins
NM	nuclear matrix
PR	progesterone receptor(s)
PS	pubic skin
PSF	pubic skin fibroblast(s)
SF	skin fibroblast(s)
SR	steroid receptor(s)
SS-BG	sex-steroid binding globulin
T	testosterone

triamcinolone 9α-fluoro-11β,16α,17,21-tetrahydroxy-pregna-1,4-diene,3,20-dione

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